PHYSIOLOGY OF BACTERIA

PHYSIOLOGY
OF
BACTERIA

BY
OTTO RAHN
PROFESSOR OF BACTERIOLOGY,
CORNELL UNIVERSITY, ITHACA, N. Y.

With Forty-two Illustrations

PHILADELPHIA
P. BLAKISTON'S SON & CO., INC.
1012 WALNUT STREET

PRINTED IN U. S. A.
BY THE MAPLE PRESS COMPANY, YORK, PA.

THIS BOOK IS DEDICATED
TO THE MEMORY OF

CHARLES E. MARSHALL

of Michigan and of Massachusetts Agricultural College, my teacher and my friend, who realized the need for theoretical bacteriology, and found ways and means to further it before its value was generally appreciated

PREFACE

This book originated from a regular course on the physiology of bacteria given to advanced students at the University of Illinois in the years 1913 and 1914, and again at Cornell University since 1927. It would have been published long ago had not the consequences of the war interfered. This required a complete rewriting of the manuscript; new facts had been discovered; new theories had been substituted for old ones. But the principal conception and the general outline have remained the same.

In the seventy years since bacteriology became a science, only a few books on the physiology of bacteria have been published. Duclaux' "Traité de Microbiologie" (1900) was followed ten years later by Kruse's "Mikrobiologie." Then came Euler-Lindner's "Chemie der Hefe" in 1915. Recently, we have the three volumes of Buchanan and Fulmer's "Physiology and Biochemistry of Bacteria," and Marjory Stephenson's "Bacterial Metabolism," the first one being meant essentially as a reference book, and the second emphasizing only one phase of bacterial physiology, namely, the metabolism, though all other functions are also discussed. It is safe to say that all but the last two books are antiquated.

My own intention in writing this book differed somewhat from that of the last named authors, and that might give it a right for existence. It is an attempt to co-ordinate the various simplest functions of life, to

study each function in itself and in its effect upon the other functions. This study is limited to the *necessary* functions of life; therefore, motility, phosphorescence, tropisms, and similar conspicuous but unimportant functions have been omitted.

By confining the discussion to the indispensable functions of the simplest forms of life, I hope to arouse an interest for this kind of work not only among bacteriologists, but among plant and animal physiologists as well. While practically all the discussion in the book refers to bacteria, the principles developed reach beyond the domain of bacteriology, and apply to biology generally. More than that, I believe that some of the principles of biology can be found and studied *only* with the simplest forms of life, and that general physiology has much to learn from the physiology of bacteria.

This book is not intended to be a review of literature on the subject. Such compilations exist already in the books mentioned above. It contains a number of unpublished experiments made with the object of deciding certain doubtful points in physiology.

The work is meant to be critical. I have tried to present all theories, and to mention their strong and weak points. To counterbalance any personal viewpoint, each chapter and subchapter contains a summary, usually separated according to facts and theories.

An effort has been made to separate logically, and, if possible, experimentally, growth from fermentation; also to differentiate between the rates of the processes and their end points. Many arguments on growth have arisen because one investigator measures growth by generation-times and another by the maximum number of cells produced. No new definitions are required; no new words have to be coined to distinguish between rates

and endpoints, or to keep fermentation separate from growth. All that is necessary is just a habit of thinking, which is easily acquired.

The literature available for the support of many points discussed herein is inadequate. Allowing that a considerable number of publications might have been overlooked by me, the fact still remains that the available material is scanty. And yet, many experiments have been made in the past which could have thrown light on some debated points had they been recorded fully instead of incompletely. Some essential facts, as, the number of acting cells, the length of time of a fermentation, or the quantity of inoculum in a growth experiment have not been determined, or at least not stated. Then, too often, data are given only in relative numbers, without the key to absolute measure; while results are given in curves only, from which it is impossible to reconstruct the data with sufficient accuracy to use them for any purpose.

The making of a book usually involves not only the author, but a number of his colleagues and associates. This has been the case with this book, too. I am obliged to Dr. J. M. Sherman, as head of the department, for providing the opportunity for completing the manuscript. I am obliged to him, as well as to Dr. F. W. Tanner of Illinois University, for reading critically some of the chapters. Several of my former and present associates, especially Mrs. Margaret N. Barnes, Miss A. Jean Ferguson, M. J. Foter, P. Arne Hansen, and J. A. Woerz, have been very helpful in correcting the style and reading proof. Further, I have to thank Dr. H. H. Boysen, Dr. D. C. Carpenter, Dr. Hermann Claassen, Mrs. V. A. Coffeen and Dr. G. L. Peltier for permission to use some of their unpublished data, and Drs. W. M. Clark, C. B.

Coulter, L. G. Gillespie, E. G. Hastings, H. R. Thornton, and R. W. G. Wyckoff, for permission to use some of their graphs.

Finally, I wish to express my special gratitude to the publisher who undertook the printing of this textbook, because he had the confidence that the book will have its place in bacteriological and biological literature.

Otto Rahn.

Ithaca, New York.

TABLE OF CONTENTS

PART C. GROWTH

APPENDIX

PHYSIOLOGY OF BACTERIA

GENERAL INTRODUCTION

(*a*) THE SIGNIFICANCE OF THE PHYSIOLOGY OF BACTERIA

Life processes are largely chemical or physical processes, and it would be wasted time and effort to try to understand the very complicated and interlinked actions and reactions, the total of which we call life, unless the best physical and chemical methods were used for their analysis and interpretation.

There may be processes other than physical and chemical involved in life. Most of those changes brought about by life processes which we, from our human viewpoint, consider most important, namely, the progress of civilization, the development of creative thoughts, cannot as yet be explained by chemistry and physics, and perhaps never can be. Whether there is a soul, an agent independent of the body, in man or animals may ever be an open question. So much is certain, however, that it will not cause any actions contrary to the laws of physics or chemistry; else, its existence would have been established long ago.

Just as physics is more than mere mathematics, and chemistry cannot be explained altogether by physics, so biology cannot be accounted for exclusively by physical and chemical equations.

This book deals only with those aspects of biology that can be treated from the physico-chemical viewpoint. Its intention is to give a conception of the life mechanism of the simplest organisms, and no other group of organ-

isms is more fit for this purpose than the lowest fungi which live on dissolved food. That some of the human physiologists do not agree entirely with this viewpoint, may be seen from the following statement in Bayliss' "Principles of General Physiology" (1924):

"Without denying the great value of the comparative method in eliminating merely incidental phenomena, it must be pointed out that this very simplicity is, in the majority of cases, a disadvantage. The same organ, or even cell, fulfils a variety of purposes which, in the higher organisms, are relegated to distinct groups of cells. Moreover, the size of the organism is of much importance. The science of nutrition would be almost impossible without the larger, warm-blooded animals. The advantage of the increased rate of reactions, owing to the higher temperature, is not to be undervalued.

The physiology of unicellular organisms, although of considerable importance in special aspects, is not to be regarded as a "general physiology." Indeed, if the choice had to be made between the investigation of simple or complex organisms alone, there is no doubt that a much more general and fundamental body of doctrine would be obtained from the latter."

Of course, it would be ridiculous to try to belittle the value of the physiology of man and of mammals as a guide to the understanding of the functions of the body of all kinds and types of animals and bacteria, for it is quite evident that the original conception of the nutrition of bacteria was based exclusively upon its analogy with animal nutrition. There are, however, certain facts which could not be observed in the highly specialized physiology of animal tissues, consequently, new conceptions have been brought into general physiology through the study of bacteria; e.g., the conceptions of anaerobiosis; of thermophilic organisms; of nitrogen fixation; of fermenting endo-enzymes; the understanding of the organic chemistry of alcoholic fermentation with its intermediate stages; and a new understanding of the laws of death. In fact, the conception of higher plants

and animals as well organized cell federations was possible only after the existence and physiology of unicellular beings had been established. Considering the very little attention paid hitherto to the physiology of bacteria, it seems quite possible that many new contributions to general physiology might originate from the study of the simplest forms of life.

Most favorable to such studies is the attitude of Pütter in his book on Comparative Physiology (published as early as 1911):

"It might perhaps surprise many to find so much space given to the discussion of the biochemistry of molds, yeasts and bacteria; however, with just these organisms, a clear conception of the different metabolic processes can be obtained, and the aim of a comparative physiology seems to me to consist rather more in working out the various principles with the organisms best suited for this purpose than in enumerating a larger number of experiments from all the groups of organisms existing."

It is not conceivable that any scientist would consider it possible to substitute physiological experiments with the liver or the thyroid gland by experiments with bacteria. Nor is it likely that anybody would believe nutrition experiments with higher animals superfluous and replaceable by experiments with lower fungi. The very simplicity of the metabolism of some microorganisms makes them fit for a good many experiments which cannot be carried out with higher plants or animals. That is why the physiology of microorganisms is of considerable importance in general physiology.

Some of the advantages of using microorganisms in physiological experiments of a more general nature, are: (1) the absolute control of temperature during the entire experiment, and the possibility of working at several different temperatures; (2) the absolute control of food which may be chosen from chemically pure and well

defined compounds only; (3) the control of the concentration of food; (4) the complete analysis of all metabolic products; (5) the circumstance that all experiments are averages of millions or billions of individual cells which are all of the same kind and the same age; (6) the very rapid rate of growth; (7) the possibility of separating to a considerable extent the food for energy supply from the food needed for building material.

There is one disadvantage in all experiments with lower fungi, i.e. their minute size. (A general conception of the meaning of the size of microorganisms can be obtained from Table 132 of the "Appendix.") This small size makes the determination of the "crop" difficult, especially with bacteria, while with molds and yeasts, it is fairly easily accomplished. But even with bacteria, direct and indirect methods have been devised which will be found in the chapter on Growth.

Not only from the viewpoint of general physiology is the physiology of microorganisms important; it is also of great value in applied bacteriology. There can be no doubt that future developments in the latter field will depend to a large extent upon a better understanding of the physiology of microorganisms. The canning industry, as well as chemical and physical disinfection, need urgently a deeper knowledge of the laws of death of bacteria, while the fermentation industries require more detailed information regarding the different types of fermentations, and of the factors influencing them; the bread yeast industry is likewise interested in further advances in the studies of growth and nutrition of microorganisms.

(*b*) PARTIAL FUNCTIONS

Primitive Functions.—The only way we can study the mechanism of life is to separate life into a number of

individual processes, and then study each process, or group of processes, by itself (if this be at all possible) and to determine also the relations between various processes. The number of separate processes, distinguishable to the scientist, is practically unlimited, owing to the fact that we can subdivide, again and again, and consider as a separate process each detail of a chemical reaction, as well as the diffusion of each compound from one cell to another. For scientific study, we divide life manifestations into groups which are logically comparable, and which fulfil the same purpose. Such groups of processes, we call functions, or more correctly partial functions, since each of them is only part of the complete life process. The eating of meat by a cat and the eating of grass by a cow are such separate processes, classified under the same function; while the running of a horse, the flying of a bird, and the swimming of a bacterium give us an idea of the variability of the function which we ordinarily call motility.

The distinction of functions is primarily a matter of convenience or viewpoint, and this varies with one's personal attitude, as well as with the progress of knowledge. The viewpoint of Pasteur that fermentation and respiration were but two modifications of the same function has been contested most persistently for a long time. Further, it must be stated that we distinguish more functions, ordinarily, in the higher organisms; in other words, what is only one function in the protozoon, may be divided into four or five functions in man. The terms function and partial function relate, therefore, not to well defined groups of processes; and they cannot be well defined, because they are interlinked in such a way that some of the processes are necessary prerequisites for the others,—e.g., respiration is the prerequisite for

heat formation in the body. We must distinguish, therefore, between dependent and independent functions. If the death of a cell by poison is independent of the rate of growth, or of the rate of fermentation, we may call it an independent function. Growth, however, depends upon the amount of available energy. Further progress in knowledge may disclose to us that the assumedly independent functions are, after all, dependent ones.

A most natural question arises in this connection: Which functions are found in all living beings, i.e., which functions are essential for life? This question leads us immediately to the simplest forms of life. The most characteristic quality of a living thing which distinguishes it from all dead matter, is growth. Growth, meaning the construction of complex molecules from less complex molecules, is, however, a dependent function and requires energy, and energy is, with chlorophyll-free organisms, provided by a function of the body which we call respiration, or, in the case of bacteria, fermentation; here we are using the term in its widest meaning.

Growth is not identical with multiplication. However, bacteriologists commonly use these terms as synonyms, probably because growth of a culture is identical with multiplication of bacteria. The difference is very great cytologically, but in these biochemical studies, no emphasis shall be placed upon the difference, and the two functions shall be treated as one because we have hardly any means to differentiate them chemically (see also Introduction to "Growth" p. 162).

Besides energy formation and growth, all living organisms share one more property: they all must die. Starvation, poison, excessive heat will kill all living cells, and we know that organisms will die of old age in a natural way. Though there are great variations in

resistance and longevity, a living cell that cannot die is unthinkable.

The mere absence of growth and energy formation does not necessarily mean death; the same symptoms are characteristic for dormancy. We can imagine conditions where we have neither growth nor fermentation and yet no death. This is probably the case with dry bacteria in a vacuum at very low temperatures. The discussion of the causes of death will show that dying is a chemical process.

Another function is also found regularly, or at least assumed for all cells, i.e. endogenous catabolism. Catabolism is assumed for all cells because all cells die from starvation, death by starvation meaning that a deterioration of some cell compounds occurs which, in normally nourished cells, is counteracted by constructive processes.

Endogenous catabolism shall mean here the deterioration of cell compounds, but not of the food taken in. This definition may eventually lead to difficulties, for we may assume that the food combines with some cell compounds before being decomposed. But we shall try to avoid hair-splitting as much as possible.

We have, then, endogenous catabolism, death, energy formation and growth (and, eventually, multiplication) as the four functions which are indispensable for life, the primitive functions of life, so to speak. This book is limited entirely to the discussion of these four functions.

Of these four functions, endogenous catabolism is the least dependent of all. The process of dying may, in some cases, be interlinked with catabolism, as in death by starvation. Growth is the most dependent function, being possible only when energy is furnished, and inseparable from catabolism. It would be impossible to discuss growth without having first studied energy

production. For this reason, the discussion begins with the independent, though least-known function of endogenous catabolism. The chapter on growth is preceded by a discussion of the energy liberation. The causes of death might have been placed before the chapter on growth, but it seemed too odd to discuss death before growth. Hence, the chapter on death has been placed at the end.

PART A

ENDOGENOUS CATABOLISM, OR NORMAL DETERIORATION OF LIVING MATTER

(*a*) ENDOGENOUS CATABOLISM AND AUTOLYSIS

As all living cells will die if kept without food for a varying number of days (except when in a dry state), it must be assumed that deteriorating changes in living cells take place continuously, and can be counteracted only by assimilation of food. These changes will ultimately cause death of the cell if continued for too long a time. Bacteria are no exception to this rule as will be shown in the chapter on Death by Starvation (p. 377).

As defined by Folin (1905), this gradual and constant decomposition of living matter may be called endogenous catabolism. All measurements are limited to protein changes, but other compounds may undergo similar changes. Endogenous catabolism has been studied for more than one hundred years with man and animals. It is usually measured by the quantity of nitrogenous matter excreted during starvation or with protein-free diet. The quantity of nitrogen excreted by a starving man seems to vary considerably, but the average is about 6 to 10 gm. of nitrogen per person per day. This means about 80 mg. per kg. of body weight, or, estimating on 10 kg. of protein per average person, it amounts to about 1 gm. of nitrogen for each kg. of dry protein, or to 0.6% of the protein nitrogen.

Normally chlorophyl plants do not excrete protein cleavage products. There are many observations indicating that green plants in the dark not only break down their carbohydrates, but also their proteins. Yet, their synthetic action seems to be so powerful that all the products of nitrogenous catabolism are re-utilized somewhere in the plant, and do not leave it.

Differing from this endogenous catabolism of the starving organism is the autolysis of the dead organism. When tissues of a dead animal are held at 37°C. under aseptical conditions, the protein readily breaks down to simpler compounds. This self-digestion or autolysis of tissues is due to proteolytic endoenzymes. The products of autolysis are in a general way comparable to the products of peptic or tryptic digestion, and are quite different from the products of endogenous catabolism.

Another difference between the two processes of protein decomposition lies in the rate of the process. As an example, Jacoby (1900) observed that normal liver of a dog, after twenty-four hours of autolysis, had lost 17.9% of the total nitrogen, or 20% of the protein nitrogen. This is an enormous rate compared with the 0.6% of nitrogen loss by endogenous catabolism by a starving man per day.

It is quite necessary to differentiate sharply between these two different processes. This distinction was not made by Rubner (1913), but is drawn very sharply by Euler (Euler-Lindner, 1915):

"Yeast contains plenty of proteinases and peptases. In the living cell, the destructive action of these is in adjustable equilibrium with the construction of protein. If, however, synthesis is retarded in comparison with the cleaving action of the digestive enzymes, we observe the phenomenon which is usually called autolysis . . .

Autolytic action occurs, as far as is known, after the 'death' of all cells and tissues; but it is not only in the dead cells that the own protein is attacked . . .

. . . Several investigations tell us about the behavior of yeast in relatively large amounts of water, with or without disinfectants. If no nitrogenous food is offered . . . the yeast loses its own protein. This loss may be temporary and need not lead to the death of the cell. It may be supposed that the same reactions take place even under normal conditions, but are covered up partly or entirely by synthetic processes. This process shall be called 'endoproteolysis.'

If this endoproteolysis is not compensated by constructive processes, then, finally, those protoplasmic groups which are indispensable for cell life will be attacked. The cell loses the power of reproduction and undergoes complete destruction. This latter process of dissolution shall be called autolysis. This autolysis which starts at the death or afterwards, is essentially an irreversible process initiated by endoproteolysis."

(*b*) ENDOGENOUS CATABOLISM OF YEAST

The amount of nitrogenous living matter changed per day has been found with man to approximate 0.1 gm. of nitrogen per kg. of body weight. If this same ratio would hold with microorganisms, the task of determining catabolism would seem hopeless, for the total solids of the cells in one liter of broth culture of *Bact. coli* amount to only 220 mg. and their catabolic products per day would total only 0.2 mg. of nitrogen per liter.

But this ratio does not hold. Rubner (1908) has pointed out that in animals, the relative amount of endogenous catabolism increases with the decrease in size, and Rubner believed the surface to be the important factor determining the rate of endogenous catabolism. But even under these favorable circumstances, the amount of catabolized nitrogen is so small, owing to the minute quantity of living matter in a bacterial culture,

that, so far, endogenous catabolism has been studied only in yeasts.

As early as 1803, Thénard observed that yeast fermenting in pure sugar solutions loses protein. Yeast may lose 30% or more of its nitrogen, and finally loses the ability of rapid fermentation (Schützenberger, 1876). Pasteur and many others have observed a decrease of nitrogen in yeast, but few of these experiments have been made quantitatively.

Though endogenous catabolism of yeast is more rapid than with higher organisms, autolysis is still swifter. Rubner (1904) observed in living yeast kept in a 20% sugar solution, a loss of nitrogen of 58% in six days, while the same yeast suspended in water + toluol, i.e. after being killed, lost 90% of its nitrogen in four days. Effront (quoted from Euler-Lindner) found that 500 gm. yeast in distilled water lost 39 mg. of nitrogen in twenty-four hours while the same yeast in water + alcohol + hydrofluoric acid lost 72 mg. After five days, the latter liquid, from the dead yeast, contained ten times as much nitrogen as the liquid from the living yeast.

Rubner (1913) made extensive experiments on the nitrogen catabolism of fermenting yeast cells; the main results are given in Table 1. The first two experiments refer to yeast kept in 20% sugar solution at 38°C. for three and six days. Different yeast was used in these two experiments. For the third experiment, the yeast remained in a 10% sugar solution at 28°C. for twenty-four hours, was then centrifuged out, and suspended in new sugar solution; after another twenty-four hours, the sugar solution was renewed in the same way, and this was repeated five times.

In connection with the last experiment, it will be of interest to know that the total quantities of alcohol

produced by the same yeast on each of the successive days were 6.70%, 4.16%, 2.30%, 1.19%, 0.74% and

TABLE 1.—NITROGEN LOSSES BY FERMENTING YEAST

Time	Mg. nitrogen in yeast	Relative N quantities	Microscopic counts (millions)	Plate counts (millions)	Relative numbers of viable cells
Yeast in 20% sucrose solution at 38°C. for three days					
Start	62.7	100	72,000	51,850	100
After 3 days	52.2	83	24,100	40,600	78
Yeast in 20% sucrose solution at 38°C. for six days					
Start	430	100	176,200	133,000	100
After 6 days	65	15	173,000	4,975	3.7
Yeast changed every twenty-four hours to a new solution of 10% sucrose, at 28°C.					
Start	93.5	100	84,600	20,355	100
After 1 day	78.9	84.4	62,845	20,898	103
2	66.3	70.9	66,605	13,728	67
3	55.9	59.8	66,950	824	4
4	47.1	50.3	88,600	218	1
5	39.6	42.3	105,400	8.2	0.04
6	28.2	30.0	101,600	6.2	0.02

0.52%. Towards the end, the yeast had almost entirely lost the power to ferment.

A similar observation was made by Meyerhof (1916a) in his studies of the Nitrobacter. This organism builds its entire cell from nitrite, minerals, oxygen and CO_2. Meyerhof observed that the rate of oxidation slowly decreased if CO_2 was removed from the atmosphere.

The nitrate bacteria were starving for CO_2, and that prevented a normal formation of the oxidizing agent in the cells.

TABLE 2.—OXYGEN CONSUMPTION BY NITROMONAS, WITH AND WITHOUT CARBON DIOXIDE

(mm.^{3} oxygen consumed per 2 hour period by 2 c.c. of culture)

	After 4 hours	After 6 hours	After 8 hours
With CO_2.....	45.5	49	50 mm.^{3} O_2
Without CO_2.....	44.5	39	31 mm.^{3} O_2

The chemical nature of the nitrogenous compounds excreted by living yeast is quite unknown while the autolytic products have been studied extensively. It is very probable that most of the excretions of living yeast cells cannot again be assimilated by the same yeast while by far the largest amount of the autolytic products can be used again for cell construction (Lindner, 1905).

Probably, all data on the endogenous catabolism of yeast have quite a high degree of error, because they may represent not only the products of living cells, but also of a certain percentage of dead cells. This percentage is practically unknown in all experiments. Since autolysis of dead cells proceeds much more rapidly than that of living cells, a relatively small number of dead cells will cause a considerable error.

The contention of Rubner (1908), that the more rapid rate of endogenous catabolism in small organisms is caused by the relatively larger surface, seems rather improbable since the compounds to be decomposed are already within the cells. Two further explanations seem possible: either the higher organisms re-utilize a large amount of the decomposed protoplasm, or the yeast

protoplasm is more readily decomposed than that of mammals.

Both explanations have some facts in their favor. In starving animals, different organs suffer in different degrees; the heart does not lose weight at all (Bayliss p. 274), and seems able to use part of the catabolic products from other tissues. Creatine appears in the urine during complete starvation. It disappears, i.e., it is re-utilized when carbohydrates are given, but not when fats are given. The carbohydrates enable a synthesis which retains the creatine in the body.

With yeasts, we need not consider different tissues. All cells are alike physiologically, and what has been excreted as useless waste by one cell cannot be utilized by any other cell of that same culture.

TABLE 3.—LOSS OF NITROGEN BY 5 GM. OF YEAST AT 28°C.

	Mg. N in yeast			Loss of nitrogen		
	I	II	III	I	II	III
Fresh	108.0	109.1	113.0			
24 hours in water	93.9	100.0	99.8	14.1	9.1	13.2
24 hours in sugar solution	90.8	92.4	103.7	18.8	16.7	9.3

Rubner (1913) believed that endogenous catabolism of yeast goes parallel with the rate of fermentation. He gives some data on the loss of nitrogen by yeast in water and sugar solution, and in two of the three experiments, the loss in water is larger. But they were not carried out with pure cultures; there was putrefaction in the culture without sugar, i.e., a synthesis of bacterial protoplasm from yeast excretions. This makes the experiment quite doubtful.

In another place, Rubner mentions that yeast in sugar solution lost 58% of its nitrogen, while the same yeast without sugar lost 89%.

(c) CHEMICAL CONCEPTION OF "WEAR AND TEAR"

The constant excretion of nitrogenous products by the starving organism has been explained as the result of the continuous "wear and tear" of the cells. It seems that most physiologists consider this to be a mechanical wearing of the organism, and what originally was to be just a simile is now considered the true explanation. The books of Bayliss and of Cathcart give no other.

Yet, this interpretation seems rather unsatisfactory. A mechanical wearing of a resting man or animal during a starvation experiment is difficult to conceive. Many experiments have shown that mechanical labor, unless it is overdone, does not increase, to any marked degree, the wear and tear, as measured by the nitrogenous excretions. This is quite contradictory to any mechanical conception of the wear and tear (Bayliss p. 267, Cathcart p. 133).

Euler (Euler-Lindner, 1915) offers a biochemical theory already quoted on p. 10. It is essentially the same as that of Rahn (1915) who explains endogenous catabolism as the result of the instability of living protoplasm or parts of it. All cells contain enzymes. All enzymes are unstable and deteriorate with time.

While it is generally admitted that enzymes in solution cannot be kept permanently active, it is often stated that they do not undergo deterioration inside of the cell. The only evidence to substantiate this claim is the fairly constant enzyme content of the cell. This proof is not conclusive since we know that all cells can produce the enzymes which are found in them.

Rahn assumed that the enzymes within the cell behave as they do outside. The deficiency in the cells from enzyme deterioration is made up through the synthesis of new enzyme by the cell itself. The cells have the power to produce enzymes not once only, during growth, but as often as is necessary, to keep the supply normal, as has been shown by many experiments with yeast zymase by Buchner, Buchner and Hahn.

A certain amount of the cell enzymes thus deteriorates at a constant rate under constant conditions. The deteriorated enzyme, being a waste product, is decomposed by an autolytic enzyme; perhaps parts of the products are utilized for the synthesis of new enzyme; the rest of the cleavage products leaves the cell. Other unstable cell constituents will have a similar fate, and the total sum of all such deterioration is what we call endogenous catabolism.

Meyerhof's experiment with the Nitrobacter (see Table 2) is very interesting in this respect. The oxidizing enzyme seems to be very readily deteriorated, and can be rebuilt only from CO_2, not from its own cleavage products.

This chemical interpretation of the wear and tear gives a simpler explanation to the endogenous catabolism of the working muscle. The work of the muscle requires additional energy, i.e., a faster combustion of food in the cells. This combustion is brought about by enzymes in the cells, but it is a well known rule of enzyme action that they do not deteriorate faster when acting than when resting. Whether the muscle works or rests, the rate of enzyme deterioration is not influenced.

But the rate is greatly influenced by an increase in temperature (see p. 124). As soon as the work of the muscle comes to a point where there is a considerable

increase in temperature, we should expect a much more rapid deterioration of the enzyme. This is actually the case. And it has also been found that endogenous catabolism increases in fever, when the body temperature is raised without any increase in mechanical work (Bayliss p. 272).

If it can be shown that endogenous catabolism has a very high temperature coefficient, the above theory would be well substantiated. Rubner's few data give little hope that this may be the case. However, the results of a more exhaustive study must be waited for before conclusions can be drawn.

A decided reduction of the nitrogen content of starving Aspergillus mycelium has been observed by Terroine, Wurmser and Montané. In 5 days, it dropped from the normal 6% N of the mycelium solids to about 2.6% in the presence of glucose, and to 2.3–2.8% in its absence.

(*d*) AUTO-OXIDATION OF THE CELL

It seems most probable that oxygen will play a considerable role in the normal breakdown of living matter. Most cells have the ability to use oxygen for the production of energy, and it is likely that starving cells will apply oxidation to the less essential cell constituents in order to obtain energy. Oxidation seems a simple way of using waste products of endogenous catabolism to greatest advantage.

It will be shown later that the life mechanism is an intricate combination of oxidation and reduction processes. Growth is essentially a reduction process, energy formation is primarily an oxidation process, though the oxygen used for oxidation need not be molecular oxygen. Part of the energy obtained by direct or indirect oxida-

tion is required to furnish the energy necessary for reduction.

TABLE 4.—INFLUENCE OF OXYGEN UNDER VARIOUS PRESSURES AT 37°C. UPON DIFFERENT BACTERIA (24 HOURS' EXPOSURE)

	Oxygen pressure in atmospheres												
	1	1½	2	3	4	10	20	30	40	50	60	70	75
Effect on growth (0 means no growth during twenty-four hours' exposure)													
B. anthracis	0	0	0	0	0								
Bact. alkaligenes, 3 strains	0	0	0	0	0								
Vibrio cholerae	+	0	0	0	0								
Ps. pyocyanea	+	0	0	0	0								
Bact. typhosum, 3 strains	+	+	0	0	0								
Bact. paratyphosum	+	+	0	0	0								
Proteus vulgare	+	+	0	0	0								
Bact. coli, 4 strains	+	+	+	0	0								
Bact. enteritidis	+	+	+	0	0	0							
Micr. pyogenes aureus	+	+	+	+	0	0	0						
Effect on viability (0 means dead after twenty-four hours' exposure)													
B. anthracis	+	0	0	0	0	0	0	0	0	0	0	0	0
Bact. alkaligenes, 3 strains	+	+	+	0	0	0	0	0	0	0	0	0	0
Vibrio cholerae	0	+	0	0	0	0	0	0	0	0	0	0	0
Ps. pyocyanea	+	+	+	+	+	+	+	+	+	+	+	+	0
Bact. typhosum, 3 strains	+	+	+	+	+	+	+	+	0	0	0	0	0
Bact. paratyphosum	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgare	+	+	+	+	+	+	+	+	+	+	+	+	+
Bact. coli, 4 strains	+	+	+	+	+	+	+	+	+	+	+	+	+
Bact. enteritidis	+	+	+	+	+	+	+	+	+	+	+	+	0
Micr. pyogenes aureus	+	+	+	+	+	+	+	+	+	+	+	+	0

The cell disposes of molecular oxygen for two reasons: to obtain energy, and to prevent oxidation of vital cell constituents. If the oxygen concentration is increased, the cells may not be able to prevent oxidation of the essential parts, and the cell dies. Table 4 gives some

experiments by Berghaus (1907) showing how different organisms can resist different oxygen concentrations to which they had been exposed for twenty-four hours.

Many years later, Callow (1924) measured quantitatively the amounts of oxygen taken up by washed living bacteria suspended in buffer solution. The amounts varied from 5 to 25 c.c. of oxygen (7–35 mg). per gram of bacteria solids per hour, and remained constant for many hours. Even after drying in vacuo and re-suspending, three species took up as much oxygen as before, while *Ps. pyocyanea* used very much less oxygen. The experiments included

Ps. pyocyanea, Ps. fluorescens
Bact. prodigiosum, alkaligenes, coli, proteus
B. megatherium, subtilis
Micr. pyogenes aureus, Sar. aurantiaca
Mycobact. phlei

The experiments were also extended to *Strept. lactis* and *Clostr. sporogenes;* these took up less than 0.5 c.c. during the first hour, and practically none thereafter. This seems to contradict the fact that these organisms are very sensitive to oxygen. An explanation will be tried on p. 74.

(*e*) SUMMARY OF FACTS

Yeasts and bacteria if left in moist condition without food for a number of days will lose nitrogenous compounds, which can be found in the liquid surrounding them. The same process takes place if the organisms are given carbohydrates, but no nitrogenous food. The decrease of nitrogenous material in the yeast cell is accompanied by a decrease in its fermenting power. Ultimately, the cells die. Nitrobacter loses very promptly part of its power to oxidize if carbon dioxide is

withheld. This is the only carbon compound from which this organism can build its cells.

Aerobic and facultative bacteria, suspended in buffer solution after washing, will take up from 7 to 35 mg. of oxygen per gram bacteria solids per hour for two, and even for ten hours at a constant rate.

Streptococcus and Clostridium took up less than 0.5 c.c. during the first hour, and practically none later.

SUMMARY OF THEORIES

The loss of nitrogenous matter by starving cells is supposed to be a normal process of all living cells which is not ordinarily conspicuous because the opposite processes, growth and reconstruction, are more prominent.

The nitrogenous products of endogenous catabolism are the cleavage products of some thermolabile compounds in the cell, such as enzymes, and living protoplasm. These compounds deteriorate at a definite rate inside the cell as well as outside.

The cell has the power to replace these compounds from new food. In the absence of food, or in the absence of appropriate food, the cell has ultimately not enough of these essential molecules left, and dies.

In the case of Nitromonas, the lack of non-nitrogenous food caused the same symptom because with this bacterium, all the carbon in any of the cell constituents must come from carbon dioxide.

PART B

ENERGY SUPPLY OF THE CELL

I. PHYSICAL CONSIDERATIONS

(a) THE TWO SOURCES OF ENERGY FOR LIFE

In the entire living world, only two sources of energy are known to be available to living cells, namely light and chemical energy. Light can be utilized only by the chlorophyl plants, the bluegreen algae and a small number of prototrophic bacteria. Chemical energy is utilized by all living organisms.

Light is of importance in the growth of certain plants which possess no chlorophyl; but it acts there only as a stimulus. *Rhizopus* grows better in diffuse light than in dark, but it requires the same amount of food to produce the same amount of mycelium; no energy of light is "stored" in the cells of the mold. Such stimulation by light causes the daily rings in cultures of bacteria and molds (Hutchinson, 1907).

Heat, electricity, or other forms of energy cannot be utilized by organisms as available energy. A higher temperature may cause a more rapid functioning of the cell, yet it is merely a difference in the rate of the process, and a cell cannot economize on food by utilizing the heat energy of the surrounding medium. A starving cell is not benefited by an increase of temperature.

Probably the cells lack the "transformers" or "activators" for all these forms of energy. Electricity, heat, and probably even light are not used as such by the cells. They must be transformed into another kind of energy, and we know only the transformer for light, i.e. the chlorophyl.

From this, we must conclude that energy liberated by one cell cannot be utilized by other cells, and that even in tissues, neighboring cells have no means of exchanging any surplus energy directly. It follows further that the chemical energy must be liberated *within* the cell, for if it were liberated outside, it would have to assume one of the forms mentioned above (heat most probably, or eventually light or electricity) which cannot be utilized by the cells. Whether the mitogenetic rays (Gurwitsch, 1929) act merely as stimulants, or whether their energy can be actually utilized is entirely unknown.

This must be kept in mind with bacteria acting upon insoluble food. Only that part of the energy becomes available which is produced in the cell; the energy liberated outside of the cell merely raises the temperature of the medium, without any further benefit to the organism.

TABLE 5.—ENERGY LIBERATED BY ENZYMES FROM 1 GM. OF SUBSTRATE

Secreted enzymes		Endo-enzymes	
Pepsin, Trypsin, Rennet	0 calories	Lactacidase	82 calories
Lipase	4 calories	Alcoholase	149.5 calories (Rubner)
Invertase	9.3 calories (Rubner)	Urease	239 calories
Maltase	10 calories	Vinegar-oxidase	2,530 calories
Lactase	23 calories		

Since only that part of the food, which is decomposed in the cell is of benefit to the bacterium, it is essential that the food pass through the plasma membrane. The compounds need not necessarily be water-soluble to accomplish this; a lipoid-soluble compound might

pass through also. As a rule, we find that the cells change insoluble compounds into a soluble form by secreted enzymes. The energy liberated by secreted enzymes usually is small, that from endo-enzymes is quite large. (Table 5.)

The chemical decomposition of compounds within the bacterial cell, for the purpose of liberating energy, shall be taken in this book as a definition of fermentation. It is practically the same definition which was given by Hugo Fischer (1902):

1. Fermentation is an intracellular process.
2. It yields products essentially different from the materials fermented and which are not mere parts of this material.
3. The end products are useless or harmful to the cell.
4. It produces "vital" energy (i.e., energy available for cell processes).

The definition includes all processes, organic and inorganic, aerobic and anaerobic, with and without gas formation, of proteins, fats, carbohydrates, acids, etc., if only they fit the above definition.

That fermentations provide cell energy as respiration does with animals, was recognized by Pasteur (1876), though he did not furnish absolute proof for this statement. He proved that yeast cells grew in the absence of oxygen, and under these conditions caused fermentation, but he did not exclude other possible sources of energy, especially that from the proteins. Since then, several cases have become known where fermentation is the only source of energy, under conditions where no other source is imaginable. To this group belongs the fermentation of a mineral-ammonia-sugar solution by yeast, of a mineral-urea solution, with traces of organic acids, by urea bacteria, and of a mineral-ammonia solution by nitrate bacteria. The growth of *Oidium lactis* and of *Mycoderma* in a lactic acid-mineral solution $+NH_3$, and the growth of most prototrophic organisms are examples of simple oxidations furnishing the energy for growth.

In discussing the energy supply of cells, it must be considered that most cells can utilize various kinds of food, and may even be able to use the same kind of food in two different ways. It is easily possible to cultivate some typical sugar-fermenters in the absence of sugar. Yeast will grow in sugar-free broth; lactic acid bacteria can grow in peptone solutions; Hydrogenomonas as well as urea bacteria are commonly cultivated on ordinary nutrient gelatin. Most molds seem to be omnivorous, while some of the prototrophic bacteria are limited to only one source of energy. Yeast will ferment sugar to alcohol, but can oxidize it completely if an abundance of oxygen is at hand. Most organisms show certain preferences or, more accurately speaking, certain compounds are more readily fermentable by certain organisms than others. It is a common experience that protein decomposition (e.g. gelatin liquefaction) is retarded by addition of sugar. Apparently, the energy needs of a cell can be more readily supplied from sugar than from protein (see p. 205).

(*b*) AMOUNTS OF ENERGY

The amounts of energy liberated by the various fermentations differ widely. As a rule, oxidations yield the greatest quantities, while hydrolyses yield but very little, if any. Only in one case, cells provide for their energy by hydrolysis, namely in the urea fermentation. Hydrolysis is very commonly caused by bacteria, but in practically all cases, it merely serves as a food-preparing process. The enzymes causing these hydrolyses are mostly soluble. Table 5 (p. 23) shows the soluble and the endo-enzymes arranged according to heat-units produced from 1 gm. of substance. It is very evident that the enzymes with small heat liberation are soluble

while those with a large energy yield are endo-enzymes. Lactase with twenty-three calories is sometimes soluble; sometimes, it is an endo-enzyme. Miquel's claim that urease is a soluble enzyme will be discussed on p. 36.

Attention should be called to the fact that the entire amount of liberated energy does not always become available for growth, for thermodynamic reasons. These will be discussed in the chapter on Growth. Ordinarily, in organic fermentations, the difference between available and total liberated energy is small; furthermore, only a small fraction of the available energy is used for growth, even under ideal conditions.

(*c*) METHODS OF MEASURING ENERGY YIELDS

We have various means of measuring the amount of energy liberated in a fermentation. It is possible to compute it as the difference of the heats of combustion of the fermented material and of the products. This computation is not very accurate since the error in combustion heat determinations becomes greatly increased if we have to consider the difference of two combustion heats of nearly the same magnitude. The inaccuracy is increased by the necessary corrections for the heats of solutions of products and for gases escaping. A good compilation of combustion heats is given by Kharasch (1929).

This method may be illustrated by the following example:

The combustion heat of glucose is 3,739 calories per gram; if a mold in a very well ventilated culture oxidizes glucose directly to carbon dioxide and water, the total amount of energy liberated amounts to 677.2 cal. per gram molecule. If oxygen is not available, yeast could change the sugar to alcohol $+CO_2$ according to the equation

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

There is a considerable amount of potential energy left in the two molecules of alcohol formed, and the yeast can liberate only the

difference between the potential energies (or combustion heats) of sugar and alcohol.

1 gm. molecule of glucose = 677.2 cal.
2 gm. molecules of alcohol = 651.2 cal.
26.0 cal.

The difference of 26.0 cal. is all the energy liberated in the alcoholic fermentation. It is only one-twenty-sixth of the amount obtained by oxidation. This means that for the production of 3,739 cal., the mold will need only 1 gm. of sugar if plenty of oxygen is available, but that 26 gm. will be needed by yeast if no oxygen is available. This is one of the reasons for the surprisingly great rate of decomposition of food by microorganisms.

The equation of the alcoholic fermentation should be written more completely and more correctly:

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2 + 26.0 \text{ calories}$$

But even that would not be really correct. If sugar is dissolved in water, the temperature decreases, and 2.25 cal. are disappearing for every gram molecule; if alcohol dissolves in water, heat is liberated to the extent of 2.54 cal. per gram molecule. These corrections should also be included in the above equation.

Tangl and Rubner tried to get more accurate data by measuring the amounts of energy liberated in the cultures themselves. Tangl (1903) used the combustion heat method, as shown in Table 6. A broth culture was dried on standard cellulose blocks and its combustion heat determined directly. Part of this culture was filtered and treated in the same way. The difference of solids gives the solids of the bacteria grown in this culture; the difference of combustion heats gives the potential energy or combustion heat of these bacteria. The potential energy of the sterile medium is 4.39 cal. for each gram of solids, which is very nearly that of the bacteria themselves. In spite of this similarity in com-

TABLE 6.—CALORIES AND SOLIDS IN 100 C.C. OF 20 DAY OLD CULTURES

	Series III (20 days)						Series IV (18 days)			
	B. anthracis		*B. subtilis*		*Bact. suipestifer*		*B. anthracis*		*B. subtilis*	
	Solids	Calories	Solids	Calories	Solids	Calories	Solids	Calories	Solids	Calories
Sterile broth	3.17	13.90	3.17	13.90	3.17	13.90	2.83	12.21	2.83	12.21
Total culture	2.99	12.75	2.53	11.13	2.56	10.88	2.79	11.94	2.42	10.19
Porcelain filtrate	2.73	11.65	2.18	9.51	2.30	9.58	2.74	11.69	2.36	9.31
In bacteria cells	0.26	1.10	0.35	1.62	0.26	1.30	0.05	0.25	0.06	0.88
1 gm. bacteria solids contains	....	4.25	....	4.65	....	4.05	....	4.9	....	14.7*
1 gm. bacteria solids required for production	....	4.4	....	11.9	....	7.9	....	5.6	....	33.7*

* Tangl believes the last two numbers, 14.7 and 33.7, to be wrong. Perhaps they might have been caused by a much larger surface of the culture, or by spores with much fat formed in this culture, but not in the twenty day old culture of series III.

bustion heats, all experiments show that the energy actually required was in every experiment at least as large again as the potential energy of the cells produced. Why so much energy is needed, will be explained on p. 32.

Rubner in his extensive experiments on the energy requirements of microorganisms used, occasionally, the

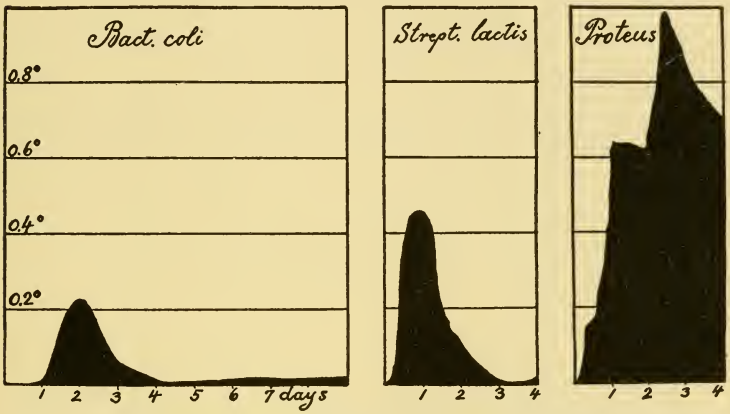


FIG. 1.—Daily temperature increases in cultures of *Bact. coli* (6% peptone solution), *Strept. lactis* (milk) and *Bact. vulgare* (6% meat extract solution).

same method employed by Tangl, but in most cases considered the direct method of temperature measurement to be more accurate. By keeping large amounts of the culture, usually 250 c.c., in a thermos bottle which was placed in a carefully controlled incubator, the temperature increases could be read accurately by means of a Beckmann thermometer. The specific heat of the medium and the heat conductivity of the system were determined and this permitted the thermometer readings to be computed into calories.

Some typical curves are given in Fig. 1, representing the lactic fermentation in milk, the growth of *Bact.*

coli in 6% peptone solution, and the decomposition of meat extract by *Bact. vulgare*, taken from Rubner (1906b and c).

The results obtained by this method show a fairly good agreement. The average of twelve determinations of the alcoholic fermentation

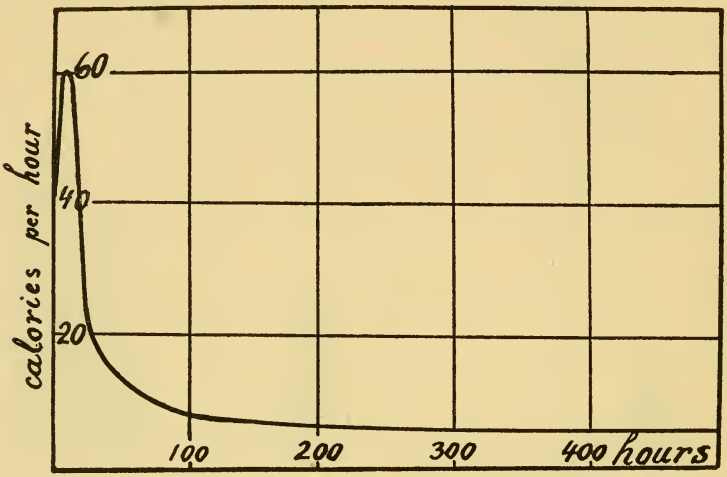


FIG. 2.—Rate of heat formation in 1 liter of soil.

at different temperatures, with sucrose or maltose, with large and small sugar concentrations, with large and small amounts of yeasts, gave the heat liberated from 1 gm. of sugar decomposed by yeast to 149.5 cal., the largest deviations being +2.4% and −2.81% (Rubner 1904b). This energy corresponds to 26.9 large calories per gram molecule.

Van Suchtelen (1923, 1927, 1929) improved this method by measuring the temperatures thermo-electrically, and by reducing heat conduction. His primary object was the determination of the availability of humus and the rate of its deterioration. He used about 1 liter of soil for each experiment, and observed for the first four days a rapid increase in temperature, due to the excess of oxygen introduced by the stirring of the soil (see Fig. 2). After twenty days, the temperature had come down to a constant, but was still slightly above the outside temperature, and stayed about 0.3 to 0.6°C.

above normal through the entire duration of the experiment which was continued for fifteen more days. This meant about 5 or 6 gm.-calories per hour and per liter of soil. At this rate, about 4% of the total humus would be decomposed during the first year.

A similar method, has been applied by Bayne-Jones and Rhees (1929) to study the energy production by *Bact. coli* during the first hours of growth. The cells were counted every hour, and this, together with the calorimetric measurements, gives the best data available for direct energy measurements. Some of the data are reproduced in Table 7.

The energy per cell, either calculated by dividing the total calories produced by the average of the acting cells, or by applying the formula of Buchanan (see Appendix p. 407) shows a sharp maximum during the first hours, and soon drops to a low and fairly constant level similar to that found by van Suchtelen (Fig. 2). Bayne-Jones and Rhees believe that this is characteristic for young cells. It may be, however, that the great amount of heat at the start is due to an oxidation process; after a few hours, the oxygen dissolved

TABLE 7.—CALORIES PRODUCED PER CELL PER HOUR

Time	Calories produced in the medium		Cells per flask	Calories per cell per hour by the formula	
	Total	Increase per hour			
Hours	S	ΔS	a, b_1 and b_2	$\frac{2\Delta S^1}{\Delta t(b_2 + b_1)}$	$\frac{2.303\Delta S\left(\log \frac{b_2}{b_1}\right)}{\Delta t(b_2 - b_1)}$
0	0	0	360×10^6		
1	1.485	1.485	420×10^6	3.81×10^{-9}	3.72×10^{-9}
2	17.68	16.20	$2{,}160 \times 10^6$	12.56×10^{-9}	15.25×10^{-9}
3	50.29	32.61	$9{,}600 \times 10^6$	5.55×10^{-9}	6.54×10^{-9}
4	76.65	26.36	$33{,}000 \times 10^6$	1.22×10^{-9}	1.36×10^{-9}
5	92.73	16.08	$96{,}000 \times 10^6$	0.25×10^{-9}	0.27×10^{-9}
6	108.60	15.87	$129{,}000 \times 10^6$	0.14×10^{-9}	0.14×10^{-9}

in the medium will have been used up, and no more oxidation takes place except on the very surface of the culture. According to A. Müller (1912), multiplying cells of *Bact. coli* require from 0.10

to $0.16 \cdot 10^{-9}$ mg. of oxygen per cell and hour, in a very poor medium (see p. 188). Figuring on 20,000,000 cells per c.c. as the average during the first three hours of the experiments of Table 7, the amount of oxygen consumed by these cells per hour would be $20 \times 10^6 \times 0.1 \times 10^{-9}$ mg. $= 2 \times 10^{-3}$ mg. per c.c., or 2 mg. per liter. Since at 37°, water can dissolve only 6.8 mg. of oxygen, it seems quite probable that in about two to three hours, the oxygen of the medium is all used up, and energy can be produced subsequently only by anaerobic processes which yield much less energy (see p. 27).

(*d*) THE NEED FOR ENERGY

It seems justifiable to question why microorganisms need energy for growth. Yeast cells are made from the malt protein and eventually from sugar, and the combustion heat of these foods is about the same as that of the new cells. The same is evident in the examples of Tangl's experiments given in Table 6 p. 28.

Since the cells are not built directly from the peptone or protein, these materials must be first reduced to much smaller units, amino-acids or, sometimes, even smaller molecules. The architecture of yeast protoplasm is quite different from that of barley protoplasm, and the architecture of protoplasm of *B. anthracis* is widely different from that of a commercial peptone. The breaking down will produce some energy, but this small amount is not nearly sufficient to weld the small particles into the new structure. Extra energy must be added to bring about new construction. This accounts for some of the energy wasted by bacteria, as well as by all other organisms. However, it does not account for *all* the waste.

After growth has ceased, the cells continue for a considerable period to liberate energy. Meyerhof (1914) has shown that life, as such, is not based upon a higher potential energy in the living cells. He killed a thick

suspension of living red blood corpuscles instantaneously by adding acrolein, and observed no change of temperature.

Nevertheless, life processes depend upon a source of energy. Blood corpuscles do not multiply *in vitro*, but show distinct respiration. The energy thus produced must be used for some purpose because all vital processes come to a standstill if oxygen, the source of energy, is excluded.

Pfeffer (Pflanzenphysiologie II, 885) assumed that the cell is like a heated engine, using up coal all the time in order to be ready to supply energy the moment it is wanted. It seems probable, however, that the cell is more nearly comparable to an electric engine than to a heat-propelled machine. This would make the simile less appropriate. More probable seems the assumption that energy is needed continuously to prevent, or make up for, certain chemical processes which would destroy the living protoplasm. Warburg (1912) believes that this destruction is due largely to diffusion processes while Meyerhof (1914) considers chemical processes more probable.

Meyerhof formulates his theory briefly as follows: For life functions, growth and cell activity, a mixture of substances within the cell must be prevented from ever reaching their physical and chemical equilibrium, and this cannot be done by merely slowing up the rate of reaction; it requires cyclic changes to re-establish again and again the continuously destroyed energy potentials.

While we know very little about these processes, attention should be called to the reduction-potential of living cells, which is discussed in detail on p. 82. The maintenance of this potential requires a considerable amount of energy. Since potentials of this type in the cell are continuously destroyed, all the energy used for their

maintenance will ultimately be transformed into heat, and this becomes evident by a temperature increase of the medium.

In most fermentations, our customary methods of cultivation show no increase of temperature because the heat of fermentation is conducted and radiated away almost as fast as it is produced. Even if we use thermos bottles, the increase of temperature rarely exceeds 2°C. (see Figs. 1 and 2). Exceptions are only the oxidative fermentations. In these, the temperature can rise very high if the heat has a chance to accumulate. The heat produced from the oxidation of alcohol by vinegar bacteria is sufficient to keep the temperature in the vinegar generators high, therefore care must be taken to control the oxygen supply lest the temperature rise so high as to kill all bacteria. This would correspond, biologically speaking, to death by fever. Such temperature increases due to bacteria seem especially high when we consider that less than 0.1% of the liquid is bacteria bodies.

Some bacteria produce light. This is another form in which energy leaves the bacterial cell. The amounts of energy are very small when compared with the amounts that leave the cells in the form of heat. Ultraviolet radiation is claimed by Baron (1928) for several yeasts and bacteria.

The energy required by bacteria to move through a liquid has been calculated by von Angerer (1919). He considers his estimates, based on Stokes formula, only as very rough approximations which give merely the order of magnitude. The calories required for one hour's continuous swimming are calculated for one cell of *V. cholerae* to be 216×10^{-16} cal., for *Bact. typhosum* 10×10^{-16} cal., for *Bacillus subtilis* 25×10^{-16} cal. The total energy liberated by one cell in one hour (calculating from lactic acid fermentation) is of the order of 10^{-10} calories. The energy required for motion is of the order of 10^{-14} cal., and is negligible in comparison with the total energy liberated.

(*e*) SUMMARY

The potential energy (combustion heat) of yeast and bacteria cells is frequently similar to that of the food from which they are made. Nevertheless, a considerable amount of energy is required to produce these cells from food.

Fermentation continues, and energy is liberated, after growth has ceased. The surplus energy thus produced appears largely in the form of heat. Motility and production of light do not require measurable amounts of energy.

The surplus energy which appears ultimately in the form of heat should not be considered as useless waste. It may have served a purpose in the cell before appearing as heat. Probably, energy potentials of some kind must be maintained in the living cell to prevent detrimental reactions. The continuous equalization of these potentials would result in the ultimate transformation of the utilized energy into heat.

II. CHEMICAL CONSIDERATIONS

(*a*) ENZYMES OF FERMENTATION

It has been proved in at least four different cases that fermentation is caused by enzymes. These examples are the urea fermentation, the alcoholic fermentation, the lactic fermentation and the vinegar fermentation. It is assumed by most bacteriologists that all fermentations are due to enzymes, though it seems to be extremely difficult to bring about the experimental proof.

The statement that fermentation is caused by enzymes, means primarily only the achievement of a mechanical separation of the cause of fermentation from the living cell. Even under the most favorable circumstances, this separation has been very incomplete. The cell juice pressed out from yeast is quite weak in fermenting power as compared with a corresponding volume of living cells. The significance of the separation first accomplished by Buchner (1897) lies in the proof that the energy-yielding processes are mere chemical processes.

They are brought about by a chemical compound (zymase), which is produced by the cell and which, after once having been produced, acts independently of the cell. The cell may control to a certain extent the *amount* of enzyme produced, but the *action* of the enzyme once produced is beyond the control of the cell. The enzyme follows always the laws of enzyme action, even if it should be against the vital interests of the cell. The fermentation of sugar to alcohol and carbon dioxide will continue for some time after the multiplication of yeast cells has ceased; there is already too much alcohol present to allow growth, and very little energy is needed by the resting cells, yet the enzyme is present, and it continues to act until it is checked by the maximum amount of alcohol, or by the disappearance of the sugar. This is against the best interests of the cell (as we understand them), but is in accordance with the laws of enzyme action. Another example is the excessive heat production by vinegar bacteria mentioned before.

(*b*) THE KNOWN ZYMASES OR FERMENTING ENZYMES

Urease.—The oldest known zymase is doubtless the urease discovered in 1876 by Musculus who noticed it in the slimy urine of some patients and believed the enzyme to be of human origin. Miquel (1904) summarizes his own earlier experiments in the statement that cultures of urea bacteria can be brought to the point where they decompose in one hour 100–120 gm. of urea per liter of culture.

Such cultures can be filtered through porcelain filters without much loss of urease if large amounts of culture are used. From this, it would seem that the urease were no endoenzyme. This is very improbable however because the cells would derive no benefit from urease outside of the cell. It is more probable that a large number of

cells are dead in a fairly old culture (at least three days at 30–35°C.) and that the enzyme which is quite stable leaves the dead cells, but not the living ones.

The proof that this fermentation can be used as the only source of vital energy by the urea bacteria, has been given by Söhngen (1909).

Alcoholase.—Much more interest was displayed when Buchner (1897) finally proved that alcoholic fermentation could be separated from the living yeast cell. The old famous dispute between Liebig and Pasteur had not been forgotten and the discovery of the yeast-zymase, or alcoholase, was a great step forward in biochemistry.

Buchner obtained the zymase by tearing the cell walls of the yeast cells through grinding with quartz sand, and by pressing the ground yeast + quartz under high pressures. Thus he obtained the cell sap of the yeast, and this had retained the power of producing alcoholic fermentation. Even after filtration through porcelain, it was still able to bring about the formation of alcohol and carbon dioxide from sugar.

Later, another method was used more commonly. The yeast was treated with alcohol and ether, or with acetone; this killed the cells, i.e. it destroyed definitely the power of yeast to grow and multiply, but not the power to ferment.

Lactacidase.—After the establishment of the enzymatic nature of the alcoholic fermentation, it was natural that the same technique was applied to other fermentations as well. Herzog (1903) was the first to grind the cells of *Bact. acidi lactici* Hueppe, and to obtain a cell sap which would change lactose to lactic acid. This was confirmed later by Buchner and Meisenheimer (1903) who worked with *Lact. Delbrücki.*

Alcohol-oxydase.—The efforts of Buchner and Meisenheimer (1903) to obtain from ground vinegar bacteria an enzyme which would oxidize alcohol to acetic acid were without success. Treatment of vinegar bacteria with acetone gave, however, dead cells which would convert alcohol to acetic acid to a small extent. This was confirmed later by Rothenbach and Eberlein (1905). Buchner and Gaunt (1906) gave more detail regarding this enzyme. The enzyme nature of this fermentation seems to be established, but the enzyme itself appears to be even more sensitive than the alcoholase.

(*c*) COMPLEXITY OF ZYMASE ACTION

The enzyme content of the yeast cell is not limited to the alcoholase. Almost simultaneously with the alcoholase, the endotryptase was discovered, a proteolytic enzyme which can liquefy gelatin. This proves its nature as endo-enzyme since living yeast cells of the Saccharomyces type do not attack gelatin. This enzyme gradually destroys the alcoholase, especially in the absence of sugar.

The alcoholase requires special conditions for its action. An essential factor is the presence of phosphate. Ordinarily, the cell juice of the yeast contains enough phosphate to bring about a visible fermentation, but the addition of phosphate often accelerates the reaction greatly and increases the total production of CO_2. It is generally assumed that the alcoholase does not act upon the glucose molecule directly, but upon the hexose-phosphate, a phosphoric acid ester of the sugar. Probably, an enzyme of the yeast brings about the esterification.

Another essential substance is the so-called *co-enzyme* of alcoholic fermentation. It was observed that heated

yeast juice increases the rate of fermentation of a normal yeast juice. Normal yeast juice can be separated by ultrafiltration, or by dialysis, into two fractions neither of which can cause the fermentation, while, combined, they again have nearly their original fermenting capacity. The nature of this co-enzyme has not been agreed upon. Kluyver and Struyk (1927) believe that different experimenters meant entirely different phenomena when speaking of co-enzyme effects. These authors think it to be a hydrogen acceptor which is necessary to start the first production of acet aldehyde. After this has been done, the fermentation can go on automatically as will be shown on p. 49. Nilsson (1930) believes the co-enzyme enters into various phases of the fermentation process. Thus we have 4 factors connected in the alcoholic fermentation.

1. Alcoholase
2. Phosphate-carbohydrate esters
3. Co-enzyme
4. Endotryptase

The latter causes a deterioration of the alcoholase as well as of the co-enzyme. Heat will destroy alcoholase and endo-tryptase, but not the co-enzyme. To complicate matters still more, the endotryptase is inhibited to some degree by the presence in the yeast juice of another compound:

5. Antiprotease

and this compound No. 5 is destroyed by compound No. 6

6. Lipase

which also attacks the co-enzyme.

The actual amount of alcohol and CO_2 formed by the enzyme is very small compared with that of the yeast itself. Harden (1923 p. 30) estimates that the living yeast produces about forty times as much alcohol as the

equivalent of cell juice. The acetone yeast is more active, and yields about one-eighth of the amount produced by an equivalent amount of living yeast cells.

For this reason, the reality of a fermenting enzyme is, occasionally, questioned again. Rubner (1913) could obtain no appreciable fermentation by treating living yeast in sugar solution with toluol. He believes that only a small portion of the enzyme is separated from the living protoplasm while most of it is interlinked with protoplasm and is inactivated by the death of the cell. Euler and his associates have proved this in a number of publications.

The essential thing, from the energy viewpoint, is the endo-enzymatic nature of the fermenting agent; it must work within the cell in order to make the liberated energy available for cell purposes. It is doubtless a complex mechanism which brings about this change of sugar to alcohol and carbon dioxide. Alcoholic fermentation occurs in several steps, with intermediate products, some of which have been actually isolated (see p. 49). Whether fermentation is separable from the protoplasm or not, has no great bearing upon the problems treated in this volume. A further discussion of the enzymatic nature of fermentations will be found on p. 64.

(*d*) EQUATIONS OF FERMENTATION

If fermentation is nothing but a chemical process activated by the influence of an enzyme, it is necessary that it obey strictly chemical laws, and that we may represent the process accurately by a chemical equation. A considerable amount of effort has been made to prove this for a number of fermentations. Most attention has been given to the alcoholic fermentation; the lactic and propionic fermentations and the acid-

gas fermentations of the colon group are also well studied. Data upon other sugar fermentations are rather incomplete.

One single equation to cover the entire action of any species of bacterium upon the entire available food is evidently impossible. It is even impossible to cover the decomposition of just the sugar by yeast in one formula because Pasteur found that about 5% of the sugar is used for the construction of new yeast cells, and we could not possibly write this in a chemical equation.

It seemed possible for a while to describe, in one equation, that part of sugar dissimilation which is not used for constructive purposes, but even that is inaccurate and incomplete, as a number of old and new experiments have shown. Most organisms have the ability to decompose sugar in more than one way, and this is due to the fact that all sugar fermentations are the result of a number of interlinking reactions. By a change of environment, the one or other of these reactions might be prevented, or changed, and the result is a different product, or a different proportion of the products obtained. Besides, interreactions between sugar and nitrogenous material are always possible, so that any equation that does not consider the nitrogenous material may not show the real facts (see p. 62).

A type of fermentation may be described by one general equation with the reservation that the formula holds true only for the conditions of the one experiment, and that even then, it accounts only for about 90% of the sugar decomposed. If a more accurate understanding of the process is wanted, the different successive stages of the change from sugar to the final product must be worked out, and their interrelations must be studied.

The general formula for the alcoholic fermentation is

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

Pasteur hesitated to accept this formula because of other compounds found regularly among the fermentation products which were not given by the above equation. The following list gives the approximate amounts of products found in normal industrial alcoholic fermentations:

TABLE 8.—PRODUCTS OF ALCOHOLIC FERMENTATION FROM 100 PARTS OF SUGAR

Alcohol	48.4 parts		
CO_2	46.5		
Acetic acid	0.05–0.25	isobutyl glycol	−0.158*
Lactic acid	0. −0.2	amyl alcohol	−0.051
Fusel oil	0.05–0.35%	n-propyl alcohol	−0.002
Succinic acid	0.5 −0.7	isobutyl alcohol	−0.0015
Glycerol	2.5 −3.6		
Acetaldehyde	0 −0.08		
Furfural	traces		
Yeast cells	1.2 −5		

Boussignault had the correct conception that in order to establish equations of fermentation processes, it is essential to find out which products are always formed in the same ratio, because only these could derive from the same type of decomposition.

It was shown much later by Ehrlich that some of these products, though nitrogen-free, derive from protein material. He found (1905) that leucin was the mother substance of iso-amyl alcohol, and that iso-leucin could be converted into optically active amyl alcohol. This was accomplished by a hydrolysis of the amino acid liberating NH_3, and by a decarboxylation of the resulting α-hydroxy acid.

* According to Duclaux III, 434 (1900). The amount of by-products is smaller with yeast juice than with living yeast.

$$(CH_3)_2{\cdot}CH{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H + H_2O \rightarrow$$

Leucin

$$+NH_3$$
$$(CH_3)_2CH{\cdot}CH_2{\cdot}CH(OH){\cdot}CO_2H$$
$$\downarrow$$
$$(CH_3)_2{\cdot}CH{\cdot}CH_2CH_2OH + CO_2$$

Iso-amyl alcohol

$$\begin{matrix} CH_3 \\ C_2H_5 \end{matrix}\!\!>\!\overset{*}{C}H{\cdot}CH(NH_2)CO_2H + H_2O \rightarrow$$

Iso-leucin

$$\begin{matrix} CH_3 \\ C_2H_5 \end{matrix}\!\!>\!\overset{*}{C}H{\cdot}CH(OH){\cdot}CO_2H + NH_3$$
$$\downarrow$$
$$\begin{matrix} CH_3 \\ C_2H_5 \end{matrix}\!\!>\!\overset{*}{C}H{\cdot}CH_2OH + CO_2$$

Optically active amyl alcohol

Ehrlich proved this theory by demonstrating that an increase of leucin or isoleucin in the medium caused an increase of fusel oil, while asparagin or ammonium carbonate did not cause such increase.

In a similar way, Ehrlich (1909) showed that succinic acid is a protein derivative. It is not a reduction product from aspartic acid, however, but derives from glutamic acid by de-amidization and oxidation.

$$\begin{matrix} CH_2{-}CO_2H \\ | \\ CH_2 \\ | \\ CH{-}CO_2H \\ | \\ NH_2 \end{matrix} + H_2O \rightarrow \begin{matrix} CH_2CO_2H \\ | \\ CH_2 \\ | \\ CH{-}CO_2H \\ | \\ OH \end{matrix} + NH_3 \qquad + O_2 \rightarrow$$

Glutamic acid — Glutaric acid

$$\begin{matrix} CH_2{-}CO_2H \\ | \\ CH_2 \\ | \\ CO_2H \end{matrix} + CO_2$$

Succinic acid

Other α-amino acids also are changed to alcohols, e.g. tyrosin $OH{\cdot}C_6H_4{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$ gives $OH{\cdot}C_6H_4{\cdot}CH_2CH_2OH$, tyrosol, a bitter tasting compound, probably the cause of the bitter taste of certain beers (Ehrlich, 1911). Tryptophane is changed to tryptophol by the same process, and these and similar compounds or their esters may be one of the causes of the differences in the taste of different wines and beers.

After this evidence that some of the products of yeast fermentation are of protein origin, the products derived from sugar can be traced with more certainty.

(*e*) SUCCESSIVE STAGES IN THE FERMENTATIONS OF SUGARS

The first great advance in the study of intermediate products and successive steps in fermentation was Neuberg's work on the fermentation of pyruvic acid by yeast (Neuberg and Hildesheimer, 1911) and his further developments from this starting point. The English biochemists with Harden studied, in great detail, the Colon fermentations as well as those by yeast. At the same time, Warburg concentrated on the chemistry of the respiration process, and Wieland developed his theory of the activated hydrogen as the essential part in all organic oxidations as well as reductions. Oxygen is a "hydrogen acceptor," but in many cases it can be substituted by other hydrogen acceptors, such as nitrate or methylene blue. Warburg had found that oxygen can oxidize only if "activated," and the activator in biological oxidations is iron. A combination of Warburg's and Wieland's theories proved possible; Kluyver and Donker (1926) have applied this combined theory to fermentations and respiration in general, and the discussions of fermentations offered here follows closely their interpretation.

As an illustration of Wieland's mode of presentation, the Cannizzaro reaction between methyl glyoxal and

acetaldehyde shall be shown here because it is part of the mechanism of alcoholic fermentation. Methyl glyoxal is written in the hydrated form; the activated H-atoms are shown in heavy type. This compound is the "hydrogen donator" giving two H-atoms to the acetaldehyde which is the "hydrogen acceptor."

$$\underset{\text{Methyl glyoxal hydrate}}{CH_3\cdot CO\cdot C(H)(OH)(OH)} - \mathbf{H_2} + \underset{\text{Acet aldehyde}}{CH_3\cdot CHO} + 2\mathbf{H} =$$

$$\underset{\text{Pyruvic acid}}{CH_3\cdot CO\cdot COOH} + \underset{\text{Alcohol}}{CH_3\cdot CH_2OH}$$

This is an example of the "oxido-reductions," the reduction of one molecule by the oxidation of another, which is the essential part of all fermentations.

By this emphasis on the hydrogen atoms as the essential parts of oxidation and reduction processes, the reactions which we commonly call oxidations would become more precisely "dehydrogenations," and the term dehydrogenase in place of oxidase is used by some biochemists.

The first step in the fermentation of sugar appears to be the formation of hexose-phosphate, an ester of the sugar with phosphoric acid. This esterification is probably brought about by an enzyme.

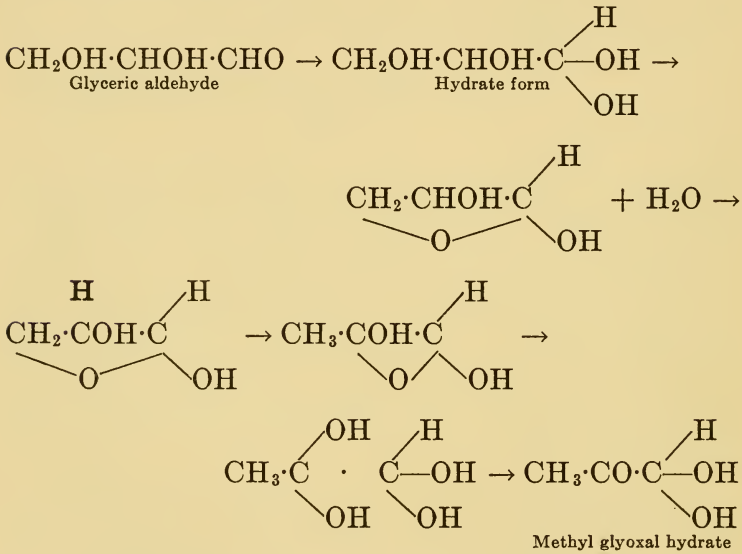
The glucose-phosphate is then split in the middle of the carbon chain, yielding two molecules of glyceric aldehyde, which, according to Kluyver and Struyk (1926), takes place in two steps:

$$C_6H_{12}O_6 + HK_2PO_4 = C_6H_{11}O_5(K_2PO_4) + H_2O$$

$$C_6H_{11}O_5(K_2PO_4) = \underset{\text{Glyceric aldehyde}}{C_3H_6O_3} + C_3H_5O_2(K_2PO_4)$$

$$C_3H_5O_2(K_2PO_4) + H_2O = \underset{\text{Glyceric aldehyde}}{C_3H_6O_3} + HK_2PO_4$$

The glyceric aldehyde, by activation of a central H-atom which then goes to the end-carbon, is changed to methyl glyoxal. This process is an oxido-reduction, just as the splitting of the sugar molecule into glyceric aldehyde mentioned above must also be considered as a process of this type.



Up to this step, to the formation of methyl glyoxal, all glucose fermentations seem to be alike. The next steps decide about the different types, and methyl glyoxal can be decomposed in a number of different ways. But the number of possibilities is limited, and Kluyver and Donker show that a few main types of oxido-reductions of methyl glyoxal and its derivatives can account for most of the well known fermentations. The same reactions are used by different organisms in

different combinations, and the number of different reactions is quite small. The following list of basic reactions is given by Kluyver and Donker:

BASIC REACTIONS IN SUGAR FERMENTATIONS

I. CHANGES OF METHYL GLYOXAL HYDRATE

Ia. $CH_3\cdot CO\cdot C(OH)(OH)H$ (Methyl glyoxal) $= CH_3\cdot CO\cdot C(OH)(O{-}H)H =$

$CH_3\cdot CHOH\cdot COOH$ (Lactic acid)

Ib. $CH_3\cdot CO\cdot C(OH)(OH)H = CH_3\cdot CO\cdot C(OH)(O{-}H)H =$

$CH_3\cdot CHO$ (Acet aldehyde) $+ HCOOH$ (Formic acid)

Ic. $CH_3\cdot CO\cdot C(OH)(OH)H = CH_3\cdot CO\cdot C(OH)(O{-}H)H =$

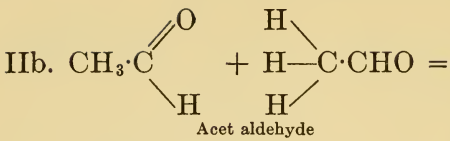
$CH_3\cdot CO\cdot COOH$ (Pyruvic acid) $+ 2H =$ Acceptor

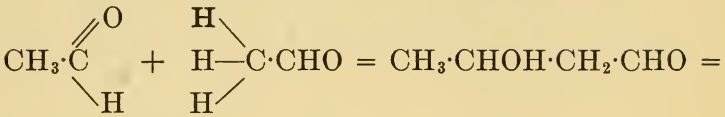
$= CH_3\cdot CHO$ (Acet aldehyde) $+ CO_2$

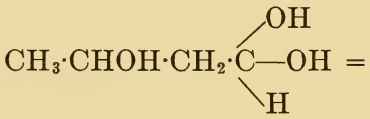
II. CONDENSATIONS

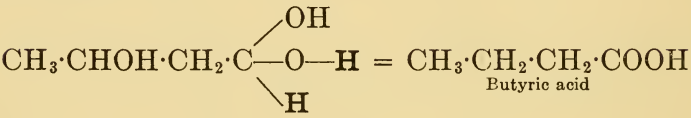
IIa. $CH_3\cdot C(OH)(OH)H$ (Acet aldehyde) $+ HC(=O)\cdot CH_3$ (Acet aldehyde) $=$

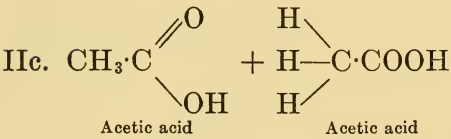
$CH_3\cdot C(OH)(OH)H + HC(=O)\cdot CH_3 = CH_3\cdot CO\cdot CHOH\cdot CH_3$ (Acetyl methyl carbinol)

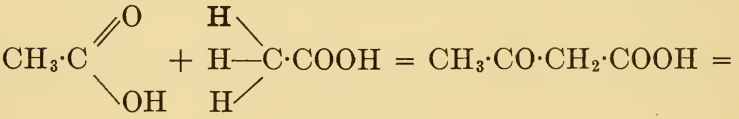
IIb. 

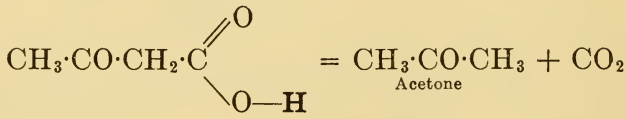






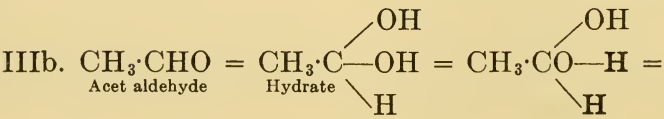
IIc. 





III. DEHYDROGENATIONS

IIIa. $HCOOH = \mathbf{H}COOH = CO_2 + 2\mathbf{H}$

IIIb. 

$$CH_3{\cdot}COOH + 2H = \text{Acceptor}$$
Acetic acid

IIIc. $CH_3{\cdot}CO{\cdot}C(OH)H = CH_3{\cdot}CO{\cdot}CO{-}H =$

$$CH_3{\cdot}CO{\cdot}COOH + 2H = \text{Acceptor}$$

(same as reaction Ic.)

IV. HYDROGENATIONS

IVa. $2H = H_2$
Hydrogen gas

IVb. $2H + O = H_2O$

IVc. $CH_2OH{\cdot}CHOH{\cdot}CHO + 2H = CH_2OH{\cdot}CHOH{\cdot}CH_2OH$
Glyceric aldehyde — Glycerol

IVd. $CH_3{\cdot}CHOH{\cdot}COOH + 2H = CH_3{\cdot}CH_2{\cdot}COOH$
Lactic acid — Propionic acid

IVe. $CH_3{\cdot}CHO + 2H = CH_3CH_2OH$
Acet aldehyde — Ethyl alcohol

IVf. $CH_3{\cdot}CO{\cdot}CHOH{\cdot}CH_3 + 2H = CH_3{\cdot}CHOH{\cdot}CHOH{\cdot}CH_3$
Acetyl methyl carbinol — 2, 3, Butylene glycol

IVg. $CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}COOH + 4H = CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2OH$
Butyric acid — Butyl alcohol

IVh. $C_6H_{12}O_6 + 2H = C_6H_{14}O_6$
Fructose — Mannitol

IV. etc.

(*f*) TYPES OF FERMENTATION

The alcoholic fermentation starts from glucose-phosphate over glyceric aldehyde to methyl glyoxal as outlined above. The next step is equation IIIc and IVe

IIIc. $CH_3 \cdot CO \cdot C(H)(OH)(OH) = \underset{\text{Pyruvic acid}}{CH_3 \cdot CO \cdot COOH} + 2H$

Methyl glyoxal hydrate

IVe. $\underset{\text{Acet aldehyde}}{CH_3 \cdot CHO} + 2H = \underset{\text{Alcohol}}{CH_3CH_2OH}$

The combination of these two equations has been given on p. 45 as an example of a typical oxido-reduction. The pyruvic acid which comes from methyl glyoxal, is changed to acet aldehyde according to equation Ic

Ic: $CH_3CO \cdot COOH = CH_3 \cdot CHO + CO_2$

The acet aldehyde thus formed can react again as acceptor for the hydrogen from a new methyl glyoxal molecule and the final sum of these reactions is the sum formula of the alcoholic fermentation. Only at the very start, a certain amount of acet aldehyde, or other hydrogen acceptor, must be present to bring about the first pyruvic acid molecules.

There are other possibilities hidden in the yeast mechanism. Small amounts of glycerol are formed, and this is due to the glyceric aldehyde acting as hydrogen acceptor in place of the acet aldehyde:

Ic: $\underset{\text{Methyl glyoxal}}{CH_3 \cdot CO \cdot C(H)(OH)(OH)} - 2H = \underset{\text{Pyruvic acid}}{CH_3 \cdot CO \cdot COOH}$

IVc: $\underset{\text{Glyceric aldehyde}}{CH_2OH \cdot CHOH \cdot CHO} + 2H =$

$\underset{\text{Glycerol}}{CH_2OH \cdot CHOH \cdot CH_2OH}$

While acet aldehyde seems to be more efficient as hydrogen acceptor, glycerol will be formed in large

amounts when the acet aldehyde is "trapped," i.e., combined with $NaHSO_3$ to a compound which does not give the above reaction. This process has been used commercially to manufacture glycerol from sugar. The general equation in this case is

$$C_6H_{12}O_6 = C_3H_8O_3 + CH_3{\cdot}CHO + CO_2.$$

There is still another possibility of reactions hidden in the yeast mechanism: acet aldehyde might act as a hydrogen donator as well as acceptor; the result is the formation of acetic acid:

IIIb: $CH_3{\cdot}C(H)(OH)(OH) - 2H = CH_3{\cdot}COOH$

Acet aldehyde hydrate — Acetic acid

IVe: $CH_3{\cdot}CHO + 2H = CH_3{\cdot}CH_2OH$

This process takes place in alkaline media. Under this condition, the above combination seems to be favored which is really nothing but a Cannizzaro reaction between two acet aldehyde molecules. This leaves no acet aldehyde to act as acceptor for the hydrogen from methyl glyoxal, and, as before, glyceric aldehyde takes its place forming glycerol. The general equation of the alkaline yeast fermentation is:

$$2C_6H_{12}O_6 + H_2O =$$

$$\underset{\text{Glycerol}}{2C_3H_8O_3} + \underset{\text{Acetic acid}}{CH_3COOH} + \underset{\text{Alcohol}}{C_2H_5OH} + 2CO_2$$

Neither the alkaline nor the sulfite fermentation of yeast follow the above formulas quantitatively because a certain amount of acet aldehyde will always react with methyl glyoxal before being trapped, and the result is a combination of two types of fermentation.

The Lactic Fermentation is essentially type Ia which is a typical intramolecular oxido-reduction.

Ia: $$\underset{\text{Methyl glyoxal hydrate}}{C_3H_6O_3} = \underset{\text{Lactic acid}}{C_3H_6O_3}$$

This describes the fermentation of about 90% of the sugar by Streptococci, and by some of the Lactobacilli. There are other Lactobacilli, however, which form also alcohol and CO_2. This means that these organisms have also the power (i.e. the enzymes) to bring about reactions Ib, IIIa, IVc and IVe producing formic acid as intermediate product which is decomposed further, liberating CO_2, ethyl alcohol and glycerol.

To which type of fermentation the volatile acids which are formed in relatively large amounts by certain groups of streptococci (Hammer, 1920, Hucker 1928) should be ascribed, cannot be stated until we know whether this fermentation is accompanied by corresponding amounts of glycerol and ethyl alcohol. The presence of propionic acid with some species suggests also the fermentation type IVd, while others producing formic acid apparently include type Ib.

The Propionic Acid Fermentation has been summarized by van Niel in a monograph (1928). Several possibilities are presented among which no experimental decision has as yet been possible. It may be that lactic is formed as such (Ia) and fermented further according to equation

IVd: $$\underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH} + 2H = \underset{\text{Propionic acid}}{CH_3{\cdot}CH_2{\cdot}COOH} + H_2O$$

where the hydrogen derives from a different mode of attack, perhaps like this:

$$CH_3{\cdot}CHOH{\cdot}COOH + H_2O = CH_3COOH + HCOOH + 2H$$

the formic acid acting also as hydrogen donator (IIIa) to reduce another lactic acid molecule. It is also possible that lactic acid is not formed as such from glucose in this fermentation, but that glyceric aldehyde or methyl glyoxal undergo an oxido-reduction directly. van Niel has made it quite probable that acet aldehyde is not an intermediary product in this fermentation.

The Colon-Typhoid Group has a much more varied way of attack. Lactic acid is always formed (Ia) and there is another process of fermentation going on at the same time, independently. This second process resembles that of the yeast fermentation to a certain extent, with acet aldehyde as an intermediary product. It starts with reaction Ib, followed up by IIIc and IVe

Ib: $\underset{\text{Methyl glyoxal}}{CH_3{\cdot}CO{\cdot}COH} = \underset{\text{Acet aldehyde}}{CH_3{\cdot}CHO} + \underset{\text{Formic acid}}{HCOOH}$

IIIc: $CH_3{\cdot}CHO + H_2O = CH_3{\cdot}COOH + 2H$

IVe: $CH_3{\cdot}CHO + 2H = CH_3{\cdot}CH_2OH$

This accounts for the formation of lactic acid, and without relation to this, for the formation of two molecules of formic acid to one each of acetic acid and ethyl alcohol. This would be the type of fermentation by *Bact. typhosum.*

Occasionally glyceric aldehyde acts as hydrogen acceptor and small amounts of glycerol are formed.

In the colon-group, reaction IIIa causes the formation of H_2 and CO_2 in equal volumes from formic acid.

Succinic acid is also formed in small amounts, and doubtless is derived from sugar (Ayers and Rupp, 1918). Kluyver and Donker believe that glucose which is not in the form of a phosphate ester might be split unevenly into a four carbon and a two carbon chain.

The origin of the extra amounts of CO_2 in fermentation by *Bact. aerogenes* (Rogers, Clark and Davis, 1914) is not so easily accounted for. From the larger total volume of gas produced, it does not seem that the increased CO_2:H_2 ratio of this group is due to a diversion of hydrogen; it appears more like an additional source of CO_2. An inter-reaction between methyl glyoxal and acet aldehyde might be assumed, as in the case of the alcoholic fermentation, but this would result in a larger amount of alcohol, for which evidence seems to be lacking.

Furthermore, this group shows the formation of acetylmethyl carbinol, probably according to reaction IIa, in the aerogenes group, and of butylene glycol (reaction IVf) in the colon group.

Acetone Fermentation by spore-forming rods of the type of *B. macerans* Schardinger, and *B. aceto-ethylicus* Northrop shows also a very varied list of products, adding acetone to all the products observed in the colon-groups. The types of reaction involved are the same as above (Ia, Ib, IIIb, IVe, IIIa) and in addition, we have IIc.

IIc: $CH_3COOH + CH_3COOH = CH_3{\cdot}CO{\cdot}CH_2{\cdot}COOH$
$= CH_3{\cdot}CO{\cdot}CH_3 + CO_2$

The subgroup of *B. asterosporus* and *B. polymyxa* Beijerinck forms butylene glycol besides alcohol, CO_2 and H_2. The origin of butylene glycol has already been mentioned in the colon-typhoid group as being due to reactions IIa and IVf while the rest of the products are mainly produced by reactions Ib and IIIa.

Butyric Acid Fermentation probably starts with reaction Ic, methylglyoxal giving acet aldehyde and formic acid. The acet aldehyde goes through the aldol stage and finally into butyric acid (reaction IIb)

IIb. $2CH_3CHO = C_4H_8O_2$

Part of the acet aldehyde reacts according to IIIb and IVa, producing acetic acid and hydrogen, or according to IIIc + IVe, giving ethyl alcohol besides acetic acid.

The formic acid from Ic is largely split according to IIIa. With some organisms, this hydrogen is liberated as such; others can activate it to reduce either butyric acid, or an intermediary stage of reaction IIb, to butyl alcohol; further acetic acid may be coupled according to reaction IIc to form acetone. It seems probable that a high hydrogen ion concentration is necessary to perform these latter changes, and this acidity seems to be prohibitive for the species forming butyric acid only.

Other Fermentations shall not be discussed here. It is beyond the scope of this book to describe all different types of fermentations. Such descriptions might be found in the book of Fulmer and Werkman (1929) or in vol. III of Buchanan and Fulmer's, "Physiology and Biochemistry of Bacteria." This chapter was meant to give a conception of how a fermentation takes place, and what an equation of fermentation does and does not mean.

Complete Oxidation of glucose is typical for certain groups of microorganisms, among which the Mycobacteria are perhaps the most outstanding. No intermediary products of any kind could be found by Merrill (1930) in a careful investigation.

In the oxidation of glucose by molds, intermediate steps are well-known, though the oxidation will finally become complete. Oxidation follows a hydration of the aldehyde group of the glucose molecule:

$$C_6H_{12}O_6 + H_2O = C_5H_{11}O_5 \cdot C\begin{matrix}\diagup H \\ \diagdown OH\end{matrix} =$$

$$C_5H_{11}O_5 \cdot COOH + 2H = \text{Acceptor}$$

A dehydrogenation follows, and the acceptor for this hydrogen is oxygen activated by the mold. The remaining gluconic acid can be hydrogenated further in various ways, and citric acid as well as oxalic acid are well-known intermediary products. The final result is CO_2 and H_2O.

Fermentations of Proteins.—The protein molecules are so large and so entirely unknown that it is entirely out of the question to present equations for the fermentation of proteins.

These large protein molecules can be attacked only by a limited number of bacteria. The common division of bacteria into those that liquify gelatin, and those incapable of doing this, is no reliable indication for their ability to attack protein molecules in general. While the specificity does not seem to be as strict as with carbohydrates, there is a certain amount of specialization the chemical foundation of which is not as yet known. (Rettger, Berman and Sturges, 1916, Berman and Rettger, 1918.)

The large protein molecule is broken up by those microorganisms which have the power to do so, into smaller molecules of similar nature which are still too large to be described by a chemical formula. These compounds, called albumoses, hemi-albumoses, peptones, peptids and various other names, are usually defined by the precipitants which will make them insoluble. A number of different compounds are used for this purpose, such as dilute acids, copper salts, lead salts, zinc salts, concentrated ammonium sulfate, tannic acid, phosphotungstic acid, etc.

The proportion of the total nitrogen contained in these fractions gives a general conception of the course of protein decomposition, and is of valuable information,

e.g., in the study of cheese ripening. In this chapter dealing with the equations of fermentation, they are of no special interest.

The next step in the degradation of proteins is the formation of amino-acids. In a very complete study of the decomposition of casein by *B. mesentericus vulgatus*, Grimmer and Wiemann (1921) found the following amino-acids: alanin, valin, leucin, tyrosin, aspartic acid, glutamic acid, prolin, arginin, lysin, histidin, tryptophane, and probably phenylalanin. Of the decomposition products of amino-acids, the following compounds could be identified: putrescin, cadaverin, tryptamin, tyrosol, ammonia, and probably p. oxybenzoic acid. In the presence of lactose, histamin and γ-aminobutyric acid were also formed. This is an example of what can be expected in an aerobic decomposition of a protein.

Fermentations of Amino Acids.—The further decomposition of these amino acids is something quite definite, chemically speaking, and the equations can be given. A good survey of all products derived from amino-acids is given by Stephenson (1930) in tabulated form.

Perhaps the most common type of decomposition of amino acids is the splitting off of NH_3. This can be accomplished in four different ways, namely by hydrolysis, by reduction, by oxidation and by leaving an unsaturated double bond (desaturation). The liberation of ammonia by hydrolysis has already been described in the formation of alcohols from amino acids by yeast, especially with the amyl alcohol (page 43). Leucin was hydrolyzed to α-hydroxy-caproic acid.

$$C_4H_9 \cdot CH(NH_2) \cdot CO_2H + H_2O = C_4H_9 \cdot CH(OH) \cdot CO_2H + NH_3$$

and then the hydroxy acid gave off CO_2 and thus became amyl alcohol.

$$C_4H_9{\cdot}CH(OH){\cdot}CO_2H = CO_2 + C_4H_9{\cdot}CH_2OH$$

In a similar way, yeast can hydrolyze other amino acids (see page 44).

The de-aminization by reduction will be mentioned on page 63 where the propionic acid bacteria reduced aspartic acid to succinic acid. This process is very probably not a source of energy.

The third possibility, the de-aminization by oxidation, has been used to explain the formation of succinic acid by yeast. It was shown on page 43 that yeast does not produce succinic acid from aspartic acid like the propionic bacteria do, but from glutamic acid. It is, of course, impossible to state whether the removal of NH_3 is accomplished by direct oxidation or by hydrolysis, immediately followed by oxidation; the result is the same.

This type of oxidation process is also found with fungi. Very probably it will yield energy.

The last possibility of deaminization, the splitting off of NH_3 leaving an unsaturated carbon chain, seems to have been observed only once, with histidine, though performed by a number of species of the colon-aerogenes group. Raistrick (1917) found the following decomposition of histidine:

$$\underset{\text{Histidine}}{(C_3H_3N_2){\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH} = \underset{\text{Urocanic acid}}{(C_3H_3N_2){\cdot}CH{:}CH{\cdot}COOH} + NH_3$$

Most probably, this process does not yield energy. Perhaps, neurin is formed from lecithin by a similar desaturation.

Another very common type of decomposition of amino acids is decarboxylation, i.e., the splitting off of the CO_2 group. This is the way in which amins are formed. E.g., glycine is decomposed into methyl amin by *Ps. fluorescens* (Emmerling and Reiser, 1902).

$$CH_2(NH_2)\cdot COOH = CH_3NH_2 + CO_2$$

In this same way, cadaverin may be produced from lysin.

$$\underset{\text{Lysin}}{CH_2(NH_2)\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH} =$$

$$\underset{\text{Cadaverin}}{CH_2(NH_2)\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot NH_2} + CO_2$$

Putrescin, or tetra methylene diamine, probably originates from arginine in a similar way.

Quite often, decarboxylation and deaminization occur simultaneously. Examples of this have been shown already (p. 43) in the decomposition of leucin, isoleucin, tryptophane and tyrosin by yeast.

Aside from these decompositions of amino acids, which can be grouped into distinct classes, many other changes may occur which are typical for one certain amino acid only. This is especially true with oxidative changes. Hydrolysis of amino acids will yield hydroxy acids, and these may be used as substrates for anaerobic fermentations (p. 76).

Another group of protein cleavage products are the acid amids. The energy yield of their hydrolysis is probably quite considerable as will be shown later (see p. 77).

Anaerobic Putrefaction.—It has been generally assumed that anaerobic putrefaction of proteins as we find it most pronounced in the group of the anaerobic spore formers, follows essentially the same type that has

been outlined in the preceding pages. The recent investigations of Sturges and his associates indicate, however, that with some species at least, the type of decomposition is different.

Parsons and Sturges (1927) studied the volatile acids produced by *Clostr. putrefaciens* from pork, and found that acetic, butyric and valeric acids were produced at the molar ratio of 85:6:9. In gelatin, the relative amount of acetic acid rose even to 91–92.5% of the total acidity. This dominance of acetic acid contrasts *Clostr. putrefaciens* to most other clostridia which show a much higher ratio of acids with longer carbon chains.

The only exception is *Clostr. histolyticum* (Sturges, Parsons and Drake, 1929) which produces acetic acid only; non-volatile, ether-soluble acids could not be detected. This holds true for such widely different media as milk, beef heart, brain, liver, gelatin and peptone.

The large amount of acetic acid formed by *Clostr. histolyticum* cannot have been derived from deamination of glycine, because even in a gelatin medium (containing 25% of glycine) the acetic acid formed is almost twice the theoretical amount from the glycine, and in a pork medium, it is more than ten times the theoretical amount.

Sturges, Parsons and Drake conclude that this organism, and possibly all putrefactive anaerobes, either have some highly specific method of splitting proteins, differing radically from the usually accepted hydrolysis, or that they can split long-chain amino acids into acetic acid and some still undiscovered residues.

Urea Fermentation.—The urea fermentation is usually summed up in the formula:

$$CO(NH_2)_2 + 2H_2O = CO_3(NH_4)_2$$

Really, the process must occur in two steps, one being the hydrolysis, and the other being the neutralization of the ammonia and the carbonic acid formed.

$$CO(NH_2)_2 + 2H_2O = CO_3H_2 + 2NH_3$$
$$CO_3H_2 + 2NH_3 = (NH_4)CO_3$$

Söhngen (1909) has proved that urea is entirely sufficient as a source of energy for urea bacteria, and also as a source of nitrogen for cell construction, but not as a source of carbon.

While urea is fermented by a large number of species from different genera and families, uric and hippuric acid are fermented mostly by spore formers. The hippuric acid is hydrolyzed into benzoic acid and glycine:

$$C_6H_5{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}COOH = C_6H_5{\cdot}COOH + NH_2CH_2{\cdot}COOH$$

Uric acid is split into its natural components, two molecules of urea and one of a tri-carbon compound which is readily oxidized.

Prototrophic Fermentations.—A large number of prototrophic fermentations are known, all of which are oxidations. The largest number of species occur in the sulphur bacteria which oxidize H_2S primarily to S, and later to H_2SO_4. While all members of this very large group behave alike in their energy acquirement, the bacteria acting upon ammonia have clearly divided their task, as the one group can only oxidize ammonia to nitrite, and the other only nitrite to nitrate; not ammonia to nitrate.

The other various oxidations of methane and higher hydrocarbons up to the paraffin, of carbon monoxide, of coal, etc., offer no special interest in their equations. The

only interesting species is the Hydrogenomonas, which requires CO_2 for the oxidation of hydrogen, and makes formic acid as intermediate product, according to Kaserer (1906)

$$CO_2 + 2H_2 = HCOH + H_2O + 6 \text{ cal.}$$

$$HCOH + O_2 = H_2O + CO_2 + 132 \text{ cal.}$$

(*g*) INTERACTION BETWEEN NITROGENOUS AND NON-NITROGENOUS FOOD COMPOUNDS

We have seen several examples of intermolecular oxido-reductions, i.e. an exchange of hydrogen atoms between molecules of different composition. The Cannizzaro reaction between methyl glyoxal and acet aldehyde is perhaps the best known case. Since these reactions are essentially caused by the activation of certain hydrogen atoms, it seems probable that many other compounds capable of reduction could act also as hydrogen acceptors, and that the hydrogen acceptors will not be limited to cleavage products of sugar.

A very simple and instructive case of this type is reported by van Niel (1928) who studied the action of twelve strains of propionic acid bacteria upon lactic acid and found that the products did not correspond to the equation:

$$\underset{\text{Lactic acid}}{3C_3H_6O_3} = \underset{\text{Propionic acid}}{2C_3H_6O_2} + \underset{\text{Acetic acid}}{C_2H_4O_2} + CO_2 + H_2O$$

The ratio of propionic to acetic acid, instead of being 2:1, was very persistently 1.8:1. Besides, some succinic acid was formed which had to be accounted for. Van Niel could demonstrate that the succinic acid did not come from the lactic acid, but from aspartic acid or some similar amino acid in the rather concentrated

nitrogenous material of his medium. The aspartic acid acted as hydrogen acceptor and was reduced to succinic acid and ammonia.

$$COOH{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH + H_2 = COOH{\cdot}CH_2{\cdot}CH_2{\cdot}COOH + NH_3$$

By adding aspartic acid to the medium, still more hydrogen could be "deviated" and the ratio of propionic to acetic acid fell to 1.6:1.

We might conclude from this that the equation for the fermentation of lactic acid is correct, and that the aspartic acid interfered. Such a statement, however, might be thought to prescribe a definite course to a biological reaction, and to establish standards rather than to give an unbiased interpretation of the facts. The unprejudiced description of the process would give each of the partial reactions (as mentioned on p. 47) and add the above reduction of aspartic acid. This would leave to the bacterium the "choice" of the hydrogen acceptor, instead of introducing the human element of "correct equations" and "deviations."

This is probably the best known, quantitatively studied example, but many others are known, in which the protein food acts either as hydrogen donator or as hydrogen acceptor.

Another very commonly found hydrogen acceptor is the elementary oxygen. In the common types of alcoholic fermentations (beer, wine, distilled alcohol), the influence of oxygen is negligible because the continuously formed CO_2 prevents appreciable amounts of oxygen from being taken up by the yeast. In the aerated tanks used for the production of bakers yeast, the ratio of alcohol to CO_2 is changed considerably (see p. 176).

A further good example of oxygen as a hydrogen acceptor is its influence upon the gas ratio of *Bact. coli*, i.e., the ratio CO_2:H_2. Keyes and Gillespie (1912,

1912a) found that a *Bact. coli* having the gas ratio 1.03–1.09 in vacuo showed the gas ratio 5.4 in presence of oxygen. Another strain, with the gas ratio 1.77 in vacuo, changed in air from 3.4 to 590.

The gas ratio of *Clostr. Welchii* belonging to the group of anaerobic butyric organisms, was not changed essentially by the presence of air (1.42–1.53 in vacuo, 1.44 to 1.85 in air) though some oxygen was adsorbed by the culture. Oxygen, apparently, could not be activated to act as acceptor.

Even in vacuo, *Bact. coli* will change the gas ratio with the medium. With ammonium lactate as the only source of nitrogen, the gas ratio was 1.03, 1.06 and 1.09 in three parallel experiments, while with 1% peptone Witte, it increased to 1.31, and with beef infusion and peptone Witte, it was 1.28.

(*h*) THE MECHANISM OF FERMENTATION

The Mechanism of Fermentation has been claimed to be essentially the activation of certain hydrogen atoms in the substrate molecule. This activation is brought about by a catalyst in the cell. The catalyst is supposed by some to combine temporarily with hydrogen and then give it up to some hydrogen acceptor. Such catalysts are used commercially; the best known inorganic hydrogen catalyst is metallic nickel, which is used in the industrial hydrogenation of fats. Palladium will dehydrogenate ethyl alcohol or hydrated acet aldehyde and transfer the hydrogen either to oxygen or to methylene blue, thereby changing the dehydrogenated product to acetic acid and acting in the same way as do the vinegar bacteria.

Possibly, the catalyst or activator only loosens the bond with which the hydrogen atom adheres to the

molecule. The catalyst in either case must be an unsaturated compound. If the hydrogen would combine with the catalyst permanently, it would lose its power as activator. A third compound steps in to prevent this, namely the hydrogen acceptor which combines with the loosened, activated hydrogen atoms thus becoming itself reduced while oxidizing the hydrogen donator. The hydrogen acceptor may have to be activated as well. We are certain of this in the process of respiration where oxygen is the final hydrogen acceptor, and will act only if activated by some complex organic iron compound.

The gradual breaking up of the carbohydrate through a number of intermediate steps leads to the question whether for each of these steps, a separate enzyme is necessary. It would seem that what we had called zymase (p. 36) must be a large group of individual enzymes. In fact, several of them have already received names, such as catalase, reductase, alcohol oxidase, lactozymase, lactacidase, carboxylase, carboligase, methylglyoxalase, mutase, dismutase, etc.

We have no criterion for the purity of an enzyme and therefore might always assume that a supposedly pure enzyme contains another enzyme if it gives a new reaction under changed conditions. However, the number of different enzymes in one bacterial cell would have to be so large under the circumstances that it seems rather impossible to concentrate so many molecules of the size of enzyme molecules in such a small space. (The dimensions of bacteria will be discussed on p. 397.)

Kluyver and Donker (1926) have endeavored to show that Wieland's theory of hydrogen activation, as the underlying cause of all oxido-reduction, brings a unifying principle into biochemistry. It does not seem necessary

to assume a different enzyme for each individual step in each fermentation.

Kluyver and Donker point out that different intensities of the affinity of the catalyst for hydrogen, i.e. different intensities of the activation of hydrogen atoms might bring about quite different reactions. They further call attention to the great influence of hydrogen ion concentration upon the reduction potential (see p. 88). This potential will very probably be interlinked somehow with the activation of hydrogen.

Another cause for differences of intensity of activation by the same catalyst might be their arrangement within the cell, and the distance between activator (catalyst) and substrate (hydrogen donator). It will be shown on p. 263 that a very definite arrangement of the essential molecules in the cell is necessary. If the distance between catalyst and substrate is larger in one species than in another, the loosening of the bond of a certain hydrogen atom will not be so great, and this may bring about a different type of reaction.

Besides, the type of reaction always depends upon the hydrogen acceptors present. If oxygen and an oxygen activator are available, the reaction must be different from that under anaerobic conditions, even with the same hydrogen activators (see the gas ratio of Bact. coli, p. 64).

While our knowledge is not sufficient to make accurate statements, the present outlook in biochemistry indicates that the complexity in fermentation processes does not lie so much in the innumerable number of different compounds reacting, but more in the adaptability of the reacting compounds to all different conditions.

Another step in this direction is the attempt of Utkina-Ljubowzowa and Steppuhn (1929, 1930) to prove that the autolytic enzymes of

different tissues and of different organisms are not specific, but that in all organs of all plants, animals and microorganisms, the same three proteolytic mechanisms are found which are characterized by their optimal pH of 3.5, 5.5 and 7.5 respectively.

(*i*) SUMMARY OF FACTS

The large majority of bacteria can exist only if they have at their disposal organic or inorganic food which they can decompose. The amounts of energy produced by such decomposition can be measured or calculated. Such decompositions, we call fermentations, using the broadest meaning of this term.

In several cases, it has been possible to separate from the living cell the agent that causes fermentation. In other cases, it could be shown that fermentation may continue after the cells have lost the power of reproduction by drying, acetone or alcohol treatment, or ultra-violet irradiation.

Ultra-filtration and dialysis have shown that fermentation is brought about by the inter-reaction of several agents.

In the fermentation by yeast, colon bacteria, butyric acid bacteria, acetone bacteria or others, intermediary products of fermentation have been isolated which demonstrate that the fermentation is not one process, but a succession of several chemical reactions.

The type of fermentation may be changed altogether by changing the reaction of the medium, or by adding sulfites, or by changing the oxygen supply, and even by changing the concentration of the substrate.

SUMMARY OF THEORIES

Bacteria require, for maintenance as well as for growth, a source of energy which, for most species, can

come only from the decomposition of organic or inorganic compounds.

Fermentation (in the broadest meaning of the term) is not absolutely connected with living organisms. The vinegar fermentation can be accomplished by finely divided palladium in the same way as by bacteria. Fermentation may be accomplished by cells after their death, i.e., after loss of reproduction. The fermenting agent could be separated from the cell in some instances. We are forced to the conclusion that fermentation is brought about by enzymes.

A number of different enzymes are necessary to bring about fermentation. We are at present quite unable to state how many different kinds are needed. Each fermentation is not one single process, but a combination of a number of different simple chemical reactions.

The fermentation mechanism of different genera and species does not involve absolutely different reactions. Different combinations of the same simple steps in the gradual breakdown of the food molecule may lead to quite different fermentation products. These differences in combination may be caused by differences in pH, by the entering of other compounds into the reaction (such as oxygen), and by differences in the arrangement of the enzyme molecules in the cell.

Most of these reactions can be explained best by the assumption that the enzymes of the cell act upon the substrate by activating certain hydrogen atoms. These then combine with a "hydrogen acceptor." The acceptor might be another part of the same molecule, or another organic or inorganic molecule, or oxygen. Oxygen, in order to function as hydrogen acceptor, must be activated by a complex iron compound.

III. THE ROLE OF OXYGEN IN ALL FERMENTATIONS

Hydrogen and oxygen are chemical contrasts to a larger degree than any other two elements. Oxidation and reduction are diametrically opposed reactions; dehydrogenation means oxidation; reduction means either the addition of hydrogen, or the removal of oxygen.

In organic compounds, hydrogen is not as conspicuous as oxygen. There are usually a number of hydrogen atoms, only a few of which can be easily activated. If an oxygen atom is present in an organic compound, it marks reactivity, a step away from the "parum affinis" of the inert hydrocarbons. Thus we have come to consider oxygen as an indicator of biochemical usefulness. The degree of usefulness of a substance for a certain organism is dependent upon the number of oxygen atoms as well as their position in the molecule.

Aside from this, the role of oxygen in respiration, the abundance of oxygen in the air, the ubiquity of it make it appear of paramount importance in life processes. Even though the basis of all oxidations and reductions may be the change of hydrogen atoms, we notice such reactions primarily through their counterpart, oxygen. There is no need, as far as the author can see, to eliminate the word oxidation from our biochemical vocabulary.

The primary purpose of fermentation is apparently the energy supply of the cell. It is brought about in practically all cases by oxido-reductions. The one outstanding exception is the urea fermentation which must probably be classified as a hydrolysis. Hydrolyses, as a rule, yield so little energy that they cannot be used as source of energy by organisms. (See however p. 76.)

In all oxido-reductions, some hydrogen is removed from the substrate, and is placed in the hydrogen

acceptor. We should distinguish, for practical purposes, three different cases:

(1) The hydrogen acceptor is molecular oxygen. Then we have a direct oxidation, as in the vinegar fermentation, or in the action of molds or mycobacteria upon sugar.

(2) Hydrogen acceptor and hydrogen donator are the same molecule. This we call intramolecular fermentation. The chemical equation shows this most simply:

$$C_6H_{12}O_6 = 2C_3H_6O_3$$

No oxygen and no hydrogen from outside are apparently needed to bring about this fermentation. It consists of nothing but a rearrangement of the H- and O-atoms (see p. 75). The same is true with all the typical sugar fermentations.

As a matter of fact, the case is not quite so simple because we must consider the separate stages of fermentation. But the term "intramolecular fermentation" indicates that *one* substance alone is sufficient as source of energy.

(3) Hydrogen acceptor and donator are different molecules. Two different compounds are required to bring about the change. The acceptor may be oxygen; this proves type (1) to be just a special case of type (3), sufficiently specialized, though, to remain classified by itself. Examples of type (3) have been given in the discussion of interaction between proteins and carbohydrates. Another example which brings the oxygen into the foreground again is the anaerobic fermentation at the expense of oxygen from nitrates, sulphates, methylene blue and similar, highly oxidized compounds. Allyn and Baldwin (1930) could make

Rhizobium grow as far as 10 mm. below the surface in an agar medium oxidized by addition of 0.015% $KMnO_4$ while in the untreated, normal medium, they grew on the surface only.

For thermodynamic reasons, processes of type (3) can take place only if the energy liberated by dehydrogenation (oxidation) is greater than that required for hydrogenation, or if the total energy gain of the acceptor is smaller than the total energy loss of the donator. This eliminates *a priori* a number of processes as impossible.

Pütter, in his book on comparative physiology (1911) has computed the calories produced by 1 gm. of oxygen in oxidizing various organic compounds. Table 9 is an extract from Pütter's data. The energy liberated is very uniformly 3.3 calories per 1 gm. of oxygen, regardless of the type of compound oxidized. Only oxalic acid deviates considerably from this average.

With inorganic compounds, the liberated energy varies greatly. Table 9 gives a few examples, computed also on the basis of energy per gram of oxygen. It is very evident which compounds are good hydrogen

TABLE 9.—CALORIES LIBERATED BY 1 GM. OF OXYGEN

A. In the oxidation of inorganic compounds

	Kg. calories per gm. oxygen
$H_2 + O = H_2O$	4.25
$H_2O + O = H_2O_2$	−1.44
$C + O = CO$	1.80
$CO + O = CO_2$	4.24
$H_2S + O = H_2O + S$	3.87
$H_2S + 4O = H_2SO_4$	3.18
$S + H_2O + 3O = H_2SO_4$	2.96
$NH_3 + 3O = HNO_2 + H_2O$	1.63
$HNO_2 + O = HNO_3$	1.16
$H + N + 3O = HNO_3$	1.02

B. In the oxidation of organic compounds

	Kg. calories
Methane	3.20
Acetic acid	3.272
Oxalic acid	3.80
Palmitic acid	3.259
Dextrose	3.508
Maltose	3.520
Starch	3.530
Asparagin	3.218
Uric acid	3.198
Legumin	3.291
Elastin	3.240
Fat (animal)	3.293
Glycerine	3.5
Alcohol	3.4

acceptors if we consider only the energy viewpoint. The best is doubtless hydrogen peroxide because energy could be gained by reducing it, i.e. by adding hydrogen to it. The other compounds offer a certain resistance to reduction. The energy required to remove 1 gm. of oxygen from these compounds is lowest in the nitrates. Let us assume a bacterium under anaerobic conditions in a medium containing oxalic acid and nitrate. Nitrate could act as hydrogen acceptor, oxalic acid as hydrogen donator.

$$C_2O_4H_2 \rightarrow 2CO_2 + 2H \quad + 3.80 \text{ cal. per gram oxygen}$$
$$HNO_3 + 2H \rightarrow HNO_2 + H_2O \quad - 1.16 \text{ cal. per gram oxygen}$$
$$C_2O_4H_2 + HNO_3 \rightarrow 2CO_2 + H_2O + HNO_2 + 2.64 \text{ cal. per gram oxygen}$$

Such an oxido-reduction is possible because it leaves a positive energy balance. The energy required to fix the hydrogen to the acceptor is less than the energy gained by dehydrogenation. This latter value (energy from dehydrogenation) is quite uniformly 3.3 cal. per gram of oxygen added (or for $\frac{2}{16}$ gm. of hydrogen removed), provided that the oxidation is complete.

Table 9 shows that H_2O and CO_2 and sulfur could not be considered as hydrogen acceptors, and that H_2SO_4 is a very poor acceptor, while nitrate and nitrite are excellent. Better than all of these (except H_2O_2) is oxygen gas which requires no energy whatever and, therefore, gives the highest energy yields.

The computation of the energy balance shows us which reactions are possible, and which reactions can take place more easily than others. But it is not sufficient that a reaction is thermodynamically possible. It is the type of catalyst which decides whether the possible processes actually happen. Very few organisms can reduce nitrates to nitrogen gas, though this seems the most economical type of reduction from the energy viewpoint. No organisms are known to reduce carbon monoxide, though it would be quite profitable with the proper mechanism.

Eventually, the catalysts for such oxido-reductions are already present in the culture medium. Demeter (1930) has shown that sterile broth, asparagin solution, etc. will reduce nitrates to nitrites at 45°C.

The importance of chemical structure in addition to energy considerations is most striking in the anaerobic species. Though their energy supply depends upon oxido-reductions like that of all other organisms, they cannot utilize the best of all hydrogen acceptors, i.e. oxygen. This is not merely due to their sensitivity to oxygen because some species which are rather indifferent to it show the same lack of oxygen utilization. The author cultivated *Strept. cremoris* at 30°C. in a flask half full of milk and closed by a rubber stopper with a large inverted U-tube whose other end opened into a glass of water. While with *Bact. coli* and *B. cereus* in peptone solution, the water was always drawn into the tube as a result of consumed oxygen, the level of water never changed with milk cultures of *Strept. cremoris*, not even when a tube of soda-lime was suspended in the flask to absorb eventual CO_2 formed. Addition of methylene blue made no

change, though the streptococcus reduces methylene blue readily. The organism is in need of a hydrogen acceptor, as this reduction proves, but cannot make use of oxygen gas. Methylene blue should have acted as a catalyst, but the small amount of this compound used (0.5 mg. in 100 c.c. of milk) could transfer to the bacteria or their products only 0.028 mg. of oxygen = 0.01 c.c.; the process would have to be repeated several hundred times before the oxygen consumption could be detected by this method.

This agrees with the findings of Callow (see p. 19) that cells of *Clostr. sporogenes* and *Strept. lactis* suspended in buffer solution, without food, take up hardly any oxygen, while aerobic and facultative organisms consumed 5 to 25 c.c. of oxygen per hour. The anaerobic organisms lack the faculty to activate oxygen so that it can serve as hydrogen acceptor.

Quite different was the experience of Meyerhof and Finkle (1925) with *Lact. Delbrücki.* This organism, though practically anaerobic, consumes oxygen and produces an equal volume of CO_2. For each molecule of sugar oxidized, the fermentation of about six molecules of sugar to lactic acid is prevented. This corresponds with experiences on the metabolism of the muscle.

As in most respiration mechanisms, oxygen consumption was greatly increased by methylene blue, and was retarded by cyanides. With small amounts of cyanide, oxidation could be repressed so that almost equal amounts of lactic acid were formed with air and without air. The relative amounts were

	Millimols KCN			
	Exp. I		Exp. II	
	0	0.7	0	1.0
Without air	112	33	72	30
With air	20	32	7	28

The cyanide had also damaged the fermentation mechanism, but not nearly to the same extent as the respiration mechanism.

The data of Table 9 can be used only with complete oxidations. For incomplete oxidations, and especially

for intramolecular oxido-reductions of type 2 (p. 70) where acceptor and donator are the same molecule, the above data cannot be applied.

In intramolecular fermentations, energy is liberated by shifting the oxygen towards the end-carbon, and by massing the oxygen on one carbon rather than having it distributed evenly to the various carbon atoms. As an example, we can write the glucose molecule and its products thus:

```
 H  H  H     H  H  H
HC . C . C . C . C . C   glucose
 O  O  O     O  O  O
 H  H  H     H  H

 O  H  H     H  H  O
 C HC . CH  HC . CH C   alcohol + CO2
 O  O  H     H  O  O
    H           H

 O  H  H     H  H  O
 C . C . CH  HC . C . C lactic acid
 O  O  H     H  O  O
 H  H           H  H
```

The shifting of oxygen from the inner to the outer carbon atoms liberates energy. A compound with oxygen on the outer carbons only can not undergo an intramolecular oxido-reduction. All the simple alcohols, all fatty acids (and, of course, all hydro-carbons), as well as the divalent acids like oxalic, malonic and succinic acid can not be fermented except by a different type of hydrogen acceptor, such as oxygen gas or nitrate or perhaps certain protein products. Organic acids may undergo decarboxylation, but that does not, as a rule, lead to a gain

of energy, as may be seen from the following combustion heats per gram molecule:

$$\underset{\substack{326\text{ cal.}*\\ \text{Lactic acid}}}{CH_3{\cdot}CHOH{\cdot}COOH} \rightarrow \underset{\substack{326\text{ cal.}*\\ \text{Ethyl alcohol}}}{CH_3{\cdot}CH_2OH} + CO_2$$

$$\underset{\substack{287\text{ cal.}*\\ \text{Tartaric acid}}}{COOH{\cdot}CHOH{\cdot}CHOH{\cdot}COOH} \rightarrow \underset{\substack{287\text{ cal.}*2\\ \text{Ethylene glycol}}}{CH_2OH{\cdot}CH_2OH} + 2\,CO_2$$

The uniform distribution of oxygen in the carbohydrates makes them especially fit for intramolecular oxido-reductions. The same holds for glycerol. Hydroxyacids (e.g., lactic, tartaric) and amino acids which by hydrolysis may be changed to hydroxy acids, can also be fermented anaerobically according to type 2.

The organisms (i.e., the catalysts) which have the faculty to bring about intramolecular changes of this type, can exist anaerobically only if the medium contains compounds of the just-mentioned type. *Bact. coli* dies without oxygen in a solution containing lactate only, though with access of air, it can utilize the lactate by using oxygen as a hydrogen acceptor (p. 187). Some peptones contain a compound which permits anaerobic growth of *Bact. coli*. According to Treece (1928), *Bact. coli* and *aerogenes* formed acid and gas in anaerobic solutions of Difco's, and Parke, Davis' peptone, but not with Armour's or Witte's peptone. The products do not seem to be derived from carbohydrate radicals.

A peculiar exception to the oxido-reductions of organic and inorganic compounds is the urea fermentation which is a hydrolysis. As a rule, the energy yield of hydrolysis is almost negligible (see p. 25). The cause of this exception of urea fermentation is the circumstance

* Combustion heat.

that by hydrolysis, an acid and an alkali are formed. We can write the urea fermentation in two steps:

$$CO(NH_2)_2 + H_2O = CO_2 + 2NH_3$$

$$2NH_3 + CO_2 + H_2O = (NH_4)_2CO_3$$

The energy yield of the second process has been determined, and Landolt-Börnstein mentions two values, 10.7 and 15.9 cal. per gram molecule of ammonium carbonate. The energy yield of the first process, the real hydrolysis, can not been measured separately. Combustion heats of urea are given only with nitrogen gas as final product. By combining the values for heats of formation of urea, ammonia and water, the computed energy yield of the hydrolysis proper is negative, -1.5 cal. This value is quite uncertain, representing the difference of various, none too exact, heats of formation; but it is probably near zero. The only energy available to the cells of urea bacteria is that of the second process; namely, the heat of neutralization. This does appear a strange source of energy.

The same principle probably applies to the organic acid amids which are contained in the protein linkages. The heat of neutralization of acetic acid by ammonia approximates 12 calories per gm. molecule. These values are quite considerable if we realize that the energy yield obtained from the alcoholic fermentation of sugar is only about 26 cal. per gm. molecule. The heat of neutralization also must enter into some of the inorganic fermentations (formation of nitrous acid from ammonia, of sulfuric acid from hydrogen sulfide or sulfur, and in denitrification and sulfate reduction).*

This discussion of the role of oxygen for the energy requirements leads us to wonder to what extent our distinction between aerobic, facultative and anaerobic bacteria is justified. Since we judge this property primarily by the growth of bacteria, it really should

* Another peculiar type of oxygen-free fermentation is the splitting of fatty acids into CH_4 and CO_2 (Thayer, 1931).

not be discussed in this part of the book. However, the source of energy is one of the most important factors in growth, and so the two might well be combined and considered here.

It might seem that we could define strict aerobes as bacteria which must have oxygen for hydrogen acceptors. One may object that this definition will fail as soon as new hydrogen acceptors are found which permit growth without oxygen gas. Since vinegar bacteria might obtain their oxygen for fermentation from methylene blue, and since oxidation of a medium with permanganate makes aerobic bacteria grow farther down below the surface (see p. 71), we are warned against making our statements too positive.

Another factor enters in, namely, the growth requirements. Söhngen (1909) has shown that urea fermentation (which needs no hydrogen acceptors) is entirely sufficient for the energy requirements of some urea bacteria, and that very small amounts of organic acids suffice for the carbon of the cell construction; no growth will take place, however, in the absence of oxygen. In the construction of cell compounds from organic acids, dehydrogenation must become necessary for certain parts of the building material, and oxygen is the only acceptor for this hydrogen. Urea cannot possibly act as hydrogen acceptor.

When it comes to the differentiation between facultative and strict anaerobes, another factor enters, namely, the toxic effect of oxygen. Really, the only definition that holds with all anaerobes is that oxygen of the concentration found in air is toxic, and prevents growth. Anaerobiosis has no direct connection with the source of energy, and some Beggiatoa species have been claimed to grow only with reduced atmospheric oxygen pressures,

while they obtain their energy from the oxidation of hydrogen sulfide.

On the other hand, the Streptococci are not able to utilize oxygen (p. 20), but are not especially sensitive to it; we do not ordinarily consider them as anaerobes. Further, the sensitivity of bacteria to oxygen depends upon the number of cells present and upon their physiological condition. Large inocula of anaerobes will frequently grow when small inocula of the same culture in the same medium will not.

Finally, the kind of medium is of great influence. It has been shown that by oxidizing a medium, or by adding appropriate hydrogen acceptors, aerobic bacteria may develop without oxygen gas. It has also been shown that the addition of pyruvic acid or cysteine to a medium will permit growth of some strictly anaerobic species without special protection from oxygen.

Our differentiation between aerobic, anaerobic and facultative organisms is quite useful as a working definition, but it involves a number of different unrelated principles, such as the need of oxygen for energy liberation, the possibility of substitutes for this free oxygen by organic or inorganic hydrogen acceptors, the requirement of oxygen for growth when it is not required for energy, the toxicity of oxygen to the cell, and the overcoming of toxicity by special media. There are no clear-cut groups set off distinctly by their reaction towards oxygen; all are allied in varying degrees, according to the composition of the culture medium.

In discussing oxygen requirements, it should be remembered that the solubility of oxygen is very small, as Table 10 shows. There is only between 0.0007 and 0.0010% of oxygen in solution, and considering oxygen as a food, a medium with such a small amount of food

would be considered very poor. It is evident that this supply must soon be exhausted.

Table 10.—Solubility of Oxygen in Water

Temperature, °C.	Oxygen atmosphere, %	Air, %
0	0.006941	0.00145
5	0.006067	0.00126
10	0.005364	0.00112
15	0.004799	0.00100
20	0.004335	0.00090
25	0.003928	0.00082
30	0.003585	0.00075
35	0.003312	0.00069
40	0.003078	0.00064
45	0.002857	0.00060
50	0.002654	0.00055
100	0.000000	0.00000

A. Müller (p. 187) has shown that *Bact. coli* growing in a poor medium requires about 1.6×10^{-10} mg. of oxygen per hour; *Pseudomonas fluorescens* needs considerably more oxygen. A full-grown culture of *Bact. coli* contains about 10^9 cells per c.c., or 10^{11} cells per 100 c.c., and these cells would require $1.6 \times 10^{-10} \times 10^{11} = 16$ mg. of oxygen per 100 c.c. of medium per hour. Since only 1 mg. is available in the medium, the supply will be exhausted in a short time. Diffusion of oxygen is far too slow to provide the bacteria in the lower layers.

There can be no doubt that in a testtube culture or flask culture of aerobes, all cells will exist under practically anaerobic conditions except those in the very top surface layer.

SUMMARY OF FACTS

The processes that yield energy to the cell involve shifting of oxygen, either within the molecule of the substrate, or between two different kinds of molecules.

The only exception is the urea fermentation, and, possibly, some similar decompositions of acid amids in protein cleavage.

Oxygen exchange between two different types of molecules depends upon the special cell mechanism as well as upon the energy liberated. Oxidation does not always take place when oxygen is available.

Some strictly aerobic bacteria can be made to exist anaerobically by supplying them with highly oxidized compounds acceptable to the species.

Anaerobic growth of facultative organisms is possible only with special substrates. Many substances not permitting anaerobic growth will allow abundant growth of the same species in air.

Some strictly anaerobic bacteria may be made to grow in unprotected testtube cultures if certain chemical substances are added.

SUMMARY OF THEORIES

Fermentation depends upon activation of certain hydrogen atoms of the substrate as well as upon an appropriate acceptor for the activated hydrogen. The acceptor may be the same substrate, or part of it, or a different substance, or elementary oxygen.

An exception to this type of fermentation is the urea fermentation. Energy in this process is not liberated by the hydrolysis proper, but by the heat of neutralization between ammonia and carbonic acid.

Streptococci and Clostridia cannot activate oxygen to function as hydrogen acceptor. This lack has no direct relation to anaerobiosis, for streptococci grow fairly well in air.

The distinction between aerobic, facultative and anaerobic species is indefinite. Besides the properties of

the species, the composition of the medium decides whether an organism can grow without oxygen, and whether it can tolerate the toxicity of oxygen.

IV. THE OXIDATION—REDUCTION POTENTIAL

(*a*) ELECTROMETRIC MEASUREMENTS OF POTENTIALS

In 1911, Potter conceived the idea that, since biochemical manifestations are frequently accompanied by electrical phenomena, bacterial cultures might also show a change of potential. He tested this assumption by filling a porous cylinder with a liquid medium and placing it into a glass jar filled with the same medium. One of the two portions of medium was inoculated with a microorganism. Platinum electrodes were then placed into both liquids and an electric current of a certain voltage was actually observed, the whole resembling a galvanic cell.

The differences in potential were found to be with

	Volts
Bact. coli in tartrate-asparagin solution	0.308
Bact. coli in tartrate-starch solution at 30°C	0.349
Bact. coli in tartrate-starch solution at 20°C	0.534
Yeast in glucose or sucrose solution	0.300–0.400

Potter concluded from this evidence that the disintegration of organic compounds by microorganisms is accompanied by the liberation of electrical energy.

Nine years later, Gillespie (1920) showed that the electric potentials observed by Potter were only a special case of the oxidation-reduction potential. If two platinum electrodes are placed in the two open ends of a U-tube filled with acid, and one electrode is saturated with hydrogen and the other with oxygen, the difference in potential of the two electrodes would be 1.23 volts.

Instead of using hydrogen and oxygen, less extreme reagents might be used, and the potential difference would be smaller.

An oxidation-reduction potential is established when an electrode is placed in a liquid which does not react with it chemically. This potential indicates the intensity of reduction. It does not tell anything about the amount of substance that can be oxidized or reduced. This potential is comparable, as a simile, to the hydrogen ion concentration which tells us the intensity of the acid, but not the amount of acid present.

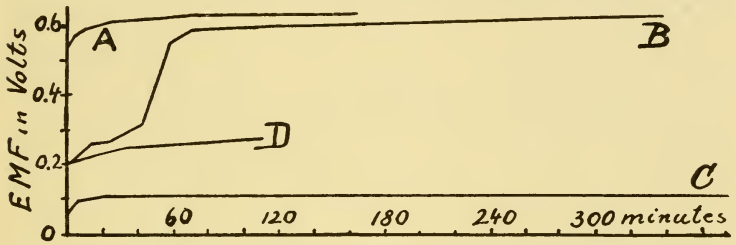


FIG. 3.—Differences in potential between a mercury electrode in bacteria cultures, and a calomel half cell: A, B, and C: a mixed culture with different amounts of oxygen; D: *B. subtilis* in dextrose-peptone solution.

A single potential cannot be measured; we have to combine two potentials and thus obtain an electric current which gives us the difference between the two potentials. Potter in the above experiments measured the differences between the oxidation-reduction potential of the sterile medium and that of the culture. Gillespie coupled the electrode in the culture with a calomel half cell, and thus measured all his potentials against a standard electrode of definitely known potential. The differences are recorded in volts. Figure 3 represents a putrifying solution of peptone in a deep layer, containing a mixed culture of bacteria carefully protected against agitation. Curve *A* gives the potential difference of an old culture when the access of air was carefully avoided.

In *B*, a little air entered accidentally, causing a low start of the potential; after about 1 hour, the original potential was again established. Curve *C* shows the result of vigorous shaking with air; the potential remains low and does not recover. The culture was too old for new growth to occur and those old cells which produced the original potential difference (probably anaerobes) were apparently unable to function.

In some other experiments, Gillespie measured also the hydrogen ion potential by bubbling hydrogen through the medium. The following values were obtained:

TABLE 11.—HYDROGEN ELECTRODE AND REDUCTION ELECTRODE POTENTIALS OF VARIOUS CULTURES OF BACTERIA

	Hydrogen electrode, volts	Reduction electrode, volts	Difference, volts
Mixed culture in Peptone Witte, 17 days	0.707	0.630	0.077
Mixed culture in Peptone Witte, 20 days	0.715	0.616	0.099
B. subtilis in Peptone Witte.....	0.753	0.314	0.439
B. subtilis, in Peptone + sugar.....	0.622	0.275	0.347
Bact. coli, in Peptone + sugar.....	0.594	0.580	0.014
Bact. coli, in Peptone + sugar.....	0.554	0.516	0.038
Bact. coli, suspension in 0.9% NaCl....	0.594	0.594	0
B. mycoides, suspension in broth.....	0.700	0.370	0.330
Soil aerobe, suspension in phosphate sol.	0.652	−0.040	0.692

The reduction potentials of the aerobic spore-formers, *B. subtilis*, *B. mycoides* and one unnamed species, are quite low in comparison to the facultative *Bact. coli* and the putrifying solution containing anaerobes.

The hydrogen electrode potential is the potential we measure in determining pH, except that it is recorded here in volts. We can also define it as the reduction potential of hydrogen gas at 1 atmosphere of pressure

at the pH of the culture under test. If the hydrogen pressure is less than 1 atmosphere, the reduction intensity will be lower, and it can be computed how much lower it will be for any given hydrogen pressure. This leads to the conception of r_H (see p. 90).

Gillespie's data show that in one case, *Bact. coli* produced the same potential as the hydrogen electrode; in other words, this culture reduced as strongly as hydrogen gas at 1 atm. pressure, and, consequently, could develop hydrogen gas. The differences between hydrogen potential and reduction potential of the other two cultures of *Bact. coli* correspond to hydrogen pressures of 0.34 and 0.052 atm. respectively.

The potentials mentioned above are *differences* in potential between the culture and an arbitrary standard, in one case the sterile medium, in the other case a calomel half cell. There is no absolute zero point for electric potentials, but in order to compare potentials, a zero point is fixed arbitrarily. The potential of the normal hydrogen electrode is generally accepted as the standard zero point. This may be compared to fixing the zero point of the thermometer scale by the freezing point of water. The difference in potential between the normal hydrogen electrode and the saturated calomel electrode is 0.247 volts, and this amount must be added to measurements with the saturated calomel electrode to make them comparable with the standard scale. The EMF on the standard scale is usually expressed as E_h.

The E_h of most sterile media in air is about +0.2 to 0.3 volts, and becomes negative by the action of bacteria. Therefore, the curves of potential change in bacterial cultures will show a decrease of potential if plotted on the standard or E_h scale. The lower the potential, the lower is the oxidizing intensity, and the stronger is the reducing intensity. If we plot potential

differences, however, as in Figs. 3 and 8, the difference becomes larger as the E_h potential drops. This accounts for the two different types of curves representing reduction potentials.

Cannan, Cohen and Clark (Clark, Cohen and assoc. 1928, X) obtained the curves shown in Fig. 4 representing the reduction potentials of a culture of *Bact. coli* and of *Bact. vulgare*, on the standard or E_h scale. The potential of the colon culture drops considerably

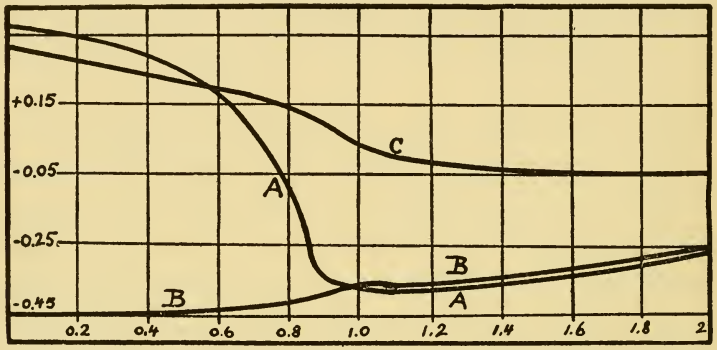


FIG. 4.—Reduction potential of *Bact. coli* (A); hydrogen potential of *Bact. coli* (B), and reduction potential of *Bact. vulgare* (C).

lower than that of the proteus culture. The time is plotted logarithmically. The third curve, *B*, is the potential of a hydrogen electrode in the colon culture. After ten hours (log 10 = 1.0), the reduction potential of the colon culture drops slightly below that of the hydrogen electrode. This is in agreement with Gillespie's data on p. 84. Hydrogen gas can be formed only if these two potentials are the same.

Several interesting potential-time curves are also given by Thornton and Hastings (1929a and b). Raw milk showed a potential between +0.200 and +0.300 volts when fresh, and was finally reduced to about −0.200 volts, probably by the action of streptococci (see Fig. 9).

The change of potential with time, with the medium, with aeration and with different species has been studied in some detail by Hewitt (1930a, b, c, d). *Streptococci* and *Pneumococci* produce peroxide; with the latter, aeration brings the potential above that of the sterile

medium. Neither of these two species maintain the reduction potential very long; it rises slowly in ordinary aerobic cultures. *Micr. pyogenes aureus* and *Corynebacterium diphtheriae* have a lower

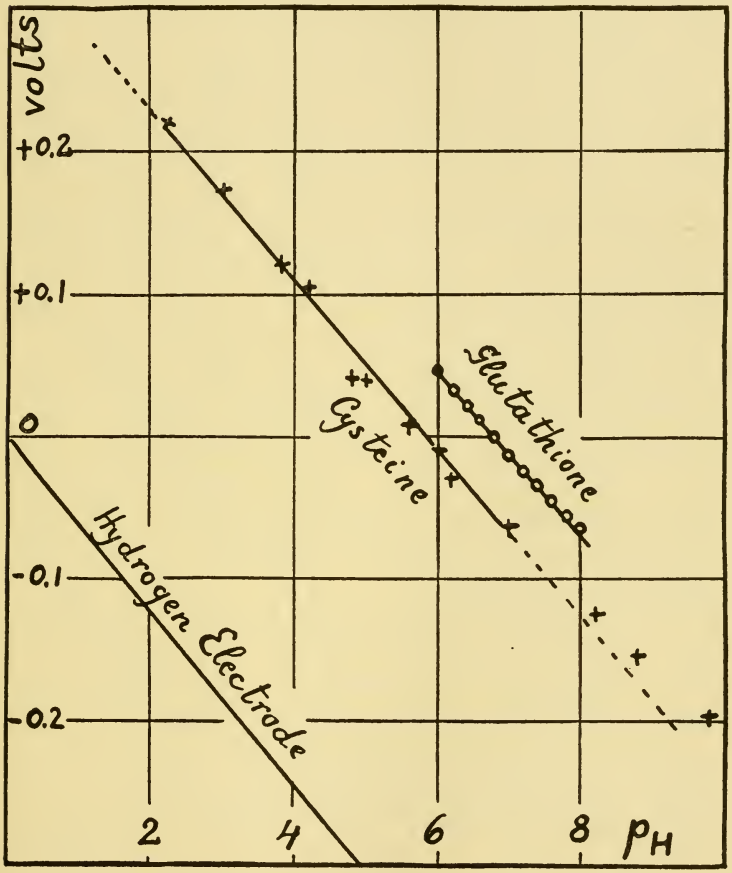


FIG. 5.—Relation between reduction potentials and acidity.

potential in aerobic cultures than in anaerobic. Perhaps they need oxygen to produce the energy required for the maintenance of the potential. With glucose, the potential is not as low as without. Serum prevents a low potential; it probably acts as an oxygen carrier.

(*b*) REDUCTION POTENTIAL AND ACIDITY

The interpretation of reduction potentials is made quite difficult and complicated by the fact that it varies with the hydrogen ion concentration.

This variation is not always regular. It depends upon the state of ionization in the system, and different systems are not affected in the same way by the same change in pH. The system $FeCl_2 - FeCl_3$ is practically uninfluenced by acidity. Ordinarily, however, the potential drops with increasing pH, i.e., with increasing alkalinity. This is especially true with the hydrogen and the oxygen electrode.

The system cysteine-cystine, which will interest us later, has been investigated by Dixon and Quastel (1923), and they found a simple rectilinear relation between potential and pH up to pH 9.5, as may be seen from Fig. 5. The same is true with glutathione though the range investigated by the authors is smaller. The effect of pH upon the potential of the hydrogen electrode is also indicated in this figure.

The relation between potential and pH is not always linear, as may be seen from Fig. 6, which represents the system methylene blue—methylene white taken from Clark, Cohen and associates (1928, VIII). A definite potential is required to change the blue dye to the colorless leuco-compound, but this potential varies greatly with the acidity of the solution. At pH 7, a potential equal to our arbitrary zero point is sufficient to turn the color, at pH 10 it requires a potential of -0.100 volts to do the same, while at pH 4, a potential of $+0.150$ volts would be sufficient.

This figure also shows short stretches of the potentials of the hydrogen electrode and the oxygen electrode and demonstrates the

linear relation of these potentials to pH. The hydrogen electrode indicates at what potential hydrogen can be formed. At pH 0, the potential 0 is sufficient; at pH 4.5, which is about the acidity in cultures of *Bact. coli*, the required potential for hydrogen liberation

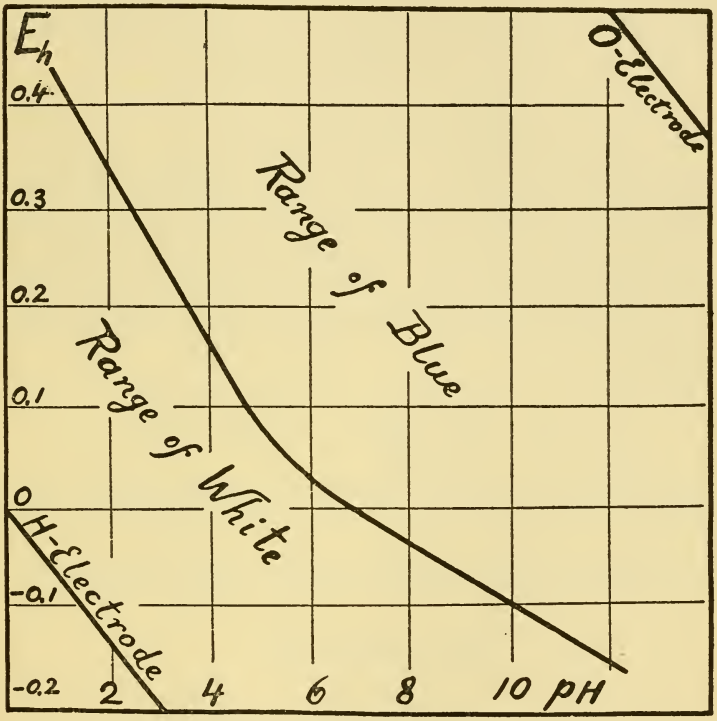


FIG. 6.—Reduction potential of methylene blue at different acidities.

is about −0.4 volts and in alkaline solutions, it becomes so strongly negative that hydrogen formation by alkali-forming bacteria appears very difficult.

The reduction potential as such, even if expressed in volts on the E_h scale, tells us very little about the reducing power if we do not also know the pH of the culture. This was the aim of Gillespie in determining the hydrogen electrode potentials (Table 11). The

hydrogen ion potential is probably the lowest potential a bacterial culture can reach, and the last column of Gillespie's table indicates how far removed the reduction intensity of the culture is from that of hydrogen. If the potential is measured in this way, it is independent of the pH, or rather, it is already corrected for pH.

The same principle was employed by Clark, Cohen and associates (1928, II) to elaborate the reduction potential, rH. This might be best illustrated graphically. In Fig. 5, the change of potential of the hydrogen electrode with pH is given. The hydrogen electrode corresponds to a hydrogen pressure of 1 atmosphere. If the pressure is less, the reducing tendency will be less, the oxidizing intensity will be stronger, and so we obtain, for lower hydrogen pressures, parallel lines above that for the hydrogen electrode. This pressure can be calculated from the formula for the potential of the hydrogen electrode:

$$E_h = -\frac{RT}{F} \ln \frac{\sqrt{P}}{[H^+]} = -\frac{RT}{0.434F} \log \frac{\sqrt{P}}{[H^+]}$$

where P is the hydrogen pressure, R the gas constant, F the Faraday constant, T the absolute temperature. For a constant temperature, the expression $\frac{RT}{0.434F}$ is constant, and for 30°C., it becomes 0.06.

$$E_h = -0.06 \log \frac{\sqrt{P}}{[H^+]}$$

$$= -0.06(\log \sqrt{P} - \log [H^+])$$

$$-\log [H^+] = \text{pH by definition}$$

$$E_h = -0.06(\log \sqrt{P} + \text{pH})$$

For pH = 0, we obtain

$$E_h = -0.06 \log \sqrt{P}$$

$$\frac{E_h}{0.06} = -\log \sqrt{P}$$

$$2\left(\frac{E_h}{0.06}\right) = -\log P = \log \frac{1}{P}$$

This formula makes it possible to express the reduction potential in terms of hydrogen pressure.

As an example, the cysteine potential (Fig. 5) is 0.36 volts above the hydrogen electrode (measured at pH 5). Assuming that the two curves are parallel up to pH 0, we have

$$\log \frac{1}{P} = 2\left(\frac{0.36}{0.06}\right) = 12 = r_H$$

$$P = 10^{-12} \text{ atm. pressure}$$

This figure seems quite meaningless. But the reduction potential rH is the negative logarithm of this number, in strict analogy to the definition of pH. Thus, the rH of the cysteine-cystine system is 12 or $\ln \frac{1}{P}$. The general formula for the computation of rH from measurements at any pH is derived from the above

$$E_h = -\frac{RT}{0.434F} \log \frac{\sqrt{P}}{[H^+]} = -\frac{RT}{0.434F}\left(\log \sqrt{P} - \log [H^+]\right)$$

$$= -\frac{RT}{0.434F}\left(\log \sqrt{P} + \text{pH}\right)$$

$$\frac{0.434\ E_h \cdot F}{RT} + \text{pH} = -\log \sqrt{P}$$

$$2\left(\frac{E_h F \cdot 0.434}{RT} + \text{pH}\right) = -\log P = \log \frac{1}{P} = \text{rH}$$

$$\text{rH} = 2\left(\frac{E_h \cdot 0.434F}{RT} + \text{pH}\right)$$

$$\text{for } 30°\text{C., rH} = 2\left(\frac{E_h}{0.06} + \text{pH}\right)$$

for pH 5, the cysteine potential is $E_h = +0.06$ volt

$$\text{rH} = 2\left(\frac{0.06}{0.06} + 5\right) = 12$$

The higher the rH, the lower is P, and the farther is the system removed from the reduction intensity of the hydrogen electrode.

The computation of rH is permissible only if the potential—pH curve is parallel to that of the hydrogen

electrode. It is nothing but an expression of the distance of the system from the hydrogen electrode. Therefore, no rH can be computed for methylene blue which does not go parallel to the hydrogen electrode, nor for ferrous-ferric systems. Clark, Cohen and associates (1928, supplement) warn against the general use of the rH because it may not always be useful, and may be even wrong.

(*c*) REDUCTION POTENTIAL INDICATORS

The electrometric measurement of reduction potentials requires a number of carefully adjusted instruments, and there are occasions, such as living protoplasm, where direct measurements could not be made at all.

It is a very old experience that bacteria have reducing properties; indigo, litmus and methylene blue are the best-known test substances for microbial reduction. But little attention had been paid to differences in the reduction of these compounds. The conception of reduction intensity as contrasted against reduction capacity was lacking then.

Clark, Cohen and various associates studied the reduction potentials of a number of dyes. They measured the potential of the dye in the completely reduced stage, and in the various stages of oxidation. It was found that the oxidation took place within a comparatively small range of potential. Decolorization occurred when about 70–95% of the dye is reduced. With different dyes, the relative position of the potential differed widely. The reduction potentials of a few of the tested indicators are shown in Fig. 7. It is seen that 50% of the methylene blue is reduced at an electrode potential of −0.002 volts. A bacterial culture which produces exactly this potential, will show a blue color

with methylene blue because only half of the dye is reduced. But if the potential goes down to −0.020 volts, the culture will be colorless. It would also reduce

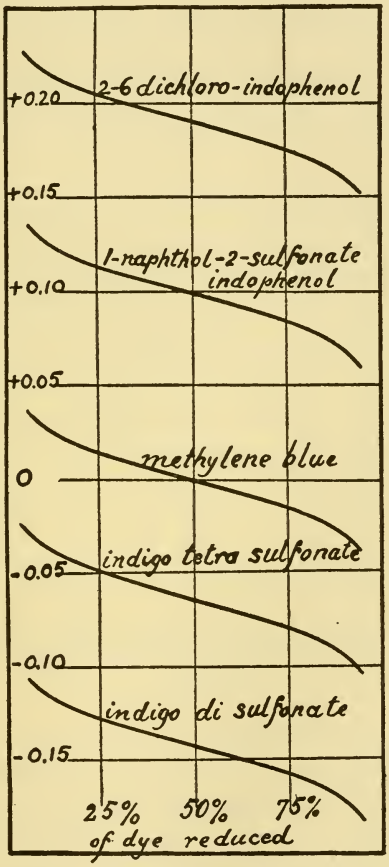


Fig. 7.—Reduction potentials of different indicators at pH 7.4.

dichloro-indophenol and naphthol-sulfonate indophenol, but would not change indigo tetra sulfonate nor disulfonate.

Thornton and Hastings (1929b) compared methylene blue with janus green *B* both of which have been recommended for the reductase test in milk, and found that janus green *B* indicates a potential about 0.1 volts lower than methylene blue. (See points *A* and *B* in Fig. 9.) They also found that methylene blue in milk was reduced at a potential higher than that given by Clark, Cohen and associates.

The indicators are greatly influenced by the acidity of the medium, as can be seen from Fig. 6 illustrating the influence of pH on methylene blue.

(*d*) BUFFERING OR POISING OF THE POTENTIAL

It has been stated before that the reduction potential measures the intensity, but not the quantity of reduction. The term quantity is now usually substituted by the

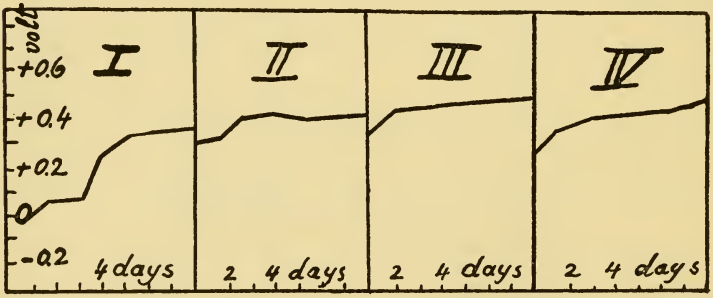


FIG. 8.—Differences in potential between 4 water-logged soils and a calomel electrode.

more significant term "capacity." A bacterial culture will not only show a certain intensity of reduction (which might be measured by reduction indicators) but it has a definite capacity for reduction. We can imagine a medium containing a large amount of a substance which acts as hydrogen acceptor at a E_h of about +0.05 volts or less. The potential of a bacterial culture will remain near this point until all of this compound is reduced, and then will fall to a lower potential. This corresponds

exactly to the buffers observed and used extensively in working with hydrogen ion concentrations. In reduction potentials it is spoken of usually as the "poising" effect.

The first example for such buffering or poising is probably that by Gillespie (1920) who measured the reduction potentials of various waterlogged soils. The

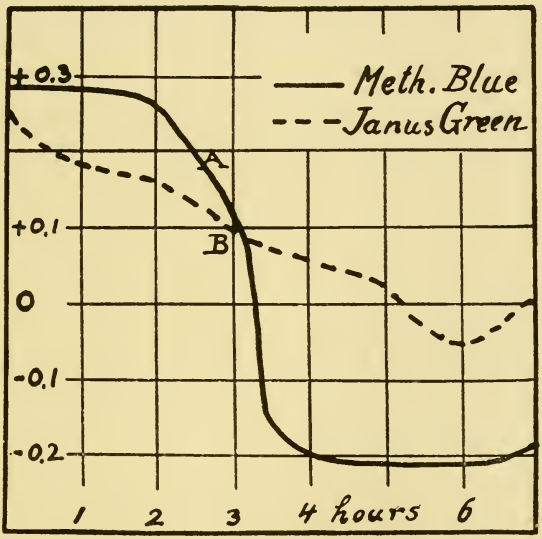


FIG. 9.—Reduction potential of raw milk with two different indicators.

results of the daily measurements for the calomel electrode as zero are shown graphically in Fig. 8. While the last three soils show a potential greatly differing from that of the calomel electrode, with a strong reducing intensity on the second day, the first soil remained much less reducing for four days, and on the fifth day was still less reducing than any of the others on the second day. Repeated experiments proved this to be a constant character of this one soil which is evidently well poised.

Another example of poising is shown in Fig. 9 which represents potentials in the same milk with two different dyes as measured by Thornton and Hastings (1929b). Methylene blue has only a negligible poising effect as was shown in other experiments. Janus green *B*, however, though it stimulated reduction at first, did not let the potential drop as suddenly as is the custom in milk, or in milk with methylene blue; it came down very slowly, and not to as low an end point as with methylene blue.

Cohen (1931) added substances of the type of potassium ferricyanide or benzoquinone which, after partial reduction by the bacteria, maintained a readily reversible oxidation-reduction system in the medium.

(*e*) THE FACTORS INVOLVED IN REDUCTION

All living protoplasm seems to have the power to effect chemical reductions, and this holds true also with bacterial cells, and consequently with bacterial cultures. With some of them, this power is very pronounced, as with anaerobic bacteria.

The power to reduce is identical with the power to oxidize, both being accomplished simultaneously by the transferring of hydrogen from one molecule to another. The reduced substance may eventually be the oxygen gas dissolved in the medium.

Aerobic bacteria will soon exhaust the oxygen dissolved in a medium, and the medium will then remain free from oxygen as long as the metabolism of the bacteria remains active, excepting a very thin surface layer into which the oxygen diffuses from the air (see p. 80). Many anaerobic bacteria can accomplish the same if they are present in sufficient quantity at the start to overcome the harmful effect of the oxygen.

Most culture media are reducing solutions, even when sterile. All organic compounds can be oxidized, and it might be expected that they all produce a reduction potential. Only reversibly oxidizable compounds can produce a measurable potential, however. Most organic compounds are quite stable; a sterile solution of sugar or of amino acid remains unchanged. This is only a false equilibrium, however, for Warburg (1921) could demonstrate that amino acids are slowly oxidized in aqueous solution at body temperature if Merck's blood charcoal is added, and Meyerhof and Weber (1923) could oxidize, in the same way, not glucose as such, but the glucose phosphate.

At the same time, Hopkins (1921) had found glutathione to be the cause of auto-oxidation in cells, and the *SH* group to be the essential part of the oxidizing property of this molecule. In the presence of glutathione, or cysteine, or thioglycollic acid (all possessing the *SH* group) organic matter, such as yeast juice, or other cell debris, takes up much more oxygen than corresponds to the amount of sulfhydryl-compound added. In other words, these compounds act as oxygen-catalysts, and overcome the false equilibrium of organic compounds in solution. Culture media are mixtures of many organic compounds, and it has been observed quite early (Th. Smith, 1896) that sterile broth will combine with oxygen, and will reduce methylene blue if protected from air. Coulter (1929) measured the reduction potential of a meat infusion broth (pH 7.6) electrometrically, and found that the potential in air was between +0.150 and +0.250 volts. When H_2O_2 was added, it rose to more than 0.400 volts. When nitrogen gas was bubbled through the broth to remove the oxygen, the potential gradually decreased (see Fig. 10) and in all of his experi-

ments with different specimens of broth, it approached a value between −0.050 and −0.060 volts. This is the potential of cysteine which is likely to be present in meat infusion in very small quantities.

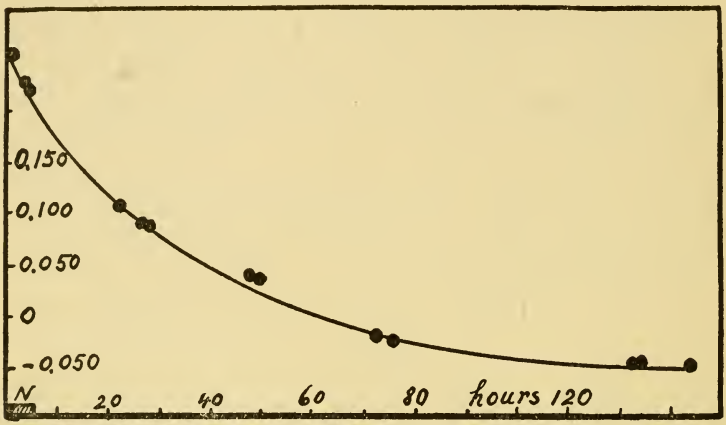


FIG. 10.—Gradual drop of potential in sterile broth after 5 hours of deaeration with nitrogen gas.

Dubos (1929) determined by means of indicators the reduction potential of meat infusion broth made with Fairchild's peptone, and he observed decolorization as far as indigo disulfonate (see Fig. 7) which means a potential lower than −0.125 volts. The quantity of dye that could be reduced was only 0.0017 molar. Thornton and Hastings (1929) found the potential of fresh milk to be between +0.200 and +0.300 volts. The final potential of deaerated milk is sufficiently low to decolorize methylene blue. The addition of cysteine brought the potential of raw milk from 0.2 to 0 volts in fifteen minutes, and to −0.2 volts in three hours. At this time, the check sample without cysteine had just started on the decrease, and had gone from +0.2 to +0.15 volts. The final potential of both samples was −0.2 volts, but this may have been due to bacteria and not to the milk as such.

Since each medium has a characteristic reduction potential which establishes itself some time after the removal of dissolved oxygen, and since all aerobic and

facultative microorganisms will remove dissolved oxygen from the medium in which they grow, the potential difference between a sterile medium and a culture in the same medium may be due only to the removal of oxygen which brings the natural reducing properties of the medium into play. Thus, Thornton and Hastings (1929) confirm the conclusion of Barthel that the reduction of methylene blue in the so-called reductase test of milk takes place in two stages: (1) the removal of dissolved oxygen by bacteria, (2) the reduction of the dye *by constituents of the milk.*

Only if the potential of a culture drops below that observed in the oxygen-free medium, are we justified in stating that we are dealing with a reduction potential characteristic for that microorganism.

It seems, from all these experiments, that the oxidation-reduction potential in bacterial cultures is comparatively simple. The cells of bacteria, as such, do not establish the potential. If cells are washed once and suspended in salt solution, they are still able to hold a distinct potential, but after repeated washing, it becomes indefinite. The potential must be produced by some metabolic products of the bacteria. It cannot be doubted that a definite potential is established inside of the cells, but this cannot be measured in the cultures.

An oxidation-reduction system must contain a reversibly oxidizable substance. This substance may act as a catalyst and transfer the oxygen to another substance which is irreversibly oxidized.

The reversibly oxidizable compound is represented by compounds like glutathione, cysteine, thioglycollic acid, or perhaps by a dye (all oxidation-reduction indicators being reversible) or even by an oxidizing enzyme. Methylene blue is a very active catalyst of cell respiration.

The *kind* of the compound determines the *potential.*

The *quantity* of this compound is one of the main factors in determining the *rate* of reduction.

The *reduction capacity* of the system is determined by the sum of the reversibly and the irreversibly oxidizable substances.

This might be illustrated by an experiment of Dubos (1929) who was the first to emphasize the rate of reduction as an essential factor. He measured the rate of methylene blue reduction by cysteine in vaseline-sealed tubes at 37°C.

Table 12.—Time in Minutes Required for the Reduction of Methylene Blue (0.0001%) by Cysteine

Concentration of cysteine	0%	0.01%	0.02%	0.03%	0.05%
Phosphate buffer (pH 7.5).	No reduction	Partial reduction	150	120	60
Phosphate buffer (pH 7.5) + 2% broth.	No reduction	Partial reduction	40	30	12
Phosphate buffer (pH 7.5) + 2% yeast extract.	No reduction	Partial reduction	40	25	12

The rate of reduction was determined by the quantity of cysteine, but also by the amount of irreversibly oxidizable organic compounds from broth and yeast extract.

The meaning of the reduction potential of a bacterial culture is, at the present stage of developments, rather uncertain. If a bacterium produces a potential lower than the final potential of the oxygen-free medium, it indicates primarily nothing but the presence of a cell product with a lower reduction potential.

Next comes the question: If the cell forms a compound which creates a potential of −0.100 volts in the medium, must this cell produce within its protoplasm this same or a still lower potential in order to produce such a compound? Must the reduction potential

within the cell of *Bact. coli* drop to −0.4 volts in order that hydrogen can be formed from sugar? If not, then the low reduction potential of *Bact. coli* does not really indicate that it has strongly reducing powers, but it is low because the bacteria form hydrogen, and the reduction electrode is simply changed to a hydrogen electrode by the gas formed. Or the potential −0.06 simply indicates that cysteine has been formed by the bacterium under test. There seems to be some reason for doubt as to what is the cause, and what, the effect.

The introduction of oxidation-reduction potentials, though not quite clear as yet in all its aspects, will influence quite considerably our conceptions of the mechanism of life, and of biological oxidations. It will prove useful to explain many reactions hitherto difficult to account for. It will tell us why certain reactions take place in the cells, and our conception of microbial activity will be shifted to a somewhat different basis.

(*f*) SUMMARY

The common culture media made from meat extract have a reduction potential of about +0.2 to +0.3 volt taking the normal hydrogen electrode potential as zero. Milk has a similar potential.

If these media are deaerated by vacuum or by a neutral gas, the potential drops below zero. They contain reducing compounds which come into play if the dissolved oxygen is removed from the medium. They will then decolorize methylene blue.

This reducing property of our common media might be caused by traces of glutathione, cysteine or similar compounds in the meat extract or milk.

Bacterial cultures will usually bring about a decrease in potential. There is not sufficient material on hand to say whether all bacteria will produce a potential lower than that of the deaerated sterile medium.

With some anaerobic and facultative bacteria, the potential is very low. With those organisms producing hydrogen gas, the reduction potential equals or approximates that of the hydrogen electrode at the same pH.

The reduction potential is quite meaningless unless we also know the pH of the culture.

V. RATE OF FERMENTATION

(*a*) METHODS OF MEASURING THE RATE OF FERMENTATION

The very rapid rate of multiplication of bacteria makes it difficult to perform experiments with a constant number of acting cells. The respiration of a leaf or the oxygen consumption of a mouse can be measured over a period of several hours without the organism changing considerably in size or weight during the observation. With bacteria, we may have a doubling of the active mass in less than half an hour. It requires special precautions, therefore, if the rate of fermentation, i.e. the amount of products per cell, or per gram of cells, or per gram of cell solids, is to be determined.

The choice of the unit is rather difficult to make. Physiologists working with larger organisms usually take the weight of the organism or tissue as the basis of measurement. Feeding experiments with animals are computed on the basis of kilogram body weight. Since this includes bones and fat, the proportions of which vary greatly, the basis is not really ideal, but it is more accurate than the basis of the single organism.

With microorganisms, the basis by weight would be possible, but rather difficult to ascertain. It is comparatively easy with yeast, but much more difficult with bacteria. The determination of the weight of bacterial cells is possible only if they can be filtered or centrifuged from their medium. It is impossible with cultures in

milk or blood or on solid media. In these cases, the single cell is the best unit. Since the determinations will always be averages of many billions of cells, individual differences will be eliminated entirely. The number might be ascertained by plate count, direct count, turbidity measurements or other methods.

A simple method for studying the rate of fermentation undisturbed by multiplication of cells is to use such large numbers of cells that they will not multiply. It is a rule to use more cells than will naturally develop in such medium. This method was suggested by Duclaux (1901, vol. IV p. 328), and applied by Rubner (1904), Slator (1906), and others. Rubner improved upon this method by studying the alcoholic fermentation with large quantities of yeast in pure sugar solution; since there was no nitrogenous food available, the yeast cells could not multiply to any appreciable degree. Slator (1906) found that the presence or absence of nitrogenous food did not influence the rate of fermentation. He measured the decrease of vacuum through CO_2 produced by the yeast; this method made it possible to determine the rate of fermentation in a very short time, five to twenty minutes. Slator's technique was later slightly changed by Rahn (1929) who measured pressure increase instead of vacuum decrease.

Another method which was used occasionally by Chassevant and Richet (quoted after Duclaux, 1901) was to add slight amounts of a weak disinfectant to the culture, sufficient to suppress growth, but not sufficient to retard fermentation. This method does not seem very advantageous because the disinfectant is likely to affect gradually the fermenting enzyme.

An entirely different principle is involved in the computation of the average number of acting cells by

considering their mode of multiplication. The formula for the "fermenting capacity" of the average single cell i.e., the amount of products formed per cell and per hour, is

$$s = \frac{2.301 \cdot \Delta S(\log b - \log a)}{\Delta t(b - a)}$$

where ΔS is the increase in products during the time interval under consideration, a the number of cells at the beginning, b the number of cells at the end of this interval, and Δt the time of the interval. The origin of this formula is developed in Appendix p. 404. This formula holds true only as long as the bacteria are multiplying rapidly. When the rate of growth decreases, the arithmetic average gives more nearly correct rates than the above formula (see p. 407).

(*b*) THE RATE OF FERMENTATION OF DIFFERENT ORGANISMS

The rate of fermentation is not constant during the entire development of a culture. The rate is fastest when the culture is young; with increasing age, the rate gradually decreases until it reaches zero. The causes will be discussed on p. 107. The rates of fermentation mentioned and compared here refer only to young, actively fermenting cells.

The rate of fermentation is of great practical importance in the bread yeast industry. Several different methods are used for determining the fermenting capacity for baking purposes. One of the old methods still in use is that of Kusserow where 10 gm. of yeast, 40 gm. of sugar and 400 c.c. of water are mixed, and where the developing CO_2 forces water out of a container; this water is measured; its volume corresponds to the CO_2 volume produced. The total gas formed in two hours

at 30°C. by a good compressed yeast varies from 570–660 c.c. (Henneberg, 1926, I). This corresponds to 30 c.c. of CO_2 per gram of yeast per hour, or about 120 mg. of CO_2 (or of alcohol) per gram per hour.

Henneberg gives also the following data for other types of yeast:

TABLE 13.—VOLUME OF CO_2 PRODUCED BY 10 GM. OF YEAST IN 400 C.C. OF A 10% SUCROSE SOLUTION

	Beer yeast, bottom yeast		Distiller's yeast		Wine yeast	
First half hour	55	70	60	60	63	38
Second half hour	210	100	160	260	153	142
Third half hour	400	200	280	310	340	330
Fourth half hour	400	260	280	280	324	408
Total	1065	630	780	910	880	918
Mg. CO_2 per gram yeast per hour	210	125	154	180	174	182

For further data with bread yeast see p. 238, Table 65.

Rubner's (1913) experiments give about 0.4 gm. of sugar fermented per gram of yeast per hour; this corresponds to 200 mg. of CO_2 per gram yeast per hour (see Table 15, p. 108).

From Rubner's data that 5 gm. yeast = 38,700,000,000 cells, we obtain a CO_2 production of 258×10^{-10} mg. or a sugar consumption of 510×10^{-10} mg. of sugar per cell per hour.

Another fermentation whose rate has been studied in some detail is the lactic fermentation. Rahn (1911) mentions a number of experiments by Marshall and Farrand (1908) from which he computed the fermenting capacity by the formula mentioned above. He also made experiments of his own. The data given in Rahn's

paper are here recalculated for the more correct Buchanan formula. More recent experiments by Baker, Brew and Conn (1919) are based on microscopic counts, and are, therefore, to be multiplied by 1.8 if they were to

TABLE 14.—FERMENTING CAPACITY OF LACTIC STREPTOCOCCI
(Mg. of acid formed per average single cell per hour)

Investigator	Number of experiments	Average fermenting capacity	Limits of fermenting capacity
Marshall & Farrand			
Strain 1	7	12.2×10^{-10} mg.	$9.1–20.7 \times 10^{-10}$ mg.
Strain 2	10	5.2	2.3–10.9
Strain 3	8	9.2	6.2–18.7
Strain 4	8	13.6	5.9–17.5
Rahn			
Dairy starter	8	23.4	10.4–34.7
Strain II	14	15.8	9.5–23.5
Strain IV	3	11.4	7.3–13.8
Baker, Brew, Conn			
Strept. lactis	6	8.8	6.55–11.4
Grand average	64	12.45	2.3–34.7

be compared with the others calculated from plate counts. This has not been done in Table 14.

This Table 14 shows that the average cell (by plate count) of *Strept. lactis* produces between 10 and 15×10^{-10} mg. of lactic acid per hour. This amount seems ridiculously small, but it is enormous if compared with the volume of the cell. The average cell of *Bact. coli* has been calculated to weigh about 8×10^{-10} mg. (see Appendix). The cells of streptococci are considerably smaller and cannot weigh more than 5×10^{-10} mg.

This means then that each cell produces two to three times its own weight of lactic acid per hour, or that each cell ferments two to three times its own weight of lactose every hour. This is a really enormous rate of fermentation, and is possible only by the enormous surface of the cells (50 square feet is the surface of the bacteria in 1 liter of a full grown culture, see Appendix) which allows an almost immediate

discharge of fermentation products into the medium (see also Slator and Sand, 1910, on the rate of diffusion into yeast cells).

We can further compute the rate of fermentation of *Lact. acidophilus* from the observation of Rahn (1929b) that 0.045% of lactic acid are formed per hour by 420,000,000 cells (microscopic count) of *Lact. acidophilus* per c.c. This amounts to 45 mg. produced by $42 \times 10^7 \times 10^2$ cells, or almost 10×10^{-10} mg. per cell per hour. The count probably included many dead cells, which would increase somewhat the fermenting capacity per viable cell.

Only one datum on another fermentation is known to the author, namely, the rate of urea fermentation by *Micr. ureae*. Burchard (1899) used a wrong formula to compute fermenting capacities, but his data show 66×10^{-10} mg. of urea per cell and hour, four to five times as much as the fermenting capacity of lactic streptococci.

SUMMARY

The rate of fermentation is known for only a few microorganisms. It is enormously high if compared with larger organisms. High-bred commercial yeasts (beer, wine or bread yeast) ferment about 30–40% of their own weight of sugar per hour. Bacteria being still smaller decompose still more food. *Lactob. acidophilus* uses at least 50% of its body weight of sugar per hour, the lactic streptococci about two to three times their own weight, and *Micr. ureae* at least five times its own weight of urea per hour.

(*c*) THE DECREASE OF THE RATE OF FERMENTATION

It is a general experience that fermentations come to end, sometimes even before all the fermentable substrate

is used up. There are various explanations for this gradual retardation and ultimate cessation of the rate of fermentation; it may be due to lack of substrate, or to toxic action of fermentation products, or to some biological change of the cells. While all these reasons might occasionally fit, the common cause of the decrease in rate ordinarily is either the accumulation of fermentation products, or the decrease of fermentable substrate.

TABLE 15.—ALCOHOLIC FERMENTATION OF SUGAR SOLUTIONS OF DIFFERENT CONCENTRATION BY 5 GM. OF YEAST

Sugar concentration	Per cent sugar					Fermentation constant $k = \frac{1}{t} \log \frac{2L}{2L - x}$			
	20 %	10 %	5 %	2.5 %	1.25 %	20 %	10 %	2.5 %	1.25 %
Hours									
2	1.88	1.69	1.83	1.50	0.98	0.0207	0.0187	0.0203	0.0165
4	3.33	3.32	3.29	1.97	1.17	0.0193	0.0192	0.0181	
6	4.63	4.64	4.18	2.16	1.24	0.0187	0.0187		
8	5.72	5.90	4.46	2.23	1.28	0.0179	0.0185		
10	6.68	7.01	4.55	2.31	1.30	0.0172	0.0183		
12	7.57	7.73	4.70	2.41	1.33	0.0168	0.0172		
14	8.34	8.34	4.80	2.48	1.35	0.0163	0.0163		
16	9.05	8.78	4.81	2.54	1.37	0.0160			
18	9.63	9.11	4.86	2.60	1.38	0.0154			
20	10.27	9.32	4.89	2.63	1.39	0.0152			
22	10.88	9.45	4.92	2.64		0.0151			
24	11.42	9.52	4.93			0.0144			
30	12.90	9.55				0.0119			
40	14.98					0.0144			
50	16.56					0.0145			
60	17.70					0.0147			
70	18.34					0.0142			
80	18.71	Average				0.0160	0.0181	0.0192	0.0165
90	18.80								

The concentration of the substrate must become very low before it exerts a retarding influence because the rate of fermentation is almost independent of the concentration of the substrate, as will be shown in the next subchapter, p. 114.

Some very good material on the gradual decrease of the rate of fermentation we owe to Rubner (1913). Rubner's original data are expressed in calories, as he studied the heat produced by yeast in sugar solutions free from nitrogenous materials. Rahn (1929b) computed from these data the amount of sugar fermented, and Table 15 shows one of these sets, where 5 gm. of yeast fermented 250 c.c. of sugar solution. This table shows the same yeast in sucrose solutions of 20%, 10%, 5%, 2.5% and 1.25%.

The retarding effect of alcohol upon the fermentation can be formulated definitely.

The simplest form would be the assumption that decrease in the rate is proportional to the amount of alcohol, or that the rate is proportional to the original rate decreased by a certain amount which is proportional to the alcohol already present. This might be written in the following way:

$$\text{Rate at any time} = \text{Initial Rate } (1 - k \times \text{alcohol concentration})$$

Since a fermentation comes to a stop at a fairly definite concentration, which is characteristic for the organism used, the alcohol concentration can be best expressed in terms of this limiting concentration which we will designate as L.

$$\text{Rate at any time} = \text{Initial Rate } \left(1 - \frac{\text{alcohol}}{L}\right)$$

If the alcohol concentration has reached the limiting concentration, the fraction $\frac{\text{alcohol}}{L}$ equals 1, and the rate of fermentation equals Initial Rate $\times$ (1 − 1) which is zero; the fermentation comes to a stop.

The rate of fermentation can be written in its mathematical form:

$$-\frac{dx}{dt} = k \cdot Y\left(1 - \frac{\text{alcohol}}{L}\right)$$

which means that the rate with which the sugar is decreased, is proportional to the amount of yeast Y, and is independent of the sugar concentration which, therefore, does not enter the equation at all, and is retarded by alcohol in the manner discussed above. Since one gm. of sugar yields practically one-half gm. of alcohol, we can substitute:

$$\text{alcohol} = \text{one-half of sugar, or}$$

$$\text{alcohol} = \frac{x}{2}$$

because x was the amount of sugar decomposed. Thus we get the equation

$$-\frac{dx}{dt} = k \cdot Y\left(1 - \frac{x}{2L}\right)$$

$$= \frac{k}{2L} Y(2L - x)$$

Y, the amount of yeast, is constant in Rubner's experiments because more yeast was added than would normally grow in such a solution. Therefore, the entire expression $\frac{k}{2L} \cdot Y$ is constant, and may be substituted by another constant, K. The equation

$$-\frac{dx}{dt} = K(2L - x)$$

gives upon integration

$$Kt = \ln\frac{2L}{2L - x}$$

The accuracy of this equation can be tested by computing the K-values for all the above data. The fermentation constants in the right section of Table 15 have been computed with the value $L = 10.2\%$ alcohol.

They express the rate of reaction per hour, corrected for the retarding effect of alcohol, and they should be constant during the entire course of the fermentation until the sugar concentration falls below 1.5%. This is not quite the case. The computed data show a slow but distinct decrease. Probably this is not due to the inaccuracy of the data on fermentation, but to a gradual deterioration of the zymase in the nitrogen-free sugar solution.

Other experiments by Rubner also show a fair agreement with this formulation (see Table 18). Rahn (1929b) verified it in a different way. He measured the rate of fermentation in the presence of increasing amounts of alcohol, using the CO_2 pressure as indicator. Table 16 shows the average pressures obtained in three experiments, and, further, the relative rates, based on the unretarded rate as 100.

TABLE 16.—RATES OF ALCOHOLIC FERMENTATION AS INFLUENCED BY ADDED ALCOHOL

Experiment II			Experiment III			Experiment IV		
Alcohol in solution, per cent	mm. mercury per minute	Relative rates	Alcohol in solution, per cent	mm. mercury per minute	Relative rates	Alcohol in solution, per cent	mm. mercury per minutes	Relative rates
0	6.58	100.0	0	8.20	100.0	0	6.10	100.0
4.93	3.95	60.0	9.7	2.41	29.4	4.77	4.28	70.4
7.40	2.52	38.3	12.15	1.10	13.4	9.55	1.48	24.3
9.86	1.79	27.2	13.35	1.23	15.0	11.92	0.96	15.7
12.5	0.47	7.1	.....	....	.....	13.12	0.87	14.3
						14.31	0.74	12.1

If the percentage decrease of rate of fermentation is proportional to the concentration of alcohol present, there must be a straight-line relationship between the

relative values. Figure 11 shows that this is actually the case until very high values are reached. It seems that from 12% alcohol upwards, there is a very slow yet noticeable rate of fermentation which is almost as high with 12% as with 14.5% alcohol.

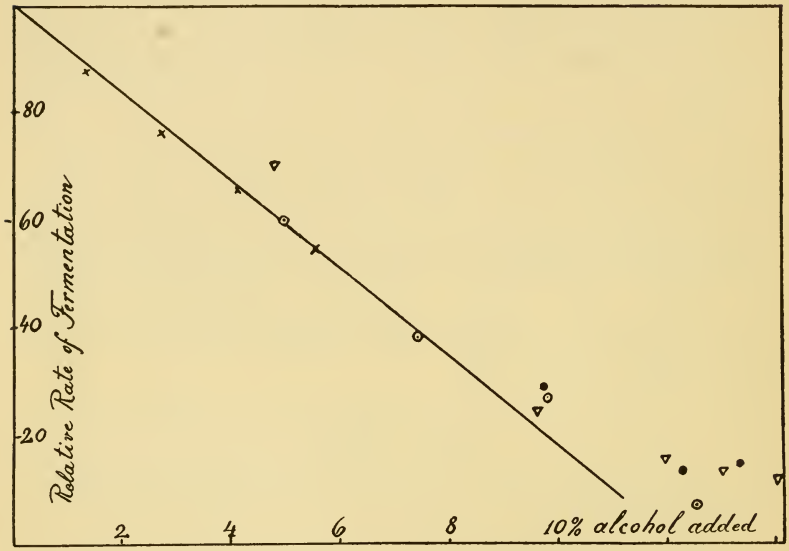


FIG. 11.—Rate of alcoholic fermentation in the presence of alcohol.

Several series of experiments on the lactic fermentation by Rahn (1911) and by Baker, Brew and Conn (1919) can also be used for a test of the general principle. Here again, the computed values are not absolutely constant, but are sufficiently close to support the principle involved. Rahn (1929b) repeated the experiment shown in Table 16 and Fig. 11 with *Lactob. acidophilus* and lactic acid. The results in Table 17 show the rate, i.e., the percentages of acid formed by 420,000,000 cells per c.c. If plotted against the amount of acid added, a straight line is obtained, as in Fig. 11. Even the

continued small amount of fermentation at very high concentrations is obvious.

TABLE 17.—RATE OF LACTIC FERMENTATION PER HOUR
(By 420,000,000 cells of *Lact. acidophilus* per cubic centimeter)

Initial acidity, per cent	Rate of fermentation in per cent lactic acid	Initial acidity, per cent	Rate of fermentation in per cent lactic acid
0.16	0.045	0.63	0.0065
0.25	0.0342	0.73	0.0054
0.35	0.0288	0.80	0.0018
0.44	0.0216	0.89	0
0.54	0.0162	0.90	0

SUMMARY OF FACTS

The rate of fermentation decreases with the age of the culture. If sufficient substrate is available, the decrease in the rate is caused by the fermentation products. Addition of these to a rapidly fermenting culture will decrease the rate of fermentation.

If the added fermentation products are as high as the limiting concentration, there still seems to take place a slow but noticeable fermentation provided that the number of fermenting cells is very large.

SUMMARY OF THEORIES

The retardation of a fermentation by its own products has been expressed in a simple formula on the assumption that the decrease of the rate of fermentation is proportional to the concentration of products divided by the limiting concentration; in other words, the decrease is proportional to the products already formed, expressed in terms of the total possible products.

This assumption leads to a formula for a "fermentation constant" representing the rate of fermentation corrected

for any retardation by its own products. The computation of this constant from available data on the alcoholic and lactic fermentations gave values of sufficient constancy to consider them a proof of the general principle involved.

(*d*) THE INFLUENCE OF CONCENTRATION OF MICROORGANISMS

It is generally understood that the fermentation produced in any medium is the sum of all fermentations going on in each individual cell. This assumption is the basis of the formula for the fermenting capacity (see p. 404).

A number of data are available to show such proportionality of cells and products. Slator (1906) found by his method (p. 103) the following relative rates of fermentation:

Relative yeast concentrations =
1 : 3 : 5 : 10 : 20 : 25

Relative rates of fermentation =
0.99 : 3.04 : 4.94 : 10 : 19.8 : 24.7

The same can be seen from Rubner's experiments in Table 18 showing the fermentation of nitrogen-free sugar solution by varying amounts of yeast. The fermentation constants, computed with the formula on p. 110, give the rate of fermentation corrected for retardation by alcohol. These rates show good proportionality with the quantities of yeast used.

(*e*) INFLUENCE OF THE CONCENTRATION OF SUBSTRATE

Considering that fermentation is brought about by enzymes in the cells, we should expect fermentation to

TABLE 18.—FERMENTATION BY DIFFERENT QUANTITIES OF YEAST

	Percentage of sugar fermented						
	20 per cent sucrose				10 per cent sucrose		
	8 gm. yeast	4 gm. yeast	2 gm. yeast	1 gm. yeast	10 gm. yeast	5 gm. yeast	1 gm. yeast
After 2 hours.....	1.60	0.80	0.25	0.03	1.61	1.09	0.46
After 4 hours.....	2.96	1.64	0.76	0.28	3.32	1.85	0.76
After 6 hours.....	4 12	2.30	1.20	0.48	4.64	2.58	0.98
After 8 hours.....	5.13	2.96	1.61	0.74	5.92	3.26	1.18
After 10 hours.....	6.08	3.59	2.03	0.99	7.03	3.92	1.38
After 12 hours.....	7.00	4.18	2.46	1.19	7.75	4.54	1.56
After 14 hours.....	7.86	4.75	2.84	1.40	8.35	5.10	1.73
After 16 hours.....	8.67	5.22	3.21	1.60	8.80	5.64	1.92
After 18 hours.....	9.47	5.76	3.57	1.80	9.12	6.12	2.09
After 20 hours.....	10.18	6.20	3.92	1.97	9.32	6.58	2.24
After 22 hours.....	10.80	6.62	4.22	2.12	9.45	7.01	2.39
After 24 hours.....	11.45	7.01	4.53	2.29	9.57	7.42	2.54
	Fermentation constants						
After 2 hours.....	0.0177	0.0087	0.0028	0.0002	0.0177	0.0118	0.0045
After 4 hours.....	0.0170	0.0092	0.0039	0.0014	0.0193	0.0102	0.0041
After 6 hours.....	0.0163	0.0087	0.0044	0.0016	0.0187	0.0104	0.0035
After 8 hours.....	0.0157	0.0085	0.0044	0.0020	0.0187	0.0094	0.0032
After 10 hours.....	0.0154	0.0084	0.0045	0.0021	0.0183	0.0092	0.0030
After 12 hours.....	0.0163	0.0083	0.0046	0.0022	0.0183	0.0091	0.0029
After 14 hours.....	0.0151	0.0082	0.0046	0.0022	0.0163	0.0089	0.0028
After 16 hours.....	0.0150	0.0081	0.0046	0.0022	0.0154	0.0087	0.0027
After 18 hours.....	0.0151	0.0080	0.0046	0.0022	0.0143	0.0086	0.0026
After 20 hours.....	0.0150	0.0078	0.0045	0.0022	0.0132	0.0085	0.0025
After 22 hours.....	0.0148	0.0077	0.0045	0.0021	0.0123	0.0083	0.0025
After 24 hours.....	0.0149	0.0076	0.0045	0.0021	0.0114	0.0082	0.0024
Averages.....	0.0157	0.0083	0.0043	0.0021	0.0182	0.0093	0.0031

follow the laws of enzyme action. There might be a possible deviation due to the time it takes the substrate to diffuse into the cell, and the products to diffuse out.

Slator and Sand (1910) have shown it to be very unlikely that conditions could be experimentally realized under which diffusion becomes a controlling factor of the rate of fermentation.

The influence of the concentration of the substrate upon enzyme activity is rather complex as may be seen from the discussion by Waldschmidt-Leitz (1929). There is no need of going into the details of this discussion because it concerns largely the rate at very low concentrations of the substrate, while in experiments with rates of fermentation, we are dealing mostly with fairly high concentrations. Under these conditions, the rate of enzyme action is almost independent of the substrate concentration. The enzyme combines rapidly with the substrate, and this compound decomposes but slowly; therefore, the concentration of the enzyme-substrate compound determines the rate of products formed. As long as there is enough substrate available to combine readily with any enzyme molecule that is set free, the concentration of the enzyme-substrate compound will be constant, and consequently the rate of enzyme action is constant.

Nelson and Vosburgh (1917) found the rate of invertase action constant if the sucrose concentration was above 5%. Northrup (1923) found the rate of trypsin action upon casein constant if the casein concentration was above 2%.

The rate of alcoholic fermentation has been found to be independent of the sugar concentration by Dumas, as early as 1874, and this result has been verified by a number of investigators (Literature, see Slator, 1906). More accurate measurements were made by Slator (1906) who found the following relative rates of fermentation at 30°C. by the same amount of yeast:

TABLE 19.—INFLUENCE OF SUGAR CONCENTRATION UPON THE RATE OF ALCOHOLIC FERMENTATION

Sugar concentration, per cent	Cm. mercury column in 10 minutes	Sugar concentration, per cent	Cm. mercury column in 10 minutes
0.16.....	2.9	4.0.....	5.5
0.28.....	4.05	5.0.....	5.4
0.52.....	4.7	8.0.....	5.05
0.66.....	5.1	12.0.....	5.05
1.00.....	4.8	20.0.....	4.4
2.00.....	5.2		

There is a certain degree of proportionality between the rate of fermentation and the sugar concentration

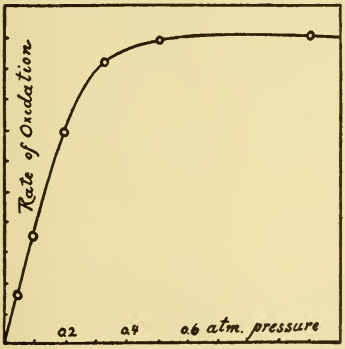


FIG. 12.—Influence of the concentration of oxygen upon the rate of oxidation of Nitrosomonas.

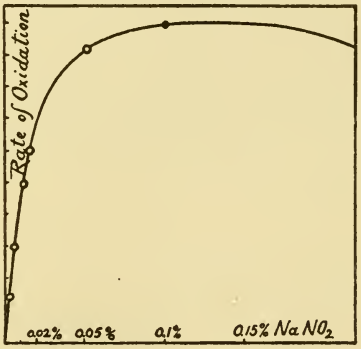


FIG. 13.—Influence of the concentration of nitrite upon the rate of oxidation of Nitrobacter.

when the concentration is low. Above 1.5%, the rate is constant and it decreases very slowly when the concentration goes above 5%.

Very nearly the same result was obtained by Meyerhof (1917) with a culture of Nitrosomonas by varying the oxygen concentration. The rate of oxidation was constant above 0.5 atmospheres of air pressure while below

0.3 atm., it seemed nearly proportional to the oxygen concentration. The results are shown graphically in Fig. 12. The influence of the nitrate concentration upon the rate of oxidation by Nitrobacter, measured by the same author, is shown in Fig. 13. The curves are nearly alike, and agree well with those from Slator's experiments.

A good verification of the same principle can also be found in Table 15. The sugar concentration was varied while the yeast concentration was constant. The amount of sugar fermented was the same in all concentrations until so much had been fermented that less than 1.5% of sugar remained. This moment is indicated in the table by a cross line.

No corresponding data for other fermentations have been found in the literature by the author. The unpublished experiments by Peltier which are mentioned in more detail on p. 207 bring out the same independence as regards the lactic fermentation. Peltier studied the lactic fermentation in milk diluted with varying amounts of water, the highest concentration being twice that of normal milk. From the number of cells and the amounts of acid formed, he computed the fermenting capacity per cell. The following data were obtained:

Milk concentration	2	1	0.5	0.25	0.125	0.062
Lactose concentration	10%	5%	2.5%	1.25%	0.6%	0.3%
Fermenting capacity 12–24 hrs.	4.7	5.4	4.2	6.5	1.9	1.6
Fermenting capacity 24–36 hrs.	2.7	3.7	4.0	2.4	2.8	2.5
Average	3.7	4.6	5.1	4.5	2.4	2.1

The result is the same as in the alcoholic fermentation: the rate of fermentation is fairly constant above 1% of lactose, but decreases as the sugar concentration falls below 1%.

SUMMARY

The rate of fermentation depends upon the concentration of the substrate only if this concentration is low.

In solutions containing more than 1% of sugar, the rate of alcoholic and of lactic fermentation is practically independent of the sugar concentration. The rate of oxidation of ammonia by Nitrosomonas is also constant if the oxygen pressure is more than one-half of that of the atmosphere, and the rate of nitrite oxidation by Nitrobacter is constant above 0.1% nitrite.

(*f*) INFLUENCE OF TEMPERATURE ON THE RATE OF FERMENTATION

1. INFLUENCE OF TEMPERATURE UPON CHEMICAL PROCESSES

Those chemical processes of which the rate is measurably slow, are accelerated by an increase in temperature. The rate of the processes is increased, and the rate of increase is called the temperature coefficient, usually designated by the letter Q. It is obtained by dividing the rates of reaction measured at different temperatures, K_1 at T, and K_2 at $T + \Delta T$:

$$Q_{\Delta T} = \frac{K_2}{K_1}$$

For most chemical processes, at temperatures of biological activity, this coefficient for 10°C. increase, Q_{10}, lies between 2 and 3.

van t'Hoff formulated this temperature relation

$$K_1 = K_0 e^{\frac{A(T_1 - T_0)}{T_1 T_0}}$$

where T_1 and T_0 are *absolute* temperatures (degrees Centigrade +273). This shows that the temperature coefficient is an exponential function, and this must be taken into consideration when Q_{10} is computed from any temperature intervals other than 10°C.

The following reasoning will demonstrate the principle: Assumed that the rate of a process is increased by the coefficient Q_1 for 1°C. increase.

At 20°C..... rate = K_0
At 21°C..... rate = K_0Q_1
At 22°C..... rate = $(K_0Q_1)Q_1 = K_0Q_1^2$
At 23°C..... rate = $K_0Q_1^3$
At 30°C..... rate = $K_0Q_1^{10}$

The temperature coefficient for 10°C. increase equals the temperature coefficient for 1°C. increase raised to the 10th power.

$$Q_{10} = Q_1^{10}$$

For any temperature interval ΔT, Q_{10} can be found from the formula

$$Q_{10} = \sqrt[\Delta T]{\left(\frac{K_{T+\Delta T}}{K_T}\right)^{10}}$$

The van t'Hoff formula shows that the temperature coefficient is not really constant. The expression

$$\frac{T_1 - T_0}{T_1 T_0}$$

will be different at different temperatures. If $Q_{10} = 2.0$ at 20 to 30°C., it must change as follows:

Q_{10} between 0 and 10°C. = 2.218; $A = 6153$; $\mu = 12306$
20 and 30°C. = 2.000; $A = 6153$; $\mu = 12306$
40 and 50°C. = 1.838; $A = 6153$; $\mu = 12306$
90 and 100°C. = 1.575; $A = 6153$; $\mu = 12306$

In fact, at the absolute zero, at −273°C., Q_{10} = infinite, and at very high temperatures, Q_{10} approaches 1.

The van t'Hoff equation may be written in the form of a differential equation:

$$\frac{d \ln K}{dT} = -\frac{\mu}{2T_2}$$

Upon integration between the limits T_1 and T_2, the van t'Hoff formula is obtained, with $\mu = 2A$. The general integration yields the equation

$$\ln K = -\frac{\mu}{2T} + C$$

which means that the rate is a logarithmic function of the reciprocal of temperature, or the logarithm of the rate is a linear function of the reciprocal of temperature. Fig. 14 shows the rate of alcoholic fermentation to be a simple logarithmic function of $\frac{1}{T}$, according to data by Aberson (1903).

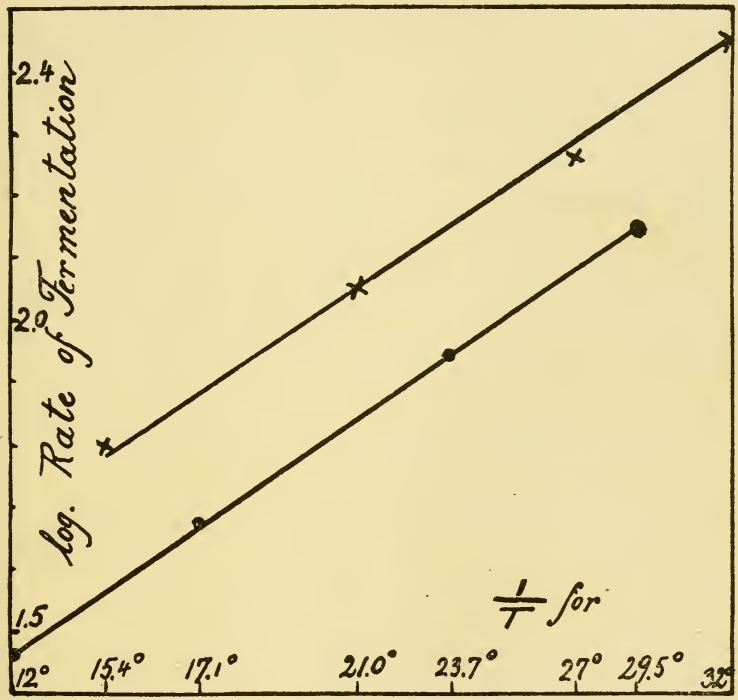


FIG. 14.—Relation between the rate of alcoholic fermentation and the reciprocal of the absolute temperature.

In biological literature, the statement is frequently found that biochemical processes of organisms, such as motility, respiration, growth, etc., follow the same temperature laws as chemical processes. This statement has its very great limitations as the following reasoning will readily show: *Strept. thermophilus* will

produce measurable amounts of acid at +10°C., but not at 0°C. The temperature coefficient of fermentation at this point equals the rate at 10°C. divided by zero, which is infinite. The same organism will produce acid at 45°C., but no more at 55°C. The temperature coefficient at this point is zero divided by the rate at 45°C. which is zero. Thus, in the very narrow range between 0 and 55°C., the temperature coefficient of fermentation varies from infinite to zero. It is unavoidable that in this range, there will be a point where it is between 2 and 3 as in normal chemical reactions, but this enormous range is anything but a resemblance to that found in chemical processes.

That the temperature coefficient of fermentation really increases with decreasing temperature, is shown by Table 20 and Fig. 15 representing the data obtained by Slator (1906) with different yeasts. The curve for

TABLE 20.—TEMPERATURE COEFFICIENTS OF ALCOHOLIC FERMENTATION BY DIFFERENT YEASTS AT DIFFERENT TEMPERATURES

	5°C. temperature coefficients				Average Q_{10}
	Brewery yeast	Distilling yeast	Wine yeast	Average Q_5	
5–10°C.	2.65	2.50	2.30	2.50	6.25
10–15°C.	2.11	1.97	1.85	1.98	3.92
15–20°C.	1.80	1.98	1.96	1.91	3.64
20–25°C.	1.57	1.62	1.62	1.60	2.56
25–30°C.	1.43	1.47	1.41	1.44	2.07
30–35°C.	1.35	1.36	1.33	1.35	1.81
35–40°C.	1.20	1.26	1.24	1.23	1.51

Q_{10} shows that extrapolation to lower temperatures must lead to infinity while extrapolation to higher temperatures must lead to zero. A similar situation

will be found in the relations between growth and temperature.

Fermentation is a chemical process, however, and if it does not follow the temperature laws of chemical processes, the reason for it must be found. It seems

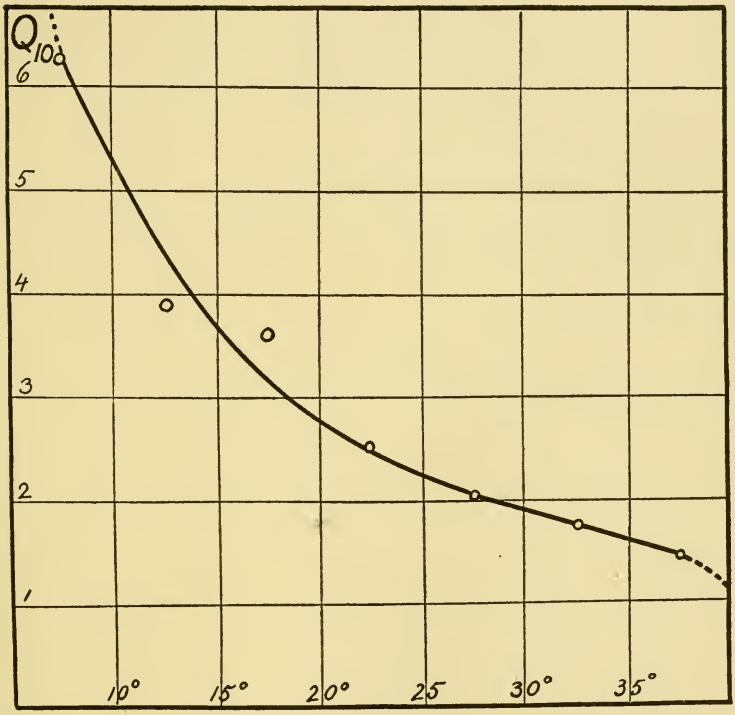


FIG. 15.—Variation of the temperature coefficient of alcoholic fermentation with temperature.

simplest to assume that the general law really applies here as well, but that above the optimum temperature and near the minimum temperature, a superposition by other processes causes the observed deviations from the rule.

2. INFLUENCE OF TEMPERATURE UPON ENZYME ACTION

The cause of the deviation at high temperatures is known. It has been expressed in physico-chemical terms by Tammann (1895) who studied the temperature relations of the action of emulsion upon salicin. He pointed out that increase of temperature not only increases the rate of enzyme action, but also, and to a much greater degree, the enzyme deterioration. The temperature coefficients are 1.26 for enzyme action and 6.36 for enzyme deterioration.

The consequence is that at high temperatures, we have a more rapid decomposition of salicin as long as the enzyme is not destroyed. But with increasing tempera-

TABLE 21.—PERCENTAGE OF ACTIVE ENZYMES AT DIFFERENT TIMES AND TEMPERATURES

Temperature..	25°C.	35°C.	45°C.	55°C.	65°C.	75°C.
$K_{65°}$.....	1.84	1.84	1.84	1.84	1.84	1.84
B.....	20,000	20,000	20,000	20,000	20,000	20,000
t = ½ hrs....	99.92	99.28	94.74	69.18	10.67	t = 1 min. 66.70
1 hrs....	99.84	98.61	89.76	47.85	1.14	2 min. 44.49
2 hrs....	99.68	97.24	80.58	22.90	0.01	5 min. 13.20
3 hrs....	99.52	95.89	72.33	10.96	0	10 min. 1.74
4 hrs....	99.36	94.56	64.92	5.24		20 min. 0.03
5 hrs....	99.20	93.04	58.25	2.51		
10 hrs....	98.42	86.96	33.96	0.0006		
20 hrs....	96.86	75.62	11.54	0		
30 hrs....	95.33	65.75	3.92			
40 hrs....	93.82	57.18	1.33			
50 hrs....	92.33	48.59	0.45			

tures, this destruction will occur more and more rapidly as shown in Table 21 until at 65°C., 90% of the

enzyme is destroyed in half an hour, and at 75°C.; 98% is destroyed in ten minutes. The quicker rate of action, combined with the more rapid rate of destruction, produces the result shown in Table 22 and in Fig. 16 which is a plaster model of the first five hours of Table 22.

Table 22.—Amount of Salicin Decomposed by Emulsin at Various Times and Temperatures, in Percentages

Hours	Amounts calculated					
	25°C.	35°C.	45°C.	55°C.	65°C.	75°C.
½	6.91	8.89	10.46	**14.30**	6.35	0.0002
1	13.41	16.45	**21.18**	17.92	7.22	0.0002
2	25.02	30.01	**33.49**	25.31	7.29	0.0002
3	35.08	41.22	**44.01**	28.62	7.29	0.0002
4	43.79	50.51	**53.13**	30.15	7.29	0.0002
5	51.33	**59.34**	58.39	30.86	7.29	0.0002
10	75.88	**81.48**	75.00	31.52	7.29	0.0002
20	94.08	**95.72**	84.40	31.52	7.29	0.0002
30	98.50	**98.81**	86.70	31.52	7.29	0.0002
40	**99.99**	99.75	87.40	31.52	7.29	0.0002
50	99.99	99.98	87.64	31.52	7.29	0.0002
∞	100.00	100.00	87.76	31.52	7.29	0.0002

Hours	Amounts found by Tammann				
1	13	.....	**18**	.....	11
3	32	.....	**51**	.....	16
5	58	.....	**61**	.....	19
8	65	.....	**68**	.....	21
12	**76**	.....	73	.....	23
26	**91**	.....	84	.....	28
50	**98**	.....	89	.....	30
74	**99**	.....	92	.....	31

The physico-chemical equations are given by Tammann as follows:

The rate of decomposition of the substrate (salicin) is proportional to the concentration of unchanged substrate $(a - x)$, and also proportional to the concentration of the enzyme, E.

$$-\frac{dx}{dt} = k \cdot E(a - x)$$

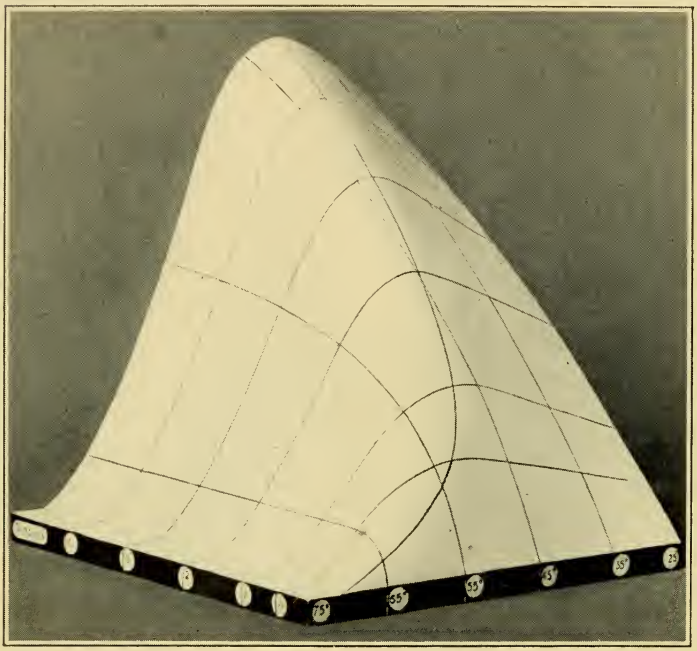


FIG. 16.—Influence of temperature and time upon enzyme action; the percentage of salicin decomposed by emulsin at different temperatures during the first 5 hours.

This equation is correct for any constant temperature. If the temperature changes, the general law remains unchanged, only k reveals itself as a function of the temperature. This is expressed by the van t'Hoff rule

$$k = k_0 e^{A\frac{T - T_0}{T \cdot T_0}}$$

when k_0 is the reaction constant at the standard temperature T_0, recorded as absolute temperature. T is the experimental temperature, A is a constant characterizing the process involved.

Thus, the equation:

$$-\frac{dx}{dt} = k_0 e^{A\frac{T-T_0}{TT_0}} E(a-x)$$

expresses the rate of enzyme action at any temperature.
For simpler calculation, we substitute

$$q = e^{\frac{A(T-T_0)}{T \cdot T_0}}$$

$$k = k_0 q$$

$$-\frac{dx}{dt} = k_0 q E(a-x)$$

This would be a simple formula if the amount of enzyme remained constant during the experiment. But this is not the case. Enzymes are unstable compounds, readily decomposed in watery solution, and, therefore, decrease during the experiment. In most cases, their deterioration follows the monomolecular reaction

$$-\frac{dy}{dt} = K \cdot (b-y)$$

where b is the original enzyme concentration, y the amount of enzyme decomposed. Here again, K is a function of the temperature, following the same law, but with a different constant.

$$K = K_0 e^{B\frac{T-T_0}{TT_0}}$$

For easier calculation, we introduce again the temperature coefficient.

$$Q = e^{B\frac{T-T_0}{TT_0}}$$

$$K = K_0 Q$$

$$-\frac{dy}{dt} = K_0 Q(b-y)$$

Integrated, this equation changes to

$$K_0Qt = \ln \frac{b}{b - y}$$

$$\frac{b}{b - y} = e^{K_0Qt}$$

$$\frac{b - y}{b} = e^{-K_0Qt}$$

$$b - y = b \cdot e^{-K_0Qt}$$

The expression $b - y$ is the amount of enzyme still active after the time t, and is the same as the letter E in the first equation.

Table 21 gives the calculated amounts of enzyme which remain active after exposure to different temperatures for different times. The data $K_{65°} = 1.84$ and $B = 20{,}000$ are taken from the experimental data of Tammann.

Substituting this value for E in the equation representing the rate of enzyme action, we obtain

$$-\frac{dx}{dt} = k_0qE(a - x) = k_0qbe^{-K_0Q't}(a - x)$$

Upon integration, the amount of salicin decomposed at any time and temperature is found to be

$$x = a\left[1 - e^{\frac{k_0q}{K_0Q}b(1 - e^{-KQt})}\right]$$

This expression has been used by Rahn (1915) for the computation of the data in Table 22 which also gives the experimental data obtained by Tammann.

Tammann explains the disagreement between calculated and measured amounts at 65°C. by the protective action of the substrate upon the enzyme. The E-values of the formula have been calculated from data obtained by heating emulsin in water. In the presence of salicin, the emulsin is more resistant. That is the reason why the calculated amounts are a little too low, even at 45°C. The principle remains unaltered by the protective action of the substrate.

Computation of the temperature coefficients reveals $Q_{10} = 1.26$ for enzyme action, and $Q_{10} = 6.36$ for enzyme deterioration. Raising

the temperature from 25 to 65°C., i.e. 40°C., would result in a rate of enzyme action $1.26^4 = 2.52$ times as rapid. The same increase in temperature would cause the rate of enzyme deterioration at 65°C. to be $6.36^4 = 1{,}640$ times as high as at 25°C. Only 10.67% of the enzyme would be left intact after half an hour's exposure to 65°C. The increased rate of enzyme action is of no avail at very high temperatures because there is no longer any enzyme. At 45 and 55°C., where the enzyme destruction is not quite so rapid, the result of the increased rate of action becomes very noticeable. But as all enzyme is destroyed before all salicin is decomposed, the reaction never becomes complete. This is accomplished only at the lowest temperatures.

As a result of this interlinking of two opposite effects, the optimum temperature of enzyme action is not definite. At one-half hour, the salicin is most rapidly decomposed at 55°C.; after two hours, 45°C. is plainly the optimum; after ten hours, 35°C. shows the largest amount of decomposed salicin; and after forty hours, the lowest temperature appears to be the best. This is also shown in Fig. 16. The heavy ascending line indicates the optimal rate at different temperatures. It is seen to shift towards the lowest temperature with increasing time. This is a very characteristic property of the Tammann principle.

The high temperature coefficient of deterioration is the cause of the thermolability of enzymes. For most enzymes, this coefficient is still higher than with emulsin. Arrhenius (1907) gives the following values for Q_{10}:

Table 23.—Temperature Coefficients of Enzymes

	Of enzyme action	Of enzyme destruction
Rennet	$Q_{10} = 2.61$	$Q_{10} = 65.8$
Trypsin	1.61	17.8
Pepsin	2.06	33.7

The decrease of optimal enzyme action with time is very apparent also in the action of pressed yeast juice upon sugar. The following table shows two experiments

TABLE 24.—INFLUENCE OF TEMPERATURE UPON FERMENTATION BY PRESSED JUICE OF YEAST
(Total CO_2 in grams)

Time, days	5–7°C.	12–14°C.	22°C.	28–30°C.	35–37°C.
1.....	0.09	0.25	0.56	**0.71**	0.53
2.....	0.18	0.49	**0.93**	0.76	0.57
3.....	0.26	0.70	**1.03**	0.80	0.58
4.....	0.31	0.85	**1.06**	0.82	0.60
6.....	0.49	**1.11**	....	0.86	0.64
8.....	0.62	**1.20**	....	0.88	0.64
10.....	0.75	**1.22**			
14.....	0.91				
16.....	0.97				
1.....	0.06	0.21	0.41	**0.44**	0.34
2.....	0.13	0.42	**0.59**	0.47	0.35
3.....	0.19	0.57	**0.62**	0.47	0.36
4.....	0.29	**0.65**	0.63	0.49	0.37
6.....	0.39	**0.71**	0.63	0.49	0.39
8.....	0.48				
10.....	0.58				

by Buchner, Buchner and Hahn (1903) in which the change of the optimum from 30°C. during the first day to 12–14°C. after six days is very distinct.

INFLUENCE OF TEMPERATURE UPON FERMENTATION BY LIVING CELLS

The result of Table 24 that the optimum temperature of fermentation by pressed juice of yeast approaches 12°C. seems to be contradictory to the old established fact that living yeast ferments best near 30°C. The necessary conclusion must be drawn from this dis-

crepancy that the temperature law of enzymes does not apply to living cells.

All enzymes deteriorate, even at low temperatures. Endogenous catabolism in all living cells (p. 16) is almost certainly due in part to deterioration of enzymes in the cell. In normally nourished cells, deterioration probably takes place at the same rate and by the same processes as outside, but new enzyme is being formed continuously to keep a fairly constant enzyme concentration in the cell.

Deterioration will proceed with greater speed at higher temperatures, and as far as enzymes are concerned, we know that their temperature coefficient of deterioration is enormously high. The production of new enzyme in the cell will also be accelerated by a temperature increase, but its temperature coefficient will be normal, i.e., low. In a fermenting liquid heated slowly, the enzymes deteriorate more rapidly, and are likewise being formed more rapidly. There must always be a temperature at which the originally very slow rate of deterioration overtakes the rate of reconstruction.

Heating above this temperature must cause a gradual decrease of the enzyme content of the cell. The higher it rises, the greater will be the difference between the rate of deterioration and the rate of reconstruction, the quicker will be the decrease of enzyme in the cell. If the rate of deterioration is only slightly greater than that of reconstruction, the cell may come to a constant enzyme concentration below normal, but if the difference is considerable, the enzyme will soon be completely deteriorated. Because of the great difference in temperature coefficients, an increase of only a few degrees will change a rapidly fermenting culture into one that has lost the power of fermentation altogether.

As long as the enzyme content of the cell remains constant, the increased temperature will cause an increased rate of fermentation corresponding to the normal temperature coefficient of the enzyme action. The highest temperature at which the production of enzyme is still equal to its deterioration, is the optimum for fermentation. Beyond this point, the enzyme content of the cell must gradually decrease and the result is the Tammann principle, i.e. the shifting with time of the optimum from high to lower temperatures. In fermentation by living organisms, the optimum does not shift to the lowest temperature, but to the optimum temperature at which the rate of fermentation remains constant.

The Tammann principle at temperatures above the optimum can be seen in Table 25, which presents some unpublished results by the author, and shows the CO_2 pressure produced by bakers' yeast in pure sugar solution. The result is not very clear because the rate of fermentation increased during the first hour. But the gradual shifting of the optimum temperature from 44° to 32.5°C. is quite conspicuous.

TABLE 25.—RATES OF FERMENTATION AT SUPER-OPTIMAL TEMPERATURES

(mm. of mercury pressure produced by yeast in sucrose solution in 5 minutes)

Average rates	29°C.	32.5°C.	35.5°C.	40°C.	44°C.
From 5–30 min	27.7	33.2	38.2	51.4	**55.7**
From 60–70 min	32.0	39.5	40.5	**57.0**	56.0
From 185–200 min	34.0	42.7	50.0	**56.7**	55.3
From 360–385 min	32.0	40.6	47.4	**50.4**	44.8
From 540–565 min	31.2	42.2	48.2	**50.2**	33.8
After 22 hours	33.0	**41.6**	31.0	11.4	0

It is also worth noting that at 29°C. and 32.5°C., the rate of fermentation remains really constant during 22 hours, and does not decrease as in the case of zymase (p. 130). This constancy of the optimum temperature after the Tammann principle has been overcome by time, differentiates the fermentation by living cells from that of a mere enzyme action.

Below the optimum, the temperature coefficient is normal. From the rates at 29°C. and 32.5°C. in the above table, we obtain $Q_{10} = 1.98$. Rubner (1913) found lower values, 1.40 and 1.58, between 23°C. and 38°C. because the latter temperature is already too high. Aberson (1903) observed an average coefficient of 2.72 between 12°C. and 30°C. Slator's data (1906) have already been given in Table 20, p. 122.

The assumption has been made above that the temperature coefficient of fermentation is that of a normal chemical reaction except for a superposition of another process near the maximum and the minimum temperatures. This assumption seems justified as far as supra-optimal temperatures are concerned.

No good explanation has as yet been given for the existence of a minimum temperature. It is considered an established fact that with most organisms, fermentation ceases at a temperature well above the freezing point. This might be a wrong conclusion, however. If no fermentation products can be found in a medium inoculated in the ordinary way, and held at low temperature, it is proof only that the bacteria did not grow. The number of transferred cells is too small to produce sufficient fermentation products to be detected in the ordinary way.

The minimum temperature of fermentation can be established only if very large numbers of cells are trans-

ferred to the cold medium. The author has tested the minimum temperature of fermentation of streptococci by transfering the centrifuged bacteria from 250 c.c. of a full-grown lactose broth culture to 150 c.c. of sterile milk, after both milk and bacteria had been cooled to the temperature of the experiment. The acid produced by these bacteria is given in Table 26.

TABLE 26.—LACTIC ACID PRODUCED IN MILK BY LARGE NUMBERS OF CELLS

(*Strept. cremoris* No. 23)

Days.....	0	3	4	7	10	14	21	26	34
At −1°C...	0	0.05%	0.06%	0.07%	0.08%	0.09%	0.09%	0.07%	0.08%
At +5.5°C.	0	0.27	0.37						
At +10°C.	0	0.42	.....	0.55	0.54	0.56	0.60	0.59	0.58

(*Strept. cremoris* No. 18)

Days.....	0	3	6	10	17	22	31	45	59	73
At −1°C.....	0	0.05	0.07	0.08	0.10	0.10	0.10			
At +5.5°C.....	0	0.19	0.34	0.44	0.48	0.49	0.43	0.49	0.495	0.51

(*Strept. lactis*)

Days.....	0	9	14	23	37	51	65
At −1°C.....	0	0.07	0.09	0.11	0.13		
At +5°C.....	0	0.24	0.30	0.33	0.40	0.40	0.39
At +10°C.....	0	0.54	0.55	0.54	0.55	moldy	

All three streptococci produced acid at +5.5°C. and even at −1°C. *Strept. lactis* and *Strept. cremoris* 23 multiplied at 5.5°C. if inoculated into milk in small numbers, at the rate of about one generation in from twenty-four to forty-eight hours; *Strept. cremoris* 18 did not multiply.

At −1°C., all strains decreased slowly in plate count (see p. 224). From the first period of observation, the temperature coefficients Q_{10} were found:

	Between −1°C. and 5.5°C.	Between 5.5°C. and 10°C.
Strept. cremoris 23.....	13.2	2.7
Strept. cremoris 18.....	6.9	
Strept. lactis.....	5.7	6.1

The experiment was discontinued on account of a breakdown of the lowest incubator. The few experiments are not sufficient to draw conclusions regarding the cause of the minimum temperature. They suffice entirely, however, to demonstrate that fermentation will take place at temperatures considerably below the minimum for growth. Whether these results can be generalized so far as to state that fermentation takes place at all temperatures until the freezing of the medium, seems very doubtful.

An explanation may perhaps develop from Crozier's (1924) analysis of the temperature relations of biological oxidations. He demonstrated graphically, by plotting rates against the reciprocal of absolute temperature (see p. 121) that at different points, different processes may dominate the rate of life functions. The critical thermal increment $\mu = 2A$ for biological oxidations is frequently 11,500 above 15°C., and 16,100 or 16,700 at the lower temperatures. The reduction of methylene blue by *Bact. coli* in the presence of succinic acid shows $\mu = 16{,}700$ (Quastel and Whetham, 1924).

For the alcoholic fermentation, the μ-values are quite different. There is apparently no oxidation connected with alcoholic fermentation. Slator's data, according to Crozier's computation, show $\mu = 12{,}250$ above 21°C., and $\mu = 22{,}200$ below 21°C. which means a higher temperature coefficient at the lower temperature. Why this is the case, and why 21°C. is the critical temperature, is still unknown.

No other data on rates of fermentation are known which could be used for a similar analysis.

SUMMARY OF FACTS

The rate of enzyme action is accelerated by an increase of temperature. At high temperatures, the deterioration of the enzyme becomes also an important factor.

The optimum temperature of enzyme action is nothing definite; it shifts gradually, as the experiment is prolonged, to lower temperatures. This holds true for all isolated enzymes, including yeast juice.

For living cells, this holds true only above the optimum temperature. At and below this point, the rate of fermentation is constant.

Fermentation will take place at very low temperatures where growth has ceased altogether. Large numbers of cells must be used to prove this.

With some organisms, e.g., with thermophilic bacteria, it does not seem probable that fermentation will occur at very low temperatures, though no experiments have been made.

SUMMARY OF THEORIES

The temperature coefficient of the rate of enzyme action is normal. It is independent of that of enzyme deterioration.

The temperature coefficient of enzyme deterioration is much higher than normal.

The simultaneous action of these two processes brings about the complex of symptoms briefly described as "Tammann principle," i.e., high initial rates of enzyme action at very high temperatures rapidly decrease with time and soon come to an end; slower rates at medium temperatures, lasting longer, decrease more slowly, but give

an incomplete decomposition; quite slow rates at low temperatures decrease but very little, and bring about a practically complete decomposition.

In living cells, the deterioration of the enzyme in the cell is counteracted by the cell's faculty to produce new enzyme. The normal cell works with a constant enzyme concentration.

In such a cell, an increase of temperature will increase the rate of fermentation, without any later decrease, until a point is reached where the enzyme deteriorates more rapidly than the cell can replace it. Then, the Tammann principle becomes evident.

The existence of a true minimum temperature, i.e., a temperature above the freezing point of the medium below which no fermentation can take place, has not as yet been proved. Fermentation will continue at temperatures considerably below the growth minimum.

(*g*) CHEMICAL STIMULATION AND INHIBITION OF FERMENTATION

It is quite generally claimed and has been proved in many instances that some poisons in very small quantities act as stimulants to certain life functions. In 1915, Rahn offered an explanation for this by an analogy between the actions of temperature and of chemical agents.

In the case of enzyme action, we may assume that the toxic compound acts as a catalyst accelerating the action of an enzyme as well as enzyme deterioration. Enzyme action (see p. 126) may be expressed as

$$-\frac{dx}{dt} = kE(a - x)$$

and under the influence of the catalyst whose concentration may be given as c, the acceleration could be represented by c^n where n may

vary for each enzyme and each chemical compound, but is constant under a given set of conditions. Thus we get

$$-\frac{dx}{dt} = k \cdot E(a - x)(1 + c^n)$$

At the same time, the deterioration of the enzyme is increased by the catalyst at the rate of c^m. The deterioration is given by the equation

$$-\frac{dy}{dt} = k'(b - y)$$

and, in the presence of the poison, or catalyst,

$$-\frac{dy}{dt} = k'(b - y)(1 + c^m)$$

$b - y$ is the amount of enzyme present at any given time, it is the same as the expression E in the first equation. By substituting the value for E from the last equation in the second, we have a mathematical formulation which will essentially work out like the Tammann principle. There appears a false optimum, shifting with time from higher to lower concentrations, and an increased rate of reaction immediately after the poison begins to act, followed by a more rapid destruction of the enzyme, which, ultimately, leads to complete inactivation.

No data sufficiently complete to try the mathematical treatment are known, but the stimulation of enzyme action by poisons is known and the shifting of the optimum with time from the higher to the lower concentrations of the poison has also been established in some cases. A very striking one is the addition of arsenate to zymase (Buchner, Buchner and Hahn).

The respiration of mold mycelium seems to be readily stimulated by chemicals. Watterson (1904) observed the CO_2-production by *Aspergillus niger* to rise from 439 to 868.5 mg. and from 885.4 to 1,138.2 mg. by addition of 0.004% of zinc sulfate to the medium. Gustafson

TABLE 27.—INFLUENCE OF ARSENIC UPON THE FERMENTATION BY ZYMASE
(Total CO_2 in grams)

Experiment No.	I		II		III	
Arsen added	0	2%	0	2%	0	2%
After 16 hours	0.50	0.89	0.16	0.35	0.16	0.34
After 24 hours	0.73	0.95	0.24	0.37	0.24	0.36
After 40 hours	1.15	0.99	0.42	0.38	0.42	0.36
After 64 hours	1.73	1.01	0.73	0.41	0.73	0.38

(1920) found the rate of respiration of Penicillium to be constant between pH 8 and 4. If the acidity was increased beyond pH 4, the rate of respiration increased at once, but dropped soon below normal. As the acidity increases, the time to drop below normal becomes shorter and shorter. Though Gustafson gives only curves and no data, there is an indication of the Tammann principle.

A. Koch (1912) found decided stimulation of the yeast fermentation by ether. The Tammann principle could not be established with certainty. In some experiments, the flasks with the largest doses of ether gradually gained in fermentation, and finally fermented most rapidly. Koch believed that the yeast became gradually accustomed to the ether, but it may well be that part of the ether had evaporated, and thus the concentration of the stimulant approached the optimum concentration.

Stimulation of the rate of alcoholic fermentation by using metal vats instead of wooden ones has been claimed repeatedly. Zikes (1913) found that the CO_2 produced by 1,000,000 cells was 0.748 in aluminum vessels against 0.700 in glass vessels.

A large number of experiments on stimulation of alcoholic fermentation by poisons has been published by Branham (1929). These data do not show the Tammann principle. Perhaps the time intervals chosen in these experiments were not appropriate to bring forth this effect.

SUMMARY

The action of enzymes, zymases and living cells can be stimulated by certain poisons, i.e., by compounds which, in slightly higher concentrations, would be distinctly toxic.

In some instances, a shifting of the optimum concentration for stimulation with time is quite evident, as in the Tammann principle. It seems possible to explain chemical stimulation on the same basis as temperature effects, assuming the poison to act as a catalyst for enzyme action as well as for enzyme deterioration.

VI. THE ENDPOINT OF FERMENTATION

(*a*) CHEMICAL CONSIDERATIONS

All fermentation in a given volume of fermentable solution will ultimately come to an end. This endpoint is an important and usually quite definite property of a culture. It is caused by one of two possibilities; either the fermentable substrate is exhausted, or the accumulation of products prevents any further enzyme action (or possibly enzyme regeneration). In most fermentations, we can bring about at will the first or second type of endpoint, by varying the concentration of the fermentable material. It still remains to be shown what happens in the case where all fermentation products are volatile, as in complete oxidation of lactic acid by *Mycoderma*.

In the first case, if fermentation ceases through lack of fermentable material, there is not much to be said concerning the endpoint. It will be in proportion to the amount of fermentable material present, and we can easily compute the maximum amount of fermentation products if we know the initial concentration of the substrate. We know, that from 1 gm. of sugar, we cannot possibly expect more than 0.49 gm. CO_2 in an alcoholic fermentation. The few experiments in dilute solutions with which the writer is familiar, indicate that the amount of CO_2 or alcohol is considerably less than should be expected, and this is probably due to the utilization of a comparatively large amount of fermentable material for cell construction in a poor medium. This may not be the case, however, if an abundance of non-fermentable construction material is supplied while the fermentable material is scarce.

More important and more interesting is the question of the endpoint of fermentation when an excess of fermentable material is present, and the fermentation

TABLE 28.—INFLUENCE OF THE SUGAR CONCENTRATION UPON THE ENDPOINT OF FERMENTATION OF STREPTOCOCCI IN PEPTONE SOLUTION
(Numbers mean per cents of lactic acid formed)

Organism	Glucose concentration					
	0.5%	1.0%	2.0%	5.0%	10.0%	15.0%
Strept. lactis 4	0.41	0.41	0.41	0.38	0.38	0.34
Strept. faecium 8	0.41	0.38	0.38	0.36	0.34	0.27
Strept. liquefaciens 1	0.43	0.45	0.43	0.38	0.37	0.32
Strept. mastitidis 3	0.34	0.34	0.34	0.34	0.20	0.14
Strept. cremoris 1	0.18	0.20	0.20	0.23	0.21	0.18

ceases before all of it is consumed. Since we are dealing with enzyme reactions it seems logical to discuss first

the general laws of the endpoint of enzyme action. A brief review will soon convince us, however, that the material on hand is of little help. Even though, on general principles, enzyme reactions are considered incomplete, the endpoint of the action of most enzymes is reached only when the decomposition is practically complete. In the case of maltase and lipase, we have true reversible action. However, in the fermentation processes, there is no reversion known. It seems quite impossible that streptococci produce dextrose from an excess of lactic acid, or that yeast produce sugar from an excess of alcohol and CO_2. It is probably safe to assume that the process is irreversible.

This assumption that the mass law does not enter into consideration is well substantiated by the fact that a certain strain of *Strept. lactis* will produce 0.4% of lactic acid in broth and no more, regardless of whether the nutrient medium contains, 2, 5, 10 or 20% of lactose (see Table 28 by Orla-Jensen, 1919). According to the mass law, an increase of substrate should always bring about an increase in the products, because the equilibrium would require the coefficient: $\frac{[\text{substrate}]}{[\text{products}]}$ or $\frac{[\text{substrate}]}{[\text{products}]^n}$ to be constant. It is not necessary, therefore, to consider the inhibition of the alcoholic fermentation by alcohol from any different chemical view point than the inhibition by phenol or other disinfectants.

The proof that a fermentation is stopped by the accumulation of its products has been given in some instances by the observation that fermentation starts anew if the harmful products are removed. It is generally known that acid forming organisms will produce more acid if the medium is neutralized.

TABLE 29.—ACID FORMATION IN NEUTRALIZED MILK CULTURES OF *Strept. lactis*

Times neutralized	Strain II					Strain IV				
	Acidity after neutralization	Final acidity	After, hours	Increase in acidity	Fermenting capacity	Acidity after neutralization	Final acidity	After, hours	Increase in acidity	Fermenting capacity
Fresh	0.146	0.695	24	0.549		0.140	0.806	?	0.666	
Once	0.128	0.680	24	0.549	1.65×10^{-10}	0.113	0.459	29	0.346	0.73×10^{-10}
Twice	0.176	0.630	24	0.445	0.86×10^{-10}	0.142	0.452	42	0.310	0.48×10^{-10}
3 times	0.196	0.633	43	0.436	0.72×10^{-10}	0.126	0.358	96	0.232	0.36×10^{-10}
4 times	0.178	0.638	72	0.460	0.38×10^{-10}	0.103	0.128	29	0.025	1.48×10^{-10}
5 times	0.198	0.470	43	0.275	0.44×10^{-10}					
6 times	0.050	0.347	48	0.297	0.31×10^{-10}					
7 times	0.238	0.380	48	0.142	0.17×10^{-10}					
8 times	0.212	0.275	24	0.063	0.26×10^{-10}					
Total acid formed				3.216	.....	.....	.....	..	1.581	

Rahn (1911) neutralized a milk culture of *Strept. lactis* repeatedly until the acid formation became very weak. The results given in Table 29 show a gradual decrease of the fermenting capacity. The plate count showed a decrease with Strain II from about 2,200 million cells to 1,700 million after the seventh neutralization, and to 820 million after the eighth neutralization. With Strain IV, the bacteria even dropped to 40 million after the fourth neutralization. The fermenting capacities are not altogether comparable because some of them had to be computed from a period of forty-eight hours, while most of them are given for a twenty-four hour period. The last datum of each series is high because it is based on the rapidly declining plate count, and it is quite probable that many cells which have lost the power to make colonies will still continue to ferment. This has been observed by Rubner with yeast (p. 13).

By both strains, fermentation is resumed rapidly after each neutralization (see also Table 62, p. 232 for Strain II), but upon repeated neutralization, they lose gradually the power to ferment, Strain IV much faster than Strain II. The total acid formed shows, by a simple calculation, that not all of the lactose can have been fermented. At least 1% must have remained in the medium.

The limiting factor in fresh cultures is the hydrogen ion concentration. In a strongly buffered medium, such as milk, much more lactic acid is formed before the limiting pH is reached than in a medium with less buffer, such as whey or lactose broth. The titratable acidity of these latter cultures is, therefore, much lower than that of milk cultures, though the pH is the same.

Van Dam (1922) and Holwerda (1921) proved, later, that the undissociated lactic acid molecules also have an inhibiting effect. If lactates are added to milk cultures, the fermentation stops at a definite concentration of undissociated lactic acid, and not at a constant pH. This is the explanation of the decreasing rate of fermentation after repeated neutralization.

Rogers and Whittier (1928) have confirmed these explanations. They grew pure cultures of *Strept. lactis*

in skim milk to which varying amounts of sodium lactate had been added, and the fermentation stopped at a different pH, but at the same concentration of undissociated lactic acid (see Table 30), as Van Dam had shown.

Table 30.—Effect of Added Sodium Lactate on the Lactic Fermentation in Skim Milk

Sodium lactate added		Final lactate concentration, in mols	Final pH	Final hydrogen ion concentration	Final concentration of undissociated lactic acid, in mols
%	Mols				
0	0	0.0723	4.25	5.62×10^{-5}	0.0171
0.26	0.0236	0.0958	4.40	3.98	0.0171
0.53	0.0473	0.1183	4.48	3.31	0.0181
0.79	0.0709	0.1416	4.59	2.57	0.0172
1.06	0.0945	0.1625	4.65	2.24	0.0172
1.32	0.1181	0.1829	4.74	1.82	0.0165

The conclusion is that the accumulation of a certain product of fermentation will prevent the zymase of the cells from further action. When this product is removed, fermentation will go on until some other product limits it. What will happen when this is removed also, we cannot say since no means have been found as yet to remove undissociated acid from the culture without killing the bacteria.

The neutralization method is used in the commercial manufacture of lactic acid and of other acids, e.g., butyric acid. The buffer action of casein is of great importance in the ripening process of cheese to prevent too high a hydrogen ion concentration which would interfere with ripening. All manipulations to remove the whey are merely a means to control the ratio of casein:lactose; this ratio determines the ripening process.

No studies on the endpoints of other fermentations are known to the author. It is probable that in the urea fermentation, the accumulation of hydroxyl ions is the primary limiting factor, but there is no real experimental evidence for this. Nothing at all is known about the endpoints of the fermentation of proteins.

(*b*) CONSTANCY OF THE ENDPOINT

Bacteriologists assume in their every-day technique that the final concentration of fermentation products of a given culture is fairly uniform under uniform conditions. This is shown by the fact that we use the amounts of acid or of gas formed by microorganisms as a means of classification. It is generally admitted, however, that this endpoint varies with the cultural conditions, especially with the temperature, and also more or less with the medium.

Different species and varieties of yeast are characterized by the quantities of alcohol they produce. The streptococci were divided by Ayers, Johnson and Davis (1918) into high acid producers (pH less than 5) which are mostly non-pathogenic, and into low acid producers (pH = 5.4 to 6) which were largely pathogenic. In the colon group, the methyl red test is a measure of final acidity, and the customary determination of the amount of gas formed is also proof of the assumption that the endpoint of fermentation is something quite definite. This also pertains to the distinction of streptococci by the amounts of CO_2 and volatile acids formed (Hucker, 1928a and b).

(*c*) INFLUENCE OF CONCENTRATION OF CELLS

Coming back once more to the relation of fermentation to enzyme action, we will have to consider the question: Does the endpoint depend upon the amount of enzyme present? According to all the data with zymase, the percentage of fermented sugar increases with the amount of zymase. But whether or not this is true also with

living cells in which the enzyme can be regenerated can be proved only by direct experiment. Such experiments were carried out by the author with compressed yeast, as a simple source of large quantities of cells.

Different amounts of yeast were placed in flasks containing 100 c.c. of a raisin decoction to which 25% sucrose and 2% dried yeast were added. These flasks were closed by a fermentation valve sealed with concentrated H_2SO_4 which permitted the CO_2, but not the moisture, to escape. These flasks were weighed daily. The loss in weight is carbon dioxide. After the weight had become constant, the alcohol was determined by the density of the distillate.

The results of two such experiments computed for 100 c.c. of moisture are shown in Table 31. Starting from the lowest yeast concentration, the amounts of alcohol and carbon dioxide are constant until 0.6 gm. yeast is reached. This indicates that the smaller amounts of yeast will multiply to about 0.6 gm. When more yeast was added, the amounts of alcohol and CO_2 produced increased very distinctly.

TABLE 31.—ALCOHOL AND CARBON DIOXIDE FORMED IN 100 C.C. OF SUGAR SOLUTION BY VARYING AMOUNTS OF YEAST

	Grams yeast added	0.02	0.04	0.08	0.16	0.31	0.62	1.25	2.5	5.0	10.0	20.0
I	Alcohol.....	11.75	lost	11.80	11.75	12.45	lost	lost	12.60	13.35	13.35	
	CO_2.....	11.04	10.95	11.01	11.04	11.14	11.04	11.56	12.02	12.44	11.91	
II	Alcohol.....	.....	12.35	12.00	11.95	12.90	13.10	13.02	13.62	13.73	13.90	10.35
	CO_2.....	.....	10.92	11.03	10.99	11.17	11.38	11.60	12.05	12.63	12.45	11.83

The same result can be obtained with lactic streptococci. A culture of *Strept. glycerinaceus* was grown for twenty-four hours in a well-buffered lactose broth, and centrifugated after neutralization. The bacteria from 1,000 c.c. of culture were put into 100 c.c. of sterile

milk. No growth could occur in this very concentrated cell suspension. To have a normally growing culture as a check, 1 c.c. of this suspension was transferred to sterile milk. Parallel experiments were made with milk + 1% peptone.

TABLE 32.—ACID FORMATION IN MILK CULTURES OF *Strept. glycerinaceus* BY VARYING AMOUNTS OF BACTERIA

	Medium	Cells per c.c.		C.c. n/10 NaOH for 10 c.c.		pH (144 hours)
		At start	At end	At start	After 120 hours	
Exp. I	Milk	3,705,000,000	.....	2.15	7.3	4.69
	Peptone milk	4,260,000,000	.....	2.85	8.5	4.93
	Milk	37,000,000	.....	2.15	6.6	4.88
	Peptone milk	42,600,000	.....	2.85	7.8	4.87
Exp. II	Milk	7,310,000,000	.....	2.60	8.77	4.59
	Peptone milk	3,820,000,000	.....	3.35	9.20	4.72
	Milk	24,000,000	560,000,000	2.60	6.90	4.84
	Peptone milk	13,000,000	1,640,000,000	3.35	8.77	4.76

The results of two experiments are given in Table 32. The milk fermented by abnormally large numbers of cells shows more acid than the milk in which the bacteria developed normally. This holds for titrated acid as well as for pH. In peptone milk, this difference in acidities is almost negligible.

The reason is to be found in the final plate counts of Experiment II. With peptone, bacteria grow to much higher numbers than in plain milk. The cell count of the heavily seeded peptone milk was only twice as high as the final count of the peptone milk culture with small seeding (3,820 against 1,640 millions). In plain milk, the difference was much larger, 7,310 against

560 millions, or 13 to 1. While a doubling of the cell concentration may not bring about much effect, an increase more than ten-fold causes a distinct rising of the endpoint of fermentation.

TABLE 33.—ACID FORMATION IN MILK WITH AND WITHOUT PEPTONE BY DIFFERENT STREPTOCOCCI

Strept.	Peptone added	Titrated acidity after				pH after 3 days	Microscopic count in millions	
		1 day	2 days	3 days	6 days		24 hrs.	48 hrs.
glycerinaceus...	0	4.4	5.7	6.3	7.0	4.903	517	640
	0.1%	6.1	7.3	7.8	7.6	4.666	677	722
	1%	7.75	8.5	8.3	8.8	4.691	661	750
fecalis (human)	0	6.5	8.9	9.8	10.8	4.742	837	
	1%	5.6	8.0	8.9	9.3	5.021	925	
fecalis	0	5.8	7.25	7.7	8.0	4.618	785	700
	1%	9.1	9.1	8.6	8.9	4.615	1,177	845
bovis	0	4.45	5.7	7.0	8.8	4.635	565	617
	1%	10.1	10.9	11.1	11.4	4.113	1,187	1,275
lactis	0	6.3	7.2	7.8	8.1	4.514	565	
	1%	8.9	9.6	9.7	9.7	4.070	702	

This better growth of streptococci in milk with peptone accounts for the higher acidity generally observed in peptone milk. Rahn (1911) described a strain II, where addition of peptone changed neither the acidity nor the number of cells, while with strain IV, the number of cells was increased four to five-fold by peptone, and the titrated acidity was doubled.

The above table with different species of streptococci verifies all previous data. *Strept. fecalis* (human)

was a rapid liquifier, and the peptone seemed to exert a retarding influence. *Strept. bovis* showed the greatest gain in growth, as well as in titratable and potential acidity.

This experiment was repeated with *Lact. acidophilus*, but no difference in the final acidities could be observed whether 0.026 gm. or 5.000 gm. of bacteria were added to the milk. It is not permissible, therefore, to generalize from the above results.

The former results are not without analogy in the realm of enzyme action. It has already been mentioned that a concentrated solution of zymase (yeast juice) will yield a higher final alcohol concentration than a diluted one. The same is true with urease; a larger amount of urea will be changed to ammonium carbonate by a larger amount of urease.

This increased amount of fermentation products by an increased number of cells can not be accounted for by the assumption of an equilibrium. If we assume, however, that the limiting concentration of fermentation products prevents only the regeneration of the zymases by the cell, but not altogether their action, then a slow fermentation will continue for some time after this point has been reached. With large amounts of enzyme, it will take considerable time before all of it has deteriorated, and during this time, a slow fermentation will take place. A small amount of enzyme at the start, however, will soon be completely inactivated, and fermentation comes to an early stop at a comparatively low concentration of products.

This explanation is just an attempt to account for the facts, without any safe biological backing. Still, it would also account for the very slow fermentation which continues for a while when the limiting amount of fermentation products, or even a little more, has been added to a concentrated suspension of active cells (p. 112).

SUMMARY

In some fermentations, the final amount of fermentation products increases with the number of cells

present in the medium. This was the case with bread yeast and with several streptococci.

The higher acidity produced in milk by many streptococci when peptone is added, can be explained by the larger number of cells growing in peptone milk.

With *Lactob. acidophilus*, this phenomenon could not be observed.

(*g*) INFLUENCE OF TEMPERATURE UPON THE ENDPOINT OF FERMENTATION

The final concentration of fermentation products is, to a considerable degree, influenced by the temperature. A very simple explanation is usually offered for this observation: the toxic effect of the product upon the zymase being a chemical reaction, its effect is increased by a rise of temperature and thus causes a lower endpoint. Several experiments show this decrease of the final concentration of products by an increase in temperature. Müller-Thurgau (1885) gave the following endpoints for the fermentation by a wine yeast:

TABLE 34.—FINAL ALCOHOL CONCENTRATIONS BY *Saccharomyces ellipsoideus*

At 36°C	3.8% alcohol
27°C	7.5%
18°C	8.8%
9°C	9.5%

Schierbeck (1900) observed a similar difference with the lactic fermentation at 20°C. and 35°C.

The temperature relation is not quite so simple, however. According to a table in Marshall, Microbiology (1912 p. 273), lactic streptococci do not produce the maximum acidity at the lowest temperature; there is a decrease in final acidity at temperatures near the minimum of growth. This agrees entirely with the

data in Table 35 compiled from results by Orla-Jensen (1919) in glucose peptone solution. There is an increase in the endpoint with increasing temperature up to a definite optimum, and beyond that a rapid falling off. The question might be raised whether four weeks' time is sufficient to obtain endpoints at temperatures below 10°C.

All experiments agree in this observation that at the optimum temperature or above, the final concentration of fermentation products is not as high as at somewhat lower temperatures. The disagreement of different experiments appears when we approach the minimum temperature. In Table 34, 9°C. for yeast is considerably above the minimum temperature of growth or fermentation while in Marshall's and Orla-Jensen's data, the minimum has been nearly or entirely reached. Both these series are rather unsatisfactory, however, as they combine growth and fermentation. If growth is very slow, the endpoint may not be reached in four weeks.

Experiments with large numbers of cells are more useful in the study of endpoints. Table 36 summarizes the endpoints obtained with three streptococci by the author (see also p. 134). Evidently, the endpoint does not increase continuously with decreasing temperature. At −1°C. where no growth takes place, the cells ferment for a while, but the amount of total acid remains very low. With strain 23, the cells died slowly, with strain 18, more rapidly. The reason for this low endpoint does not seem to lie in the dying of the cells, nor in an accumulation of other harmful compounds, for the same cultures, after four to five weeks at −1°C., produced 0.7–0.8% of acid within forty-eight hours after being placed at 30°C.

The situation of the fermentation at temperatures below the growth-minimum appears to be this: The cells are entirely normal at the start, having been grown near their optimum temperature. Centrifugation and

TABLE 35.—FINAL ACIDITIES OF GLUCOSE BROTH CULTURES OF STREPTOCOCCI
(Percents of lactic acid in culture)

Organism	After 4 weeks				After 2 weeks							
	5–6°C.	9–11°C.	14–16°C.	19–21°C.	25°C.	30°C.	35°C.	37.5°C.	40°C.	42.5°C.	45°C.	50°C.
Strept. lactis 4	0	0.25	0.27	0.32	0.40	0.41	0.32	0.25	0	0	0	0
Strept. faecium 8	0.02	0.25	0.29	0.36	0.36	0.36	0.27	....	0.27	.	0.27	0.18
Strept. liquefaciens 1	0	0.23	0.28	0.34	0.34	0.42	0.40	....	0.23	.	0.14	0
Strept. mastitidis 3	0	0	0	0.09	0.33	0.34	0.29	0.16	0	0	0	0
Strept. cremoris 1	0	0.07	0.14	0.18	0.22	0.22	0.18	0	0	0	0	0

TABLE 36.—FINAL AMOUNTS OF LACTIC ACID PRODUCED IN MILK
(C.c. n/10 KOH)

	Large inoculum					Small inoculum			
	−1°C.	5°C.	10°C.	30°C.	37°C.	+5°C.	10°C.	30°C.	37°C.
Strept. cremoris No. 23	2.9	8.1	8.5	8.7	..	..	7.8	8.7	
Strept. cremoris No. 18	3.0	7.3	...	...	..	..	7.1	7.4	6.3
Strept. lactis	...	...	8.6	...	..	..	...	9.0	

cooling to −1°C. does not seem to injure them, and they produce lactic acid at a slow rate, as would be expected, but still at a rate reasonable for such a low temperature. But the rate of fermentation soon begins to decrease; something seems to interfere. It cannot be the fermentation products because only 0.3% have been formed when the fermentation ceases altogether. Nor are other products interfering for fermentation begins again at higher temperatures and reaches a normal endpoint. It must be that some change in the cell prevents fermentation. This may be the deterioration of the fermenting enzyme which cannot be regenerated at temperatures below the minimum of growth. Above this temperature, the enzyme is gradually reconstructed, and normal fermentation starts again.

Some doubt in this explanation is justified. The endpoints of growing cultures, or of heavily seeded cultures just above the growth-minimum, seem to have a lower endpoint than at higher temperatures while according to the above assumption, it should be at least as high or higher.

This is shown by an experiment with *Strept. lactis*. Four milk cultures were kept at 30°C. until they were just beginning to coagulate. Then, they were cooled rapidly in water, one to −1°C., one to 5°C., one to 10°C., while the last one remained at 30°C. They required 5.5 c.c, 5.8 c.c., 5.7 c.c. and 6.1 c.c. of n/10 normal KOH for neutralization, immediately after cooling. Fourteen days later, the neutralization required 6.1 c.c., 6.8 c.c., 8.0 c.c., and 8.8 c.c. respectively. Later titrations showed no increase in acidity. This strain of *Strept. lactis* grew distinctly at 5°C. in cultures with small seeding. The low minimum of 6.8 c.c. n/10 KOH at 5°C. against 8 c.c. at 10°C. and 8.8 c.c. at 30°C. cannot be explained simply by lack of reconstruction power of the bacteria.

SUMMARY OF FACTS

At temperatures well above the growth-minimum, the final amount of fermentation products decreases with

increasing temperature. Above the growth-optimum, the drop of endpoint with rising temperature is very rapid.

At temperatures near the minimum, the final concentration decreases with decreasing temperature.

At temperatures below the growth-minimum, the endpoints are quite low.

SUMMARY OF THEORIES

The decrease of the endpoint by an increase in temperature is well accounted for by the assumption that the products which inhibit further fermentation are more harmful to the cells or to the enzymes in the cells at higher temperatures. This is in accordance with the action of disinfectants.

The explanation of the decrease of the endpoint below the minimum as due to deterioration of the enzymes and lack of formation of new enzymes, is not entirely satisfactory, as it leaves some facts unexplained.

VII. CHANGES IN TYPE OF FERMENTATION

(*a*) CHANGES DUE TO CHEMICAL COMPOSITION OF MEDIUM

Many microorganisms, perhaps all except the strictly prototrophic species, can use more than one kind of chemical compounds for the production of the necessary energy. Quite often, an organism will produce different fermentations in different foods. Yeast growing in a sugar-free medium will form products different from those in a sugar-containing medium. In solutions of dextrose or fructose or maltose or sucrose, however, a beer yeast will form the same endproducts from different

substrates. These substrates are similar, but not identical. Other microorganisms produce different fermentations in different sugars: mannite bacteria reduce fructose, but not glucose, to mannitol; *Leuconostoc* produces slime only in sucrose solutions; the Pneumococcus group ferments different sugars to different products.

On the other hand, the same substrate may be fermented by the same organism to different products, owing to outside conditions. Some of these are due to the chemical composition of the medium. The best known example is that of the different fermentations by yeast (p. 50), in a normally acid medium, in an alkaline medium, and in a medium containing $Na_2S_2O_3$. The change of the gas ratio of *Bact. coli* by the presence of oxygen (p. 64) might also be included here. A change of acidity effects some other fermentations as well, as might be seen from the importance of pH control in the acetone fermentation (Arzberger, Peterson and Fred, 1920).

(*b*) CHANGES DUE TO TEMPERATURE

It has been shown before (p. 151) that the endpoint of fermentation is greatly influenced by the temperature. Pronounced changes of the type of fermentation by temperature are not known, but the by-products of fermentation are influenced. It is well known that the ripening at high temperatures of the cream for churning gives a sharp, undesirable flavor. Bioletti (1908) stated that the quality of California wines could be greatly improved if the wine manufacturers would employ lower temperatures. Sarkaria and Hammer (1928) observed distinct but not very pronounced changes in the type of metabolism of proteolytic bacteria if they were cultivated at different temperatures.

Changes of type might be brought about by the fact that the same organism has different minimum temperatures for different substrates. *Monilia sitophila* grows well with citric acid at higher temperatures, but not at 15°C. or lower, where it still thrives with dextrose (Went, 1901). Similarly, *Penicillium*, according to Thiele (1896), cannot grow with dextrose if the temperature rises above 32°C., but can still live there with glycerol, while at lower temperatures, sugar is by far the better food. For *Aspergillus*, the same author found the minimum temperature to be 6°C.–8°C. if grown on dextrose, but it was 10°C.–12°C. if grown on formic acid salts.

(*c*) INFLUENCE OF CONCENTRATION OF SUBSTRATE

A rather unexpected experience is the more recent observation that the same substrate at different concentrations yields different products, or at least different ratios of the products. The first experiment carried out on a large scale is that of Mendel (1911), who studied the products formed by various gas producing bacteria from dextrose, maltose, lactose and sucrose in concentrations from 1 to 20%. He found that the $\frac{CO_2}{H_2}$ ratio was in some cases very distinctly influenced by the concentration.

These data, interesting as they are, cannot be taken at full value because the gas ratios $CO_2 : H_2$ contradict all other experiences. The hydrogen and carbon dioxide headings of the tables probably became confused in printing. But whatever it be, there is some influence of concentration upon the gas ratio in some instances, and upon the ratio between volatile and non-volatile acids as well, which deserve attention.

A much clearer case is that of the citric acid fermentation by *Aspergillus niger*. This Aspergillus forms oxalic acid only when the sugar concentration is low, and citric acid besides oxalic acid when the sugar concentration is high. Currie (1917) who was the first to make this observation, gave, along with others, the data compiled in the following table. The best medium for a good citric acid formation is one high in sugar and low in nitrogen.

TABLE 37.—FORMATION OF OXALIC AND CITRIC ACID BY ASPERGILLUS NIGER

1,000 c.c. of medium contain				Products after 7–8 days			
Sucrose, gm.	Nitrogen, gm.	KH_2PO_4, gm.	$MgSO_4$, gm.	CO_2	Oxalic acid	Citric acid	Mycelium
50	1.5 $NaNO_3$	1.0	0.2	25.08	9.82	2.44	7.44
50	2.0 $NaNO_3$	1.0	0.2	27.28	12.09	0.88	10.40
50	3.0 $NaNO_3$	1.0	0.5	31.03	12.80	0.00	12.82
50	1.2 NH_4NO_3	1.0	0.2	25.44	4.33	6.08	13.52
50	2.0 Asparagin	...	0.2	10.51	3.48	8.23	
100	3.0 $NaNO_3$	1.0	0.25	39.51	8.26	11.62	15.50
150	2.5 NH_4NO_3	1.0	0.25	24.12	0.19	70.00	19.38

In butyric acid fermentation, a gradual change takes place with time. Perdrix found that the formation of acetic acid ceases after five days, while butyric acid continues to be formed in large quantities.

(*d*) SELECTION OF FOODS

The selection of foods by microorganisms must be discussed in two entirely separate chapters, one dealing with food for energy purposes, and the other dealing with food as construction material.

The study of food selection with microorganisms was started by Pasteur's observation (1860) that *Penicillium* used d-tartrates in preference to the l-form. Duclaux (1889) and Pfeffer (1895) studied the choice which *Aspergillus* and *Penicillium* made from different sources of energy offered, such as salts of organic acids, glycerol and sugars. They found that, as a rule, both foods were attacked, but usually the one more completely than the other. Acetates were used more readily than either butyrates or tartrates. Dextrose was used in preference to glycerol. Pfeffer found that in a 4.5% peptone solution, glycerol was not touched by *Aspergillus*.

A very highly specialized case of food selection is that of *Penicillium palitans* which, according to Stokoe (1928) destroys practically all the caprylic acid of coconut oil while comparatively little of the other fatty acids had disappeared. The caprylic acid is oxidized at the β-carbon as usual, but then, instead of complete breakage of the carbon chain at this point, the keto-acid is decarboxylized leaving amyl methyl ketone which is the cause of the rancid odor of coconut oil.

$$\underset{\text{Caprylic acid}}{C_5H_{11}\cdot CH_2\cdot CH_2\cdot COOH} + O_2 = \underset{\text{Keto acid}}{C_5H_{11}\cdot CO\cdot CH_2\cdot COOH} =$$

$$\underset{\text{Amyl methyl ketone}}{C_5H_{11}\cdot CO\cdot CH_3} + CO_2$$

While molds have been used very extensively for such experiments, we know a good deal less about the preferences of bacteria with regard to their sources of energy. The most typical and best known case is the protection of proteins by sugar. There is much less protein decomposition in media containing sugar (Literature and explanations, see Berman and Rettger, 1918b).

Most of these facts lack a good explanation. It does not seem that the available energy of a food compound determines the preference. Some compounds are simply

"less digestible," though this term is rather meaningless with bacteria acting upon dissolved food.

The so-called selection of foods is probably linked closely with the molecular structure of the cell, and a comprehensive quantitative study of food selection by bacteria might give us a clearer conception of the cell mechanism.

(*e*) SUMMARY OF FACTS

The type of fermentation produced by a certain species of bacteria depends above all upon the substrate. Similar substrates, e.g., different sugars, may give the same products with one species, and may give entirely different products with another species.

The same substrate may be decomposed by the same organism in different ways, which depend upon the environment. Such differences in type of fermentation may be brought about by changes in acidity. Yeast in alkaline solution forms large amounts of glycerol. *Clostr. aceto-ethylicum* produces more volatile acids, more alcohol and less acetone if the medium becomes alkaline.

Temperature changes cause variations in the by-products, but not in the main type of fermentation.

Differences in the concentration of the substrate may bring about a different type of fermentation, as in the citric acid fermentation by Aspergillus in sugar concentrations above 10%.

SUMMARY OF THEORIES

The basis for many of the possible different types of fermentation has already been given extensively in the chapter on Equations of Fermentation.

Why one type changes into another, can not usually be explained. It is understood only in the case where

the change is brought about by a change in acidity. But how a change in concentration, or in temperature, can bring about a different type of fermentation, is not easily conceived.

There remains always the assumption that intermediate products are excreted under special conditions which ordinarily would be decomposed further.

Here as well as in the general problems of food selection, we are probably dealing with phenomena relating to the chemical structure of the bacterial cell which is practically unexplored.

PART C

GROWTH

I. GENERAL CONSIDERATIONS

(*a*) MEASUREMENT OF GROWTH

Growth, here, shall be understood to mean the total increase of the cell substance of living matter. No distinction will be attempted between the two processes comprised in the increase in size of one cell, and the splitting of this enlarged cell into two smaller cells. It would clear up matters if such distinction could be made, but it cannot, as the factors concerned in the process of cell division have not, as yet, been approached by chemical analysis. All we may do is measure growth by the increase in total weight or volume of living matter, or by counting the increase in the number of cells, with the assumption that the newly grown cells weigh as much in the average as the ones at the start. We know that the young and old cells differ in volume (Henrici, 1928), but we know nothing about their moisture contents.

Other indirect methods have been used to measure growth. Rubner (1906a) determined the nitrogen content of the bacteria as a measure of the "crop," and in one experiment, even the sulphur content of the cells was taken as the measure. Turbidity has been used to estimate the number of bacteria (Strauss, 1929), and so has the volume of the cells obtained by centrifugation (Richards, 1928).

None of these methods gives an absolutely correct conception of the increase of living matter. Nor can any method be suggested

which would give a true measure of living matter because we do not know enough about it. Cells may increase in weight by adsorbing more water, or by depositing fat, without increasing the living matter. They may increase in numbers without increase in weight. They may increase in nitrogen content by storing reserve substances of protein nature.

In measuring growth, it should be kept in mind that all living cells undergo endogenous catabolism. Therefore, the measured growth is the difference between total growth and endogenous catabolism. The latter, however, is usually so small as to be neglected without appreciable error; but this may not be true at super-optimal temperatures.

Growth of cells requires material for cell construction; it also requires a definite amount of energy to weld together the building material which is of a simpler nature than the living matter; finally, it requires a very intricate building plan to have all the construction done in the correct way and at the correct place. It is possible to study the materials needed for cell construction though it is rather difficult in some cases, on account of the extremely small quantities needed; the advance of this line of research depends primarily upon the improvement of the methods of analytical chemistry. It is also possible to study the sources and amounts of energy available to the cells; the entire preceding section on fermentation was devoted to this study. It does not seem possible that we shall ever understand the actual process of growing, the mechanism of the production of new protoplasmic molecules, the principles which keep order in the microcosmos of the cell, arranging all molecules in their proper places and proper sequences after they have been created.

(*b*) BUILDING MATERIALS OF CELLS

A study of growth should begin with the materials of cell construction. Unfortunately, the requirements are

different for every species of bacterium, and while some general statements may be made concerning the necessity of K, P, Ca, S, *also* probably of Fe, Mg and Na, *and* possibly of Mn *and* eventually even of Zn and Cu, the organic requirements are much more complex.

The minerals are usually resorbed as ions, eventually as salts, and it makes little difference in what form they are given as long as they are at all soluble. Carbon and nitrogen on the other hand are capable of forming innumerable different compounds which do not ionize. A good deal of attention has been given to the finding of good, synthetic media, i.e., media of which every component is chemically well defined and known. While good growth has been obtained with some bacteria in certain media containing amino acids and sugars, the media containing peptone, meat extract, or milk are still the only standard ones.

It is probable that the reason for this is not the unfitness of the food as such, but the lack of something else. It is difficult to see why bacteria should not grow in synthetic media to the same large numbers as in peptone media because we can be certain of providing the same source of energy. A systematic study considering hydrogen donators and reduction potentials (see p. 82) might lead to better results now than the efforts of 20 years ago.

(*c*) SOURCES OF CARBON

The element **carbon** may be used by one species of bacterium, according to Potter (1908). **Carbon monoxide** is a source of carbon for the *Carboxidomonas;* it oxidizes CO to CO_2, and can grow in the absence of any organic matter.

Carbon dioxide can be changed to cell material by all prototrophic bacteria. This group of bacteria, simpler in their metabolism than any other known

organisms, includes the nitrite and nitrate bacteria, sulphur bacteria, the thiosulphate bacteria and the Hydrogenomonas group (see also p. 168).

Generally speaking, these prototrophic bacteria are very similar to algae, the difference resting mainly in the substitution of the chlorophyl mechanism by a chemical method of energy supply. The amount of energy required to make organic matter from inorganic is quite large, much larger than that needed by the common saprophytes.

Another compound with but one carbon atom is the **formic acid.** This is often stated to be a rather unsatisfactory source of carbon for growth, though it can be used by some molds and bacteria. Most experiments on the decomposition of formic acid, (and of many other compounds) have been carried on in a medium containing peptone as a "source of nitrogen"; these results indicate the utilization of the acid for energy, but not for cell construction. The fitness of a compound as building material is not dependent upon the amount of energy needed to reduce it to cell constituents, but is largely determined by its chemical constitution.

There is for example the carbonic acid, with three oxygen atoms coupled to the carbon. This compound can be used by a goodly number of bacteria. Most of these can use neither carbon-monoxide nor methane, though these compounds are much more easily reduced, and methane would even liberate energy if changed to carbohydrate or organic acid. The structure seems to play the essential role.

Formaldehyde is a strong disinfectant, much stronger than formic acid. But there is evidence of its being used as a source of carbon. Kaserer (1906) observed that *Hydrogenomonas* required both hydrogen and carbon

dioxide besides oxygen for growth, and that the bacteria first reduced CO_2 to HCHO which could be found to exist as such in the culture.

Urea does not seem to be suitable as a source of carbon. At least, Söhngen (1909) could not cultivate urea bacteria on urea alone, while very small amounts of organic acids (10–20 mg. of ammonium malate or asparagin in 50 c.c. solution) sufficed to bring about normal development of *Bact. erythrogenes* and of *Urob. jackschii.*

Methane can be used only by the *Methanomonas* (Söhngen, 1906) and it is not certain that the methane as such is the building stone to be hewn. It might well be that these bacteria use CO_2 as the starting point for shaping the parts of molecules for growth as do all other prototrophic bacteria. This carbon dioxide might originate from methane oxidized completely by the same cells.

The organisms which can use compounds with only one carbon atom must possess the ability to couple carbon atoms. It is interesting to notice that while the coupling of single carbon atoms is possible to all chlorophyl plants, but only to a very limited number of chlorophyl-free organisms, a large number of the latter are able to grow on compounds with two carbons, e.g., alcohol or acetic acid. Many molds can do this; also vinegar bacteria, some yeasts, Oidium, all Mycodermae and many bacteria can live on acetates. It appears to be much easier to couple two carbons with two carbons than to couple one carbon with another. How this coupling is actually done we do not know with certainty, but it seems possible that we have processes similar to the formation of aldol from aldehyde:

$$CH_3{\cdot}CHO + CH_3{\cdot}CHO = CH_3{\cdot}CH(OH){\cdot}CH_2{\cdot}CHO$$

We must consider in the further discussion that the number of carbon atoms in most of the compounds of the cell is an even one. We find most dominantly six carbons (carbohydrates, leucin, benzene ring) but four carbons are not uncommon in bacterial metabolism (butyric, succinic and hydroxy-succinic acids) and the fats, as is well known, contain only the acids with even numbers of carbon atoms. The most common exceptions to the dominance of even numbers of carbons in cell compounds are lactic acid and glycerol.

The compounds with three carbons are very commonly used, perhaps because they give, when coupled, the carbon skeleton of the sugars. Lactic acid is sufficient for the entire carbon and energy supply of Mycoderma, Oidium and probably of many other molds and bacteria like *Bact. coli.*

The assimilation of higher alcohols and higher organic acids is shown in two tables by Buchanan and Fulmer (II p. 431 and 438) after data from de Jong (1926).

Even with sugars, we have not in all cases the proof that they are sufficient as a source of carbon, for in most experiments, an organic source of nitrogen is offered, which may supply carbon as well as nitrogen. In order to be really certain that sugar is sufficient for the carbon supply, we must give the nitrogen either in mineral form, or as an amino-derivative of sugar, or eventually as amino-derivative of the decomposition products of sugar (e.g., amino acetic acid in the case of *Bact. coli*, or of vinegar bacteria). That yeasts can grow with ammonia nitrogen from sugar alone has already been shown by Pasteur. Most molds behave in the same way. The difficulty in settling the question of carbon supply really rests with the nitrogen source. If an organism cannot use ammonia, nitrate or urea, but requires an organic source of nitrogen, we can tell but little about the carbon supply.

The customary conception of carbon requirements for growth as presented above has become quite questionable

by the observation that complete removal of carbon dioxide prevents the growth of all bacteria. This has been asserted by many investigators and denied by many others, but the work of Valley and Rettger (1927) leaves hardly any doubt of the fact.

The role which CO_2 plays is entirely unexplained. Since the amounts present in air are sufficient for normal growth, it would seem that the carbon dioxide enters in the process of cell construction rather than in energy liberation. With the latter process, larger amounts would probably be required to produce noticeable effects. The author is of the opinion that we stand here at the already opened door to an important biological principle.

(*d*) SOURCES OF NITROGEN

Elementary nitrogen is used by the three well-known groups of nitrogen-fixing bacteria, the *Rhizobium* group, the *Azotobacters* and certain *Clostridium* species. Less intensive nitrogen fixation has also been claimed for some other bacteria, and even for yeast (Fulmer and Christensen, 1925). All organisms mentioned can take the nitrogen in other forms.

Ammonia and **nitrates,** also nitrites in low concentrations, are used by many molds and many bacteria. Considerable discussion has taken place concerning the preference of ammonium salts and of nitrates, especially for the growth of molds. The results by different investigators are in some cases contradictory. It seems quite probable that we have the superposition of another factor which was not generally recognized at the time when most of these experiments were conducted, i.e., the antagonism of certain salts, the change of hydrogen ion concentration or the oxidation-reduction potential.

In comparing nitrates with ammonia, it is customary to add to the same mineral-sugar solution in one case a nitrate (KNO_3 or $NaNO_3$ most commonly) and to the other solution an ammonium salt (NH_4Cl or $(NH_4)_2SO_4$). If the mineral solution had been a balanced salt solution, the addition of a potassium salt might make it slightly poisonous; if it had been unbalanced, the potassium salt might balance it more perfectly and cause a better growth. The same explanation probably accounts for the fact that some investigators find $Ca(NO_3)_2$ to produce a much better growth than KNO_3 while others find the opposite. It is not very probable that the ion of a soluble ionized salt has different food value because it was originally combined with a different base.

Nitrates and especially ammonia are for some bacteria the only source of nitrogen, and organic nitrogen compounds cannot be utilized by them. In this group we find the organisms with a very simple, mineral form of fermentation, such as the nitrifying organisms, the sulphur bacteria, and, perhaps, the iron bacteria. They are the same species which can build their cells only from CO_2 and not from organic compounds. Nitrate-assimilating bacteria are known in great numbers. The denitrifying bacteria, *Ps. fluorescens, Ps. pyocyanea, B. subtilis* are able to live on nitrates and some nitrogen-free organic matter. In soil, such species are found abundantly. Some representatives of the colon group can live with nitrate as the only source of nitrogen (Kisch, 1919).

Ammonia is also used commonly (e.g., by the colon-paratyphoid group, and by many soil bacteria) and, perhaps, more commonly than nitrates. It is a fairly good source of nitrogen for yeasts which refuse nitrates as a rule (Lindner, 1905). Many of the bacteria which use nitrates, can also use ammonia, though some exceptions are mentioned. In experiments with ammonium salts, it must be kept in mind that the consumption of

ammonia will make the neutral solution acid, and Wehmer found that a *Penicillium* species was readily killed by prolonged growth on ammonium sulphate merely by accumulation of acid. Accumulation of alkali by nitrate-consuming organisms has never been reported harmful, perhaps on account of the formation of the organic acids.

As a curiosity, *B. hiltneri* which can oxidize HCN to CO_2, H_2O and N, according to Kaserer (1907), should be mentioned.

The organisms which depend upon organic matter for their energy supply, seem to prefer organic nitrogen also. The usefulness of amines for cell construction has not been studied extensively, but we know that methylamine can be used by *Aspergillus* and *Penicillium;* that the mono-amine is the best, and trimethylamine the poorest source of nitrogen, and that tetramethyl ammonium hydroxide is not suitable for cell construction. den Dooren de Jong (1927) has given the genus name *Protaminobacter* to a group of bacteria which were able to assimilate amines.

Urea can be used by quite a large number of bacteria as a source of nitrogen. This faculty is not limited to the urea-fermenting organisms. Some yeasts and many molds can also live on urea as the only nitrogen source (Lindner, 1905).

Next in simplicity of composition are the **amino-derivatives.** Alkaloids do not seem to be sufficient for growth, at least not for the common molds, though they are decomposed, perhaps for energy purposes only. Amino-acids such as glycol, leucin, aspartic acid etc. are good nitrogen sources for many organisms but some thrive well only if they have a better source of energy (or of carbon) such as dextrose or mannite. That the

amino-acids are not always used as such, is quite evident from Ehrlich's observation that the leucin assimilation by yeast is decreased in the presence of ammonium salt.

Amino-acid organisms in a strict sense of the word are microorganisms which can utilize nitrogen in no simpler form than that of an amino-acid. This would mean, chemically speaking, that these organisms cannot synthetize the group $-NH\cdot CH\cdot CO-$ which is so common in the proteins of all living beings. They must obtain this combination ready-made, but can do the rest of the synthesis themselves, eventually only with an especially good source of energy. Most commonly used for experiments of this nature is asparagin, and the good results are perhaps due to the fact that we have here not only the amino-acid group, but, at the same time, the very frequent acid-amid group preformed.

If we consider the great variety of nitrogenous cleavage compounds of protoplasm, we realize at once that the synthetizing power of a cell that can grow on *one* amino-acid only, is very remarkable, since all other amino-acids, carbohydrates, and the cell wall are all formed from the one material.

The term "peptone bacteria" is sometimes used and is meant to indicate that these organisms can use nothing simpler than peptones. That such organisms exist, is questionable. If we consider that even the mammals can be kept alive, and probably be made to grow with amino-acids only, and that probably all the body cells are fed with amino-acids and not with peptones, it seems quite probable that this would be true also with bacteria. If they refuse to grow on tyrosin, asparagin or leucin solutions, this is no proof that they require peptone. It shows only that the proper amino-acid had not been offered. Certain species may require a mixture of two

or more amino-acids, because they cannot form one from the other. In this connection it should be mentioned that Abderhalden and Rona found no influence of the source of nitrogen upon the composition of the protein of *Aspergillus niger;* whether potassium nitrate, glycocoll or glutaminic acid was given, the protein of the mold gave the same cleavage products. There is no doubt, however, that the properties of yeast are greatly influenced by the source of nitrogen (p. 238).

It is imaginable, that certain parasitic forms take large molecular complexes out of their host's native protein, and have lost the power of synthesizing these groups.

(*e*) SOURCES OF OXYGEN

In the discussion of the needs of oxygen for fermentation, the requirements of oxygen for cell growth have been already mentioned for the sake of a more accurate definition of anaerobic and aerobic bacteria (p. 78). It was pointed out there that an analogy exists between the carbon, nitrogen and oxygen requirements, and that the structure of the molecules is probably quite as essential, or eventually more so than the energy relation.

Carbon, nitrogen and oxygen may be compared in regard to their fitness for growth by arranging them according to the energy required to change them into organic material.

	Carbon	Nitrogen	Oxygen
Completely oxidized	CO_2	HNO_3	O_3, H_2O_2
Partly oxidized	CO	HNO_2	
Molecular	C	N_2	O_2
Partly oxidized / Partly reduced	HCOOH	$NH_2CH_2CO_2H$	HCOOH
Completely reduced	CH_4	NH_3	H_2O

The analogy is quite close between carbon and nitrogen. While the completely oxidized and the completely reduced compounds are used by some microorganisms, and the partly oxidized, partly reduced compounds by many others, the elementary stage is least suited for assimilation, though less energy would be required to build cells from N than from HNO_3. It seems very difficult for cells to break up the affinity between the atoms of the same element. With oxygen, however, the opposite seems to be true; at least this is the case in fermentation, and we may well assume that it holds true for growth.

Elementary oxygen is readily used by most living beings. Oxygen fixation is the most common biological reaction. There are exceptions, however, such as the lactic organisms and some strictly anaerobic bacteria which depend exclusively upon oxygen from organic compounds, and cannot use elementary oxygen if it is offered them. We may assume in these cases an analogy to the nitrogen and carbon sources, i.e., the inability to break up the oxygen molecule into oxygen atoms.

There is no use in speaking about hydrogen sources, because hydrogen is so common in water as well as in practically all organic compounds that its source could not be traced. Attention should be called only to the hydrogen-fixing bacteria (Kaserer, 1906).

(*f*) WASTE PRODUCTS OF CONSTRUCTION

When prototrophic bacteria build their cells from carbon dioxide as the only carbon source and from nitrates as the only nitrogen source, the growth process is entirely a process of reduction and condensation. The only part of the building material that is not used is a good share of the oxygen in these compounds. This oxygen is most probably used for the oxidation of the energy furnishing substrate and cannot be traced.

Other organisms build their cells from very complex material, such as amino acids, peptones, etc., and these

large molecules are not always put into the cell as such, but are split and reshaped before fitting into the cell architecture. This shaping must cause some waste products. In many cases, these waste products of cell construction may be used as food for energy; but there are some instances known where this is not the case. The only definitely established example is probably that of the fusel oils in alcoholic fermentation which is discussed in detail on p. 43. The yeast needs ammonia for building its own proteins, and takes it from leucin, leaving the rest of the molecule in the form of amyl alcohol.

The main reason why more such examples are not known, is the small amount of these products. The total dry weight of the cells of *Bact. coli* in 1 liter of a full grown broth culture is only 220 mg. (Appendix p. 397). The waste products from the construction of 220 mg. of cells are likely to be less in quantity than the cell weight; this means less than 0.02% of the weight of the medium.

It seems probable that the carbon dioxide produced by some streptococci belongs into this category of products. Hucker (1928a) mentions that *Strept. lactis* produces about 70 mg. CO_2 per liter. The dry weight of the cells probably does not exceed 200 mg. The CO_2 comes from peptone, and not from sugar; it must originate from the decarboxylation of amino acids. With other species of streptococci which produce more carbon dioxide, there seems to be a distinct protein metabolism established, and more amino-acids are split than are needed for growth.

Balls and Brown (1925) and Claassen (1927) during the production of bread yeast observed a small amount of a reducing substance which was not sugar, nor was it

assimilated by the yeast (see p. 211). This product amounts to about 1% of the fermented sugar, or to about 4% of the yeast solids. The small amount, in comparison to the crop, makes it appear possible that it might be a waste product of growth.

(*g*) BALANCE SHEETS OF FEEDING EXPERIMENTS

While an enormous literature on growth of microorganisms exists, there is very little material available which shows complete balances of all foods used, or of all products, including growth. The most complete data available are those of the bread yeast industry. This is the only large industry which is interested in growth as such, and not in the products of fermentation.

Even in this industry, the number of complete experiments is not very great. It is regrettable that the carbon dioxide and the quantity of the evaporated alcohol are measured only exceptionally. But enough material is available to get a good conception of the balance.

A first attempt at such an account of all foods used and all products formed is the study of yeast metabolism by Brown and Balls (1925) who determined alcohol, CO_2, decrease of sugar and amount of yeast growth in a well aerated culture. About 750 c.c. of the mash or wort (consisting of 48 gm. molasses, 1.2 gm. $H_2(NH_4)PO_4$ and 1.6 gm. $(NH_4)_2SO_4$ per liter) were kept at 28°C. and aerated with 5 liters of air per minute, or about seven times the volume of the liquid per minute. The products formed were determined after 3, 6, 8, 16.5 and 24 hours.

The results of some experiments are given in Table 38. There is a noticeable amount of sugar not used for the formation of either alcohol or CO_2, and this amount, in the aerated cultures, is about one-half of the yeast

growth. We shall see later, from Claassen's data, that about one-half of the yeast solids is non-protein in nature.

An interesting observation is the alcohol: CO_2 ratio. This should be according to the simple fermentation formula (p. 42), 1.04. In the experiments without aeration, this ratio is a little higher,

TABLE 38.—BALANCE SHEET OF YEAST GROWTH
(All data are gm. per liter)

	Aerated			Not aerated	
Experiment number	I	II	III	I	II
Duration of experiment	8 hours	24 hours	28 hours	8 hours	48 hours
Total carbon dioxide formed	11.39	14.78	12.96	6.58*	7.03*
Total alcohol formed	8.02	5.17	4.83	8.00	8.15
Alcohol plus carbon dioxide	19.41	19.95	17.79	14.58	15.18
Sugar used	23.34	23.76	17.96	19.01	22.20
Sugar minus alcohol and CO_2	3.93	3.81	0.17	4.43	7.02
Yeast growth	5.48	8.85	6.73	2.40	(1.21)†

* These CO_2 values are too low.

† This is the value for 6.75 hours; no later value is given.

1.21 and 1.16, owing to an incomplete elimination of CO_2 from the wort. In the aerated experiments, it is much smaller, 0.70, 0.35 and 0.37 respectively. The reason for this is either the complete oxidation of part of the sugar, or the utilization of alcohol by yeast, or both. Doubtless, some alcohol is used by the yeast. In Expt. II, all sugar had been used after the eighth hour; but during the next sixteen hours, 5 gm. of alcohol per liter disappear, 1.25 gm. CO_2 are produced and the yeast crop increases 2.5 gm. In Expt. III, 1.2 gm. alcohol are used after the eighth hour, together with the remaining 0.3 gm. sugar, to produce 2.4 gm. of yeast and 3.0 gm. of CO_2. Special experiments showed that yeast in sugar-free aerated alcoholic solution with ammonium salts gained in weight 25 to 29%, while the alcohol decreased and CO_2 was produced.

More detailed are the data of an experiment under plant conditions by Claassen (1927) with 77,000 kg. of wort which consisted of molasses, malt sprout extract

Table 39.—Balance Sheet of Yeast Growth

	Total weight of products kg.	Solids			Of the organic matter				
		Total kg.	Inorganic kg.	Organic kg.	Sugar kg.	Protein kg.	Not protein kg.	Alcohol kg.	CO_2 kg.
Composition at start									
Water	70,000								
Molasses	3,000	2,475	336	2,139	1,545	318	276		
Malt sprouts	525	200	20	180	44	60	76		
Phosphate	120	61	61						
Seed yeast	350	82	6	76	.....	49	27		
Wort of seed yeast	4,400	57	22	35	2	19	14	26	
Total	.....	2,875	445	2,430	1,591	446	393	26	
Composition after 11 hours									
Yeast crop	1,875	516	36	480	..	229	251		
Fermented wort	77,000	1,011	393	618	22	223	373	350	
Carbon-dioxide	823	.....	...	.....	..	...	...	...	823
Alcohol evaporated	98	.....	...	.....	..	...	...	98	
Total	.....	1,527	429	1,098	22	452	624	448	823

and acid phosphate. To this, after filtration and sterilization, the seed yeast was added with some wort. The balance sheet of Table 39 shows the composition of these materials. The air forced through the medium totaled 44,800 cubic meters, or six hundred times the volume of the medium, = 12,800 kg. oxygen, for the entire time of growth.

The lower half of this table shows the composition of the medium and yeast crop, eleven hours later. The balance is not perfect, owing to the enormous bulk of the liquid, and to inaccuracies involved in determining the evaporated alcohol and carbon dioxide. There is an unaccounted loss of 16 kg. of inorganic matter, and of 87 kg. of organic matter, or about 4% of the total organic matter involved.

A summary of all analyses made by Claassen in short intervals bears out the claim of Balls and Brown that alcohol can be used for growth by the yeast. After eight hours, the wort contains only 0.054% sugar. During the next three hours considerable growth still takes place, while the alcohol concentration decreases much faster than can be accounted for by evaporation.

TABLE 40.—UTILIZATION OF SUGAR AND ALCOHOL DURING YEAST GROWTH

	4 hours, kg.	6 hours, kg.	8 hours, kg.	11 hours, kg.
CO_2 formed	227	520	721	823
Alcohol formed	230	395	531	423
Sugar used for yeast and by-products	185	189	304	329
Total sugar used	642	1,104	1,556	1,575
Yeast and by-products in % of sugar used	28.8%	17.1%	19.5%	20.9%

The alcohol:CO_2 ratio, for this reason, is only 0.52 instead of the theoretical 1.04 for alcohol fermentations.

The yeast solids (516 kg. − 82 kg. seed yeast) contain 229 − 49 kg. of protein which may derive from the nitrogenous matter of molasses and malt sprouts, and 251 − 27 = 224 kg. non-nitrogenous

matter which may derive from the sugar. The subtraction of alcohol and CO_2 from the total sugar leaves 329 kg. to be accounted for by "growth and by-products." Of this amount, 224 kg. are found in the non-nitrogenous part of the yeast. The remaining 105 kg. may have been at least partly used in cell construction, but it cannot be proved.

The observation that of the 1,575 kg. sugar used, at least 224 kg. = 14.2% had been used as construction material, in addition to a similar quantity of nitrogenous matter, is of considerable interest, as not nearly such large amounts have ever been found to be assimilated by bacteria. The efficiency of energy utilization must be quite high.

According to Lindner and Unger (1919), yeast will produce fat from alcohol if plenty of oxygen is available. If the increase in non-nitrogenous matter were largely fat, produced from alcohol, it is evident that fairly large quantities of alcohol must be used because, according to the simplest formula

$$\underset{\text{Alcohol}}{8C_2H_5OH} + O = \underset{\text{Palmitic acid}}{C_{16}H_{34}O_2} + 7H_2O$$

it takes about 3 kg. of alcohol to produce 2 kg. of fatty acid.

Both authors give special nitrogen balances for their experiments. That of Claassen is the following:

TABLE 41.—NITROGEN BALANCE IN YEAST GROWTH

Nitrogen in medium:		
3,000 kg. molasses with 1.67% N =	50.10 kg. N	
525 kg. malt sprouts 1.70% N* =	8.93 kg. N	
	59.03 kg. N	
Nitrogen in seed yeast:		
360 kg. yeast with 2.18%	7.85 kg. N	
4,400 kg. wort of seed yeast with 0.0682% N	3.00 kg. N	
	10.85 kg. N	
Total N at start		69.88
1,875 kg. total yeast crop 1.95% N	36.56 kg. N	
77,000 kg. fermented wort 0.045% N	34.65 kg. N	
Total N at finish	71.21	71.21

* Soluble nitrogen only.

There is a nitrogen gain of 1.33 kg., doubtless due to the great probable error in analysis.

Of the total nitrogen offered, 59.03 kg., the new growth of the yeast contained 36.56 − 7.85 = 28.71 kg. or 48.6%.

II. RELATIONS BETWEEN GROWTH AND FERMENTATION

(*a*) EFFICIENCY OF UTILIZATION OF ENERGY

Most bacteriologists hold the viewpoint that fermentation (as defined by Hugo Fischer, see page 24) is the source of energy which is needed by the cell for repair and for new construction, i.e., growth. The amounts of energy necessary for growth will vary considerably with conditions. It might seem that bacteria growing in a peptone solution would need no energy at all, because the combustion heat of the food is as high as that of the bacteria (see Table 42 from Rubner, 1904a); and bacteria growing on fat would even have a surplus by just converting every bit of the fat into carbohydrate and protein.

TABLE 42.—COMBUSTION HEATS OF 1 GM. OF SOLIDS

	Calories		Calories
Peptone	5,492	*Penicillium glaucum* with spores	5,359
Gelatine	4,992	Top yeast	4,475
Meat extract	3,514	Bottom yeast	4,554
Dextrose	3,740	*Proteus vulgare*	4,643
Sucrose	3,950	*Bact. prodigiosum*	4,764
Fat, about	9,200	*Bact. prodigiosum*, old culture	4,442
Penicillium glaucum	4,753		

This assumption has two errors. It does not take into consideration that the architecture of the bacterial

protoplasm is quite different from that of the peptone molecule, and that the peptone molecule has to be torn into very small units before it can be used in the construction of protoplasm. The tearing down will yield energy which may not be available, and the building up will require energy. The combustion heat of the protoplasm tells only how much energy is contained, and not how much energy was necessary to construct it. Growth processes do not work with 100% efficiency as we shall see later.

The other error is made by the assumption that the combustion heat (or the balance of combustion heats in anaerobic fermentations), represents *available* energy. According to thermodynamic laws, the "free energy," i.e., the energy that might be used to do work, is not identical with the combustion heat, or differences of these. These values represent the total energy liberated by the fermentation, but part of this goes towards the increase of entropy, and is lost for any work. This amount varies greatly with the kind of process.

Let the combustion of glucose serve as example (Baas-Becking and Parks, 1927)

$$\underset{\text{Solid}}{C_6H_{12}O_6} + 6\,\underset{\text{Gas}}{O_2} = 6\,\underset{\text{Gas}}{CO_2} + 6\,\underset{\text{Liquid}}{H_2O}$$

Under the above conditions (solid sugar; oxygen and CO_2 at pressures of 1 atm.; water of combustion in liquid form), we have

	Calories
Heat of combustion	685,800
Free energy	674,000
Entropy increase	11,800

The heat going towards the increase of entropy which cannot be used for any work, is 1.7% of the combustion heat. A mold oxidizing one

mol of sugar has not the full benefit of the 685,800 calories, but can use only 98.3% of that amount. In the oxidation of methane to CO_2 and H_2O, the loss is 9%.

While these losses towards the entropy may eventually be fairly large in inorganic processes, it seems that they are rather small in the organic reactions ordinarily met with in biological processes. Thus, the error in assuming the free energy to equal the differences of combustion heats between substrate and products is not very great, and amounts perhaps to a few per cents of the total energy liberated. For most fermentations, this percentage is not known. A good survey on the autotrophic bacteria in their energy requirements is given by Baas-Becking and Parks (1927) and a detailed discussion of the theory is offered by Buchanan and Fulmer (1928–30).

Realizing that the energy liberated is not identical with the available energy for construction purposes, but in most cases approximately so, the next question will be that of efficiency: how much of the available energy is actually used for growth? This has been computed for some of the autotrophic bacteria by Baas-Becking and Parks (1927). The percentage of the available energy stored in the new growth is the efficiency. This efficiency seems very low. The following maximum efficiencies were found by Baas-Becking and Parks:

Table 43.—Amount of Available Energy Utilized for Growth

	Per cent
Hydrogenomonas	26
Methanomonas (maximum efficiency)	33.8 and 25.5
Methanomonas (minimum)	0.6
Nitrobacter and *Nitrosobacter*	6
Thiobacillus denitrificans	9
Sulfur bacteria, probably about	8

Linhart (1920) found for Azotobacter an efficiency of only about 1%, but Baas-Becking and Parks believe that 7% is more probable.

Similar calculations based on the total energy liberated rather than on the "free energy" have been made by Tangl as early as 1903. One compilation of his data has already been given on p. 28. From his data on *B. anthracis*, we obtain an energy utilization of 49%. This seems very high in comparison with the above data calculated by Baas-Becking and Parks. It must be remembered, though, that the former data referred to synthesis of protoplasm from CO_2, H_2O and either NH_3 or HNO_3 while here, the food has even a higher energy level than the cells. The energy content of 1 gm. of broth solids was 4.4 calories while 1 gm. of bacteria solids represented only 4.23 calories. It is quite evident that even with the same species of bacteria, the energy required for synthesis will vary with the kind of building material used. More data on this subject may be found in Pütter's book (1911, p. 121–129).

(*b*) EFFICIENCY OF YOUNG AND OLD CELLS

All these experiments, from Tangl to Baas-Becking and Parks, disregard the time factor. It is generally known that fermentation continues for a considerable time after the multiplication of cells has stopped. This must be of considerable influence upon the efficiency of energy utilization. If one experimenter discontinues his experiments with beer yeast after 2% of alcohol has been formed and multiplication has practically ceased, and the other waits until the fermentation is over, and 6% of alcohol has been formed, the former's efficiency will be three times that of the latter, both using the same organism in the same medium. After growth has ceased, fermentation will be needed for nothing but repair and upkeep; after completion of growth, the efficiency must be practically zero.

This would be the one extreme; we are more interested in the other extreme, i.e., the maximum efficiency.

Most probably, this would be found in the very young, actively growing cells.

Quite instructive are three series of experiments on the nitrogen fixation by *Azotobacter*, made by Koch and Seydel (1912). They determined the nitrogen fixed and the sugar used by *Azotobacter* at different times, and computed the mg. N_2 fixed per gram of dextrose. Nitrogen is not a direct measure of growth, but is as good as any of the other methods.

TABLE 44.—NITROGEN FIXATION AND SUGAR CONSUMPTION BY AZOTOBACTER

Experiment II; 2.383 gm. dextrose in 50 c.c.

Days	1½	2	3	4	5	7	8	9	11	13	17	32
Mg. N fixed	3.29	3.7	5.3	6.6	7.3	6.7	9.1	8.0	10.0	7.3	8.8	7.6
Mg. dextrose used	0	69	72	162	323	283	706	1,276	1,335	1,287	1,401	1,351
Mg. N per 1 gm. dextrose	∞	53.6	75.0	48.6	22.9	24.7	15.0	6.3	7.5	5.6	6.2	5.6

Experiment III; 7.445 gm. dextrose in 50 c.c.

Days	6	7	8	9	11	47
Mg. N fixed	1.17	1.80	1.49	1.62	2.11	2.47
Mg. dextrose used	70	224	191	344	435	494
Mg. N per 1 gm. dextrose	17.0	8.7	7.4	5.2	4.8	4.3

Experiment IV; 2.316 gm. dextrose in 50 c.c.

Days	6	8	9	11	47
Mg. N fixed	1.85	3.90	2.06	2.73	3.56
Mg. dextrose used	41	181	747	918	1,016
Mg. N fixed per 1 gm. dextrose	30.3	21.1	4.5	3.0	3.5

Table 44 shows how the growth produced (= mg. nitrogen fixed) by the utilization of 1 gm. of dextrose, decreases rapidly with the age of the culture. The amounts of the first two days are too small to be determined accurately, but the efficiency measured after six days is three to ten times as large as that measured after the twelfth day, in all three experiments.

The absolute efficiency can be estimated from the data of Gerlach and Vogel (1902) that 1 mg. of N = 9 mg. of dry cells of *Azotobacter*, and that 1 gm. of dry cells has a combustion heat of 4.6 Cal. (Table 42). This gives a utilization of about 50% for the first four days of Experiment II, and of 30% for Exp. IV. After ten to twelve days, the efficiency is only 3 to 8% (see also Linhart, p. 183).

In well aerated cultures of *Azotobacter*, Hunter (1923) found a constant utilization for the first eight days. The mg. N fixed per gram of glucose consumed were, for four successive two-day periods, 6.9, 9.37, 8.64, and 9.74. The average efficiency of utilization of energy in this culture is much smaller than that of the previous experiment.

From Rahn's data on the lactic fermentation (see p. 400), it is possible to compute the amounts of lactose required for one genera-

TABLE 45.—MG. SUGAR FERMENTED DURING ONE GENERATION OF *Strept. lactis*

Hours	Culture I, mg.	Culture II, mg.
9–12	.....	12.5×10^{-10}
12–15	.....	14.6×10^{-10}
15–18	9.1×10^{-10}	17.6×10^{-10}
18–21	14.2×10^{-10}	49.8×10^{-10}
21–24	12.4×10^{-10}	$173. \times 10^{-10}$
24–27	24.9×10^{-10}	∞
27–30	37.2×10^{-10}	∞
30–33	∞	∞

TABLE 46.—DEVELOPMENT AND OXYGEN CONSUMPTION OF *Bacterium coli*

Experiment No. 9					Experiment No. 10				
Time in hours	Plate count per mm.³		Dissolved oxygen per liter, mg.	Oxygen consumed by 10^9 cells per hour, mg.	Time in hours	Plate count per mm.³		Dissolved oxygen per liter, mg.	Oxygen consumed by 10^9 cells per hour, mg.
	Open flask	Closed bottle				Open flask	Closed bottle		
0	23	23	9.072		0	287	287	9.084	
2½	.....	22	9.088		5	357	316	9.020	0.043
7	26	41	9.105		10	742	868	8.765	0.097
12	57	105	9.028	0.14	15	1,179	2,398	8.071	0.096
17	102				20	2,500	10,028	5.351	0.110
22	208	1,818	8.345	0.16	22	.....	15,573	3.748	0.064
24	.....	2,482	8.046	0.07	24	3,758	20,587	1.293	0.068
25½	485				26	.....	22,240	0.015	(0.030)
26	.....	6,078	6.772	0.16	28	10,917	24,333	0.033	
28	.....	7,142	5.854	0.07	30	.....	25,917	0.030	
30	614	17,534	2.178	0.16	33	20,417	33,167	0.015	
32	.....	22,743	0.000	(0.05)	38½	35,851	31,167	0.000	
35	1,325	30,583			43½	51,256	24,750		
40½	2,133	26,750			48½	56,298	25,417		
45½	3,208	23,417			53½	59,379	28,417		
50½	7,193	19,083			94½	79,265	25,796		
55½	19,250	26,333			145½	80,105	23,892		
96½	70,022	26,188			193½	63,300			
147½	59,379	28,485			337½	68,342			
195½	63,300	25,083			409½	67,221			
243½	57,166	17,478							
339½	62,460	10,667							
411½	65,821	6,022							

TABLE 47.—OXYGEN REQUIREMENTS OF BACTERIAL CELLS PER HOUR AND PER GENERATION

Pseudomonas fluorescens

Generation time		Mg. oxygen required		Generation time		Mg. oxygen required		Generation time		Mg. oxygen required	
Hours	Minutes	Per generation	Per hour	Hours	Minutes	Per generation	Per hour	Hours	Minutes	Per generation	Per hour
2	23	$5.0\ 10^{-9}$	$1.87\ 10^{-9}$	3	34	$0.8\ 10^{-9}$	$0.13\ 10^{-9}$	4	13	$0.4\ 10^{-9}$	$0.059\ 10^{-9}$
1	47	2.3	0.99	2	8	2.5	0.93	3	33	1.1	0.22
1	27	2.8	0.80	1	33	1.5	0.81	3	26	1.7	0.35
1	24	1.1	0.60	0	58	1.1	0.69	3	1	1.8	0.42
				2	18	1.7	0.50	1	48	1.4	0.57
								0	57	0.7	0.59

Bacterium coli

5	28	$0.73\ 10^{-9}$	$0.14\ 10^{-9}$	35	59	$2.21\ 10^{-9}$	$0.043\ 10^{-9}$
2	26	0.40	0.16	3	28	0.46	0.097
1	33	0.36	0.16	3	25	0.46	0.096
1	32	0.41	0.16	2	25	0.36	0.110
				3	9	0.30	0.064
				4	58	0.20	0.068
				17	57	0.07	0.030

tion. Table 45 shows that 9 to 14 $\times$ 10^{-10} mg. are sufficient for the doubling of young cells, and that the required amount increases with increasing age of the culture, approaching infinity.

Meyerhof (1916a) determined how many mg. of nitrite nitrogen must be oxidized by *Nitrobacter* in order to increase the organic nitrogen of the culture 1 mg. This increase is a good measure of the growth rate. Young cells accomplished this with 107 mg. of nitrite while older cells required 135 mg. to accomplish the same amount of growth.

While with Azotobacter, no accurate data could be obtained for cultures younger than three days, and with *Strept. lactis*, not younger than nine hours, the very accurate methods of oxygen determination make it possible to estimate the energy utilization right after inoculation. A good material for this are the experiments by A. Müller (1912) with cultures of *Ps. fluorescens* and *Bact. coli* in a very dilute medium, containing only 0.004% of asparagin and 0.006% of ammonium lactate and minerals. The organisms were grown in closed bottles filled completely to the stopper without leaving an air space. For each test, a new bottle was used. Some of the data on *Ps. fluorescens* will be given in Table 130, p. 390 while the data on *Bact. coli* are shown in Table 46.

From these data, the oxygen consumption per generation has been computed (Table 47). Though there is a good deal of fluctuation in these data, it cannot be said that there is less oxygen needed at the start than a few hours later. This should be the case if the very young cells could use their food more efficiently than the older ones.

(c) SUMMARY OF FACTS

All microorganisms liberate energy during their life processes. Only a fraction of this energy is accounted for by the energy content of the newly formed cells: the larger part is transformed to heat.

Even when cells are formed from building material which has a higher combustion heat than the cells, energy is liberated by fermentation.

Part of the total energy liberated by fermentation can not be used for cell construction, for thermodynamic

reasons. This part is usually quite small with the type of organic changes usually met in fermentations.

During the period of rapid growth, more energy is utilized in cell construction than in the later stages of development.

SUMMARY OF THEORIES

It is generally believed that growth is impossible without a source of energy. The amount of energy required for growth seems quite considerable. In peptone media and in milk, the total energy content of the construction material is similar to that of the cells, i.e., their combustion heats are similar, but a special source of energy seems still necessary. Even when the energy content of the building stones is much higher than that of the cells (methane, amines) growth takes place only when accompanied by oxidation or fermentation.

Perhaps, the formation of certain essential cell compounds requires a definite potential which can be maintained only by a special and continuous source of energy.

Since fermentation continues after growth has ceased, young cultures must show a better efficiency in the utilization of energy than the older ones. There is no indication that during the lag phase, or at the very earliest period of rapid growth, energy is utilized better than during the immediately following period of rapid growth.

III. THE GROWTH RATE

(*a*) METHODS OF MEASURING THE GROWTH RATE

The growth rate, or more correctly, the rate of multiplication, is ordinarily measured by a computation

of the generation time (Appendix p. 399). Since the generation time means the time required by bacteria to double in number, it is smallest when multiplication is most rapid. This necessitates thinking in reciprocals. The formula is

$$g = \frac{t \log 2}{\log b - \log a}$$

where a is the number of cells at the start, and b the number after the time t.

A direct method for measuring the growth rate has been suggested by Slator (1916) who, by the application of calculus (see p. 403), derived the following formula for a growth rate constant:

$$K = \frac{\ln b - \ln a}{t} = \frac{\log b - \log a}{t \log e} = \frac{\log b - \log a}{0.434t}$$

Comparing this with the formula for the generation time, we see that the growth rate constant K of Slator is nothing but the reciprocal of the generation time multiplied with a constant factor:

$$K = \frac{1}{g} \frac{\log 2}{\log e} = \frac{0.694}{g}$$

The disadvantage of the growth rate constant is that it cannot be defined descriptively; it has no easily expressed meaning. It is just a number indicating the relative growth rate.

For comparison, the following relation between growth rate constants and generation times might be useful:

The generation time of 15 minutes corresponds to $K = 2.776$
The generation time of 20 minutes corresponds to $K = 2.082$
The generation time of 30 minutes corresponds to $K = 1.388$
The generation time of 60 minutes corresponds to $K = 0.694$
The generation time of 120 minutes corresponds to $K = 0.347$

If growth is determined by any method other than counting cells per c.c., the growth rate can be computed by the same formula, just as the formula for the generation time would apply. As an example, Slator (1916) measured the growth rate of *Lact. Delbrücki* of which plate counts would give no accurate measure, by comparing the turbidity of the culture with a standard asbestos suspension, and obtained the following results:

Age of culture, in hours	$\frac{b}{a}$	$0.434K$
2.53	119	0.82
3.48	800	0.83
5.02	18,100	0.85

The average for $0.434K = 0.84$ corresponds to a generation time of 21.5 minutes (see also the computations for yeast growth p. 211).

Slator went even further than this and claimed that, since the fermentation is proportional to the number of cells, the growth rate can be computed from the increase of the fermentation products. This will be permissible only if all conditions are entirely under control for we have seen (p. 111) that the rate of fermentation is easily influenced by the products already formed. This same principle has been used as early as 1877 by Nägeli and Schwendener to determine the number of cells. Meyerhof (1917) used it to compare the numbers of active cells in Nitrosomonas cultures.

The growth rate of bacteria under optimal conditions is very large. With common saprophytic bacteria in a suitable medium at room temperature, the generation time is about one hour. At high temperatures and in excellent media, especially with a good source of energy, the generation time may drop to fifteen minutes. This means that one cell can multiply to 16 cells in one hour. This rapid rate can be maintained only for a few hours. With yeast, the fastest rate is about 1 hour.

However, some microorganisms grow very slowly. Among the slowest is *Mycob. tuberculosis* requiring about

six hours for one generation, and slower yet is the organism of Johne's disease. No food has as yet been found to speed up the growth of these organisms to approximately the same rate as saprophytes. In the organism of the host, these bacteria also multiply extremely slowly.

The data obtained by the plate count method are averages. The growth rate of individual cells varies greatly even if the cell material, as well as the environment is very homogeneous as will be shown on p. 266.

(*b*) THE LAG PERIOD

It has become customary to speak of "growth curves" of bacteria or yeasts which means curves representing the changes in the numbers of living organisms in a given time. Often, instead of the numbers of the individuals, the logarithms of these numbers are plotted against time. Frequently, these "growth curves" include the stage where the individuals die from old age, and sometimes this period is spoken of, and even calculated, as "negative growth." The conception of death as negative growth cannot be considered a biochemical definition; it is used only as a simile, for death is not a reversed growth process. Since death in old cultures will be treated together with other causes of death, this phase will not be considered here.

In Fig. 42 of Appendix (p. 404) a growth curve of *Ps. fluorescens* is given. A set of logarithmic growth curves is shown in Fig. 17 representing the multiplication of a *Bacterium A* at different temperatures, as measured by Max Müller (1903). The data for these curves are to be found in Table 48.

In the logarithmic growth curve, the generation time is constant as long as the curve is a straight line. This

period of growth at a constant rate is therefore sometimes given the strange name "period of logarithmic growth"; the bacteria really increase exponentially and not logarithmically.

It is a common observation that the rate of multiplication of a culture is not uniform throughout the entire period of development. A decrease in the rate towards

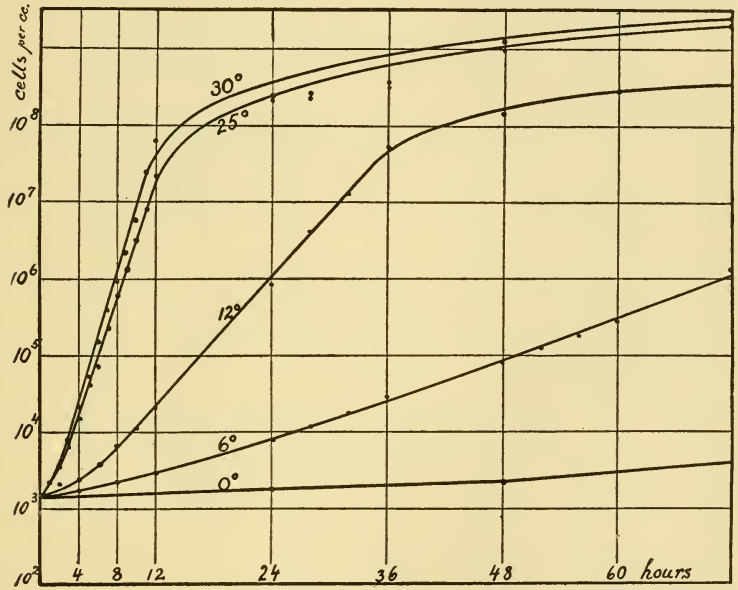


FIG. 17.—Logarithmic growth curves of *Bacterium A* at different temperatures.

the end would be expected, but there is also a period of slow development at the start, during the first hours after inoculation. This "lag period" is visible in the curves of Fig. 17 and is especially conspicuous in the high generation times during the first hours of development in Table 48.

The "lag period" has been the cause of much investigation. As early as 1895, Max Müller explained it through

TABLE 48.—MULTIPLICATION OF BACTERIUM *A*

Time, hrs.	30°		25°		12°		6°	
	Plate count	Generation time, min.	Plate count	Generation time, min.	Plate count	Generation time, min.	Plate count	Generation time, hrs.
0	1,370	.....	1,370	.....	1,370	.....	1,370	
1	2,150	93.2	2,190	88.7				
2	3,560	87.1	3,620	85.7	2,060	291.7		
3	6,580	80.1	7,140	75.5				
4	21,190	60.8	15,700	68.2	2,530	271.2	1,650	14.9
5	51,430	57.3	43,800	60.0				
6	156,250	52.7	60,900	65.8	3,500	266.1		
7	397,500	51.3	230,000	56.8				
8	930,000	51.0	600,000	54.7	6,570	212.2	2,160	12.1
9	2,160,000	50.8	1,320,000	54.5				
10	5,610,000	50.0	3,130,000	53.8	11,000	199.6		
11	25,610,000	47.6	8,000,000	51.4				
12	60,628,000	46.6	22,125,000	51.5	21,100	182.4	2,860	11.3
24	233,100,000	82.8	215,100,000	83.4	827,000	155.9	8,000	9.5
28	252,000,000	95.9	239,000,000	96.4	4,000,000	145.9	11,600	9.0
32	.....	.....	.....	.....	12,700,000	115.6	17,500	8.7
36	363,300,000	119.9	320,400,000	121.1	53,400,000	141.6	27,900	8.3
48	1,260,000,000	145.3	945,800,000	124.5	184,600,000	169.0	79,400	8.2
52	.....	.....	.....	.....	.....	.....	130,000	7.9
56	.....	.....	.....	.....	.....	.....	180,000	7.9

TABLE 48.—MULTIPLICATION OF BACTERIUM *A*.—(*Continued*)

Time, hrs.	30°		25°		12°		6°	
	Plate count	Generation time, min.	Plate count	Generation time, min.	Plate count	Generation time, min.	Plate count	Generation time, hrs.
60	.....	.....	.....	.....	266,500,000	204.9	273,000	7.9
72	1,377,000,000	215.8	1,042,500,000	221.0	350,600,000	240.5	1,353,000	7.2
76	.....	.....	.....	.....	.....	.....	1,956,000	7.2
96	.....	.....	.....	.....	805,100,000	300.5	13,545,000	7.2
120	.....	.....	.....	.....	1,225,500,000	364.1	102,000,000	7.5
144	.....	.....	.....	.....	.....	.....	187,500,000	8.4
168	.....	.....	.....	.....	.....	.....	301,000,000	9.9
192	.....	.....	.....	.....	.....	.....	500,000,000	10.4

0°

Time, days	Plate count	Generation time, hrs.	Time, days	Plate count	Generation time, hrs.	Time, days	Plate count	Generation time, hrs.
0	1,370		6	55,000	27.0	12	30,450,000	20.0
1	1,963	46.3	7	166,000	24.3	14	68,500,000	21.5
2	2,200	44.4	8	450,000	22.7	16	125,700,000	23.3
3	4,200	44.2	9	1,022,000	22.6	18	224,000,000	25.0
4	7,500	39.1	10	2,964,000	21.7	20	293,000,000	27.7
5	22,000	30.5	11	10,266,000	20.5	23	420,000,000	30.3

the age of the mother culture. He summarized his experiments in the table reproduced in Table 49 and drew from it the following conclusion:

"The previous discussions show that besides the well-known requirements (composition and reaction of medium, optimal temperature, etc.), a new, heretofore not considered factor is of importance for fastest possible growth, namely inoculation from a very young culture, not more than a few hours old."

Table 49.—Initial Rate of Growth, and the Age of the Corresponding Mother Cultures of *Bact. typhosum*

Age of mother culture, hours	Time of growth after transfer, minutes	Plate count		Generation time
		Start	End	
2½	75	1,398	5,239	about 40 min.
2¾	145	166	2,144	about 40 min.
3	150	6	87	about 40 min.
6¼	80	2,150	3,130	more than 80 min.
6¼	85	662	1,095	more than 85 min.
14	160	2,454	8,559	more than 80 min.
16	160	5	16	more than 80 min.

Kruse (1910) believed the lag to be a case of poisoning by traces of copper or other toxic substances adsorbed on the surface of the bacteria after transfer. Henneberg (1926) suggested that substances causing surface tension depression might be to blame for it. Though neither of the two investigators gives any proof, these explanations may hold true occasionally. But the phenomenon is too general to be accounted for altogether in this way.

The above explanation of Müller (1895), accepted by Barber (1908) and Penfold and Ledingham (1914), is now generally assumed to be the best explanation of the lag period. The transferred bacteria continue to multiply for some time at the rate with which they multiplied

in the original culture. This is shown very clearly by Chesney (1916) who transferred at frequent intervals bacteria from a growing culture of *Bact. coli* into a fresh medium, and found the rates of multiplication in the new culture for the first two hours after transfer to equal that of the mother culture. Fig. 18 shows in the upper

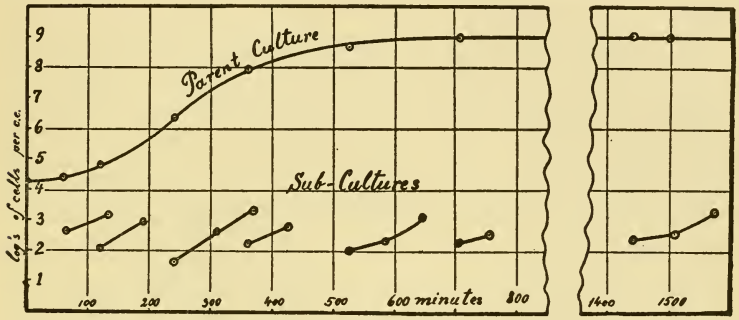


FIG. 18.—The parallelism in the growth rates of the parent cultures and the subcultures of *Bacterium coli*.

continuous line the logarithms of the numbers of cells in the mother culture. The short lines below are the two hour growth periods of the transferred organisms. The lower lines are practically parallel to the corresponding part of the upper line which indicates the same rate of multiplication. A similar agreement was obtained also with cultures of *Bact. pneumoniae*.

This explanation implies that the cell mechanism is affected as the medium is gradually changed through the action of bacteria. This change must be more than just an accumulation of fermentation products because it would require only a very short time for these products to diffuse out of the cell into the new medium. As a matter of fact, morphological changes are involved, and the form and size of young, actively growing cells are different from older, slowly multiplying cells as has been

shown especially by Henrici (1928). K. A. Jensen found (1928) that young cells of *Bact. coli* may be so sensitive that they die if transferred to a new medium. These hypersensitive cells are almost invisible, transparent "shadow forms" appearing about three hours after transfer, and changing back to normal after about six hours.

The ready growth of young cells transplanted to a new medium from a rapidly growing culture, and the slow growth of cells from an old culture simplify the problem of the growth curve greatly because the reason for the initial lag phase appears to be the same as the reason for the decreasing rate of growth. Lag is not typical for new transfers, but for old cells. Thus, the lag phase need not be discussed here as a problem in itself; it will be studied in the next chapter with the decreasing growth rate.

Many efforts have been made to express the entire growth period of bacteria and similar organisms in a limited volume by one mathematical formula. Several formulas have been developed empirically which fit the facts fairly well. Some formulas agree better with certain sets of data while others fit better for observations with other organisms.

It will be shown in the following pages that there is more than one cause for the cessation of growth in bacterial cultures. In fairly early stages, the exhaustion of oxygen in the lower strata of the medium must necessarily influence greatly the growth rate of most microorganisms. In the last stages of slow growth, the accumulation of fermentation products is likely to be another cause of growth retardation. It will be seen, from the following discussions, that the main cause of growth retardation is still entirely unknown. It seems impossible that these different causes of retardation of growth which become effective at different stages of development can be incorporated into one mathematical equation. It also seems that the empirical equations which fit the facts approximately will be of little help to find the still unknown causes of retardation of growth.

(c) THE DECREASING GROWTH RATE

Several explanations have been given for the decreasing growth rate, and probably, all of them are correct. It has been stated that increasing accumulation of fermentation products decreases the rate of multiplication. In Table 133, p. 400, the generation times for *Strept. lactis* in milk are given for each three-hour period, and also the amounts of acid formed. The generation time is slowest, i.e., growth is most rapid just before the acidity begins to become noticeable. But it does not seem probable that an increase of only 0.005% of lactic acid could decrease the growthrate 26% (culture I) or that 0.007% increase of acid could cause a drop of 40% (culture II). Nor is this beginning retardation likely to be caused by lack of food because addition of 1% peptone did not change the numbers of bacteria of this very strain appreciably (p. 210).

With other streptococci, however, cessation or retardation of growth may be due to lack of food. It has been shown on p. 149, that many streptococci do not find enough available nitrogenous food in milk to produce maximal growth. Addition of peptone increases the final number per c.c.

Attention has been called already (p. 80) to the very rapid disappearance of oxygen in cultures of aerobic bacteria, and to the fact that only on the very surface layer, cells can multiply rapidly after this stage has been reached. These facts will be discussed in more detail later, in the chapter on the concentration of food. Lack of food, then, may be the cause of retardation of growth in some cases, but cannot be considered so generally. The growth rate frequently decreases before any appreciable amounts of food have been used up,

and before any appreciable amounts of fermentation products have been accumulated.

In most cases on record, no long-continued period of constant growth is noticeable. From the initial lag phase with its high generation time, the growthrate

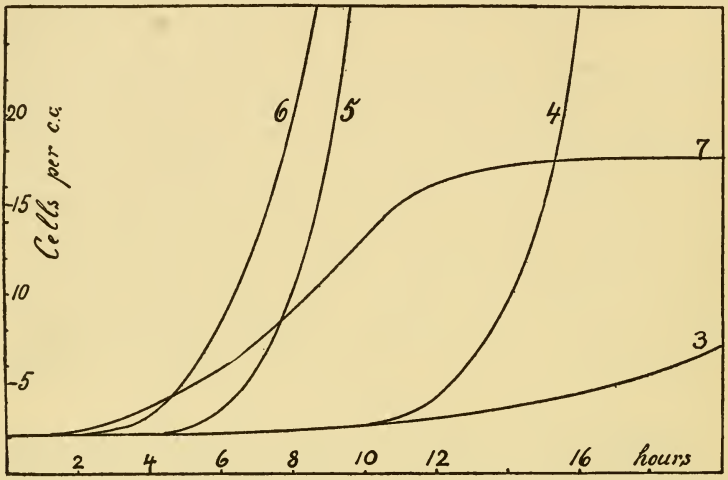


FIG. 19.—Yeast growth from different amounts of inoculum. The inoculum for curve 3 was 2,095 cells, for curve 4, it was 10 times as much, for curve 5 10 times as much again, etc. The scale of cells per c.c. is to be multiplied for curve 3 with 10^3, for curve 4 with 10^4 etc. The largest inoculum (curve 7) shows the most rapid recovery from the lag period.

gradually increases until it reaches a maximum (or the generation time comes to a minimum) and then, the rate does not remain there very long, but decreases quite rapidly to zero. This change of rate after the maximum is reached, is the cause of the point of inflexion of the growth curve.

The discussions on the cause of the "Endpoint of Growth" (p. 231) will reveal that our knowledge of this cause is still very scant. The evidence points at present to some cell secretions of a specific nature as the main

cause of a cessation of growth. These products, then, must also bring about the morphological changes and change the actively growing and fermenting cell into a resting cell, and thus cause the lag if these cells are transferred.

It cannot be, though, that small amounts of this peculiar cell secretion are transferred with the inoculum, and bring about retardation. Then, we should expect the lag period to be more pronounced with a larger inoculum, while the opposite is the case as shall be shown presently.

It can be only that the cells have gone into a resting stage, morphologically and physiologically different from that of the active cells, and that some time is required to change from one stage into the other. The assumed cell secretions will bring about this change, but have no further effect after the change of the cells has been accomplished.

This explanation does not satisfy entirely, for it does not account for the fact that the lag period is shorter when the inoculum is larger. This has been shown by many investigators, (Rahn, 1904, Penfold, 1914, Graham-Smith, 1920) and is illustrated graphically (Fig. 19) from some data by Henrici, (1928), showing the multiplication of yeast in a medium of 2% peptone + 10% glucose.

Cultures 4, 5, 6, and 7 had 10, 100, 1,000, and 10,000 times as many cells at the start as culture 3. They are all drawn here with the same starting point, and therefore, the number 5 on the ordinate scale means 5,000 cells in case of culture 3, and 50,000,000 in case of culture 7. If the cultures all grew at the same rate, their curves should fall on the same line. They differ greatly, however, and culture 7, with the largest inoculum, overcomes the lag most rapidly, while the others follow in order of their cell concentration.

This leads to another observation which belongs to the same group of unexplained facts, and which has become quite conspicuous perhaps only because it has the interesting name "bios theory" attached to it. The bios theory dates back to the observation of Wildiers (1901) that 0.25 c.c. of a yeast suspension transferred to a synthetic medium would not develop while 2.5 c.c. transferred to the same medium would grow. This is nothing else but an extreme case of lag with a very small inoculum into a poor medium.

These observations have since been repeated, contested and verified, and explained in many different ways. A literature review is given by Tanner (1925).

The decrease of lag time by a larger inoculum would speak in favor of the presence of small amounts of toxic compounds in the new medium (assumption of Kruse and Henneberg). The absence of lag in transferring young cultures forbids this as a general explanation. Where this explanation is correct, the lag period should be greatly shortened by a good adsorbent like charcoal in the medium.

It is also imaginable that the resting cells store a certain compound which allows the cells later to grow at a more rapid rate, than if they depended upon food from outside only. This would require a distinct cycle; a stage of slow growth, with storage, followed by a stage of rapid growth, partly at the expense of the stored food. There is no evidence to support this assumption.

One other explanation remains, namely, that by a required oxidation—reduction potential. A number of experiments are on record showing a better initial growth if the dissolved oxygen is partly removed from the medium. Perhaps the most typical example is the observation of Webster (1925) that *Bact. lepisepticum*

would not grow aerobically in broth when the inoculum was small; when the oxygen tension was lowered, it grew well, and without lag.

Lindner (1919) has already claimed that the bios problem was nothing but an oxygen problem. Yeasts were found to keep healthy and ready to multiply if oxygen was excluded. This is strengthened by the observation of Miss Copping (1929) that the oxidizing yeasts (*Mycoderma*, *Willia*, *Torula*) are less in need of "bios" than the fermenting yeasts (*Saccharomyces cerevisiae*, *apiculatus*). Important is her conclusion:

"The presence of bios . . . accelerates the growth, . . . but does not increase the final number of the cells as this is very probably limited by the supply of nutriment in the medium."

Bios, then, is not a food. It creates a condition favorable for growth, it influences the environment, but it is not building material or source of energy.

Old cells do not maintain the reduction potential. Litmus milk cultures of streptococci are decolorized, but gradually, the color comes back. There must be a relation between old age and loss of reduction potential.

A single cell, old and with a low power of reduction, may not be able to fight off the invading oxygen of the medium; it may be killed by oxidation. A large number of the same cells will have the oxygen distributed among them. There is less oxygen per cell. The chances for establishing a reduction potential sufficient for growth is more probable when the inoculum is large. As soon as a few cells are working efficiently, the oxygen tension in the medium will be lowered and the weaker cells, unable to fight off the oxygen by their own mechanism, will then be able to repair their cell mechanism and grow normally after a while.

The growth of large inocula of anaerobes in culture tubes under aerobic conditions, the great help of cysteine in such cultures, and the beneficial influence of CO_2, observed by Rettger and associates, all point in the same direction. The author's experiments are not sufficiently uniform as yet to consider this explanation proved.

(*d*) CONCENTRATION OF FOOD AND GROWTH RATE

The growth rate depends upon the amount of available energy, as well as upon the kind and quantity of the

building material. The total solids of most bacteria growing in the usual culture media are less than 0.03% of the weight of the medium (see Table 132, p. 397). The amount of construction material beyond this amount is not likely to influence the rate of growth to any appreciable degree. With yeasts and molds, the crops, and consequently the requirements, are larger.

The energy available for growth is proportional to the rate of fermentation, and it has been shown on pp. 114–118 that beyond a certain minimal concentration of the substrate, the rate of fermentation is almost independent of the concentration. We must conclude from this that the concentration of food is not likely to influence the rate of growth very much, except when the concentration becomes very low.

TABLE 50.—GENERATION TIMES OF *Bact. typhosum* IN PEPTONE SOLUTIONS OF DIFFERENT CONCENTRATION

	Generation time		Generation time × concentration	
Experiment	I	II	I	II
1.25% peptone	.....	42 min.		
1.0	39 min.			
0.8	49			
0.6	.....	42		
0.4	49	40		
0.2	77	51	15.4	10.2
0.1	111	84	11.1	8.4
0.05	181	.....	9.0	
0.025	450	.....	11.2	
0.0125	783	723	9.8	9.0

The amount of available energy might be greatly changed by using a different substrate, and examples for this shall be shown in this chapter. But as long as

only the concentration and not the kind of food is changed, the growth rate is not greatly altered as the following facts will bear out.

The data of Penfold and Norris (1912) show that the generation time of *Bact. typhosum* in peptone water remains practically constant if the peptone concentration is above 0.4% (Table 50). Let us try here to distinguish again between the energy formation and the building material. Above a certain concentration of fermentable material, the fermentation, i.e., formation of available energy, remains constant. In this stage, the growth process can be accelerated only by a better supply of building material, and even there, it must by necessity soon reach the maximum. There must be a concentration where neither the increase of fermentable material nor the supply of building material causes a more rapid growth. This condition is apparently reached in the above case at 0.4% peptone.

If, in this example, the *nature* of the fermentable material, or of the building material is changed, we have the possibility of an increase or decrease in the rate of growth. With peptone alone, the cells cannot exceed a definite number of calories per unit time, owing to the limited quantity of oxidase or endoprotease in each cell. However, if sugar is added to the peptone medium, a different enzyme begins to react, in addition to the previous ones, and this may yield a larger number of calories per cell in unit time, and therefore may enable the cell to grow more rapidly. This is proved by experiments of Penfold and Norris. The addition of 0.175% glucose to a medium containing only 0.1% peptone lowers the generation time about 50%, i.e., it doubles the rate of growth; with 1% peptone this effect is less marked.

What happens in a very dilute medium, can hardly be explained theoretically, as we know but very little about the rates of fermentation at very low concentrations of fermentable material. We may assume in the simplest case, that it is proportional to the amount of material; the available energy would then be proportional to the peptone concentration. This may account for the fairly close agreement between rate of growth and concentration in Table 50, causing the product of generation time and food concentration to be nearly constant. It seems that even in these low concentrations, there is still sufficient building material to allow cell growth, if only the energy suffices for the needs of the cell. But we may also assume that the cells use the energy much more economically if the food supply is scanty. The question can be settled only by experiments where the energy supply is constant, i.e., where the concentration of fermentable material is fairly high, while the building material is varied. Such experiments are still lacking.

Other examples can be found in the experiments by Curran (1925) who cultivated *Bact. coli* and *Bact. aerogenes* in solutions of 1, 2 and 4% peptone. An increase of 100% in the food concentration caused an increase of the growth rate of only 3%, and an increase of 300% in the food increased the growth rate 13%.

A similar ratio was obtained with yeast by Zikes (1919a). He studied the growth of beer yeast in different concentrations of wort by direct observation under the microscope, and obtained the following relative growth rates:

for 1 part of wort with 0 parts water: relative growthrate 100
1 part of wort with 4 parts water: relative growthrate 102
1 part of wort with 8 parts water: relative growthrate 94
1 part of wort with 12 parts water: relative growthrate 98, 95, and 92

In discussing growth, some authors do not distinguish sharply between growth rate and numbers of cells. A small increase in the growth rate will cause a conspicuous increase in total numbers if the experiment extends over a long period. The faster growth rate works all the time, and the results multiply and accumulate similar to compound interest.

TABLE 51.—DEVELOPMENT OF *Strept. lactis* IN SKIM MILK OF DIFFERENT CONCENTRATIONS

Lactose concentration in %	10	5	2.5	1.25	0.6	0.3	0.15	0.075
Milk concentration	2×	1×	0.5	0.25	0.125	0.062	0.031	0.016
Fermenting capacities × 10^{10} mg.								
12–24 hours	4.7	5.4	4.2	6.5	1.9	1.6		
24–36 hours	2.7	3.7	4.0	2.4	2.8	2.5		
Generation times in minutes								
6–12 hours	39	46	48	85	79	89	(54)	(52)
12–24 hours	(119)	77	78	64	73	70	176	130
Average	79	62	63	75	76	80	115	91
Final number in millions of cells per c.c.								
36–48 hours	888	1468	725	307	223	130	0.93	1.0

The rather incomplete data of Peltier's unpublished experiments (see p. 118) may be given here as a summary. Skim milk powder was dissolved in water to a concentration about twice the normal concentration of milk. Of this concentrated milk, part was diluted with an equal amount of water, giving skim milk of normal strength. Part of this was diluted again with an equal amount of water, and so on. The different dilutions were sterilized, inoculated with *Strept. lactis*, and the bacteria were counted, and the acid titrated frequently. From these data, the fermenting capacities and the generation times were computed. Table 51 gives the averages of there sets of experiments.

The fermenting capacity of the cells fluctuates considerably, but does not decrease distinctly until the milk is diluted 1:8; i.e., the rate of fermentation is constant until the sugar concentration falls below 1%. If we average the generation times (omitting the lag period from 0–6 hours), we find the growth rate also quite uniform, decreasing very slowly until the milk is diluted with 32 parts of water. At this point, the lactose concentration is 0.15%, and the total proteins amount to about 0.1%, of which the available proteins are only a very small fraction.

A frequently disregarded factor is the oxygen concentration. Many bacteria grow in peptone solution only aerobically, i.e. they can obtain energy from peptone only by oxidizing it. A medium containing no carbohydrates or other hydrogen acceptors can support growth of facultative organisms only as long as oxygen is available. As soon as sufficient growth has developed to make the culture distinctly cloudy, all oxygen will have been used up in the medium, and growth can take place only at the very surface (see p. 80).

This is illustrated by an experiment of Rahn (1912) who inoculated a 1% peptone solution with an unidentified aerobic soil bacterium. Part of this inoculated solution was kept in a flask, and part was poured on sterile sand so that the sand contained 10% moisture; this made the moisture films around the sand particles in which the bacteria grew about 20 to 40μ thick, and gave optimal conditions for a rapid oxygen exchange. Table 52 shows one of the three experiments.

The same experiment was repeated with *Strept. lactis* in milk. This organism does not have the faculty of utilizing oxygen, and the better oxygen supply did not increase growth nor acid formation. On the contrary, a slight retardation through the better oxygen exchange became noticeable.

It is an old experience that yeast is greatly favored by a liberal oxygen supply. Aeration not only increases the crop, but also the rate of growth, as A. J. Brown has shown (1905). Hunter (1923) showed the same for Azotobacter.

TABLE 52.—GROWTH AND AMMONIA FORMATION IN PEPTONE SOLUTIONS WITH AND WITHOUT SAND BY AN UNIDENTIFIED SOIL BACTERIUM

	100 c.c. peptone solution		100 c.c. peptone solution + 1,000 gm. sand	
	NH_3, mg.	Plate count	NH_3, mg.	Plate count
Start	0	910	0	910
After 24 hours	1.53	9,500,000	4.68	28,200,000
After 48 hours	7.48	12,000,000	23.38	180,000,000
After 72 hours	8.67	20,000,000	37.40	342,000,000
After 96 hours	12.92	37,000,000	47.60	312,000,000
After 168 hours	20.57	28,000,000	62.48	282,000,000

TABLE 53.—GROWTH AND ACID FORMATION BY *Strept. lactis* IN MILK WITH AND WITHOUT SAND

	100 c.c. milk		100 c.c. milk + 1,000 gm. sand	
	Per cent acid	Plate count	Per cent acid	Plate count
Start	0.16	700	0.16	700
16 hours	0.57	1,200,000,000	0.25	785,000,000
24 hours	0.66	1,670,000,000	0.49	980,000,000
40 hours	0.73	1,700,000,000	0.64	1,280,000,000
88 hours	0.82	.....	0.63	

Many of the experiments on growth rates have been made in peptone solutions or meat extract solutions under the assumption

that peptone is an excellent food for both energy formation and building material. However, we really know very little about either. For the non-liquefying organisms commonly used for laboratory experiments we scarcely know which compounds of peptone they can use, and which are indigestible for them. Some of these bacteria may be able to use only a very small portion of the peptone given, and though 1% peptone may seem ample food, it is very little, if 95% of it indigestible.

Most streptococci do not find sufficient food for optimal growth in milk, and grow to higher final numbers if peptone is added to the milk (see p. 149). That the rate of growth is also influenced may be seen from the following data of Rahn (1911):

Table 54.—Generation Times of Streptococci in Milk

Strain	Period of observation, hours	Generation time in		Increase in growth rate by peptone, %
		Milk, minutes	Milk + 1% peptone, minutes	
II	12	60	63	0
	24	69	69	0
IV	24	118	99	19
	24	82	70	17
	24	78	68	15

Strain II must have been able to use the nitrogenous material of milk readily for construction while Strain IV was plainly starving for available nitrogen in the midst of all the milk protein.

With liquefiers, we are more certain about their being able to utilize most of the peptone and meat extract.

Attention has already been called to the fact that a change in the type of substrate for fermentation may cause a more pronounced change in growth rate than an increase in concentration. Such a change of the growth rate by a change of *source* of energy is very typically shown in Fig. 20 representing the yeast growth and sugar consumption in aerated wort as found by Balls and

Brown (1925). The logarithms of the yeast crop plotted against time should form a straight line if the growth rate is constant (p. 192). Fig. 20 shows a constant growth rate, after one hour's lag, to the seventh hour, and then changes abruptly to another constant growth rate which is only about one-tenth of the previous. We notice

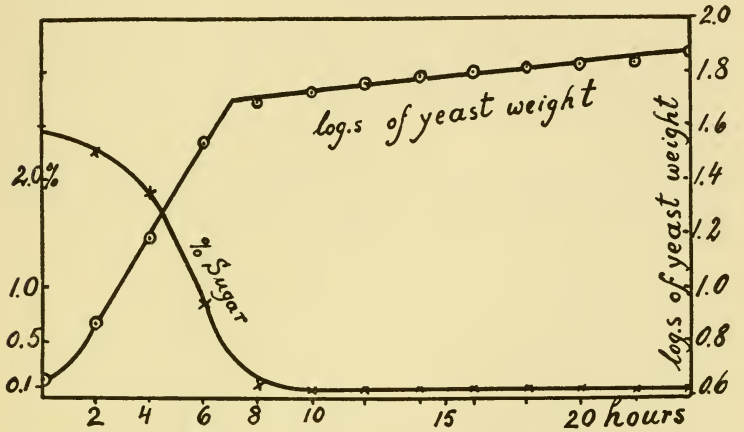


Fig. 20.—Logarithmic growth curve of a yeast culture, showing an abrupt change of the growth rate after exhaustion of the sugar supply.

that this change occurs when the sugar concentration falls below 0.09%. The yeast then uses alcohol as the only source of energy (see p. 176), and this source is not nearly so good. The 0.5 gm. sugar per liter which remain is an unassimilable reducing compound (see p. 175). Other experiments of Balls and Brown show exactly the same sudden change of growth rate at the point when the sugar is exhausted.

SUMMARY OF FACTS

The growth rate is not very greatly influenced by the concentration of food. In a peptone solution without

sugar, an increase above 0.5% did not increase the growth rate of *Bact. typhosum*. The increase from 1% to 2% peptone produced with *Bact. coli* an increase in the growth rate of about 3%, and even 4% peptone increased the growth rate only 10–13%. *Strept. lactis* in diluted skim milk grew at the same rate until the milk was diluted with 32 parts of water reducing the lactose concentration to 0.15% and the total protein to about 0.1%. Yeast in beer wort showed almost the same rate of growth whether the wort was normal or diluted with 12 volumes of water.

While the growth rate is changed very little, a slight increase over a number of hours will produce noticeable differences in the total number of cells in comparative experiments.

An important food for many bacteria is oxygen, and on account of its low solubility, the oxygen concentration is frequently the limiting factor of the growth rate. Organisms which cannot utilize oxygen, such as *Strept. lactis*, are not benefitted in their growth by aeration of the culture.

SUMMARY OF THEORIES

For theoretical considerations, we must distinguish between the energy food and the building material though in many cases, the two might be represented by the same compound.

The rate of fermentation had been found to be independent of the substrate concentration above a comparatively low limit (with yeasts and streptococci, about 1% glucose in the medium). If no increase in available energy can be expected, an increase of the growth rate through more concentrated food could come only from a concentration of building material. Cell

construction might be more rapid when more building stones are available. But no great increase can be expected. This is borne out by the facts. All increases observed are small if compared with the changes in growth rate brought about by temperature changes.

It is an interesting and significant observation that while an increase in concentration does not affect the growth rate, the addition of another substrate may. *Bact. typhosum* was not affected by peptone beyond 0.4%, but the addition of sugar doubled the growth rate. Oxygen influenced growth in a similar way. This proves that the growth mechanism can work faster if more energy is available, and that the failure to produce more rapid growth by more food is due to the impossibility of producing more energy by more substrate.

The solubility of oxygen is so low that an increase in its concentration by aeration will bring about a greater liberation of energy and accelerated growth, though it is primarily a food for energy.

(*e*) TEMPERATURE AND GROWTH RATE

It has already been pointed out, in discussing the relation between temperature and the rate of fermentation, that the temperature coefficient of all biological activities varies from infinite, at the minimum temperature, to zero at the maximum temperature. The temperature coefficient of biological activities is much less constant than that of common chemical reactions.

However, we may assume that the influence of temperature is primarily the same as in normal chemical processes, and that other processes are superposed at very high or very low temperatures. The temperature coefficient may be considered essentially normal, except for some complications at the two extremes.

Figure 21 shows the temperature coefficients of growth of three fungi, after Fawcett (1921). The curves might be explained as being essentially straight lines which are deviated at both ends, and which indicate, in their

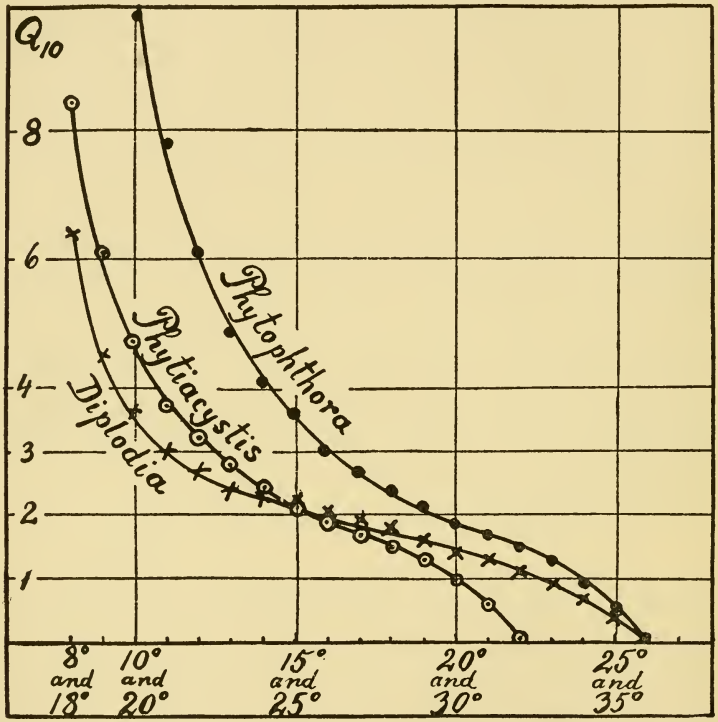


Fig. 21.—Variation of the temperature coefficient of growth with the change of temperature, demonstrated with three fungi.

unhampered path, a normal temperature coefficient of about 2 to 3 (data see Table 55, p. 218).

Some compound, or group of compounds, in the cell is the cause of growth. This agency, in higher organisms, is located in the chromosomes. If the cell is heated, this agency is destroyed; there is no more growth.

Without making any further assumptions about this growth-producing agent, we must assume that it is destroyed rapidly at high temperatures, and therefore it must be destroyed slowly at low temperatures. We have seen the same behavior in enzymes.

Just as we have assumed a continuous slow deterioration of the endo-enzymes in living cells (p. 131), so we may assume a gradual slow deterioration of the growth-producing agent in the cell. And just as under normal environment, the cell is continuously rebuilding the endo-enzymes as fast as they deteriorate, keeping the enzyme concentration of the cell constant, so we may assume that the growth-producing agent is rebuilt by some part of the cell as rapidly as it deteriorates, and that a cell, in favorable environment, maintains a constant level of the growth-producing agent.

An increase in temperature will bring about a more rapid action of the agent, i.e., accelerated growth. At the same time, it will bring about a speedier deterioration of the agent, and also a more rapid regeneration of the agent. But these three processes are not accelerated at the same rate. The temperature coefficient for growth is about 2 to 3; the coefficient for the regeneration will probably be about the same. But the coefficient of the deterioration of the agent is doubtless much greater. It must be quite high to account for the short interval between optimal and maximal temperature. We must expect here again a complete analogy with the fermentation processes.

Since deterioration is accelerated with greater speed than regeneration, there must be a temperature where the regeneration is just barely able to make up for deterioration. This is the highest temperature at which the cell can work with its normal amount of growth-

agents, and therefore, this is the optimum temperature. If we heat still higher, growth will be more rapid at first, but since the growth-agent deteriorates faster than it can be built up again, the cell loses gradually its growth-agent, and will finally work with a lower concentration of this agent which means a decrease in the growth rate.

We must expect, then, that there is a definite optimum temperature of growth, and that above the optimum, we find the Tammann principle (see p. 129) established, most rapid growth at first, decreasing rapidly with time.

The well known experiments of M. Ward (1895) with *B. ramosus* are as beautiful a proof of the above deductions as we can imagine. He states:

"At the optimum, it (*B. ramosus*) metabolizes and grows, and respires etc. at its best; but at a higher temperature, it may grow *for a short time more rapidly*, but sooner exhausts itself, and so produces a poorer crop in the end."

In his summary, Ward says on page 462:

"At the optimum temperature, the growth is very rapid, and lasts for a long time, and the organism uses the materials to maximum effect, and produces from them the maximum amount of its own substance, in other words, the largest 'crop.'

"At temperatures above the optimum, however, the growth, though at first as rapid as at the optimum temperature, lasts for a shorter and shorter time, according as the temperature is further and further removed from the optimum; consequently the curve, though equally steep in its steepest parts, begins to fall soon and growth ceases sooner, and the crop obtained from the same amount of original food material is smaller and smaller according as the temperature is higher. At length, a temperature is reached where the curve is infinitely short, i.e. no growth occurs at all. This temperature is, however, about 39°C. and indicates the death point."

(p. 459) "The maximum temperature, therefore, is not a fixed point, until we approach 39°C. to 40°C. beyond which no growth seems possible; but it differs according to the length of time the organism has been exposed to the high temperature. Thus it fre-

quently occurs that a *first* doubling period is completed at even 35°C. or 36°C. in the minimum time,—i.e., thirty minutes or so—but the *second* doubling of the same filament will require a longer time; and the *third* may occupy nearly twice as long, and so on."

A very good confirmation of the existence of the Tammann principle in the growth at high temperatures is given further by Fawcett (1921). He grew four plant-pathogenic molds on agar at different temperatures, using as inoculum a disk of 1.25 mm. radius cut out of a five day old agar culture of the mold kept at 20°C. This disk was inverted and placed in the center of the surface of a new agar plate containing 15 c.c. of solidified cornmeal agar. The rate of growth was measured by the increase of the radius of the colony. Most of the data are averages of 8–12 separate experiments.

Table 55 represents the experiments with *Phytiacystis citrophthora.* The data show the daily increment, i.e. the rate of growth. The maximum growth rate, indicated by heavier type, shifts from 27.5°C. on the first day to 23.5°C. on the fourth day and remains there. This is probably the optimum temperature for continuous growth, while the initial growth was faster at 27.5°C. The growth rate of molds is nothing very definite, and we observe an increase of the "growth rate" at all temperatures below 30°C. Whether this is a question of lag, or of adjustment, or of recovery from injury by tearing the mycelium in transplanting does not interest here especially. It is of great interest, however, that above 30°C., the growth rates of the second day are much lower than on the first day. This is even more characteristic of the Tammann principle than the shifting of the optimum temperature with time.

Phytophthora terrestria shows a shift of the optimum temperature from 34.5°C. on the first day to 31°C. on the fifth day. Above 34.5°C.

TABLE 55.—GROWTH OF *Phytiacystis citrophthora*
(Increments of radius in mm. for successive 24 hour periods)

Temp. in °C.	7.5	13.5	15.5	18.5	19.5	21.5	23.0	23.5	24.5	25.0	27.0	27.5	29.5	30.0	31.0	32.0	34.0	35.5	36.0
1st day	0.04	2.5	2.3	4.0	4.7	5.6	6.1	5.4	5.7	7.0	7.6	**8.3**	7.3	7.0	6.3	4.9	0.9	0.5	0
2nd	0.4	4.2	4.5	7.3	7.8	9.0	9.5	10.0	10.0	9.9	**10.5**	10.3	8.9	8.1	5.5	0.7	0	0	0
3rd	0.6	3.5	5.7	8.0	8.3	9.5	10.5	10.2	10.3	**10.5**	10.2	9.9	8.6	7.6	3.5	0	0	0	0
4th	0.8	3.8	6.5	7.1	8.5	9.8	9.9	**10.5**	10.2	....	9.9	9.8	8.0	7.1	1.5	0	0	0	0
5th	1.2	4.3	6.5	8.4	9.1	9.5	9.7	**10.5**	10.0	....	10.0	9.3	7.5	6.6	0.5	0	0	0	0
6th	1.9	4.4	...	8.4	...	9.5	....	....	9.5	....	....	....	....	6.0	0.5	0	0	0	0

the growth rates decrease from day to day. With *Phomopsis citri*, the maximum growth rate, i.e. the optimal temperature is constant at 27.5°C. Above 31°C. the growth rate decreases from day to day. With *Diplodia natalensis*, the optimum temperature shifts from 30°C. to 25°C. and at 30°C. and above, the growth rate decreases with time.

Considering, then, the Tammann principle established, we have a good explanation why the upper range of temperature for growth is limited, why there is a maximum and an optimum temperature which are unknown with common chemical reactions.

For the range of normal growth temperatures, the most accurate and extensive data of the older research are those of Ward, Max Müller, and Barber. All three differ in their technique. Ward measured the increase in the length of the cells of *B. ramosus* under the microscope; Müller counted his bacteria by the plating method; Barber grew one single cell of *Bact. coli* in a hanging drop and counted the number of cells under the microscope after a given time.

TABLE 56.—GROWTH OF *B. ramosus* AT DIFFERENT TEMPERATURES

Temperature, degrees	Generation time, minutes	Growth rate constant	Temperature, degrees	Generation time, minutes	Growth rate constant
10–12	300–400	0.10–0.14	26	40	1.04
14	192	0.22	28	33	1.26
15	138	0.30	31	34	1.22
16	108	0.38	34	33	1.26
17	90	0.46	35.5	31	1.34
18	76	0.55	36.8	29	1.44
19	70	0.60	38	30	1.39
21	60	0.69	39	30	1.39
22.6	52	0.80	39.5	35	1.19
24	40	1.05	40	50–∞	0.83–0
25	40	1.05			

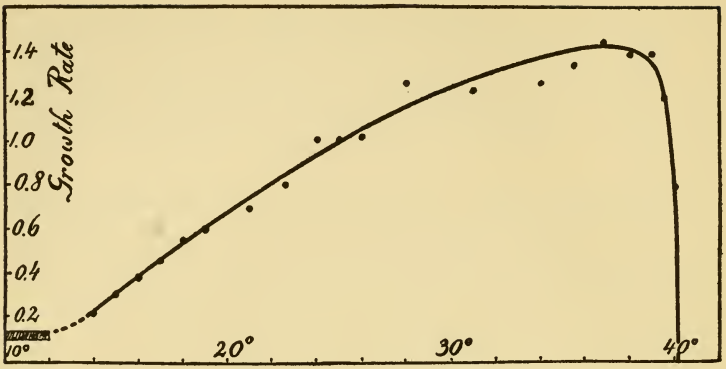


FIG. 22.—Growth rate of *B. ramosus* at different temperatures.

The data of Ward are computed in Table 56, as growth rate constants (see p. 190). These constants are plotted in Fig. 22. It shows the asymptotical approach to zero of the rate of growth at low temperatures, and the abrupt approach to zero at high temperatures.

TABLE 57.—GENERATION TIMES OF FIVE BACTERIA AT DIFFERENT TEMPERATURES

(Shortest times only)

	0°C.	6°C.	12°C.	25°C.	30°C.
Bact. A	19ʰ57′	7ʰ14′	115′38″	51′24″	46′38″
Bact. B	22ʰ18′	6ʰ25′	190′30″	53′9″	50′56″
Bact. C	33ʰ38′	7ʰ1′	145′54″	53′48″	46′15″
Bact. D	15ʰ55′	5ʰ13′	125′ 0″	45′6″	44′38″
Ps. fluorescens	21ʰ26′	7ʰ33′	161′30″	75′36″	64′11″

	Temperature coefficient Q_{10}			
Bact. A	5.4	9.1	1.9	1.2
Bact. B	3.1	2.7	1.1	1.08
Bact. C	13.6	5.8	2.1	1.4
Bact. D	6.8	4.4	2.2	1.01
Ps. fluorescens	5.7	5.4	1.8	1.4

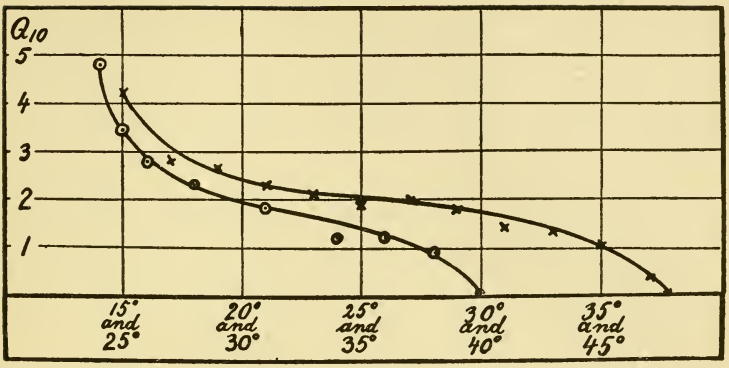


FIG. 23.—Temperature coefficients of growth of *Bacterium coli* (upper curve) and of *B. ramosus* (lower curve).

The following table shows the generation times and temperature coefficients of five bacteria at the time of most rapid growth, after Max Müller (1903). The detailed data for *Bacterium A* will be found in Table 48, p. 194. The temperature coefficient is quite evidently not constant, but decreases with increasing temperature. To emphasize this decrease of the temperature coefficient

TABLE 58.—TEMPERATURE COEFFICIENTS OF GROWTH

B. ramosus		Bact. coli	
14°–24°C.	4.8	15°–25°C.	4.2
15°–25°C.	3.4	17°–27°C.	2.8
16°–26°C.	2.8	19°–29°C.	2.7
18°–28°C.	2.3	21°–31°C.	2.3
21°–31°C.	1.8	23°–33°C.	2.1
24°–34°C.	1.2	25°–35°C.	1.9
26°–35.5°C.	1.2	27°–37°C.	2.0
28°–38°C.	0.9	29°–39°C.	1.8
		31°–41°C.	1.4
		33°–43°C.	1.3
		35°–45°C.	1.04
		37°–47°C.	0.42

with the increase of temperature, Table 58 represents the temperature coefficients of *B. ramosus* computed from Table 57, and those for *Bact. coli* obtained by Barber (1908). This relation is shown graphically in Fig. 23.

It should be mentioned that Barber in his direct microscopic observations with *Bact. coli* made no statements indicating that he ever found the Tammann principle. He never observed more than ten generations, and the generation times were

at 40°C. from 17–25 minutes
at 42°C. from 20–23 minutes
at 45°C. from 18–30 minutes
at 47°C. from 42–68 minutes

with no differences whether ten or only two generations were observed.

We had started with the assumption that the growth rate is increased by temperature increases in the same way as any chemical process, and that the deviations at the maximum and minimum temperatures (as evidenced by Figs. 21 and 23) are to be explained by a superposition of other factors. The existence of a true optimum and a less definite maximum temperature for the growth rate could be accounted for very satisfactorily by the Tammann principle. The difficulty lies in explaining the minimum temperature.

Growth is the result of many interlinking processes. If one of these many processes is extremely slow, the entire growth is slow. If one of the processes ceases to function, there will be no growth, regardless of the condition of all other involved agencies. At the minimum temperature, there is evidently some limiting factor, but we have not sufficient knowledge of the growth mechanism to locate this factor.

A possible explanation is the assumption that the available energy is no longer sufficient for synthesis. Each synthesis requires a minimum amount of available energy, not only in quantity but probably also potentially. This is furnished readily in suitable media at normal growth temperatures. Even then, a considerable amount of the available energy is not utilized for synthesis (see pp. 180–188). This apparent waste might be due to radiation or conduction of heat, or to the general effort of keeping up a certain energy potential. In the first case, the absolute loss would be greater at lower temperatures; the relative loss would be very much greater, because the amount of available energy is greatly reduced by low temperatures. If the maintenance of a certain energy potential is necessary to bring about growth, there may be a temperature well above the freezing point where certain organisms lose so much of the small amount of energy furnished them by their fermentation that the potential in the cell cannot be maintained, and the cell ceases to grow. It has been shown on p. 134 that streptococci in milk at −1°C., gradually lose the power to ferment. Their endoenzyme deteriorates and cannot be replenished at such low temperatures. If the cell ceases to be able to make repairs, it must gradually die from the slow, but certain deterioration of its essential, thermolabile components. (See also influence of kind of food, p. 157.)

The data obtained by the author with streptococci indicate that bacteria die at temperatures too low for growth. The death rate is very slow, but distinct. Table 59 shows the number of cells in milk cultures of three streptococci. *Strept. cremoris* No. 18 is the only one which does not grow at +5°C.

The generation times at 5°C. for the first time interval were 40.7 hours for *Strept. cremoris* 23 and 55.5 hours for *Strept. lactis*. It takes about 2 days for one cell to double. At 10°C., the generation times are 24.2 hours (*Strept. cremoris* 23) and 12.5 hours (*Strept. lactis*). It seems remarkable that a growth process can be so slow and yet not come to an end. At −1°C., all three cultures showed a decrease in numbers. A gradual decrease of the number of cells of *Lactobacillus acid-*

ophilus in milk at low temperatures has also been recorded by Kopeloff (1926). Graham-Smith (1920) also reports a slow decrease in the number of viable cells of *Micr. pyogenes* in broth at 8–10°C.

TABLE 59.—INCREASE AND DECREASE OF STREPTOCOCCI AT LOW TEMPERATURES
(Colonies per c.c.)

Strept. cremoris No. 18

°C.	Start	10 days	31 days	45 days
− 1	20,000	4,500	1,000	80
+ 5	20,000	40,000	(135,000)?	8,900
+10	20,000	>1,000,000		

Strept. cremoris No. 23

°C.	Start	7 days	14 days	21 days	34 days	48 days
− 1	5,400	2,000	2,300	700	240	63
+ 5	5,400	62,000	1,085,000	.....	1,500,000	5,500,000
+10	5,400	>1,000,000	75,000,000	59,000,000		

Strept. lactis

°C.	Start	9 days	23 days	37 days
− 1	77,000	22,000	113,600	20,700
+ 5	77,000	630,000	21,000,000	6,000,000
+10	77,000	835,000,000		

Another fact must be mentioned here which is still more difficult to explain biochemically, namely the adaptation of organisms to certain temperatures. This is shown very plainly in Table 60 representing some experiments of Zikes (1919b) with yeast. Of each of the six yeasts tested, one culture was grown at 8°C., and another at 25°C., for several weeks previous to the final test. The temperature of precultivation had a very decided influence upon

TABLE 60.—GROWTH RATE OF YEAST AT DIFFERENT TEMPERATURES

No. of yeast	Temperature, °C.	Generation time of yeast precultivated at	
		8°C.	25°C.
1	12	6 hrs. 19 min.	17 hrs. 31 min.
	15	4 hrs. 45 min.	9 hrs. 58 min.
	19	3 hrs. 16 min.	4 hrs. 38 min.
	24	3 hrs. 31 min.	3 hrs. 17 min.
	30	2 hrs. 19 min.	2 hrs. 16 min.
	38	33 hrs. 50 min.	24 hrs. 51 min.
2	9	11 hrs. 47 min.	30 hrs. 24 min.
	12	5 hrs. 48 min.	13 hrs. 40 min.
	15	4 hrs. 04 min.	7 hrs. 02 min.
	20	4 hrs. 13 min.	5 hrs. 02 min.
	25	4 hrs. 04 min.	3 hrs. 59 min.
	30	2 hrs. 39 min.	2 hrs. 29 min.
	39	58 hrs. 10 min.	30 hrs. 25 min.
3	8	5 hrs. 18 min.	14 hrs. 24 min.
	15	3 hrs. 29 min.	3 hrs. 52 min.
	21	3 hrs. 02 min.	2 hrs. 20 min.
	29.5	2 hrs. 02 min.	1 hr. 54 min.
4	9	16 hrs. 30 min.	24 hrs. 41 min.
	12	9 hrs. 43 min.	10 hrs. 07 min.
	14	5 hrs. 02 min.	6 hrs. 39 min.
	20	4 hrs. 43 min.	3 hrs. 55 min.
	24	3 hrs. 46 min.	3 hrs. 15 min.
	30	2 hrs. 53 min.	2 hrs. 28 min.
	40	118 hrs. 03 min.	68 hrs. 07 min.
5	10	5 hrs. 12 min.	7 hrs. 28 min.
	15	3 hrs. 40 min.	5 hrs. 12 min.
	24	3 hrs. 48 min.	2 hrs. 52 min.
	29	2 hrs. 54 min.	2 hrs. 09 min.
6	11	6 hrs. 11 min.	13 hrs. 36 min.
	15	4 hrs. 19 min.	4 hrs. 19 min.
	20	4 hrs. 19 min.	4 hrs. 00 min.
	25	3 hrs. 09 min.	3 hrs. 02 min.
	30	2 hrs. 48 min.	2 hrs. 36 min.

the rate of growth at different temperatures. Yeast No. 2 shows the greatest adaptation. At 9°C., the culture adapted to low temperatures grew almost three times as fast as the other, while at 39°C., it required almost twice as long a time to double.

To account for such adaptations biochemically seems almost impossible with our present knowledge of the cell mechanism.

SUMMARY OF FACTS

Temperature influences the growth rate greatly.

At very low temperatures, the time required for a cell to double may be more than forty-eight hours. The same organism could double at the optimum temperature in half an hour.

There is a definite low temperature at which a culture will cease to grow. Near this point, the time required for a cell to double increases rapidly with a decrease of temperature and finally becomes infinite. At temperatures below this minimum, the cells die slowly.

From a point about 10°C. above the minimum to the optimum temperature, the rate of growth will be increased from two to three times by a 10°C. rise in temperature.

Above the optimum temperature, the growth rate is not constant, but decreases with the time. During a first short interval, it may be higher than the rate at the optimum temperature, but it does not last.

A culture grown for a while at low temperatures will grow more rapidly at low temperatures than a culture of the same strain precultivated at high temperatures. Bacteria and yeasts can be adapted within limits to low and high temperatures.

SUMMARY OF THEORIES

Growth must be considered as the sum of a number of slow chemical reactions, all of which are accelerated by an increase in temperature. Growth must have a fairly normal temperature coefficient.

The maximum and minimum temperatures of growth are brought about by the superposition of other processes.

Above the optimum, we can assume the deterioration of some essential part of the growth mechanism with a high temperature coefficient. Such assumption would lead to the Tammann principle which has actually been observed in a number of cases. Growth rates are constant below the optimum temperature, and decreasing with time above the optimum, decreasing the more rapidly the farther the temperature is above the optimum. The decrease will ultimately lead to death.

The minimum temperature is not as yet explained in a generally accepted way. One possible explanation is the assumption that the slowness of energy liberation does not allow the cells to accumulate enough energy to accomplish some essential synthesis, or repairs.

(*f*) CHEMICAL STIMULATION OF THE GROWTH RATE

In the study of chemical stimulation of fermentation, an analogy was drawn between the action of poisons and the action of temperature. With isolated enzymes, an immediate acceleration of enzyme activity was found, followed by a greater decrease due to a more rapid enzyme destruction. In fermentation by living cells which can regenerate the enzyme, a continuous acceleration of the fermentation by stimulants was assumed to be possible.

In the case of chemical stimulation of growth, the analogy with temperature may well be continued. Let us assume, for the sake of simplicity, that the rate of fermentation remains uninfluenced by the concentration of the stimulant which affects growth. Two processes are stimulated by the poison acting as a catalyst: the cell synthesis, and also the deterioration of the growth-producing agent. This agent is deteriorating normally in all cells, though quite slowly, and is constantly being

regenerated by the cell. This regeneration has a limited speed. As long as the deterioration of the growth agent does not exceed its regeneration, everything goes well, and a stimulation up to this point will be well tolerated by the cell. Beyond this point, cessation of growth is inevitable if the stimulation continues. This deduction, which is merely based on analogy, leads to the conclusion that the Tammann principle should become evident at higher concentrations of a chemical stimulant.

Very little experimental evidence which includes the time factor is available. A good example is an experiment of Hüne (1909) given in Table 61 which shows, at first glance, a good agreement with the influence of temperature on fermentation and on growth. It is a fine illustration of the existence of the Tammann principle in chemical stimulation.

This example may be objected to for the reason that the bacteria are multiplying, and that, in the same culture, the same amount of formaldehyde is acting upon much larger numbers of cells as the time advances. However, the theory of stimulation by poisons assumes that the action is catalytic, and therefore, its action is independent of the concentration of the substrate i.e. the number of cells. This assumption is justified because it will be shown on p. 341 that the death rate depends upon the concentration of the poison, but is independent of the numbers of cells acted upon, within very wide limits.

The experiment quoted above is not sufficient, however, to make any further deductions. There is a very long lag period, the control requiring seven hours before the most rapid growth is reached. Besides, the generation time of all cultures but the last shows a distinct increase between two and four hours which suggests some unknown outside retarding influence. Therefore, the generation times mean little.

All other material on growth stimulation refers to final crops, and will be discussed later (p. 254). No

TABLE 61.—*Bact. coli* IN DILUTED BROTH + FORMALDEHYDE

Formaldehyde	0	0.000001%	0.00001%	0.00003%	0.0001%	0.001%	0.01%
	Plate count						
15 min.	9,720	9,610	9,820	10,800	*11,760*	11,340	8,100
45 min.	6,340	6,210	14,160	*16,200*	11,340	10,260	1,280
2 hrs.	17,300	21,260	*55,100*	44,280	15,280	7,020	440
4 hrs.	36,400	61,300	*129,600*	22,680	18,260	11,200	0
7 hrs.	140,000	2,054,000	*2,782,000*	2,090,000	1,832,000	1,555,000	0
11 hrs.	17,000,000	25,480,000	*38,920,000*	18,200,000	7,880,000	4,776,000	0
20 hrs.	50,000,000	75,210,000	*96,640,000*	48,000,000	51,000,000	45,680,000	0
	Generation times						
15–45 min.	∞	∞	58 min.	53 min.	∞	∞	Negative
45–120 min.	46 min.	42 min.	*38* min.	52 min.	160 min.	∞	Negative
2–4 hrs.	113 min.	*79* min.	98 min.	∞	448 min.	155 min.	
4–7 hrs.	93 min.	*36* min.	41 min.	28 min.	27 min.	26 min.	
7–11 hrs.	*35* min.	66 min.	63 min.	77 min.	114 min.	123 min.	

further data concerning the stimulation of the rate of growth are known to the author.

SUMMARY

The rate of growth can be increased by certain concentrations of chemical poisons.

If a number of different concentrations are tried, stimulation is greatest at first with the higher concentrations, but the optimal stimulating concentration becomes smaller and smaller as the time passes.

This behavior suggests that poisons effect organisms similarly as high temperatures, accelerating catalytically the rate of action, as well as that of deterioration of some essential growth factors.

IV. THE ENDPOINT OF GROWTH

All growth in a given amount of medium will ultimately come to an end. The final amount of growth, i.e., the total crop, will depend upon numerous factors. With the same organism in the same medium, it will be proportional to the volume of the medium (unless the volume has had an influence upon the rate of oxygen penetration into the medium). It will be independent of the size of the inoculum unless this has been very near to or above the number of cells that could develop in the medium. (Rahn, 1906; Graham-Smith, 1920; Buchanan and Fulmer, 1928.)

The composition of the medium will have the greatest influence. In our standard media, we are likely to get quite uniform crops. It is generally considered a rule that most of the common saprophytic bacteria, the organisms of the colon-group, the streptococci (if sugar is present) and quite a large number of other parasites

will grow in nutrient broth to about one billion cells per c.c. This may be a question of size or total surface: Table 132 on p. 397 gives the following final numbers of cells per liter: Yeast: 50,000,000; *Bact. coli:* 1,000,000,000; Bacteriophage: 100,000,000,000. But the weight of these cells ranges in the reverse order. The total crop in 1 liter of medium weighs in moist condition: with aspergillus, 7,000 to 19,000 mg.; with yeast, 6,500 mg.; with *Bact. coli,* 800 mg.; with bacteriophage, 0.42 mg.

The concentration of food will influence the final crop. Addition of chemicals (not food) may influence the crop favorably, by acting as buffer, or providing a more suitable osmotic pressure, or they might decrease the crop if they have a toxic action.

(*a*) THE CAUSES OF THE ENDPOINT OF GROWTH

Before the factors of concentration of food, of temperature and of chemical influences can be discussed, it seems essential to try to come to a clearer understanding of the cause of cessation of growth. This question has already been raised regarding the decrease of the growth rate (p. 199). It was not answered in a satisfactory way. Many conflicting experimental data have to be analyzed, and many conflicting theories must be considered.

It should be realized from the beginning that different organisms may cease to grow from different reasons, and that the growth of the same organism in different media may stop from different reasons. Theories on the limiting factor of Streptococci cannot be disproved by experiments with *Bact. coli* or with yeasts.

It must be further kept in mind that the rate of growth is independent of the final amount of growth. While the rate was found to be but slightly influenced by the

concentration of food, the total crop shows distinct relation to the quantity of food (see next chapter).

Probably the most common assumption is that the accumulation of the products of fermentation prevents further growth. This can be most easily proved by removing the fermentation products from a full-grown culture. This has been done by Rahn (1911) by neutralizing the acid in milk cultures of two streptococci. The result was increased growth with Strain II, but no increase with Strain IV.

TABLE 62.—INCREASE IN NUMBERS OF CELLS OF ACID FORMING STREPTOCOCCI AFTER NEUTRALIZATION OF CULTURE
(Numbers indicate millions per c.c.)

Strain	II	II	II	II	IV	IV
Just before neutralization	1,790	1,190	640	765	1,340	2,065
After 8 hours	3,270	3,290				
After 24 hours	.....	.....	2,235	2,010	1,195	1,590

Strain II is not affected by peptone (see p. 210). It is capable of utilizing the protein matter of the milk, and its growth limit is primarily fixed by the hydrogen ions. If these are removed, growth continues until another inhibiting factor intervenes.

Strain IV does not find sufficient building material in milk; the final number of cells is increased if peptone is added. Neutralizing a culture which contains no peptone does not further the growth because there is no building material. Lack of food is the limiting factor of Strain IV, fermentation products are the limiting factor of Strain II.

Rogers and Whittier (1928) give the following final crops for their strains of *Strept. lactis:*

	Cells per c.c.
Unbuffered broth	60,000,000
Buffered broth	480,000,000
Broth with pH held constant	1,000,000,000
Milk	1,000,000,000
Milk with pH held constant	3,600,000,000
Milk, aerated and pH held constant	5,200,000,000

If the inhibiting product is removed, growth will continue until another product of the cells inhibits growth. In the case of lactic acid bacteria, the growth-inhibiting compound after removal of the hydrogen ions might be thought to be undissociated lactate which was found to be the cause of cessation of fermentation (p. 145). But the experiments of Rogers and Whittier show no influence of lactates upon growth.

With yeast in malt wort, alcohol is not the limiting factor of the final amount of yeast, according to A. J. Brown (1905). He found that it required very high amounts of alcohol to decrease the final crop, as may be seen from Table 63.

TABLE 63.—AVERAGE NUMBER OF YEAST CELLS PER C.C. IN WORT WITH INCREASING AMOUNTS OF ALCOHOL

Experiment I		Experiment II	
Inoculum	15,600,000	Inoculum	6,880,000
Wort alone	72,800,000	Wort alone	79,200,000
Wort + 0.7% alcohol	78,400,000	Wort + 1.4% alcohol	68,800,000
Wort + 1.4% alcohol	72,800,000	Wort + 2.8% alcohol	70,400,000
		Wort + 4.2% alcohol	57,200,000
		Wort + 5.6% alcohol	38,400,000
		Wort + 7.0% alcohol	24,800,000
		Wort + 8.4% alcohol	9,200,000

The added amounts of alcohol do not represent the total alcohol concentration because some more is formed

by the yeast. The final concentration in normal wort was 3.6% alcohol.

Special experiments demonstrated that no other volatile or non-volatile fermentation products were the cause of growth inhibition. Good aeration, however, increased the final crop considerably, and it seems certain that the first limiting factor of yeast growth in wort is lack of oxygen which is nothing else than lack of food. Another example where lack of food is the cause of cessation of growth will be shown on p. 248. It concerns the growth of *Micr. pyogenes* in a meat infusion medium.

Aside from these two factors, accumulation of fermentation products, and exhaustion of food supply (including oxygen as a food), a third possibility has been repeatedly claimed, and has been disclaimed as often by others; namely, the existence of a more or less specific cell excretion which is thermolabile and of colloidal nature. Eijkman (1905) claimed such a compound for *Bact. coli.* Full-grown gelatin cultures, after steam heating, allowed normal growth to occur again while the same gelatin, remelted without heating to high temperatures, showed no growth upon re-inoculation. This experiment was modified in various ways, always with the same result. Conradi and Kurpjuweit (1905) enlarged upon this theory. Later authors (e.g., Manteuffel, 1907) disproved this by the claim that the absence of growth was merely due to lack of food. Wherever large numbers of living cells were left, the food was divided between so many cells that none could multiply visibly. If, however, heat destroyed the old cells, the newly inoculated cells had sufficient food for growth (see also Henrici, 1928).

Rahn (1906) observed with *Ps. fluorescens* that new growth developed in old cultures upon re-inoculation after

steaming or filtering. This process could be repeated several times with the same culture, thus producing

TABLE 64.—REPEATED GROWTH OF *Ps. fluorescens* IN BROTH

	Hours after inoculation	Cells per c.c.		Generation time, minutes	
		Heated to 100°C.	Heated to 68°C.	100°C.	68°C.
Pure culture	48 hours	2,200,000,000	2,000,000,000		
		Heated			
Second growth	0	100,000	37,000		
	2	300,000	70,000	76	135
	6	1,620,000	650,000	99	76
	12¼	31,000,000	16,000,000	89	82
	24¼	760,000,000	800,000,000	156	128
		Heated			
Third growth	0	8,000	10,800		
	3	44,500	35,000	73	106
	6	100,400	98,000	153	121
	12	12,000,000	6,000,000	52	61
	24	470,000,000	250,000,000	136	134
		Heated			
Fourth growth	0	1,000,000	1,000,000		
	3	2,570,000	2,200,000		
	6	4,070,000	1,940,000		
	12	40,000,000	26,000,000		
	24	.....	976,000,000		
	48	2,800,000,000	4,200,000,000		
		Heated			
Fifth growth	0	28,000	42,000		
	24	39,000,000	72,000,000		
	48	600,000,000	1,800,000,000		
	72	800,000,000	1,700,000,000		
		Heated			
Sixth growth	0	4,800	12,700		
	24	45,000,000	189,000,000	182	173
	48	400,000,000	1,100,000,000		
	72	500,000,000	900,000,000		
Total number of cells grown in 1 c.c. of broth		7,600,000,000	10,100,000,000		

again and again a normal crop from the same amount of medium (Table 64). After sterilization of the culture with ether, no growth occurred; growth in the ether-sterilized culture became possible after steam heating, or after adding pieces of a Chamberland filter to adsorb the inhibiting substance.

Avery and Morgan's (1924) inhibiting substance of pneumococcus culture finally proved to be hydrogen peroxide, a well-defined cell product. Hajos (1922) obtained an inhibiting substance from cultures of *Bact. coli* and related bacteria by repeatedly centrifuging the bacteria out of the culture and re-inoculating the medium until no further growth occurred. The chemical constitution remained unknown so far. Since a few drops of it prevented growth in 10 c.c. of fresh broth, the toxic compound seems to be quite concentrated. But this substance is heat-resistant and is perhaps a fermentation product.

Rogers and Whittier (1928) appear to have established a growth-inhibiting factor in *Strept. lactis* which is a cell product, but not acid. They grew *Strept. lactis* in double strength broth in a collodion sac suspended in a flask of the same medium, the volume of the outside medium being about ten times that in the sac. After two days of growth, the outside liquid was also inoculated with *Strept. lactis*, and the bacteria were counted from hour to hour. The cells developed to about 70–71 millions per c.c. while in a normal check, they grew to 300 millions. Repeated addition of concentrated broth brought the number of cells in the double culture up to 98 millions, while the normal culture increased to 550 millions. Rogers and Whittier believe this compound to be identical with, or similar to, the agent by which *Strept. lactis* inhibits *Lactobacillus bulgaricus;* it is also believed to be the substance controlling the final amount of growth in solid or liquid media which contain but little carbohydrate.

SUMMARY

There are various reasons why growth of a bacterium will cease in a given volume of culture medium. It has been shown that certain streptococci in milk cease to grow because of too high acidity. It has also been

demonstrated that other streptococci cease to grow in milk for lack of available nitrogenous food. It has further been found that *Ps. fluorescens* in broth ceases to grow on account of accumulation of a thermolabile cell secretion, and the same is true for streptococci in broth.

(*b*) ENDPOINT AND CHEMICAL COMPOSITION OF FOOD

The final crop or the endpoint of growth will depend largely upon the kinds and amounts of construction material if a reasonably good and continuous source of energy is taken for granted. The type of construction material seems quite essential. Most streptococci grow to much larger numbers in milk if peptone, meat extract, or yeast extract is added (see p. 149).

Very extensive quantitative studies on the influence of the kind of building stones upon the crop have been made by the yeast industry. The yeast factory tries to obtain the largest possible amount of cells from a given amount of sugar. Since molasses is the cheapest source of sugar available, the manufacture of yeast from molasses is a common process. The building material consists of different organic or inorganic nitrogenous compounds, including the nitrogenous material in molasses. While most of the results of these studies are considered trade secrets, the following two tables show a number of unpublished experiments which I owe to the kindness of Dr. H. Claassen, of the "Rheinischer Aktienverein für Zuckerfabrikation," Dormagen, Germany.

These experiments were carried out with 5 to 7 liters of wort under plant conditions, being well aerated from perforated copper tubes at the bottom of the vessel. The normal aeration was 15 to 20 liters of air per minute during the first hour, 50 to 60 liters during the next seven hours, and 15 to 20 liters during the last hour. The

TABLE 65.—YEAST CROPS OBTAINED UNDER DIFFERENT CONDITIONS

	Experiment number	1	2	3	4	5	6	7	8	9	10	11	12
Food per 7 liters	Gm. molasses: 49% sugar, 1.83 % N	300	300	300	300	300	300						
	Gm. sucrose	.....	.....	.....	.....	.....	.....	200	200	200	150	150	100
	C.c. peanut meal extract, 0.94 % N.	.....	.....	405	405								
	C.c. malt sprout extract, 0.155 % N.	.....	.....	.....	.....	2,445	2,445						Used
	Gm. ammonium sulfate, 20.9 % N.	6	6	.....	.....	.....	.....	12					wort
	C.c. ammonia water, 20.4 % N	6	6	.....	.....	.....	.....	7.1					from
	Gm. Asparagin, 18.44 % N.....	.....	.....	.....	.....	.....	.....	.....	21.7				Exp. 11
	Gm. Aspartic acid, 10.52 % N.....	.....	.....	.....	.....	.....	.....	.....	.....	38.0			
Seed	Seed yeast per 100 gm. of sugar (moist weight, 75 % moisture)...	32	32	32	32	32	32	25	25	25	37	34	51
	Nitrogen in yeast solids, in %.....	8.63	8.63	8.63	8.63	9.33	9.33	8.23	8.23	8.23	8.87	8.09	8.09
Air	Duration of experiment, in hours..	9	9	9	9	9	9	12	12	12	9	10	10
	Liters of air per minute: start	15	30	15	30	15	30				10	10	10
	Liters of air per minute: middle ..	30	60	30	60	30	60		20–50–30–20		30	30	30
	Liters of air per minute: end.....	15	30	15	30	15	30				20	20	20
Products	Yeast crop, (75 % moisture) per 100 gm. of sugar consumed.....	98.8	113.2	139.8	216.8	110.4	144.7	69.0	83.5	93.9	23.6	23.8	28.6
	Alcohol per 100 gm. of sugar.....	22.9	12.0	24.5	11.0	33.9	19.8	17.4	23.2	17.9	24.1		
	Nitrogen of yeast solids.....	8.53	8.38	7.84	6.63	8.25	7.17	9.06	8.86	6.50	4.83	4.33	5.13
	Of the total nitrogen in wort, the yeast assimilated %.....	39.0	44.8	44.5	55.4	36.1	39.7	80.3	93.0	71.2	loss 11.2	loss 8.6	
Yeast properties	Yeast softens at 35°C. after hours.	72	48	100	72	102	82	72	72	48	72	96	96
	Fermenting power after Kusserow												
	1st half hour: c.c. CO_2.....	71	30	59	39	52	30	31	38	22			
	2nd half hour: c.c. CO_2.....	259	180	241	154	256	190	171	216	153			
	3rd half hour: c.c. CO_2.....	362	264	312	228	366	275	261	292	207			
	4th half hour: c.c. CO_2.....	324	275	336	232	398	300	269	341	234			
	Total CO_2 in 2 hours.....	1,016	749	948	653	1,072	795	732	887	616			
	Dough fermentation, time in minutes	69	75	65	87	55	68	90	89	114	120	112	100
	Purpose of experiment.....	Influence of air upon yeast crop and nitrogen assimilation; influence of different sources of nitrogen upon yeast crop and its properties						Influence of different sources of nitrogen			Yeast in nitrogen-free medium		Yeast in wort of experiment 11

TABLE 65.—YEAST CROPS OBTAINED UNDER DIFFERENT CONDITIONS.—(*Continued*)

	Experiment number	13	14	15	16	17	18	19	20	21	22	23
Food per 7 liters	Gm. molasses: 49% sugar, 1.83 % N	300	300	300	250	250						
	Gm. sucrose	.....	.....	.....	.....	.....	125	125	125	125	125	150
	C.c. peanut meal extract, 0.94 % N	240	240	.....	.....	.....	.....	556	.....	243		
	C.c. malt sprout extract, 0.155 % N	.....	.....	.....	.....	.....	.....	.....	3,516			
	Gm. ammonium sulfate, 20.9 % N											
	C.c. ammonia water, 20.4 % N											
	Gm. urea, 46.0 % N	.....	.....	.....	3.25	3.25						
	Gm. ammonium phosphate, 12.1 % N	.....	.....	.....	.....	.....	30	.....	.....	15		
Seed	Seed yeast per 100 gm. of sugar (moist weight, 75 % moisture)	58	58	24	24.5	24.5	31	30	30	31	31	33
	Nitrogen in yeast solids, in %	8.90	8.90	9.25	9.96	9.96	8.95	9.10	9.10	8.95	9.30	9.30
Air	Duration of experiment, in hours	10	10	10	9	9	10	9	9	10	10	10
	Liters of air per minute: start	20	20	20	20	20	20	20	20	20	15	10
	Liters of air per minute: middle	50	50	50	40	40	50	50	50	50	30–20	30–20
	Liters of air per minute: end	20	20	20	20	20	20	20	20	20	10	20
Products	Yeast crop, (75 % moisture) per 100 gm. of sugar consumed	49.1	48.7	90.8	84.2	85.0	75.8	140.2	104.5	136.7	42.7	43.8
	Alcohol per 100 gm. of sugar	4.7	5.1	24.6	.....	.....	12.7	16.8	21.0	10.1	15.4	
	Nitrogen of yeast solids	7.91	7.86	7.16	10.0	8.58	8.94	7.96	7.23	7.94	4.20	5.61
	Of the total nitrogen in wort, the yeast assimilated %	52.1	50.2	38.9	47.8	39.7	60.1	62.5	43.3	80.2	3.3	12.2
Yeast properties	Yeast softens at 35°C. after hours	115	115	120	10	24	72	96	96	96	96	72
	Fermenting power after Kusserow											
	1st half hour: c.c. CO_2	68	71	36	.....	.....	57	37	37	52	12	
	2nd half hour: c.c. CO_2	224	226	138	.....	.....	212	132	157	173	59	
	3rd half hour: c.c. CO_2	308	301	276	.....	.....	235	248	252	254	101	
	4th half hour: c.c. CO_2	308	305	343	.....	.....	273	252	252	253	132	
	Total CO_2 in 2 hours	908	903	793	.....	.....	777	669	698	732	304	
	Dough fermentation, time in minutes	63	63	69	.....	84	84	101	77	81	120	105
	Purpose of experiment	Influence of temperature: 24°–32°C.	24°–28°C.	Molasses nitrogen only	Assimilation of urea, completely changed to $(NH_4)_2SO_4$ with H_2SO_4 in Exp. 16 and two-thirds changed in Exp. 17		Comparison of purely organic and purely inorganic sources of nitrogen and of mixed diet				Assimilation of nitrogen from a used wort from the plant, with sugar. Total N in 7,000 c.c.: 3.43 gm. in Exp. 22 and 3.829 gm. in Exp. 23.	

temperature of fermentation was 24°C. at the start, and gradually increased to about 31°C. The acidity of the solution was kept at pH 4.8 to 5.2, and was allowed to increase towards the end of the fermentation to pH 5.5 to 5.8; if necessary, it was regulated by H_2SO_4 or Na_2CO_3.

The wort was allowed to run slowly into the container filling it in five to seven hours, as is the custom in plant procedure. Acid calcium phosphate is added to molasses at the rate of 2.0 gm. P_2O_5 per 100 gm. sugar; the molasses is diluted, heated to 85°C., acidified with H_2SO_4, and filtered. Malt sprouts are extracted with hot water. Peanut meal is heated with dilute H_2SO_4 until about 90% of the nitrogen has become soluble.

In order to offer equal quantities of assimilable nitrogen, the following assumptions, based upon long experience, were made:
Molasses: 0.7–0.8% of the molasses weight is assimilable N.
Peanut meal extract: 65–70% of the soluble nitrogen is assimilable N.
Malt sprout extract: 65–70% of the soluble nitrogen is assimilable N.
Ammonia, asparagin, urea etc: 100% assimilable.
When sucrose was offered in place of molasses, K_2SO_4 and $MgSO_4$ were added in appropriate amounts.

The washed yeast and the wort were analyzed at the end of the experiment, and the yeast was tested for its fermenting capacity because all these experiments aimed at the production of a good bakers' yeast. The volume of CO_2 produced by 10 gm. of yeast was determined as described on p. 104, and the time which 5 gm. of yeast required to raise a standard flour dough to a standard volume was measured. The keeping quality was tested by observing the time required for the yeast to soften in a 35°C. incubator.

In all these experiments, the substrate, i.e., the sugar, is limited, and is used up completely during the nine to twelve hours of the experiment, the initial concentration varying between 1.5 and 2.5%. The biochemical problem is then to explain why yeast, with the same energy supply, will give different crops with different building materials.

The crops vary considerably. The first six experiments contain the same amounts of assimilable nitrogen in the wort, 2.4 gm. from molasses and 2.5 gm. from

other sources. All conditions being exactly alike, the crops obtained per 100 gm. of sugar used were:

	From molasses + ammonia, gm.	From molasses + peanut meal, gm.	From molasses + malt sprouts, gm.
With slow aeration....	98.8	139.8	110.4
With rapid aeration...	113.2	216.8	144.7

With ammonia as the only source of nitrogen, molasses being substituted by sucrose, the crop was only 69.0 gm. (Exp. 7). Asparagin (Exp. 8) and aspartic acid (Exp. 9) gave better crops, 83.5 and 93.9 gm. The total available nitrogen was only 4 gm. against 4.9 gm. in the six previous experiments.

Another set of comparable data are experiments 18 to 21, with 3.6 gm. of total assimilable nitrogen and 1.8% sucrose instead of molasses. The crops were:

	Gm.
With ammonium phosphate	75.8
With peanut meal	140.2
With malt sprouts	104.5
Half ammonium phosphate, half peanut meal	136.7

These results probably mean that the protein material of the peanut extract was most similar to that of the yeast, and that with the same amount of energy, the yeast could grow more rapidly, and therefore reach a higher final crop. The surprising fact is that with half the amount of peanut extract, and the rest of nitrogen substituted by ammonia, the yeast reached practically the same weight, 136.7 gm., and had about the same nitrogen content, the same keeping quality and a better fermentative action. This shows that while it is uneco-

nomical to feed the yeast on ammonia alone, it is quite advantageous to feed ammonia besides organic nitrogenous matter. A great drop in the yeast crop, if the last of the organic nitrogen is taken away, is very conspicuous in Table 67.

These results are enlarged by a number of other experiments. Numbers 16 and 17 are directly comparable with the above, having a total available nitrogen of 3.5 gm. Numbers 7, 8, and 9 are comparable with each other, showing the differences in the availability of asparagin, aspartic acid and ammonia.

In nitrogen-free sugar solution, (Exps. 10, 11, and 12) the yeast loses nitrogen. Exps. 22 and 23 show that in "spent" wort, practically all available nitrogen had been assimilated by the first crop, and that after addition of more sugar, only a small amount of the total nitrogen could be used by the second crop.

When ammonia is used as the only source of nitrogen (Exp. 18), the crop is small, but the yeast is high in protein content. This is typical for all yeasts grown with ammonia.

Table 66.—Nitrogen Content in Yeast Solids

Yeast with ammonia	Yeast without ammonia
Exp. 1: 8.53% N	Exp. 3: 7.84% N
Exp. 2: 8.39	Exp. 4: 6.63
Exp. 7: 9.06	Exp. 5: 8.25
Exp. 16: 10.00	Exp. 6: 7.17
Exp. 17: 8.58	Exp. 8: 8.86
Exp. 18: 8.94	Exp. 9: 6.50
Exp. 21: 7.94	Exp. 13: 7.91
Exp. 24: 9.11	Exp. 14: 7.86
Exp. 25: 8.00	Exp. 15: 7.16
Average 8.73%	Exp. 19: 7.96
	Exp. 20: 7.23
	Average 7.58%

Whether this higher nitrogen content is stored protein, we have no means of telling. It does not mean an increase in zymase, for the

amounts of CO_2 produced average 800 c.c. for yeast grown with ammonia, and 813 c.c. for yeast without ammonia. The average time for softening varies greatly; it is fifty-two hours for yeast grown with ammonia, and ninety-two hours for yeast without ammonia. The two samples with urea are the worst ones to soften.

These results are verified by another experiment of Claassen's (1928) where increasing amounts of the assimilable nitrogen of malt sprouts were substituted by ammonia.

TABLE 67.—YEAST CROP AND PROPERTIES WITH INCREASING AMOUNTS OF AMMONIA

Ammonia in % of assimilable nitrogen	Yeast crop per liter, gm.	% N in yeast solids	Dough fermentation, minutes	Keeping quality at 35°C. after 72 hours
0	19.56	6.74	67	solid
10	18.92	6.98	64	solid
20	15.98	7.66	75	soft (in 24 hours)
30	15.88	8.60	76	soft (in 48 hours)
40	14.36	8.13	72	soft (in 24 hours)
50	17.85	8.81	59	solid
60	17.02	8.86	59	soft (in 24 hours)
70	16.30	9.15	62	solid
80	16.82	8.95	51	solid
90	17.40	8.88	51	solid
100	12.89	9.58	56	solid

Poor keeping quality and slow fermentation are characteristic for yeast with a mixed nitrogen diet of from 20 to 60% ammonia. The nitrogen content of the solids increases with the percentage of ammonia in the food, and the total crop decreases. Whether this rapid softening is due to a general weakness of constitution, or to an over-supply of endo-tryptase cannot be ascertained from these data. But there can be no doubt that ammonia, though it gives a good crop with normal fermenting capacity, does give a yeast differing physiologically from that grown on organic compounds only.

With yeast, the final crop, as well as its nitrogen content, its fermenting and keeping qualities, are decidedly influenced by the nitrogenous food. This

seems to disagree with the finding of Abderhalden and Rona (1905) that the protein of *Aspergillus niger* was the same regardless of the source of nitrogen. Terroine, Wurmser and Montané (1922), working with the same mold, found little difference in the nitrogen content of the mycelium when the source of nitrogen was ammonium sulfate, peptone, urea, or sodium nitrate; but it dropped decidedly with guanidine. Perhaps it is the proportion of the different kinds of proteins within the cell which brings about the differences in different yeasts.

A well-known illustration of the influence of the kind of food upon the final amount of growth is the appearance of pinpoint colonies on agar. Many milk streptococci give such colonies on standard, i.e., sugar-free agar, while the colonies on glucose agar are of normal size.

SUMMARY

The final number of cells which can develop in a given volume of medium depends to a large extent upon the chemical composition of the food. With different types of material for cell construction, yeast will produce not only different quantities of cells, but also cells of considerable variation of physiological characters.

(c) ENDPOINTS WITH AN ABUNDANCE OF FOOD

In all these experiments, the source of energy was used completely; the nitrogenous building material was used almost completely (see Exp. 22–23 of Table 65). It is generally taken for granted that a poorer food, even if given in abundance, will produce a smaller crop. The facts seem to bear out the assumption.

Really, the situation is not as simple as it seems at first glance. Our easy acceptance of the facts is probably based upon an analogy (perhaps subconsciously) with higher organisms. A poor soil will

give a small crop, but a poor soil is either a fair one deficient in plant food, or one which contains compounds injurious to growth, such as acid, or alkali. Such poor soil is not comparable with our case, which allows liberal amounts of a poor food.

With animals, a large supply of poor food will bring about undersized growth. But the reason is different. The capacity of the stomach and intestine is limited, and if they are filled to capacity with a food low in nutriment, the cells of the growing animal do not obtain all the nourishment they could utilize. The body does not develop to its maximum size, because the rate of growth is abnormally slow, and sexual maturity develops before the normal size is reached: After this stage, there is hardly any more growth, even with better food.

This analogy applies to our problem only if we assume that there is such a thing as an adult stage in bacterial cultures. The comparison with animals can be carried quite a long way. In this case, we would have to assume that each cell, regardless of its rate of growth, secretes into the medium some substance which, in sufficient concentration, will prevent further growth. In a well nourished culture, this limiting concentration is reached only after perhaps one billion cells per c.c. are formed. Poorly nourished cells secrete this harmful compound at the same rate, and the limiting concentration is reached long before the more slowly growing bacteria have had the chance to reach the billion mark.

This explanation would be satisfactory if the existence of this peculiar secretion could be proved. This seems possible; a full grown culture fed on ammonia as the only source of nitrogen should then show no further growth if asparagin or peptone were added. No such experiment is known to the author.

If, however, there is new growth, then the cause of cessation of growth cannot have been a secretion; it must be in the cell mechanism. Then, the cell can use only a limited amount of poor food, though there is plenty of it available, but will continue to grow with a better food, i.e., if the cell works under higher pressure, or with higher intensity. Only an experiment can decide between these two possibilities.

(*d*) INFLUENCE OF THE CONCENTRATION OF FOOD

We must again distinguish between energy-producing food and building material. To the energy-producing

foods belong not only carbohydrates and the like, but also oxygen. Attention has already been called to the experiments of A. J. Brown (p. 233) proving the oxygen concentration to be quite essential for a large crop of yeast.

Of the large amount of literature on the yields of yeast under varying conditions, very little can be used here, because it is customary in the yeast industries to record the crop in relation to the seeding, and not in absolute values; it is stated, e.,g. that the yeast multiplied five to eight times. This method of measurement is meaningless except for the direct comparison of two experiments

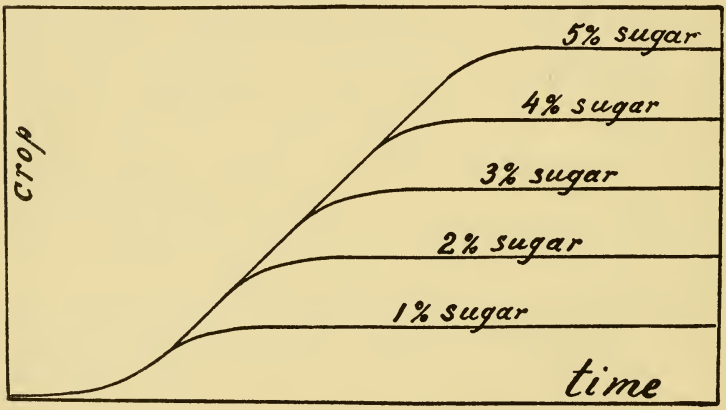


FIG. 24.—Theoretical growth curve of yeast with abundant building material and increasing amounts of sugar.

with the same seeding in the same medium. Even the scientists resort to this method when working on yeast problems (A. J. Brown, Lafar, Henneberg).

The theory of the influence of food concentration is fairly simple. Building material that does not contain a source of energy will produce no growth; nor will a source of energy that contains no building material induce growth; both are needed.

The rate of energy supply is constant as long as there is a fair amount of substrate (e.g., sugar) available. With a low amount of energy food, and sufficient building material, there will be a definite growth. If the energy food is increased, growth will not be more rapid, but will continue longer, because there is an energy supply for a longer time, which will ultimately produce a larger crop. Assuming sufficient building material, further increases of energy food will bring about further increases of the crop as is illustrated schematically in Fig. 24. In actual practice, the accumulating fermentation products or cell secretions will retard the growth rate after a while, and will make the utilization of the food less effective.

One of the most commonly quoted experiments is that of Rubner's (1906*a*) with *Bact. vulgare* grown in different concentrations of

TABLE 68.—YEAST CROPS OBTAINED WITH DIFFERENT SUGAR CONCENTRATIONS FROM THE SAME BUILDING MATERIAL

Glucose offered, %	Glucose not used, %	After 7 days		After 10 days		Increase for each additional % sugar	
		Crop, mg.	N in crop, mg.	Crop, mg.	N in crop, mg.	Yeast, mg.	Nitrogen, mg.
0	0	40	2.8				
1	0	94	8.1	...	....	54	5.3
3	0.045	166	12.9	...	....	36	2.4
5	0.14	226	17.8	...	....	30	2.5
7.5	0.24	280	20.0	...	....	22	0.9
10	0.42	314	21.6	...	....	14	0.5
12.5	0.75	352	24.6	311	23.0	15	1.2
15	0.98	382	27.5	326	23.8	12	1.1
20	7.48	248	16.7	322	25.2		
25	12.90	211	14.8				
30	19.60	160	12.2				

meat extract. Rubner determined the nitrogen assimilated by the bacteria cells as measure of the crop. His quite complicated method of determining the nitrogen of the bacteria gave rather improbable data, and his results are not only contradictory within themselves, but also with the work of others who found the law of diminishing returns established in bacteria.

An experiment very similar to the theoretical discussion above is that by Stern (1901) who grew yeast in a mineral solution with 0.3% asparagin and increasing amounts of glucose. Table 68 gives the crops from 100 c.c. of the medium. The yeast can grow without sugar. Addition of sugar increases the crop, but the increase per gram of sugar becomes smaller and smaller as the food concentration increases, and above 15%, no further gain is observed.

TABLE 69.—CELLS PER STANDARD LOOP OF *Micr. pyogenes* IN MEAT EXTRACT

	Relative concentration of meat extract				
	10	25	50	75	100
Start.....	868	868	868	868	868
22 hrs.....	3,566,000	6,296,000	8,880,000	10,664,000	11,808,000
40 hrs.....	**4,273,000**	9,336,000	13,288,000	16,960,000	18,960,000
64 hrs.....	3,392,000	**9,416,000**	**15,760,000**	20,300,000	23,424,000
88 hrs.....	.....	5,736,000	13,696,000	**20,864,000**	**25,840,000**
112 hrs.....	.....	.....	.....	17,936,000	22,816,000

Another frequently quoted experiment is that by Graham-Smith (1920) who grew *Micr. pyogenes* at 37°C. in meat extract of varying concentration. He used the extract of 400 gm. meat in 1 liter of water, filtered, without any other addition except neutralization. Compared with standard nutrient broth, this is a rather dilute medium. This meat infusion was diluted

with varying amounts of water. In Table 69, the original diffusion is given the relative concentration of 100.

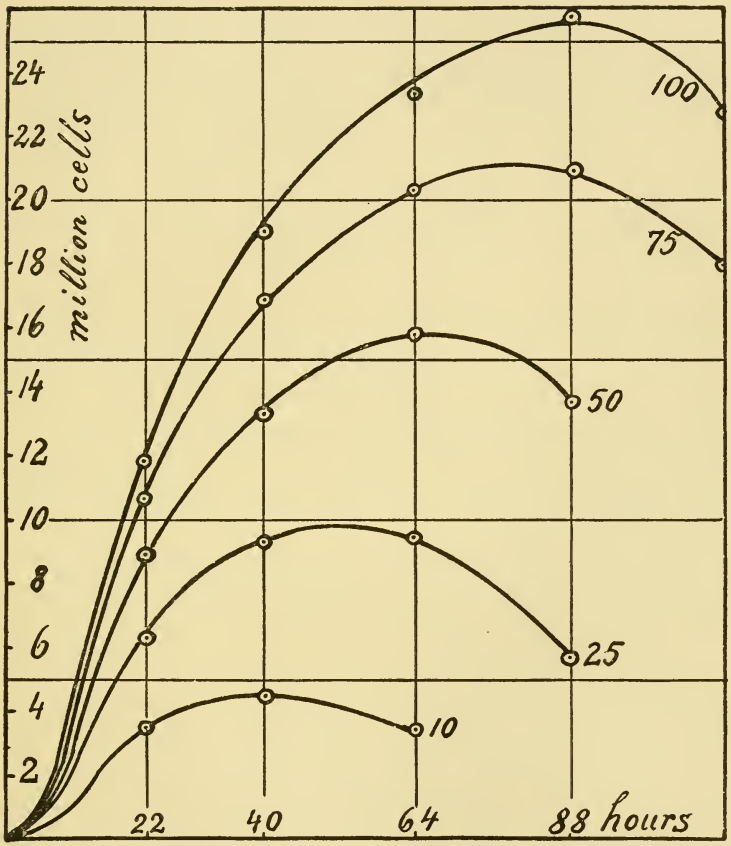


FIG. 25.—Growth curves of *Micrococcus pyogenes* in different concentrations of meat extract (the strongest concentration bearing the relative value 100).

The outstanding result is that in the more concentrated solutions, growth still continues when it has passed the maximum number (marked by heavy print) in the lower

dilutions. This is entirely in agreement with the theory (p. 246) and is graphically shown in Fig. 25.

The total crop at the different concentrations shows the following relative values:

Relative concentration of meat extract	Maximum number of cells per standard loop, millions	Relative numbers of cells	
		Highest concentration = 100	Lowest concentration = 10
100	25.8	100	61
75	20.9	81	49
50	15.8	60	37
25	9.4	36	22
10	4.3	17	10

There is a general parallelism between food and crop, but it is not a direct proportion between the two. While the food concentration drops from 100 to 10, the crop drops only to 17. Or, while the food concentration is increased from 10 to 100, the crop increases from 10 to only 61.

This is the well-known "law of diminishing returns." It is quite evident also in the yeast experiment described in Table 68 where the increase in growth became smaller for each additional gram of sugar.

There seems to be, for some bacteria at least, a limit of food below which they do not multiply at all. Carpenter and Hucker (1927) give a number of such examples. Peltier's data (p. 207) also show a very decided drop when the milk is diluted farther than 1:16. The problem of growth in very dilute media does not seem to have been investigated to any extensive degree though the bacteriology of surface waters, city water supplies, and even of soil would be greatly benefited by such an investigation.

SUMMARY

With increasing amounts of food, the crop, i.e., the final number of cells in a culture, usually increases.

With a liberal supply of building material, increase of energy food will cause no acceleration in growth, but a longer growth period, and, therefore, a larger crop. This is limited by accumulation of fermentation products or of inhibiting cell secretions.

With all food components increasing, the crop will still be limited by fermentation products or cell secretions. The law of diminishing returns becomes quite evident.

Many species require a fairly well defined minimum concentration of food for growth.

(*e*) TEMPERATURE AND ENDPOINT OF GROWTH

Our knowledge of the growth mechanism is too limited to permit general predictions regarding the influence of temperature changes upon the final crop. For temperatures above the optimum, it is easily seen that the crop must decrease with increasing temperature. This is verified by the experiments of Ward (p. 216).

In those cases where growth is limited by accumulation of fermentation products, a prediction seems possible. Since the fermentation products act as poisons, the same concentration will act more strongly at higher temperatures, or a smaller amount at high temperature will have the same inhibiting effect as a larger amount at a lower temperature. Consequently, a smaller crop should be expected at higher temperatures.

Where lack of food is the limiting factor, our basis for prediction is much less sound. The preceding

chapter showed a better utilization of food when the concentration was low. If this should mean that a *slower* fermentation causes a better utilization, we should expect a larger crop at the lower temperatures. However, there is probably a limit to the advantage of slow fermentation.

Where some specific compound (autotoxin or whatever it may be called) is the limiting factor it may be supposed that it will act similarly to the fermentation products. But because it is a specific compound, the amount produced may have a temperature law of its own.

Summarizing, the general prediction tends to the assumption that the crop will decrease rapidly as the temperature rises above the optimum, and will increase

TABLE 70.—INFLUENCE OF TEMPERATURE UPON THE "CROP" OF *Micr. pyogenes* IN MEAT EXTRACT
(Cells per standard loop)

	17°C.	27°C.	37°C.
Start	632	632	632
After 1 day	2,064	5,528,000	5,968,000
After 2 days	73,850	10,918,000	8,744,000
After 3 days	3,120,000	12,568,000	**10,448,000**
After 4 days	8,128,000	14,288,000	9,968,000
After 5 days	12,400,000	**15,918,000**	8,688,000
After 6 days	14,512,000	15,664,000	
After 7 days	17,072,000	13,568,000	
After 8 days	**18,272,000**		
After 9 days	17,592,000		

slowly as the temperature falls below the optimum. There is probably a limit to this increasing crop with decreasing temperature; we would not expect a maximum crop at the minimum temperature.

Very few data are available on the influence of temperature upon the maximum number of cells.

Two good experiments have been given by Graham-Smith (1920). Table 70 shows the slow growth to high numbers at low temperatures, and the rapid growth to to less high numbers near the optimum.

In another experiment, the maximum numbers were reached at

33°C. after 4 days..... 10,610,000
27°C. after 4 days..... 18,671,000

At 8–10°C., no growth occurred, and a very gradual decrease of the numbers of living cells was observed.

Henneberg (1926, p. 179) found that yeast will give larger crops by weight at low temperatures, but that the total amount of yeast protein formed is higher at high temperatures. The following data are given:

a. Multiplication at 20°C.: 6.4 fold, at 30°C.: 9 fold

Culture aerated	Then without aeration		Multiplication	% protein in yeast	Total N in yeast in gm.
	For	At			
30° for 8 hrs.....	12 hrs.	30°C.	7.4 fold	57.8	1.01
8 hrs.....	12 hrs.	10°C.	7.1 fold	47.7	0.88
22 hrs.....	24 hrs.	30°C.	8.8 fold	51.2	1.01
22 hrs.....	24 hrs.	20°C.	8.3 fold	50.3	0.98
19° for 24 hrs.....	24 hrs.	19°C.	8.4 fold	45.8	
24 hrs.....	24 hrs.	12°C.	8.4 fold	43.6	
24 hrs.....	24 hrs.	2.5°C.	8.4 fold	42.3	

Not quite comparable with these data is the experiment by Stern (1901) who obtained the following final yeast crops in 100 c.c. of a 10% glucose solution with 0.3% asparagin and minerals (Table 71).

The yeast cultures were not aerated. At 21°C., the maximal crop is reached, and also the maximal produc-

TABLE 71.—YEAST CROPS AT DIFFERENT TEMPERATURES

Temperature, °C.	Time of growth, days	Sugar not used, per cent	Crop weight, mg.	N in crop, crop, mg.	Per cent N in crop
12	13	1.47	252	19.4	7.7
15.5	16	0.36	291	19.8	6.8
18	12	0.47	337	22.1	6.6
21	9	0.92	339	22.4	6.6
25	7	0.44	310	21.2	6.9
30	5	0.56	243	17.3	7.2
37	3	4.01	110	8.5	7.8

tion of yeast protein, though this yeast contains less nitrogen than that grown either at higher or at lower temperatures. The temperature of the maximal crop is a good deal lower than the optimum temperature of growth.

SUMMARY

The temperature which gives the largest crop is considerably lower than that giving the most rapid growth rate, but it is still far above the minimum temperature of growth.

An increase above the optimum temperature causes a very rapid decline of the crop.

The temperature of the largest crop weight and that of the largest amount of protein in the crop are not exactly the same.

(*f*) ENDPOINT OF GROWTH AND CHEMICAL STIMULANTS

It is generally understood that the final growth in any culture will depend not only upon the amounts and

kinds of food offered and upon the temperature used, but eventually also upon other factors, e.g., upon chemical substances which might increase or decrease the crop, or upon physical factors such as high osmotic pressures in concentrated salt or sugar solutions.

The most common factor in chemical respects will be the acidity, or more correctly for most cases, the hydrogen ion concentration. Frequently, acid is a product of fermentation, and these cases fall under the general heading of the influence of fermentation products upon growth. The same holds true with alkali formers.

Some organisms, like many yeasts and molds, have the ability to adjust the medium to their optimum acidity, i.e., they can increase the acidity if sugar is present, or eventually decrease it by complete oxidation of the acid. These are not included in the treatment of this chapter.

Growth comes to an earlier end if toxic substances are present in the medium. This is readily understood. It merely means the addition of one more unfavorable factor. But with the same substances which decrease the crop, an increase is often observed if the concentration of the toxic compound is chosen correctly. In other words, the stimulation of the rate of growth by small amounts of poison, as shown p. 227, may result also in a greater crop.

Of the older research on chemical stimulation, the papers by Koch (1912) and by Fred (1911) are of special bacteriological interest. The most comprehensive modern paper on this topic is that of Hofmann (1922). By dosing the toxic compound exactly right, Hofmann obtained not only a larger number of colonies from the same amount of culture, but the colonies were also larger. Of the six experiments recording the size of the colonies, only one shall be reproduced here (Table 72).

Assuming the colonies to be spherical, the total volume of all on any plate is obtained by multiplying the number of colonies in each group with the corresponding volume, and adding all volumina. The result thus obtained is given in the lowest line of Table 72. The control, without silver nitrate, had produced a total volume of 171 mm.^{3} of bacteria. The maximum with $AgNO_3$ was 755 mm.^{3}, or 4.4 times as much. Since these last figures represent, at least approximately, the volume of all bacteria produced in the same amount of agar from the same amount of seeding, the only difference being the presence of 0.00075% $AgNO_3$ in the one medium, a great influence of chemical stimulation upon crop production of bacteria cannot be denied.

TABLE 72.—NUMBERS AND SIZES OF COLONIES OF *Bact. typhi murium*, ON AGAR + $AgNO_3$

$AgNO_3$ added.....	0	0.0005%	0.00075%	0.001%	0.002%	0.01%
Diameter of colonies, in mm.	Number of colonies of the various diameters					
1.030–1.140.....	...	8				
1.140–1.250.....	12	23	...	5	6	7
1.250–1.370.....	34	24	...	13	14	10
1.370–1.480.....	26	41	5	13	16	21
1.480–1.600.....	26	28	12	27	29	23
1.600–1.710.....	7	14	19	54	55	8
1.710–1.820.....	4	5	34	16	16	7
1.820–1.940.....	4	...	61	17	18	7
1.940–2.050.....	...	...	48	6	3	
2.050–2.170.....	...	...	19	2		
2.170–2.280.....	...	...	13			
Total colonies.....	113	143	211	153	157	83
Total volume of bacterial colonies per plate, in mm.^{3}....	171	218	755	359	352	157

Hofmann mentions a large number of substances which acted as stimulants in similar experiments. Among them

are $HgCl_2$, $CuCl_2$, $ZnCl_2$, As_2O_3, $H_2Cr_2O_7$, HCHO, C_6H_5OH, lysol, atropin, saponin, malachite green, methyl orange, methylene blue. With some toxic compounds, however, no stimulation could be noticed in any concentration; among these were $FeCl_3$, $KMnO_4$, iodine, acetate of lead and of aluminum.

Hofmann's data show further there markable fact that the number of colonies from the same amount of bacterial suspension is increased by certain doses of poison. This can mean only that certain cells too weak to reproduce on normal agar (see p. 271) can be stimulated by a small dose of toxic substance sufficiently to produce normal colonies.

A general explanation for the stimulating action of chemical poisons has been given on p. 137. The evidence given here is quite sufficient to show that even through a large number of generations, chemical stimulation does not necessarily mean a final detriment to the cell.

V. MECHANISM OF GROWTH

(*a*) MOLECULAR STRUCTURE OF THE CELL

Growth, chemically speaking, is essentially a process of dehydration and of reduction. Doubtless, breaking-down processes such as hydrolysis and oxidation will also take place, but the outstanding, dominant problem is that of construction either by removal of water, or by reduction, or by other means.

While the preceding pages have shown how the growth mechanism reacts upon certain environmental conditions such as concentration of food, temperature, kind of food and stimulants, the mechanism as such has not been discussed. In this chapter, an effort is made to obtain a conception of it. The difficulty of a clear understanding

lies primarily in the fact that the problem is not only one of physics and chemistry, but also one of cytology, of systematic arrangement.

We cannot understand growth without first understanding the energy forming processes, for we can hardly imagine growth without a supply of energy. The liberation of energy in bacterial cells is fairly well understood, comparatively speaking.

The form of energy made available through cell activity is unknown. It may be heat, or it may be radiant energy; or we may call it "chemical energy" which is merely a good word for an unknown form.

Number of Enzyme Molecules per Cell.—While the form of energy is unknown, data on its amounts are available. The average cell of *Streptococcus lactis*, during its period of most rapid growth at 20°C., ferments about 12×10^{-10} mg. of sugar per hour (p. 106). The weight of one molecule of glucose is

$$\frac{180}{6 \times 10^{23}} \text{ gm.} = 30 \times 10^{-20} \text{ mg.}$$

Therefore the number of molecules of sugar used per hour by a single cell is

$$\frac{12 \times 10^{-10}}{30 \times 10^{-20}} = 0.4 \times 10^{10}$$

or four billion molecules per hour, or about one million molecules per second. This number is so large that we must consider the fermentation to be for all practical purposes a continuous process. The number of enzyme molecules required to handle one million sugar molecules per second is not known, but it must be fairly large.

Most cells can use more than one source of energy, e.g., streptococci and yeasts can grow without sugar,

and therefore, they must have different sets of enzymes for each source, and probably a fairly large number of molecules of each kind.

By applying this same method of calculation to the data on p. 105, it can be shown that a yeast cell will ferment about fifty million glucose molecules per second, and *Micrococcus ureae* as many as eighteen million urea molecules per second.

Number of Growth Catalysts per Cell.—The real growth mechanism of the cells is centered in the chromosomes. These are the growth catalysts. They are believed to consist of sub-units, called genes, which are beyond microscopic visibility. Each gene is supposed to be the catalyst for one specific reaction or property in the organism, and therefore, each gene must be chemically different from all other genes (unless we are dealing with multiple sets of chromosomes).

The size of the genes has not been measured. We know only that it is below the dissolving power of the microscope, i.e., smaller than 100 mμ. The size of the molecules of which the chromosomes are composed is not known either, but of many other proteins, the molecular radius has been determined by the Svedberg method. The author is indebted to Dr. D. C. Carpenter, of the Geneva (N. Y.) Experiment Station, for the compilation of Table 73. The proteins marked with a star are known to have nearly spherical molecules.

The diameter of these more common proteins goes as high as 24 mμ. The molecules of the chromosomes are much more remarkable than these, because they control the most complicated synthesis of the many different cell substances from simple food. It is probable (though it is not necessary) that molecules which bring about the synthesis of protein are more complex than

protein molecules. It seems permissable to assume that the molecules of which the chromosomes are composed, are of the same order of magnitude as the genes, and that a gene consists of only a few very large molecules, perhaps only of one.

TABLE 73.—SIZE OF VARIOUS PROTEIN MOLECULES CALCULATED FROM SEDIMENTATION VELOCITY MEASUREMENTS

Protein	Molecular weight	Molecular radius in mμ
Bence-Jones*	35,000	2.18
Egg Albumin*	34,500	2.17
Insulin*	35,100	2.18
Hemoglobin	68,000	2.44
Serum Albumin	66,700	2.39
Serum Globulin	103,600	2.75
Casein 2X	188,000	....
Amandin*	208,000	3.94
Edestin*	212,000	3.94
Excelsin*	212,000	3.96
Legumin*	208,000	3.96
Phycocyan	208,000	3.94
Phycoerythrin*	209,000	3.95
Casein 4X	376,000	4.18
Hemocyanin (Limulus)	1,760,000	6.96
Hemocyanin (Helix)*	5,005,000	12.00

* Molecules nearly spherical.

The enzymes of dissimilation can be produced by the cell; a cell can control its enzyme content to a certain degree (p. 131). It seems, however, that a gene can be produced only by another gene of the same kind. If a gene is destroyed, the cell cannot usually replace it, and if such a cell can divide at all, the two daughter cells will lack this gene and will be abnormal. More commonly, the cell will not divide.

Chromosomes have never been observed in bacteria. There is some indirect evidence for their existence, however. Lacassagne

(1928) exposed bacteria to X-rays and observed that a cell could be killed by being hit by one electron, but only if it was hit in a definite part of the cell which he called the sensitive zone. Wyckoff (1930) computed the volume of this zone from experiments with X-rays of various wave lengths and found it to be not more than one-hundredth of the cell volume. Nothing can be said about the form or distribution of this sensitive zone in the cell, but the fact of its existence suggests strongly a special segregation of the growth function corresponding to the chromosomes of larger cells.

Arrangement of Molecules in the Cell.—A computation of the "intensity of energy" or the "potential of energy" will permit us a further insight into the relation between the assimilative and the dissimilative mechanism.

It has just been mentioned that the average cell of *Strept. lactis* will ferment about 12×10^{-10} mg. of glucose per hour. The heat liberated in the lactic fermentation of 1 gm. glucose is 82 calories (p. 23). The sugar fermented by a single cell produces $12 \times 10^{-10} \times 82 \times 10^{-3} = 984 \times 10^{-13}$ calories. Taking the weight of this cell to be 5×10^{-10} mg., the energy liberated in one hour is sufficient to heat the cell to 200°C. Considering the enormous surface (about 60 square centimeters per mg. of moist cells, p. 397), radiation and conduction must play a great part in relieving the cell of this amount of surplus energy. Possibly, the energy in the cell is not liberated in the form of heat, but in some other, more harmless form.

If the entire cell assumed a higher uniform temperature, there would be no potential inside the cell. In order to get a conception of the possible energy potentials within the cell, the heat produced per molecule of sugar fermented must be calculated.

A single glucose molecule weighing $\frac{180}{6 \times 10^{23}}$ gm., in

being fermented to lactic acid, will liberate $\frac{180 \times 82}{6 \times 10^{23}}$ calories $= 2{,}460 \times 10^{-23}$ cal. If this energy, in form of heat, would distribute itself only upon the two lactic acid molecules formed whose weight would be

$$\frac{2 \times 90}{6 \times 10^{23}} = 30 \times 10^{-23} \text{ gm.},$$

these two molecules would show a rise in temperature of 82°C., (assuming, for simplicity's sake, the specific heat of lactic acid to be 1). But the enzyme which brought about the change of sugar to acid must be directly connected with these molecules, and must receive its share of the energy.

If this energy is to be used at all for growth, it must be used at once, before it is radiated or conducted away. A potential of energy is probably required to bring about any synthesis in the cell. Synthesis cannot be accomplished by a uniform rise of temperature of the cell. To utilize the energy of this potential established at the "molecule tips" of the enzyme, the synthetizing mechanism (such as the chromosomes, and probably other preliminary catalysts as well) must be in close proximity to the source of energy. It does not seem probable that energy could be sent only in one direction, as a beam, to the mechanism of synthesis.

Our knowledge of the growth process would be greatly benefitted, if we could get a conception of the number of different chemical processes necessary to change the food to the compounds which make up the cell wall, the protoplasm, and the reserve substances of the cell. Many different steps would be necessary, but with a given building material, it should be possible to estimate the number of steps approximately.

It would then be possible to sort out these different steps in groups of identical or homologous reactions, such as the condensation of formaldehyde and of acetaldehyde. It is imaginable that these

two different, but homologous processes might be accomplished by the same part of the cell mechanism. Thus we could get a conception of the number of different catalysts needed for the growth process.

Remembering the enormous surface of bacteria, it seems surprising that intermediate construction products have never been observed with bacteria, yeasts, or molds. The construction of cell matter from building material appears to be a continuous process comparable to the moving belt method of automobile manufacture. The molecules singled out for building stones have no chance to escape or to get side-tracked. Once a certain CO_2 molecule has been drawn by a nitrate bacterium into the growth machinery, it cannot leave the cell; it is destined to become a part of cell wall or protoplasm.

This is perhaps the most remarkable part of the entire growth process. The absence of partly synthetized compounds in bacterial cultures makes us believe that in one continuous process, the molecules are changed at least to a state where they do not diffuse through the cell wall.

This would require a very definite arrangement of the molecules in the cell. There are the molecules of the energy producing zymase, present in quite large numbers. These enzyme molecules are probably not scattered around by chance, but are located at some definite parts of the cell, or at least in definite position relative to the synthetic catalysts. The molecules of these catalysts are not all of the same type; there are different kinds for different synthetic processes. These catalysts must be arranged in some definite order so that the molecule under construction can be "passed on." Cytologists have found from experiments on inheritance that each gene has a definite location in its chromosome. All of

these necessities make the cell appear as a factory well arranged after a carefully thought-out plan. This definite arrangement is exhibited in the cells of higher plants by the symmetry of the chromosomes in cell division. Still, there must be considerable allowance for individuality.

There will be different degrees of complexity in the different types of bacteria. A prototrophic bacterium which builds its cells from CO_2 and HNO_3 must go through more steps than a cell which uses sugar and amino acids. The higher the claims of an organism are in respect to its food, the simpler will be the growth apparatus, the less will be the number of synthetic steps.

We can imagine the food requirements to become so highly specialized with certain parasites that the food is almost identical with the protoplasm of the host. The growth mechanism of these organisms would be extremely simple. They would simply take part of the host protoplasm, and incorporate it, after a few simple changes, into their cells where it will be the parasite's protoplasm. Such parasites could not have a cell wall; it would prevent the direct "intake" of such large molecules. It would seem possible that these organisms might consist of just one giant molecule. This might fit the case of filterable viruses and bacteriophages (Rahn, 1931*b*).

The knowledge of the filterable forms of well-known bacteria is still too scanty to be included in this discussion, but entirely new viewpoints are likely to be introduced in our conception of the cell mechanism by these studies.

(*b*) THE GROWTH PROCESS

After having considered the probable arrangement of the most essential molecules in the cell, we return to the

original problem, the chemistry and dynamics of the growth process.

Some information might be obtained from the observation that growth is hardly accelerated by increases of concentration of either substrate or building materials while it is greatly influenced by a change of the substrate, or by a change of temperature.

Bact. typhosum in peptone solution had a generation time of about forty minutes, with 0.4% peptone as well as with 1% peptone (p. 204). But with the addition of glucose, the growth rate doubled. *Bact. coli* in 1% peptone solution showed a generation time of thirty-five minutes and quadrupling this amount gave only 13% increase in the growth rate; the same effect which this additional 3% of peptone produced, could be obtained by as little as 0.1% of sugar (Curran, 1925).

Quite different is the effect of temperature upon growth. It influences fermentation, increases the energy yield per unit time. Concentration of food cannot do that.

A certain food will give a definite amount of energy. The enzymes in the cell can ferment only a limited number of substrate molecules. For *Strept. lactis* at 20°C., this number was estimated to be about 1,000,000 molecules per second. Increasing the sugar concentration will not cause more molecules to be decomposed. The enzymes are working with maximum capacity. But an increase in temperature brings about the decomposition of more molecules by the same number of enzymes; the mechanism is accelerated; everything moves more rapidly.

A change of substrate changes the rate of energy formation. We have no means of telling exactly what happens when a streptococcus cell is changed from glucose

broth to sugar-free broth. The only source of energy then is some peptone or amino acid. The available amount is less per molecule of substrate. How many molecules per second of the new substrate can be utilized by the cell has not been determined as yet. Doubtless, the enzyme furnishing energy from peptone is a different one from that which ferments sugar. These new enzymes must also be arranged in definite position to the synthetic catalysts in order to make them profit from the energy potential.

We saw that *Bact. typhosum* grew twice as fast with sugar + peptone than with peptone alone. It may be that sugar gave a larger amount of energy per molecule. It may be that the energy yield per molecule was not greater, but that more enzyme molecules of this type were present, producing a larger total of energy per unit time. It may be that even the total energy per unit time was not greater, but that the enzyme attacking the sugar is situated in a more favorable position in the cell, closer to the catalysts, while with the more distant protein-attacking enzymes, which produce perhaps more energy, most of the energy is lost by conduction or radiation.

By selecting the right kind of organisms and of energy sources, and by applying calorimetry as well as chemical analysis, these questions could be decided to a certain extent. The probability of obtaining a better understanding of the growth processes is greater with bacteria than with any other experimental organism.

(*c*) VARIABILITY OF THE GROWTH RATE

Starting from the facts that in most cells, there is only one gene of each kind per cell, and that all genes must have doubled before the cell can divide, the author (1932) developed a theory of the variability of the growth rate. If a large number of uniform cells (i.e., cells with the same kinds and numbers of molecules in the same arrangement) are kept in a uniform, favorable environment, there are two possibilities for the order

of their growth: either they all multiply at the same moment, or their reacting molecules follow definite chemical rules, and the order of multiplication is governed by these laws. The latter case is more probable, and the theory is supported by the facts.

It may be assumed that the molecules in a number of uniform cells react as if all cell contents were one continuous fluid. This assumption seems permissible at least for the molecules of the genes because genes do not react with one another. In this case, the doubling of any one certain type of gene molecule must follow the mass law, and as there is only one such molecule for each cell, it can be computed in what order this gene in the different cells will double. It can also be computed in which order any of the other genes, or each molecule of each gene, will double. From this, the probability that all genes in a cell have doubled, can be ascertained. This probability gives us the variation of the rate of cell division.

The calculation has been carried out for various numbers of genes (or molecules) in the chromosomes.

TABLE 74.—FREQUENCY OF FISSION TIMES OF *Bacterium aerogenes* IN PERCENTS

Minutes	5	10	15	20	25	30	35	40	45	50	55	60	Over 60
I. 323 observations.....	...	0.3	3.4	7.8	13.0	30.0	20.7	13.9	6.2	2.5	0.6		2.1
II. 168 observations.....	0.6	...	4.1	18.5	31.0	23.8	14.9	3.6	1.2	1.8	0.6		
III. 242 observations.....	...	2.4	17.0	48.0	24.0	7.0	0.8	0.8					

Figure 26 represents one such series. It shows that uniform cells under uniform environment will not all divide at the same rate, but that for chemical reasons, i.e., on account of the mass law, they must display con-

siderable variability of the growth rate, some of them dividing four to five time units later than the others.

This computation is an over-simplified case, making several assumptions which are not probable. For example, this computation assumes, that all genes multiply at the same rate, and that a cell divides as soon as the last gene has doubled while we know that, with higher organisms at least, some time elapses after chromosome division before cell division occurs.

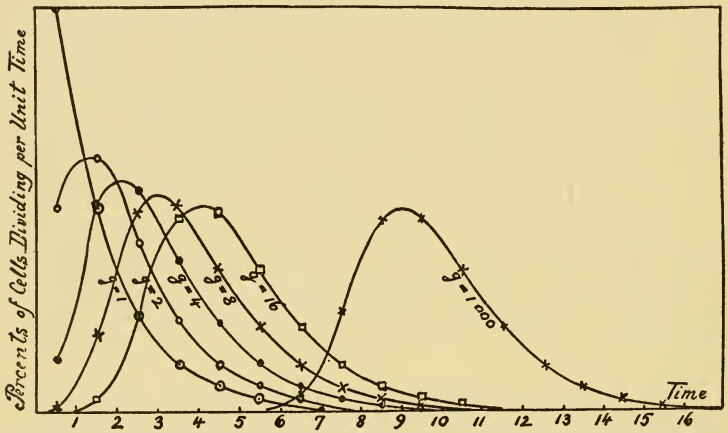


FIG. 26.—Percentage of cells dividing in successive time units computed for $g = 0.5$, and for various numbers of genes, g.

The observed facts (Kelly and Rahn, 1932) agree with the theory in a general way. *Bact. aerogenes* as well as *Sacch. ellipsoideus* showed a great variability of growth rate. Table 74 shows three series of data with *Bact. aerogenes*. The frequency curve is skewed to the left as in the theoretical curve of Fig. 26. The results with yeast corresponded to the data computed for a larger number of genes while *Bact. aerogenes* appears to have a smaller number. A special investigation showed that the offspring of the most rapidly multiplying individuals was not rapid, but average, and the same was true with the progeny of the slowest cells.

With colonies of *Bact. aerogenes*, the variability decreased as the size increased. This is due to the fact that fast and slow reproduction is a matter of chance, and not an inherent quality of the individual cell. The more generations we observe, the smaller will be the probability of the average being influenced by chance.

With mammals, the gestation period shows a relatively small variation, and no asymmetry.

(*d*) SUMMARY

Fermentation and respiration is brought about by catalysts (enzymes) of which each cell contains a considerable number. They can be regenerated by the cell.

Multiplication is brought about by very many different catalysts (genes), each cell containing usually only one of each kind. A gene, once lost, cannot be regenerated by the cell.

The molecules which bring about growth, i.e., synthesis, must be arranged in the cell in a definite position to each other to prevent the loss of partly finished building material.

The enzyme molecules that cause fermentation and liberation of energy must be in a definite position to the growth-producing catalysts to prevent loss of energy by radiation or conduction.

By making several simplifying assumptions, it can be shown that uniform cells in uniform environment will not all divide at the same time, but must have a definite frequency curve of the growth rate which is asymmetric and askew. The theory demands that the progeny of rapid and of slow growing individuals should have the same average growth rate, and that cells with only a few genes should vary more than those with many genes. All of these demands are borne out by the experiment.

PART D

MECHANISM OF DEATH

I. DEFINITIONS OF A DEAD CELL

Death has been defined in the introductory chapter as a function by itself; it consists not merely in the absence of fermentation and growth, but is a chemical process. We might perhaps define it more accurately by saying that dying is a chemical process which becomes irreversible after a while; from this time onward, the cell is considered dead.

The customary conception of dead bacteria is that they can not grow on the agar plate, nor in broth; in other words, that they have permanently lost the power of reproduction. This definition is different from those of other biological sciences; it is distinctly a bacteriological one. Cells which have lost the power to reproduce may still ferment; their protoplasm may still appear normal under the microscope, and may still be capable of plasmolysis. Such cells would be considered alive by most biologists.

It may be well to realize from the very beginning that many definitions of a dead cell exist which might give cause for considerable confusion unless their meaning is kept in mind all the time.

The bacteriological definition is not more exact than any of the others, as may be seen from the experiments of Süpfle and Dengler (1916) who dried anthrax spores on silk threads, heated them in steam for various lengths of time, and then placed each thread in broth. They took duplicate samples, and tested one in standard

broth, the other in the same broth + 3% glucose + 5% horse serum. The results (Table 75) show that, according to the standard broth test, the spores of strain SM were apparently killed between four and six minutes, and of strain Og between ten and twelve minutes of heating. However, the test in the better medium proved that this conclusion was wrong, as some of the spores revived and germinated after twenty-five minutes of heating in the richer medium. The destruction of the spores by heat was the same in both SM and Og cases because the tests were made with duplicate samples. Life in the spores was suspended irreparably in standard broth after four to twelve minutes of heating, while in the better medium, some of these spores recovered, and the fatal stage was not reached until they were heated for about thirty minutes.

TABLE 75.—VIABILITY OF SPORES OF *B. anthracis*, AFTER HEATING IN STEAM

(+ means growth; 0 means no growth; − means not tested)

Strain	Test medium	Heating time in minutes												
		1	2	3	4	6	8	10	12	15	20	25	30	40
SM	Standard broth.....	+	+	+	+	0	0	0	0	0	0	0	0	0
	Same + 3% glucose + 3% horse serum..	+	+	+	+	+	+	+	+	+	+	+	+	0
Og	Standard broth.....	−	+	−	+	+	+	+	0	−	0	0	0	−
	Same + 3% glucose + 3% horse serum..	−	+	−	+	+	+	+	+	−	+	+	0	−

From this experience, it may well be postulated that with a still better test medium, it might be proved that even forty minutes is not sufficient to really kill all spores. Similar experiments have been recorded in literature.

Death, as defined above, is, therefore, a function of the test medium. It might be recalled here that in the earlier development of agricultural bacteriology, much time was spent on finding the best medium which would give the highest plate count in milk, or in soil. Since the numbers of colonies developing from the same sample on different media are quite different, some cells are counted as "living" on one medium while they are "dead" on the other.

Attention may also be called to the observation of Hofmann (p. 256) that from the same dilution of a pure culture, a larger plate count might be obtained by adding small amounts of poisons like $AgNO_3$ or $CuCl_2$ to the medium. According to our definition of death, the chemical stimulus brought some of the "dead" cells back to life.

As long as a cell can recover from injury, the death process is reversible. It seems that the point of irreversibility is not reached very soon after the process of dying begins. This becomes evident in chemical disinfection. Bacteria treated with a poison may remain incapable of reproduction even when all poison has been washed away from the cells. An antidote that combines with the poison which has already reacted with the protoplasm, may be able to make the cell grow again (p. 358).

This discussion leads to the conclusion that our definition of death means the fixation of one point on the continuous line of a gradual chemical process. Dying is a gradual time process, of measurable velocity, and may be reversible, after removal of the cause, during the first stage by the mechanism of the cell itself. Gradually, it becomes irreversible for the cell, but may still be made reversible by some outside influences such as antidotes. Finally it reaches a state where it becomes irreversible under any condition. This may be due to secondary chemical changes which could take place because some part of the cell was not functioning sufficiently to ward it off as in normal cells.

II. METHODS OF MEASURING DEATH

(a) **The Endpoint Method.**—The oldest method of measuring the power of a disinfectant was to determine the shortest time in which all bacteria added to a certain concentration of the poison would be dead. Naturally,

the time differs with the species used. The tuberculosis organism is extremely resistant to alkali; molds can tolerate more formaldehyde than most bacteria; spores are much more resistant than vegetative cells.

The experiment is usually carried out by taking samples after certain intervals, transferring them to a good nutrient medium, and waiting for a certain length of time to see whether growth develops. The results obtained are of the nature shown in Table 75.

The accuracy of this method depends upon the frequency of the time intervals. In the above example, the interval is relatively large; the span between four and six minutes is 33% of the longest time. The last one of the four experiments of the above table is more accurate. The correct death time lies between twenty-five and thirty minutes. The span within which we know the exact time to be, is only five minutes out of thirty, or 18%. This relative accuracy will prove important where the endpoint method is used for computing death rates.

It has been known for many years that more time is needed to kill a larger number of bacteria. This is important for the technique of the endpoint method. In order to obtain comparable data, it is essential that the same number of cells is used for all data to be compared. If that proves to be impossible, the initial number of cells acted upon should be determined. It has become quite customary to count the initial number of cells.

(b) **The Plate Count Method.**—The plate count for determining the efficiency of a disinfectant has been in use for a very long time. It has the advantage of showing more of the kinetics of the disinfection process than other methods, and requires a smaller number of samples, but more time, and more media; therefore it is used mostly for studies on the laws of death, and less frequently in applied bacteriology.

(c) **Staining Methods.**—Most cells cannot be stained easily as long as they are alive, but take the dye readily

when they are dead. The bread yeast industry has made use of this fact for determining the dead cells in a batch of bread yeast, by suspending the cells in a counting chamber filled with a very dilute solution of methylene blue (Lafar V, 173). Fulmer and Buchanan (1923) applied this method to measure quantitatively the number of dead cells in a yeast culture exposed to some disinfectant.

In using this method, it should be borne in mind that it means a new definition of death. Considering the death process as a continuous curve plotted against time, the point where the protoplasm begins to take the dye is probably not identical with the point where the faculty of reproduction is lost.

Generally speaking, bacteria are too small to lend themselves to the staining technique. This applies also to most other methods for proving the death of cells in botanical or zoological experiments, such as plasmolytic reaction, loss of motility, response to stimuli, etc. The inaccuracy of the various staining methods has been pointed out by Bickert (1930).

III. THE LOGARITHMIC ORDER OF DEATH

(*a*) ORDER OF DEATH OF HIGHER ORGANISMS

When large organisms, such as green plants, or vertebrates, or insects, are exposed to a detrimental influence, some time will pass before the first organism dies. They will not all die at the same moment; some will die soon, others will be more resistant. The order of death will be represented by a chance curve, due to the variation of resistance among the individuals. This can be shown by the order of death of seeds, or of fruit flies exposed to high temperatures. Table 76

records two such experiments, the one with seeds being performed by Groves (1917), the other, with fruit flies (Drosophila), by Loeb and Northrop (1917).

If we plot the curves of the survivors, we obtain the inverted S-shape, the typical curve for chance distribution. If we plot the organisms dying per unit time as

TABLE 76.—ORDER OF DEATH BY HEAT

Wheat seeds at 87.5°C.			Fruit flies at 39.45°C.		
Time in minutes	Survivors	Per cent dying per minute	Time in minutes	Per cents of survivors	Per cent dying each interval
0	98	0	0	100	
7	74	3	25	95.5	1.8
8	60	14	30	83.0	12.5
9	38	22	35	65.4	17.6
10	11	27	40	45.4	20.0
11	5	6	45	28.0	17.4
12	2	3	50	17.1	10.9
13	0	2	55	11.8	5.3
			60	8.8	3.0
			65	2.5	5.3
			70	1.1	1.4
			75	0.3	0.8

TABLE 77.—1,000 MUSTARD SEEDS IN 0.2% $HgCl_2$

Time, min.	Survivors	Seeds killed in 15 minutes	$\frac{1}{t}\log\frac{a}{b}$
30	940.0	(30)	0.0018
45	895.8	44.2	0.0016
60	790.6	105.2	0.0023
75	486.6	304.0	0.0052
90	220.6	266.0	0.0087
105	163.8	56.8	0.0087
120	146.0	17.8	0.0080
135	39.0	107.0	0.0117

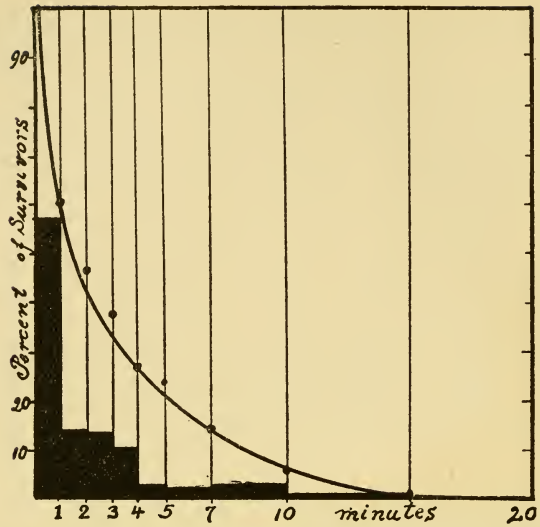


FIG. 27.—Order of death of *Bacterium typhosum* exposed to 49°.

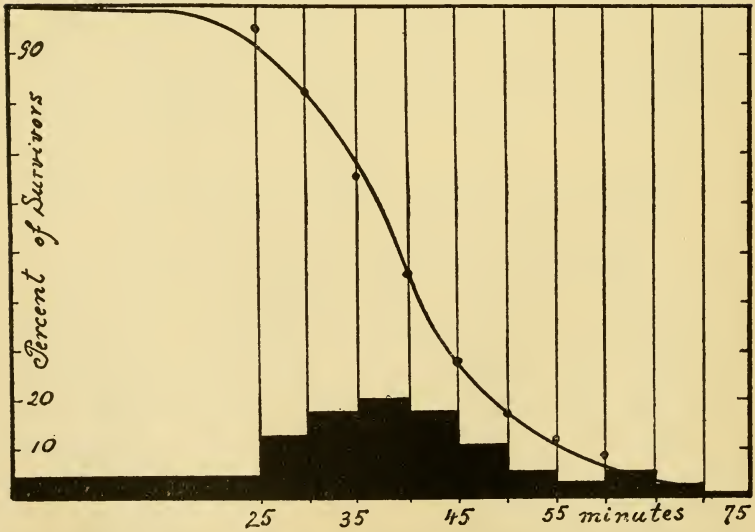


FIG. 28.—Order of death of *Drosophila* exposed to 39.45°. The black line in figures 27 and 28 demonstrates the numbers of survivors; the black blocks represent the organisms dying per unit time (1 and 5 minutes respectively).

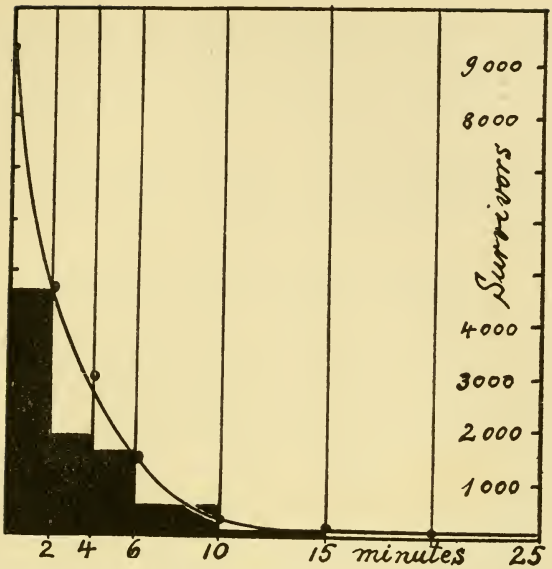


FIG. 29.—Order of death of the spores of *B. anthracis* by $HgCl_2$.

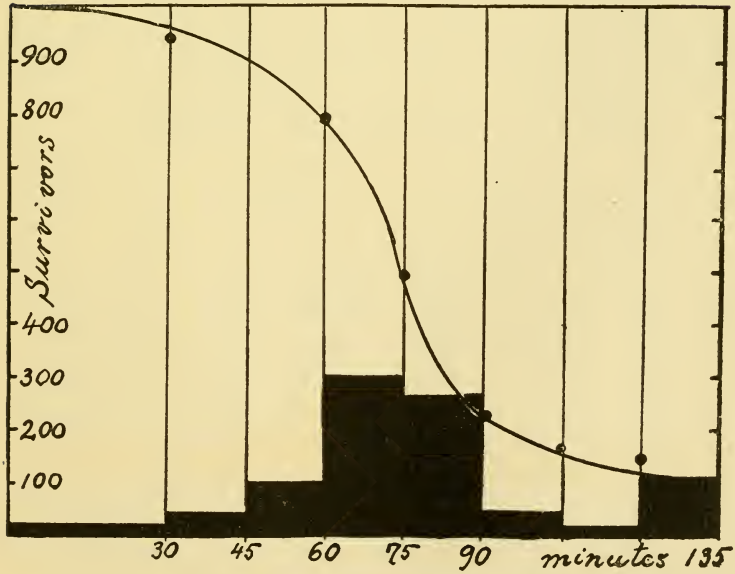


FIG. 30.—Order of death of mustard seeds by $HgCl_2$. The black line demonstrates the numbers of survivors; the black blocks represent the number of organisms dying per unit time (2 and 15 minutes respectively).

indicated by the black blocks (the unit being five minutes with fruitflies), we get a probability curve. (Fig. 28.)

Quite similar curves are obtained when death is caused by chemical poisoning, by drying, or other outside influences. As example may serve (see Table 77 and Fig. 30) the death of mustard seeds from treatment with $HgCl_2$ by Hewlett (1909).

(*b*) ORDER OF DEATH OF BACTERIA

With bacteria, the order of death is different. If the outside influence (heat, drying, chemical poison, light, etc.) is sufficient to kill the cells at all, the first unit of time will show the largest number of deaths.

Madsen and Nyman (1907) and, independently, Chick (1908) were the first to recognize that the order of death of bacteria could be described quite simply by the definition that the rate of death is proportional to the number of living cells. In other words, the percentage of the survivors dying per unit of time is constant during the experiment, regardless of the number

TABLE 78.—A THEORETICAL CASE OF DISINFECTION

Time	Survivors	Dying per unit time	Total dead
0	1,000,000	0	0
1	100,000	900,000 = 90%	900,000
2	10,000	90,000 = 90%	990,000
3	1,000	9,000 = 90%	999,000
4	100	900 = 90%	999,900
5	10	90 = 90%	999,990
6	1	9 = 90%	999,999

of cells present. Let us assume a very simple case: we have 1,000,000 bacteria; the death rate is 90%; this leaves, after the first minute, 10% or 100,000 cells alive.

During the second minute, again 90% of all living cells die, which leaves 10% or 10,000 cells alive. In this way, the number of cells decreases as is shown in Table 78.

If we compare the number of cells dying per unit time, with the examples given for seeds and fruit-flies, a difference in principle will be noticed.

That this order is typical for most causes of death of bacteria, will be shown all through this part of the book dealing with death; the evidence regarding the order of death is also summarized in Table 84, p. 295. Only two experiments shall be mentioned here (Table 79), as parallels to the afore-mentioned experiments with multi-

TABLE 79.—DEATH OF BACTERIA BY HEAT AND BY $HgCl_2$

Bact. typhosum at 49°C.				Spores of *B. anthracis* in 0.5 per cent $HgCl_2$			
Time, min.	Survivors	Per cent of total dying per minute	$\frac{1}{t}\log\frac{a}{b}$	Time, min.	Survivors	Spores killed in 2 minutes	$\frac{1}{t}\log\frac{a}{b}$
0.28	2,008			0	9,500		
1.00	1,198	56.0	0.311	2	4,860	4,640	0.146
2.05	925	13.6	0.190	4	2,964	1,896	0.126
3	755	18.5	0.156	6	1,408	1,556	0.138
4	542	10.6	0.153	10	304	552	0.149
5	488	2.7	0.130	15	2.6	120	0.204
7	289	4.9	0.125	20	1.8	0.3	0.186
10	112.8	2.9	0.129	25	2.0	0	0.147
15	24.2	0.9	0.130				
20	3.0	0.1	0.143				

cellular organisms, one on the death of *Bact. typhosum* by heat, by Chick (1910), and another on the death of spores of *B. anthracis* by mercuric chloride, by Madsen

and Nyman (1907). The fundamental difference in the order of death is well shown in the survivor curves of Figs. 27 and 29 as well as in the black blocks representing the number of organisms dying per unit time.

(c) MATHEMATICAL FORMULATION OF THE ORDER OF DEATH

The regular and simple order of death makes a general mathematical treatment possible. The initial number of cells shall be called a, and the number still living after an exposure for the time t shall be b. Since the rate of death at any time is proportional to the number of survivors, this can be written in the form

$$-\frac{db}{dt} = K \cdot b$$

$$-\frac{db}{b} = K dt$$

Integrated, this expression changes to

$$-\ln b = Kt + C$$

for $t = 0$, we have $b = a$, and consequently

$$-\ln a = C$$

We substitute this value for the integration constant into the preceding equation and obtain

$$-\ln b = Kt - \ln a$$

$$K \cdot t = \ln a - \ln b$$

$$K = \frac{1}{t} \frac{\log a - \log b}{0.434}$$

This last expression gives the formula for the death rate, K, for decimal logarithms. If the number of cells dying per unit time is at all times the same percentage of the number of living cells, K must be constant. The

computation of K in Table 79 shows that it fluctuates around the average value of K. In the experiments with mustard seeds (Table 77), the death rate is computed by the same formula; it is not constant, however, but increases continuously. This is sufficient evidence that the death follows a different law.

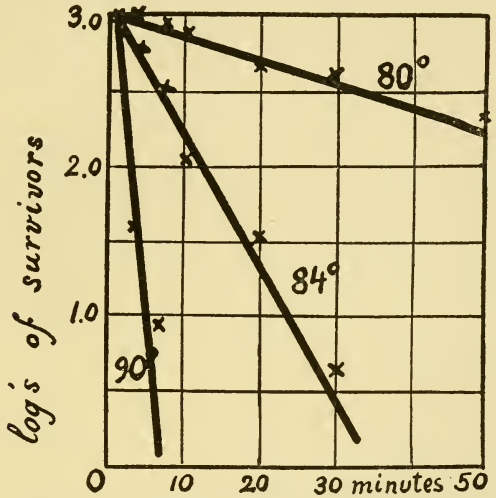


FIG. 31.—Logarithms of survivors of anthrax spores plotted against the time of exposure to different temperatures.

The computation of the death rate will prove very useful when we try to understand the mechanism of the processes bringing about death. It is practically impossible to define K in words that would mean anything more than a description of the mathematical formula. Even the relation between this death rate and P, the percentage of cells surviving per unit time, is not very simple. We find

$$P = \frac{100}{10^{0.434K}}$$

If we wish to compute the number of survivors b for any time, we have the equation

$$\log b = \log a - 0.434Kt$$

log a is constant within the experiment. Therefore, we have

$$\log b = C - 0.434Kt$$

which means that the number of survivors is a logarithmic function of the time. For this reason, this regularity of death is often referred to as the "logarithmic order of death." If we consider log b to be a variable, instead b itself, we get the general formula of a straight line

$$y = C - 0.434Kt$$

This means that if we plot the logarithms of survivors against time, all points will be on a straight line. Figure 31 shows the survivors of spores of *B. anthracis* exposed to three different temperatures plotted in this way, from an experiment by Eijkman (1912–13). The logarithmic survivor curves are straight lines, the different angles indicating different death rates.

In recent years, several investigators have been satisfied that the logarithmic order is proved whenever the logarithms of survivors are found to be on a straight line.

The most essential point in this kind of graphic proof is the inclusion of the initial number of cells in the curve. If this is not included in the graph, a straight line relationship proves nothing. As may be seen from Fig. 32, most of the curves indicating quite different orders of death are so nearly a straight line that they would be considered so from experimental data, if the first horizontal part of the curve were omitted. It is very essential to ascertain whether or not there is an initial period without deaths. The omission of the initial number makes this impossible. Only a constant death rate can then be considered proof of a logarithmic order of death.

(*d*) INTERPRETATIONS OF THE ORDER OF DEATH

This order of death is, in its mathematical aspects, a complete parallel to the monomolecular reactions. What the chemist calls "rate of reaction" or "reaction veloc-

ity," is mathematically identical with the "deathrate" $K = \frac{1}{t}\frac{\log a - \log b}{0.434}$ as computed above.

This similarity has led some bacteriologists to the belief that bacteria are sufficiently small to react like molecules. Others, however, have assumed that the logarithmic order of death is ultimately due to the logarithmic order of some chemical reaction causing the death.

Some bacteriologists and biologists have been very much opposed to such mechanistic views and claim that the order of death is due to a graded variation of resistance of the individuals. However, it is not easy to explain the logarithmic order in terms of a graded resistance. Figures 27 and 29 show in their block curves the number of deaths per unit time. The resistance should be graded in a similar way which would require that the most sensitive bacteria are present in the largest numbers. This is quite different from the gradation of higher organisms (Figs. 28 and 30).

Reichenbach (1911) tried to explain this especially graded resistance by the mode of multiplication. He assumes that from each new generation, a definite percentage ceases to multiply, and goes into a state of dormancy in which it becomes the more resistant the longer it remains in this stage. In this way, the last generation which is the largest, is also the most sensitive.

There is no biological reason to assume that part of the cells of an actively growing culture should go into a resting stage. Besides, Kelly and Rahn (1932), in their study of the fission rates of individual cells, could never observe that any cells stopped growing in the early stages of growth after they once had started multiplying. This holds for *Bact. aerogenes and B. cereus* as well as for yeast.

Another attempt at an explanation for this is that made by Henderson Smith (1921). He raises the well-grounded objection that in death by chemical poisoning, differences in resistance might come from differences in the thickness of the cell membrane. Our only measure of resistance is the killing time; but killing times will correspond to the grades of resistance only if the penetration of the poison is directly proportional to the thickness of the wall. If it would take the poison four times as long to penetrate a membrane twice as thick, the killing times could not be considered as indicators of uniformly graded resistance.

However, if retarded penetration were the reason for the logarithmic order of death of bacteria, then they must show an order equal to that of higher organisms when death is caused by agencies which penetrate with immeasurable speed, such as light or ultraviolet rays. This is not the case, however. With these agencies, we find the logarithmic order established just as plainly as in chemical disinfection (p. 371).

Quite different again is the interpretation of Rahn (1929c, 30, 31a) who started from the idea that the property of growth is condensed in the chromosomes and genes (p. 259). The genes are very small, beyond the limit of microscopic visibility. Considering the large size of the complex protein molecules of living protoplasm, each gene may contain only one, or a very few, molecules. If only one molecule of one of the genes is inactivated, the cell cannot multiply. Cell division requires the doubling of all chromosomes, and of each gene.

In computing the variability of the growth rate (p. 267), Rahn had assumed that the gene-type molecules of a large number of uniform cells under uniform environ-

ment react as if all cell contents formed one continuous fluid, as if cell-walls and separating medium did not exist. This seemed justified because the genes do not react with one another, but only with molecules which are plentiful in each cell.

Let us assume that a large number of uniform bacteria were heated to 55°C., and at this temperature, the most sensitive gene molecule would be hydrolyzed at

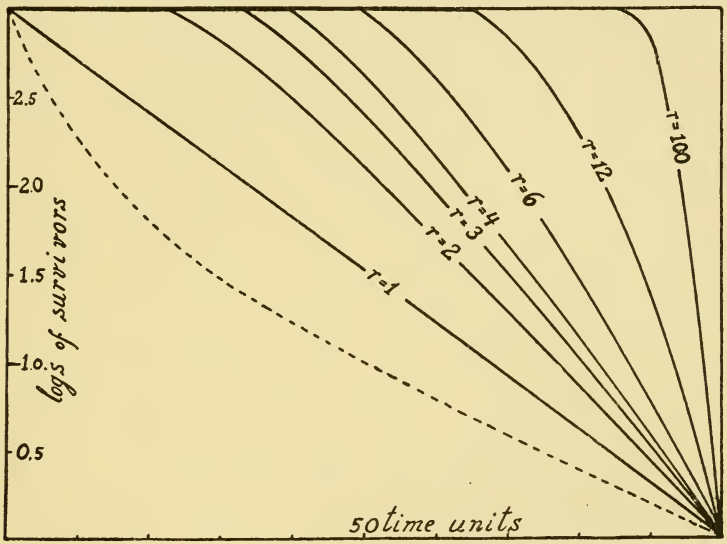


FIG. 32.—Order of death computed for organisms which are killed by the reaction of $r = 1, 2, 3, 4, 6, 12$, or 100 molecules per organism. The dotted line is the order of death for $r = 1$ molecule, but with three different grades of resistance.

the rate of 90% of all such molecules per minute. At the end of this time, only 10% of the cells will have this gene intact; the 90% will have lost the power to multiply because one of the genes is damaged beyond repair. During the second minute, 90% of the undamaged cells will again suffer the hydrolysis of the special gene, i.e.,

9% of the original number of cells will again become sterile, unable to multiply, and only 1% of the original cells is left unchanged. After the third minute, only 0.1% of all cells will be able to grow, and so on. This order of death is identical with that of a monomolecular reaction (see p. 278).

For chemical reasons, then, uniform cells cannot all die at the same time, but must follow the mass law. If the cells are not uniform, the order of death is different, as shall be shown presently. This order of death does not apply to death by physical causes (e.g., freezing). There are some other exceptions, e.g., death by starvation. Why the effect of poisons must be considered monomolecular, will be explained on p. 341.

From this one extreme, where the inactivation of only one single molecule causes death, to the other extreme of the higher organisms, where many cells must be inactivated before the organism dies, there are many intermediate steps. Rahn (1929c) computed a general formula for the order of death for any number of "reacting molecules," i.e., molecules which have to be changed to bring about death. Figure 32 gives the logarithms of survivors plotted against time. It shows a straight line only for one reacting molecule. For all other cases, the line is not straight, but shows at first a period of no deaths, and then turns sharply down in a curve which is not really straight, but might be mistaken as such.

Rahn found (1931a) that death of Colpidium by $HgCl_2$ seems to be brought about by the reaction of two molecules per cell, and that avian red blood corpuscles when exposed to ultraviolet light, also dissolve after the inactivation of two special molecules. With yeast, death was brought about sometimes by one, sometimes by two inactivated molecules. Mold spores seem to con-

tain quite a large number of reacting molecules. The logarithmic survivor curves for higher organisms are distinctly bulging as may be seen from Fig. 33 representing the death of wheat and mustard seeds.

We have seen (p. 280) that for bacteria, the death rate

$$K = \frac{1}{t} \frac{\log a - \log b}{0.434}$$

remains constant. This K value must be constant when death is brought about by the reaction of one molecule per cell. If the

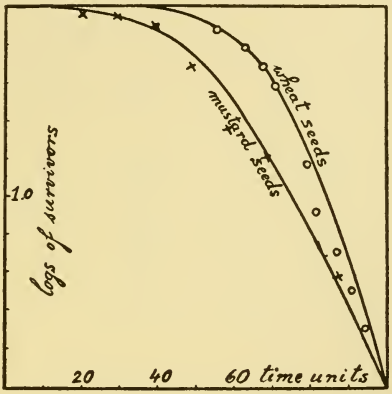


FIG. 33.—Order of death of seeds on standard scale. Wheat seeds, killed by heat, and mustard seeds, killed by bichloride of mercury.

number of "reacting molecules" is larger than one, K increases. When we compare the death rates of higher organisms with those of bacteria, as shown in Table 80, it becomes conspicuous that there is a continuous increase with all examples of the higher animals and plants while with bacteria, the rate is either constant or decreasing.

All living beings except unicellular organisms show survivor curves bulging out above the straight line, indicating more than one reacting molecule per cell (see e.g. Fig. 33). All bacteria (with three exceptions to be discussed later) show either a straight line or a survivor curve sagging below the straight line (see Figs. 31 and 34).

This latter type will be explained in the next subchapter as due to a variation in the resistance of the individuals.

If bacterial cells contained more than one reacting molecule per cell, their survivor curves should show a bulging out above a straight line, and not a straight or even a sagging curve. If they had more than one reacting molecule per cell, their death rates should be continuously increasing, and not remain constant or decrease.

TABLE 80.—DEATH RATES FOR SUCCESSIVE TIME UNITS

$$\left(\text{Computed as } k = \frac{1}{t} \log \frac{a}{b}\right)$$

Higher organisms			*Colpidium*	Bacteria			
Fruit flies	Wheat seeds	Mustard seeds		*Anthrax spores*	*Bact. typhosum*	*Bact. coli*	*Bact. paratyphosum*
0.004	0.017	0.0018	0.013	0.146	0.194	0.406	0.748
0.013	0.027	0.0016	0.011	0.126	0.185	0.383	0.355
0.026	0.046	0.0023	0.058	0.138	0.191	0.452	0.222
0.043	0.095	0.0052	0.077	0.149	0.188	0.484	0.166
0.061	0.117	0.0087	0.108	0.204	.....	0.350	0.133
0.077	0.141	0.0087	0.103	0.186	.....	0.366	0.113
0.084	.....	0.0080	0.127	0.147	.....	0.344	0.101
0.088	.....	0.0117	0.145	.....	.....	.....	0.083
0.123	.....	.....	.....	.....	.....	.....	0.070
0.140	.....	.....	.....	.....	.....	.....	0.060
0.167	.....	.....	.....	.....	.....	.....	0.051
(Table 76)	(Table 76)	(Table 77)	(Peters, 1920)	(Table 79)	(Table 79)	(Table 96)	(Reichenbach, 1911)

The pronounced difference in the order of death between bacteria and higher organisms indicates that death in the former is brought about by the change of only one molecule in each cell.

(*e*) PHYSIOLOGICAL YOUTH

Cells of the same strain show a different degree of resistance at different stages of development. The first quantitative observation in this respect was probably

that of Schultz and Ritz (1910). Similar results were obtained independently by Sherman and Albus (1923) who tested *Bact. coli* in its resistance to heat, to cold, to salt solutions, and to phenol. Some of their results are summarized in the following table.

TABLE 81.—DEATH OF CELLS FROM YOUNG AND OLD CULTURES OF *Bact. coli*

Exposure to	Time of exposure	Age of culture	Plate counts		Percentage of survivors	Death rate
			Before	After		
Distilled water	1 hour	11 days	607	606	100	0.0007
		6 days	720	710	99	0.006
		4 hours	530	155	29	0.534
		4.5 hours	373	247	66	0.179
53°C.....	20 min.	7 days	1,470,000	97,000	6.6	0.059
		3.5 hours	27,800	24.6	1.1	0.153
0.5% phenol	3 min.	24 hours	4,650,000	42,000	0.917	0.713
		4 hours	1,240,000	300	0.024	1.202
		24 hours	6,900,000	10,800	0.156	0.935
		4 hours	2,400,000	430	0.018	1.249

The differences in the percentage and in the death rate of survivors are so striking and check so well with the preceding experiment that a "physiological youth" of bacteria, as the latter authors termed it, must be considered established. It seems proved that the physiologically young bacteria are more sensitive to adverse conditions than old ones. This circumstance is probably one of the main reasons for decreasing death rates and sagging survivor curves.

(*f*) DECREASING DEATH RATES

Decreasing death rates can be accounted for by varied resistance of the cells. If we have a mixture of sensitive and resistant individuals, the death rate at the beginning of the experiment will be high because all sensitive cells will die rapidly. After most of these are killed, the rate will be largely that of the resistant cells, and this is lower.

It is most probable that in a culture which is not especially cared for, there will be organisms of different physiological conditions, and therefore of different resistance to the cause of death. Rahn (1930) computed a theoretical case for bacteria with three different grades of resistance, and the curve of survivors is shown in the dotted line of Fig. 32.

This effect of variability of resistance had already been forseen by Harriett Chick (1910) who tried to avoid this decrease of death rate by using very young cultures, three hours old, and by transplanting them repeatedly in order to get rid of old and more resistant cells. The results are seen in Table 82.

The degree of constancy or variability of K can be best compared by the variation in the relative death rates based on the average K of each experiment = 100. The lowest line of Table 82 shows that the relative spread of variation in the two experiments with twenty-four hours old cultures is quite large, 137 − 41 = 96% in the one and 140 − 56 = 84% in the other. With the three hours old culture, the spread of variation was 108%, i.e., larger than in the twenty-four hour cultures; perhaps there were many old cells left among the new growth. But upon transferring this culture repeatedly every three hours, the uniformity improved, and the spread of variation dropped to 26 and 44%.

Reichenbach (1911) tried a similar experiment with the same organism, *Bact. paratyphosum*, exposed to heat.

TABLE 82.—DEATH RATES OF *Bact. paratyphosum* IN 0.6% PHENOL

Experiment No.....	III	V	XI	XII	XIII
Age of culture, minutes	24 hours		3 hours	3rd 3 hours generation	4th 3 hours generation
1	.....	....	0.56		
2	.....	0.63	0.46	0.066	0.081
3	0.27	0.61	0.38	0.062	
4	0.27	0.42	0.33	0.064	0.086
5	0.26	0.34	0.32	0.053	
6	0.22	....	0.30	.....	0.093
7	0.22	0.25	....	0.051	
8	0.20	....	0.23	.....	
9	0.18	....	0.22	.....	0.072
10	0.15	....	0.20	0.058	
15	0.12	....	....	0.059	0.065
20	0.08	....	....	0.054	0.059
Average K.....	0.197	0.45	0.333	0.058	0.076
Relative Values of K (average K = 100)					
1	.....	.....	168		
2	.....	140	138	113	107
3	137	135	114	106	
4	137	93	99	110	113
5	132	76	96	91	
6	112	.....	90	.....	122
7	112	56	.....	87	
8	101	.....	69		
9	91	.....	66	.....	95
10	76	.....	60	99	
15	61	.....	.....	101	85
20	41	.....	.....	93	78
Greatest variation....	41–137	56–140	60–168	87–113	78–122

He did not transfer repeatedly. Only the degrees of variation shall be given here.

TABLE 83.—VARIABILITY OF DEATH RATE OF *Bact. paratyphosum*

Age of culture, hours	Temperature, °C.	Average of K	Average K = 100		
			Maximum	Minimum	Spread
5.5	49	0.179	417	25	392
8	48	0.064	178	41	137
13	50.1	0.049	113	(44) 92	21 (69)
18.5	51	0.134	105 (130)	88	17 (42)
24	50	0.046	158	46	112
28	51	0.177	109	93	13
48	49	0.066	113 (122)	92	21 (30)
55	50	0.071	109	89	20

In this experiment, the older cultures seem more uniform, with less spread than the young cultures; the only exceptional experiment

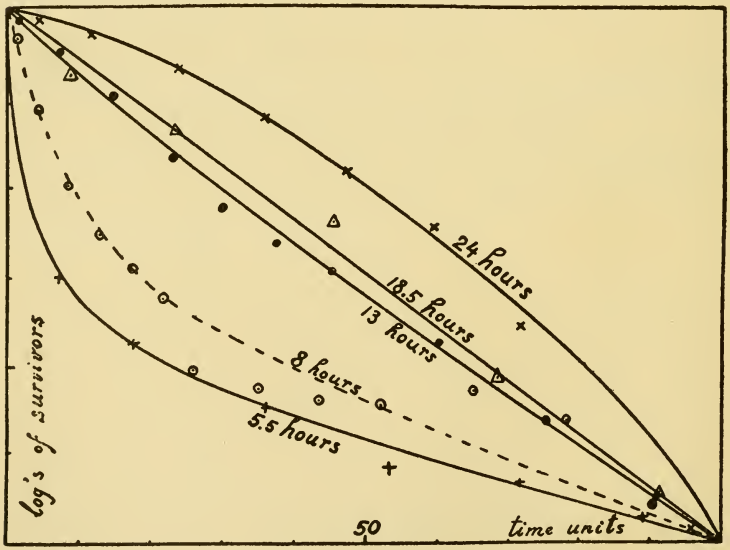


FIG. 34.—Survivors of *Bacterium paratyphosum* from cultures of different age, drawn on standard scale.

is that of a twenty-four hours old culture which was considered abnormal by Reichenbach. The figures in parentheses are unusual

values obtained during the first two minutes of the experiment; they might be caused by the change of medium, or by heat shock. The trend of the death rates in all of Reichenbach's experiments is a gradual decrease of K, and the "spread" of table 83 shows the relative amount of this decrease.

If Reichenbach's plate counts are plotted as logarithmic survivor curves on a standard time scale (Rahn, 1930) the striking curves of Fig. 34 are obtained. The youngest culture contains old and young cells which differ greatly in resistance and the survivor curve sags deeply. With increasing age of the culture, the old cells begin to multiply, and finally, after thirteen hours, all cells are in a state of rapid multiplication and fairly uniform resistance. This is depicted graphically by the gradual straightening of the curves.

(*g*) FREQUENCY OF THE LOGARITHMIC ORDER OF DEATH WITH BACTERIA

Some biologists have questioned the logarithmic order of death. Loeb and Northrop (1917) in their study of the death rate of fruit-flies (p. 275 and Fig. 28) make the following statement:

"Miss Chick has stated that bacteria are killed by disinfectants at a rate corresponding to that of a monomolecular chemical reaction, i.e., that in each interval of time the same percentage of individuals alive at this time is killed. She was probably led to such an assumption by the fact that the ascending branch of the mortality curve in her experiments was generally very steep. The agencies used by her for killing the bacteria were so powerful that the ascending branch became almost a vertical line, thus escaping attention. Hence she noticed usually only the less steep descending branch which could be interpreted as a monomolecular curve for the reason that her experiments lasted only a short time."

This same objection has been raised again and again; it is believed by many biologists that the logarithmic

order would not appear at all if the first plate counts had been made sooner.

This belief is not based on facts. It has never been proved that a logarithmic order of death would become established e.g. in the experiments p. 275 with seeds, fruit-flies or protozoa, if the first observations would have been omitted. In fact, no logarithmic order would be established if this were done, as any calculation or graphic test will easily show (e.g., see Fig. 33). The death rate of the higher organisms increases, and the curve continues to bulge, while with bacteria, the death rate is always decreasing if not constant, except in the case of some spore-formers and of staphylococci, which will be discussed on p. 296.

It is true, that in most experiments on the order of death, or on the theory of disinfection, almost half of the bacteria or more have died during the first time interval investigated. But this very fact points out that the order of death is essentially different from that observed with higher organisms.

Table 84 gives a survey of all experiments known to the author referring to the order of death. They are divided into three groups according as the death rate is dominantly constant or decreasing or increasing. Nearly one-half of all experiments cannot be used to prove or disprove the logarithmic order of death because the most important part of the experiment, the initial number of bacteria, has been omitted. Of the remaining one hundred and fifty-four experiments, only thirty-two have a constant death rate. But we have just seen (p. 290), that a decreasing death rate must be expected to be the most common occurrence because the cultures under test are rarely very uniform in resistance. It should be remembered, too, that the decreasing death

TABLE 84.—SURVEY OF ALL EXPERIMENTS ON THE ORDER OF DEATH OF BACTERIA

Author	Number of experiments			
	With initial count missing	With death rate		
		Decreasing	Constant	Increasing
Death by drying				
Paul (1909)	4			
Paul, Birstein and Reuss (1910a)	10			
Madsen and Nyman (1907)	2			
Death by heat				
Chick (1910)	21			
Reichenbach (1911)	...	3	9	2
Eijkman (1912/13)	...	3	4	2
Sattler (1928)	...	62	1	8
Death by chemical poisoning				
Madsen and Nyman (1907)	13	..	8	
Chick (1908)	12	5	2	
Chick (1910)	6	..	..	3
Reichenbach (1911)	...	..	1	2
Paul, Birstein and Reuss (1910b, c)	72			
Eijkman (1912/13)	...	1	..	1
Lee and Gilbert (1918)	...	2	2	
Winslow and Falk (1926)	...	5	4	2
Myers (1929)	...	..	1	17
Death by irradiation				
Clark and Gage (1903)	...	1		
Lee and Gilbert (1918)	...	1		
Gates (1929)	...	..	..	2
Total in each group	140	83	32	39

rate indicates that this order of death is still further removed from that of higher organisms than the constant one. It may well be stated that one hundred and fifteen of the hundred and fifty-four experiments speak in favor of the logarithmic order of death, or at least in favor of an order entirely different from that of higher organisms.

There remain thirty-nine experiments with an increasing death rate which makes them resemble the order of death of larger organisms. Increasing death rates have been observed occasionally, as exceptions, with several species (e.g. *Bact. coli*, *Bact. paratyphosum*) which ordinarily show constant or decreasing rates. But with a few species, an increasing death rate seems to be the rule. The three well established cases are the Staphylococci (Chick, 1910), the large, unnamed spore-former of

TABLE 85.—DISINFECTION OF *Micr. pyogenes aureus* BY 0.6% PHENOL COMPUTED FOR STANDARD SCALE

Chick's Table VI			Chick's Table VII		
Time units	Survivors	Death rate	Time units	Survivors	Death rate
0	100.0		0	100.0	
4.9	92.2	0.035	4.3	84.6	0.073
14.9	80.8	0.031	8.6	81.8	0.043
19.8	73.7	0.033	12.9	72.4	0.047
24.8	54.7	0.052	17.2	63.0	0.050
29.6	42.0	0.063	22.0	52.4	0.056
34.6	31.0	0.073	30.2	26.2	0.083
39.5	18.8	0.091	43.1	10.4	0.098
44.5	15.65	0.089	64.7	2.47	0.107
49.4	12.08	0.092	100.0	0.1	0.115
59.3	3.51	0.121			
74.2	1.69	0.118			
100.0	0.1	0.149			

Reichenbach (1911) and the spore-former of Myers (1929). As an example, two experiments by Chick are given in Table 85.

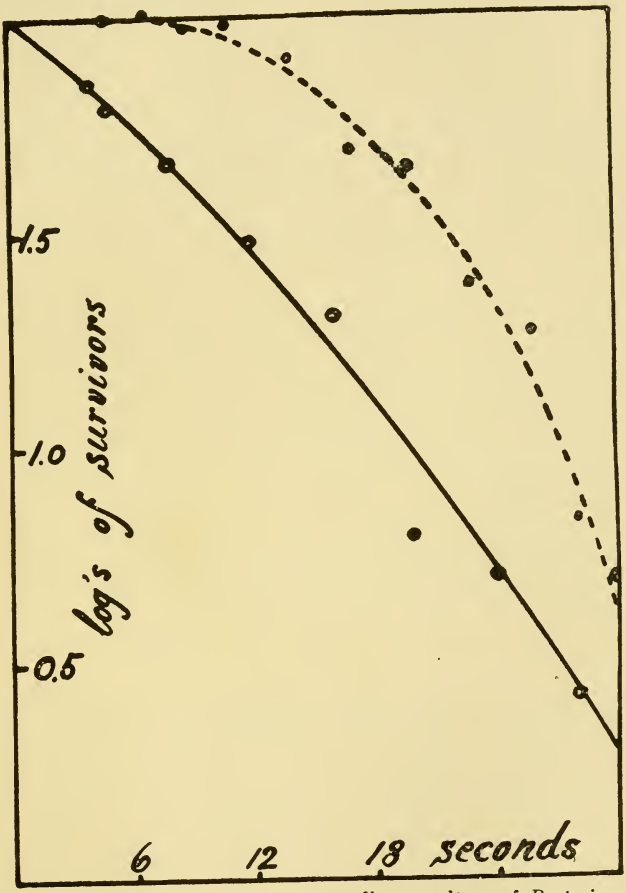


FIG. 35.—Survivors obtained by spreading a culture of *Bacterium coli* on agar and exposing it there to X-rays. The full line shows the survivors when the agar plate is exposed immediately; the dotted line shows the results when the agar plate had been incubated for a short time before exposure.

While we have here an approximation to the survivor curves of higher organisms, it is not necessary to assume

a larger number of "reacting molecules" per cell though such cases appear quite possible (see p. 367). Rahn (1930) has pointed out that the staphylococcus gets its name from the tendency to form clusters, and that spore-formers usually grow in threads. Each thread or cluster will contain several cells, yet on the agar plate, it will produce only one colony. The plate count does not give us the number of individuals in these cases, but the number of clusters, and the clusters contain several "reacting molecules," namely one for each cell. The fault lies in our inadequate method of counting living cells.

A very simple and convincing proof for this assumption is given by Wyckoff and Rivers (1930). They spread a young culture of *Bact. coli* on an agar surface, exposed it to cathode rays, and considered that the colonies developing on the agar represented the survivors. A microscopic investigation proved that the cells spread on the agar had not been clustered. The survivor curve was nearly a straight line (Fig. 35). A similar plate was held in the incubator for a short time before being exposed. The originally single cells of the bacterium had had a chance to form small groups. The survivor curve of this plate was bulging like those known for larger organisms.

(*h*) ADAPTATION AND SELECTION OF RESISTANT STRAINS

According to the strict chemical interpretation of the logarithmic order of death, the last survivor is no more resistant than the first cell that died. Surviving is merely a question of chance. According to the theory of graded resistance, the last survivor is much more resistant than the first, in fact, it is the fittest survivor. Although with higher animals and plants, this method of selecting resistant strains is almost certain to give good results (provided due consideration is given to the laws of inheritance), the experiments with bacteria have not been very successful.

This is partly due to inadequate technique. The death rates for two transfers of the same culture on different days vary greatly. Chick (1910) found the death rate ($0.434K$) of *Micr. pyogenes aureus* with 0.6% phenol at 20°C. to be, on March 13th = 0.32, March 16th = 0.114, March 30th = 0.32 and April 6th = 0.136. The same author found that *Bact. coli* died in water of 489°C. with a death rate of 0.172, and eleven days later at 49.0°C., with a death rate of 0.042. Such variations (amounting in the last case to 400%) are found in the work of other investigators as well (see e.g., Table 82). An explanation shall be tried later (p. 333 and 345). It seems that in recent experiments, it has been possible to reduce this error considerably. Myers (1929) was able to average data from different months without difficulty. But as long as the technique shows deviations within the same strain of the degree just mentioned, there is little hope of proving increased resistance. This may account for some of the failures.

It is well known that bacteria can be adapted to poisons, to higher temperatures or other adverse conditions. This is accomplished by subjecting them to gradually increasing intensities of the harmful substances, or conditions. With our problem, however, we are interested only in those cases where the last survivors show greater resistance when compared with their ancestors, or with the average.

The experiments of Magoon (1926) which are sometimes cited as such an example, are not at all convincing. Magoon transfered the last survivors of heated spores of *B. mycoides* to a new medium and determined the resistance of the newly developed spores again. The first culture survived nine minutes at 100°C.; how much longer the spores might have lived, was not determined. The second culture survived twenty minutes; it was not ascertained how much more heating it might have tolerated. Only with the third culture, it was certain that it could tolerate twenty-eight, but not twenty-nine minutes' heating. There is no evidence whatever that the other two cultures were less resistant. The fourth culture was tested for thirty-six minutes and all survived; this culture was more resistant

than the preceding one. With the fifth culture, Magoon suddenly changes his technique and holds the culture for sixty-four days instead of twenty-nine as in all previous experiments. The result is a greater resistance. If we do not use these incomparable last data, but consider the last experiment finished after twenty-nine days (Magoon's records make this possible), then it becomes evident that the fifth culture is decidedly less resistant than the fourth, and about equal to the third. The experiments do not prove an increase in resistance by selection, but, on the contrary, a fluctuation. The last survivors are not more resistant than the average.

Similar are the experiences of Arnold (1929) who sprayed several millions of cells of *Bact. coli* and *Bact. prodigiosum* into the nose, made cultures of the few remaining cells and repeated the experiment with these cultures as often as nine times. "There was no evidence of an acquired resistance on the part of those bacteria that had survived for a maximum period of time upon the nasal mucosa."

More than twenty years previous to this experiment, Gage and Stoughton (1906) tested *Bacterium coli.* Broth cultures of this organism were exposed for five minutes to temperatures ranging from 45°C. to 100°C. The survivors of each tube were counted by plating. The tube heated to 60° was used as the "second generation." Subcultures of it were again exposed to different temperatures, the survivors were counted, and the culture heated to 60° was used as "third generation." In this way, nine generations were tested, all of which had been heated to 60°C. or 55°C. The results are shown in Table 86. The numbers are the survivors in percents of the inoculum; + means growth in the tube, but no colonies on the agar plate; 0 means no growth in the tube.

Gage and Stoughton summarize their results as follows: "Experiment 195 in which we attempted to produce, by the survival of the fittest, a race of especially resistant organisms, demonstrated a diametrically opposite result."

This agrees with the experiences of Gates (1929) whose experiments on death by ultraviolet light will be recorded on p. 372. He states that "cocci from colonies of the last surviving organisms have proved to be inherently no more than normally resistant to ultraviolet light."

These experiments speak quite decidedly against the assumption that the last survivors are more resistant;

TABLE 86.—SUCCESSIVE CULTURES OF LAST SURVIVORS OF *Bact. coli* PERCENTAGE OF SURVIVORS AFTER 5 MINUTES' HEATING

Heated, °C.	Generations								
	First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	Ninth
45	136.0%	86.0%	100.0%	63.0%	72.0%	63.0%	258.0%	64.0%	243.0
50	70.0	3.6	44.0	46.0	20.0	23.0	4.5	9.7	49.0
55	4.4	0.006	0.050	2.3	0.004	0.020	0.020	0.012	0.200
60	0.001	0.008	0.010	0.001	0.009	0	0.160	0.002	0
65	0.250	0.400	0.001	0.001	+	0	0.020	0	0
70	0.030	0.001	0.002	0	0	0	0.010	0	0.022
75	0.050	0.014	0.003	+	0	0	0	0	0
80	0.015	+	0.003	+	0	0	0	0	0.018
85	0.003	0	+	0	0	0	0	0	0
90	0.003	0	0	0	0	0	0	0	0
95	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0	0

it appears to be much more a question of chance which cells survive.

(*i*) SUMMARY OF FACTS

Dying is a gradual chemical change in the cell, and any definition of death is the arbitrary assumption of one point on a time curve.

If we define a dead cell as one which has lost the power of reproduction, the death of bacteria through heat, chemicals, light, drying and some other causes proves to be an orderly process. If the logarithms of survivors are plotted against time, bacteria show either a straight line or a curve sagging below a straight line, while larger organisms give a curve which bulges out above the straight line. In only three cases, bacteria are known to show consistently bulging curves.

A "death rate" can be computed by using the formula for the rate of reaction in monomolecular processes.

This rate is increasing with higher organisms, but constant or decreasing with bacteria, except in the above-mentioned three cases.

Young cells, a few hours after having been transferred into a new medium, are more sensitive to adverse conditions than cells of the same culture after growth has ceased.

The time required to kill all bacteria in a given volume increases with the number of cells, regardless of the means used to kill them.

The last survivors of a disinfection experiment, as a rule, do not give cultures of a higher resistance than the original strain.

SUMMARY OF THEORIES

The logarithmic order of death cannot be explained by a graded resistance unless several assumptions are made which have no biological justification other than to fit this purpose.

If the order of death is computed under the assumption that the destruction of one certain molecule in the cell is sufficient to prevent growth, the resulting order of death corresponds to that observed with bacteria of uniform age. The computation for the assumption that several or many molecules must have reacted before death occurs, gives survivor curves corresponding to those observed with multicellular organisms.

The order of death of cells with only one "reacting molecule" shows a constant death-rate and a straight line in the logarithmic survivor curve. If the cells under test are not all of the same physiological condition, but show differences in resistance, (each grade of resistance having a constant death-rate) the logarithmic survivor curve will sag below the straight line and the

death rate will decrease. This must happen, e.g., with mixtures of old and young cells.

Organisms with more than one such "reacting molecule" show an increasing death rate, and a survivor curve bulging above the straight line.

Bacteria which have a tendency to clump or cluster, and whose plate count does not reveal the actual number of cells, but only the number of clumps or clusters, must give survivor curves corresponding to more than one "reacting molecule" because the only method of counting living bacteria is the plating method, and here, each colony represents a clump of cells whose total number of reacting molecules equals the number of cells in the clump.

IV. DEATH OF DRY BACTERIA

The following subchapters will treat the various causes of death. That of dry bacteria has been chosen as the first cause to be discussed because conditions here are simpler than with other causes. When bacteria are in a moist condition, endogenous catabolism is decreasing the cell substance continuously, while in dry cells, lack of moisture prevents this. Thus the uncertain factor of catabolism does not enter into the death of dry bacteria.

(*a*) ORDER OF DEATH OF DRY BACTERIA

Dry bacteria will die in the course of time; some species within a few minutes, most of them within weeks or months, and the spores of some bacilli eventually not for a decade. The viability or longevity of dried bacteria depends not only upon the species, but also upon the temperature and the stratum on which the cells are dried.

While all of this had been known for a long time, no real understanding of the cause of death was obtained until Paul (1909) started his quantitative investigations. Though he used staphylococci which are likely to give abnormal results (p. 296), he obtained a fair constancy of the death-rate in his experiments.

TABLE 87.—DEATH OF *Micr. pyogenes aureus* AT VARIOUS TEMPERATURES

Time, days	Number of living cells			Death rates for t = 2 days		
	16–18°C.	6–8°C.	−190°C.	16–18°C.	6–8°C.	−190°C.
1	90,800	88,800	65,900			
2	68,600	77,100	60,900	0.28	0.14	?
4	48,500	66,400	77,800	0.21	0.10	
6	27,200	65,100	98,400	0.24	0.06	
8	11,400	37,100	60,700	0.30	0.12	
10	5,320	18,700	61,200	0.32	0.17	
12	6,680	17,100	69,200	0.24	0.15	
14	2,630	10,200	61,500	0.27	0.17	
16	900	4,980	73,300	0.31	0.19	
18	1,500	3,000	68,100	0.24	0.20	
32	300	350	67,900	0.18	0.16	
Average..	.....	.....	.....	0.26	0.15	< 0.001

Paul dried the bacteria on small stones (garnets), of uniform size (details, see Paul, Birstein and Reuss, 1910a), which were kept in test-tubes under varying conditions. From time to time, some of them were removed and shaken with sterile water to separate the bacteria which were then counted by the plating method. The first experiment of Paul, with dry staphylococci at room temperature, at that of the refrigerator, and at the temperature of liquid air, gave the data of Table 87.

Another experiment, with another strain, gave similar results. The death rates are sufficiently constant to obtain an average. From the equation on p. 281, the percentage of survivors can be computed:

$$P = \frac{100}{10^{0.434K}}$$

$$= 77.1\% \text{ at room temperature (16–18°C.)}$$
$$= 86.1\% \text{ in the refrigerator (6–8°C.)}$$
$$= \text{over } 99.9\% \text{ in liquid air } (-190°\text{C}).$$

This gives the death rates per unit time (= 2 days) as 22.9%, 14.9% and less than 0.1% respectively.

(*b*) OXIDATION AS THE CAUSE OF DEATH

The possibilities for the cause of death by desiccation are few. Catabolism is out of the question because it requires moisture. It may be that the colloidal state of the protoplasm is changed irreversibly and irreparably. This does not seem probable because of the very slow death-rate. Such a change might occur during the drying process, and may cause death. After the bacteria are once dry, and still alive, a colloidal change is less probable.

Another possibility is oxidation. Paul, Birstein and Reuss (1910a) proved oxidation to be the main cause of death of dry staphylococci. The bacteria were kept in air (20.8% O_2), in technical oxygen (96.2% O_2), and in mixtures of oxygen and nitrogen. Each of the death rates in the following table is the average of 6–8 individually determined constants.

This table shows that the death-rate increases with the oxygen concentration. The authors found that the rate is proportional to the square root of the oxygen

concentration. This might be accounted for by assuming either an adsorption of oxygen by the bacterial surface, or by a dissociation of the oxygen molecule into atoms, $O_2 = 2O$, as in the slow oxidation of phosphorus.

TABLE 88.—DEATH OF *Micr. pyogenes aureus* AT DIFFERENT OXYGEN CONCENTRATIONS AND TEMPERATURES

Experimental series	Temperature, °C.	Oxygen concentration, %	Death-rate per hour
VIa	18.2	20.8	0.0243
VIa	18.2	96.2	0.0530
VIII	37.4	20.8	0.0157
VIII	37.4	96.2	0.0256
VIII	18.0	20.8	0.0017
VIII	18.0	96.2	0.0034
XII	37.4	20.8	0.0264
XII	37.4	54.6	0.0369
XII	37.4	96.2	0.0444
XII	24.9	20.8	0.0107
XII	24.9	63.2	0.0152
XII	24.9	96.2	0.0200

The data of Paul and his associates on the influence of oxygen on dry bacteria have been confirmed and completed by Rogers (1914) who also controlled the moisture content of dried streptococci. It seemed that the less moisture was contained in the cells, the longer they lived. Rogers dried his bacteria by placing the culture in an evacuated desiccator over sulfuric acid at freezing temperature.

After being dried, the streptococci were tested for viability in various gases. Only one count was given

by the author for each series. The death-rates have been computed in Table 89 for one day as unit time. The observation that more bacteria survived in oxygen than in air, is contradictory to the other data by Rogers as well as to Paul's results. In all other gases, the death-rate was lower than in air, as was to be expected. The vacuum is the best method of preservation.

TABLE 89.—VIABILITY OF DRY LACTIC STREPTOCOCCI IN DIFFERENT GASES

	Oxygen	Air	Hydrogen	Nitrogen	CO_2	Vacuum
At beginning.....	3,270,000	3,270,000	3,270,000	3,270,000	3,270,000	3,270,000
After 132 days.....	7,750	750	350,000	22,000	276,000	785,000
K =	0.0200	0.0276	0.0074	0.0164	0.0081	0.0047

The difference in the influence of the various "inert" gases is not explained. The average death-rate in all experiments without oxygen is 0.009 as compared with 0.0276 in air. The dry streptococci die in air three times as fast as without oxygen.

Paul's computation that the death rate is proportional to the square root of the oxygen concentration does not take into consideration this slow death without oxygen. Possibly, the rate was too slow to influence his calculations. However, the fact remains that even with the removal of oxygen, dry bacteria will still die, though more slowly. The cause of death in the absence of oxygen is unknown.

The simpler method of drying bacteria on filter paper used by Brown (1925, 1926) has not been used for quantitative experiments, but could easily be adapted to this purpose.

(c) INFLUENCE OF TEMPERATURE

Paul's data show that dry bacteria die more rapidly at higher temperatures. At that of liquid air, −190°C., death was so slow that no distinct decrease could be observed in a month.

Since death of dry bacteria has been found to be primarily due to the oxidation of some essential part of the cell, we must assume this oxidation to follow the same temperature laws as other chemical reactions (p. 119).

The data of Tables 87 and 88 can be well used for calculating the temperature coefficients of the fatal oxidation. This computation does not require the assumption that the logarithmic order of death is strictly adhered to, nor that the order is due to a chemical process. It is just assumed that the value K is a correct expression of the relative velocity at which the bacteria die.

TABLE 90.—TEMPERATURE COEFFICIENTS OF THE DEATH BY DRYING OF MICROCOCCI

Series	Oxygen concentration, per cent	Interval of temperature, °C.	Coefficient for 10°C. increase
VIII*	20.8	37.4–18.0	3.16
VIII*	96.2	37.4–18.0	2.83
XII*	20.8	37.4–24.9	2.04
XII*	96.2	37.4–24.9	1.90
Table 87.....	20.8	17 –7	1.73
Average.....	....	.....	2.33

*From Table 88.

Apparently, the coefficient is not constant, but the data are too few to warrant conclusions. So much seems certain, however, that the temperature coefficient of death through drying is similar to that of common chemical reactions, i.e., it lies between 2 and 3. Rogers (1914) found the temperature coefficient of the death rate of dry streptococci to be 3.6 between 0 and 30°C.

It seemed interesting to determine the temperature coefficient at temperatures which exceeded the range of normal growth temperatures. Upon the author's suggestion, Dr. H. H. Boysen made three series of experiments with dry yeast cells keeping them between 30 and 100°C. The distribution of cells in the strata used (infusorial earth or sand) was not sufficiently uniform to use the data directly. They were accurate enough, however, to compute the times required to kill 99%

TABLE 91.—TIME REQUIRED TO KILL 99% OF THE CELLS OF DRY YEAST

Series	Stratum	30°C.	37°C.	42.5°C.	50°C.	60°C.	80°C.	90°C.	98°C.
I	Infusorial earth.	56 d.	21 d.	11 d.	.....	76 min. 160 min.	less than 4 min.		
II	Infusorial earth....	.....	15 d.	.....	5.8 d.	.....	.....	4 min.	
III	Sand.....	110 d.	.....	.....	.....	75 hrs.	.....	.....	29 min. 60 min.

(Temperature coefficients)

I	Infusorial earth....	...	4.06	3.24	← 14.61 / 21.20 →	< 4.36 / < 6.32	
II	Infusorial earth....	..	.....	←2. 08→	←6.76→		
III	Sand.....	...	←3.18→			← 5.29 / 4.22 →	

of the initial number of viable cells. From these times, the temperature coefficients of Table 91 could be obtained.

The results are fairly consistent with the exception of one too high value in the first series. If the range from 30°C. to 60°C. is taken in toto, the temperature coefficient is 10.2 or 7.96, just which, depends on whether seventy-six or one hundred sixty minutes is used as the correct time.

The temperature coefficients for the entire range of the three experiments are 8.34, 5.77 and 3.53 respectively. This difference is very great. There are at least two possibilities that might account for this difference: the stratum on which the yeast was dried; and also, that no effort was made to dry the yeast to the same degree of dryness. The varying moisture content would very likely affect the temperature coefficient greatly, since the coefficient of death by moist heat is very high.

Despite several inconsistencies in the results, the data are entirely sufficient to show that the chemical process of dying of dry bacteria is not essentially influenced by the change from "growing temperatures" to "killing temperatures." At 30°C. and 37°C., the yeast would have shown good growth in a suitable medium, while at 50°C., in the same medium, the yeast would have died. This great contrast has been entirely eliminated by the absence of water. No inactivation of enzymes or coagulation of proteins by supramaximal temperatures takes place because these processes require moisture. Death is altogether controlled by oxidation, which is a normal chemical process.

The dry micrococci of Paul's are reduced from 100 living cells to 1 in thirty-three days at 17°C. (Table 87). Calculating with a constant temperature coefficient of 2.5, the same reduction would require

at 67°C. $33 \div 2.5^5$ days = 8 hours 7 minutes
at 97°C. $33 \div 2.5^8$ days = 31 minutes

while moist cells would be killed at these temperatures in a few minutes or even seconds. If the coefficient should decrease over this range, the resistance of dry bacteria at high temperatures would be still greater than calculated.

This also enables us to compute the time for sterilizing at lower temperatures dry glassware or other dry materials which cannot stand the customary high heat of 160°C. for thirty minutes. With a temperature coefficient of 2.5 (this coefficient has really never been determined for dry spores), it would require at 100°C. about 30×2.5^6 minutes = five days two hours to obtain a sterilizing efficiency equal to thirty minutes at 160°C.

It is generally known that the material on which bacteria are dried has a great influence on their viability. Bacteria dried on coverslips die very readily, while those dried in soil remain alive over a long period. The bacteria of legume nodules can be cultivated from herbarium specimens after years, while the attempt to use, commercially, cultures dried on cotton proved to be a failure. A large amount of time has been spent in searching for paints or finishes for walls of hospitals and operating rooms which would kill in the shortest possible time any bacteria smeared on them. But no quantitative work has been done in this respect except just a measurement of the time required to kill all bacteria spread and dried on certain strata. No experiments which try to explain the different death-rates on different strata from the viewpoint of death as an oxidation process are known to the author.

A very interesting and important question has never been solved or attacked, namely, the cause of death of dry bacteria in the absence of oxygen. It is barely possible that bacteria remain alive for infinite times in the absence of oxygen. Lipman (1931) has succeeded in demonstrating living bacteria in coal. The rate of death becomes smaller when we eliminate the main

cause of death. Cells without food live longer when dried; dry cells live longest at the lowest temperatures; dry and cold cells live longest in the absence of oxygen; but they do not live indefinitely, and the cause of death under these circumstances, though unknown, is of general biological interest.

The laws of the death of dry bacteria apply also to bacteria in non-aqueous liquids. Bartlett and Kinne (1913) compared the resistance of spores heated in water, glycerol, olive oil, cottonseed oil and paraffine, and found the bacteria in non-aqueous liquids much more resistant. Staphylococci were killed quickly at 100°C. in all media, lower temperatures not being tried. Spores of *B. anthracis* or *B. subtilis* at 100°C. died in water in a few minutes, but were still alive in glycerol after seventy-five minutes, in oil after fifty minutes. Spores of *B. vitalis* survived 120°C. (15 pounds pressure) in water ten minutes, in glycerol 90 minutes, and in oil two hours, but not longer. This again indicates that the death process by dry heat is entirely different in its nature from that by moist heat.

(*d*) SUMMARY OF FACTS

Dry bacteria die slowly, and there is a fairly definite order of death. The rate of dying varies greatly, even with the same culture dried on different days.

Dry bacteria die more rapidly in air than in a vacuum, or in an inert gas; they die more rapidly in an oxygen atmosphere than in air. Oxygen is the most essential cause of death of dry bacteria.

Dry bacteria die more rapidly at higher temperatures.

Dry bacteria probably die more rapidly when their moisture content is high.

Bacteria in oil or other water-free fluids die in the same way as do dry bacteria.

The rate of death of dry bacteria is greatly affected by the medium upon which they are dried.

SUMMARY OF THEORIES

The primary cause of death of dry bacteria is an oxidation process; the death rate is proportional to the square root of the oxygen concentration.

The temperature coefficient of this oxidation process is either normal (experiments with micrococci, range 7–37°C. and lactic acid bacteria, range 0–30°C.) or higher, but not above 10 (experiments with yeast, range 30–100°C.). The temperature coefficient probably increases with an increasing moisture content of the test organism. There is no evidence that the cause of death of dry bacteria at temperatures above the maximum for growth is different from that at temperatures of normal life.

The cause of death of dry bacteria in the absence of oxygen is still unknown.

V. DEATH BY FREEZING

Death of bacteria by freezing has been studied to a considerable extent from the viewpoint of typhoid epidemics from river ice, and also on account of food preservation by freezing. The data obtained are not very uniform, sometimes they are contradictory, and make it probable that several factors enter which had not been controlled in the experiments published.

If we consider freezing to be a solidification of water, then freezing is identical in its effect with drying, and death by freezing should follow the same general rules and laws as that by drying. However, there

is no evidence at all available that death by freezing is due to an oxidation process as is death by drying. All data on death by freezing indicate that it is not brought about by one fundamental reaction. Hilliard, Torossian and Stone (1915) enumerate the following factors which might cause death during the freezing process: interference of low temperature with metabolism; rupture of membrane by pressure of ice formation in the cell; rupture by external pressure or grinding during crystallization; pressure by expansion of the freezing medium in closed receptacle; prolonged suspension of metabolism leading to slow death by old age or by starvation.

That freezing, i.e., the crystallization of water as such, causes death was shown by Hilliard and Davis (1918) by comparison of the death rate in water which solidified, with that in sugar solutions which did not solidify, at the same temperature. Some of these results are given in Table 92, each datum representing the average of at least four experiments. The

Table 92.—Death of *Bact. coli* by Freezing Temperatures for 3 Hours

Temperature, °C.	Percentage of cells killed	
	In glucose solution, not frozen, %	In water, frozen, %
−0.5	49.6	90.3
−1.5	36.2	95.9
−2	40.8	93.1
−3	44.2	96.9
−4	49.3	98.8
−6	49.5	99.2

destructive effect of the physical process of freezing was further shown by the very rapid destruction of

cells by repeated freezing and thawing as compared with check cultures remaining frozen over longer periods of time (Table 93).

It was also found by the same authors that in milk, bacteria are not decreased as much by freezing as in water.

TABLE 93.—DEATH BY CONTINUOUS FREEZING AND BY ALTERNATE FREEZING AND THAWING
(Numbers indicate plate counts per c.c.)

Bact. typhosum

Frozen solid		Alternate freezing	
Inoculum	40,896	Inoculum	40,896
24 hrs. frozen	29,780	Frozen 3 times	90
3 days	1,800	Frozen 5 times	0
4 days	950	Frozen 6 times	0
5 days	2,490		

Bact. prodigiosum

Frozen solid		Alternate freezing	
Inoculum	339,516	Inoculum	339,516
24 hrs. frozen	36,410	Refrozen once	2,570
30 hrs. frozen	41,580	Refrozen 2 times	275
48 hrs. frozen	14,440	Refrozen 3 times	15
96 hrs. frozen	4,850	Refrozen 4 times	0

Recently, extensive studies on the freezing of bacteria and yeasts have been published by Tanner and Williamson (1928), not so much with the object of studying the fundamental reactions concerned as to test the differences of resistance of different species. The organisms were frozen in small samples and kept at −13 to −15°C.; for each test, one of these samples was thawed and a

plate count made. Parallel sets in physiological salt solution and in broth were made of all organisms.

The survivor curves are quite irregular, indicating the possibility of more than one cause of death. The indication of a period of no death at the start is noticeable in most experiments. This may be due either to clustering of the cells, (see p. 296) or to the slowness of action, for in death by very slowly acting agents, such curves have been observed.

The differences in resistance of the different species have been summarized in Table 94 by giving the time required to kill 99.9% of all cells. In salt solution, almost all species can tolerate the freezing better than in broth.

TABLE 94.—TIME REQUIRED TO KILL 99.9% OF ALL CELLS BY A TEMPERATURE OF −13 TO −15°C.

Species	Time required	
	In broth, weeks	In salt solution, weeks
Bact. coli	8	12–16
Bact. prodigiosum	8–9	12–16
B. mesentericus	Over 80	Over 80
B. subtilis	80	Over 80
Sacch. cerevisiae	Over 160	Over 160
Sacch. ellipsoideus	5	18
Sacch. pastorianus	58	Over 160
Sacch. marxianus	Over 160	14
Pichia membranefaciens	9–26	7–9
Torula rosea	9–10	10–26
Zygosaccharomyces mongolicus	10–26	46–54
Mycoderma vini	46–54	68–160

This very slow rate of death is verified by the observation of Prucha and Brannon (1926) who found that

it took one year to kill 99.9% of all typhoid bacteria in ice cream when held at approximately −19°C.

The slowness makes possible the explanation that the fundamental process of death, aside from physical destruction, might be an oxidation process, or, perhaps, a starvation process. No data are available to decide between these two and possibly other explanations.

SUMMARY

Death by freezing involves a number of different possible causes. While the low temperature, as such, is one of the factors, physical destruction by the formation of ice crystals has been shown to be important, so that repeated thawing and freezing kills more rapidly than long continued cold. The medium in which bacteria are frozen is also of significance for the rate of death.

Death by freezing is slow. The extremes recorded in literature for a reduction of 99.9% are with *Bact. coli*, eight weeks, *B. subtilis*, more than eighty weeks, *Sacch. ellipsoideus* five weeks, *Sacch. cerevisiae*, more than three years.

VI. DEATH BY HEAT

Under "heat" in this chapter is understood "moist heat," i.e., the application of high temperatures to bacteria in their natural state of living. Death by dry heat has been treated in the chapter on Drying (p. 309).

(*a*) METHODS OF MEASURING

While all that has been said about the technique of disinfection experiments in general (p. 270 to 274) applies here, some special features enter into the killing of bacteria by moist heat that deserve special attention.

The death rate is influenced remarkably by the medium in which the organisms are heated. In the earlier experiments (Chick, Eijkman) distilled water was used altogether. This introduces considerable uncertainty into the cause of death. Probably, the diffusion of cell constituents into the distilled water is too slow to kill cells in the short time of the experiment. Since the hydrogen ion concentration in distilled water is known to vary greatly, it will not be possible without pH control to obtain data which agree. If these experiments are carried out in a medium suitable for the development of the test organism, there is no chance for variation of acidity, nor for leaching out of essential compounds from the cell.

A very remarkable observation was made by Lange (1922) namely, that bacteria killed by heat and added to living bacteria, protect these against the action of heat to a remarkable degree. One of his experiments is

TABLE 95.—PROTECTION AGAINST HEAT OF LIVING CELLS BY DEAD CELLS IN BROTH

Standard Suspension		Water	Broth	Culture living (+) or dead (0) after								
Living cells	Dead cells	.....	.....	5′	10′	20′	30′	45′	60′	120′	180′	240′
Bact. coli at 60°C.												
0.02 drops	.....	1 c.c.	5 c.c.	+	+	+	+	0	0	0	0	0
1 c.c.	.....	.....	5 c.c.	+	+	+	+	+	+	0	0	0
0.02 drops	1 c.c.	.....	5 c.c.	+	+	+	+	+	+	0	0	0
Micr. pyogenes at 56°C.												
0.02 drops	.....	1 c.c.	5 c.c.	+	+	+	0	0	0	0	0	0
1 c.c.	.....	.....	5 c.c.	+	+	+	+	+	+	+	+	0
0.02 drops	1 c.c.	.....	5 c.c.	+	+	+	+	+	+	0	0	0

reproduced in the preceding table; the addition of 1 c.c. of dead cells doubles the time required to kill *Bact. coli,* and trebles the time for *Micr. pyogenes.* It is necessary that the added dead cells come from a young culture. Old cells exert no protective action.

(*b*) THE ORDER AND RATE OF DEATH AT DIFFERENT TEMPERATURES

In the killing of bacteria by moist heat, the logarithmic order is generally quite well established. Most of the examples given in the chapter on the order of death refer to death by heat. As typical examples, we may mention (see Table 96) the death of *Bact. coli* at 48.9°C. and 52.7°C., by Chick (1910) and the death of the spores of *B. anthracis* at 80°C., 84°C. and 90°C., by Eijkman (1912/13) of which only the survivor curves are given in Fig. 31, p. 281 showing straight lines.

TABLE 96.—DEATH OF *Bact. coli* AT 48.9 AND 52.7°C.

Time, minutes	48.9°C.		52.7°C.	
	Number of surviving cells	*K*	Number of surviving cells	*K*
0.25	372	.....	383	
1.0	274.3	0.406	208	0.820
2	190	0.383	89.7	0.830
4	68.25	0.452	14.5	0.874
6	23	0.484	1.1	1.017
8	.....	.....	0.55	0.845
10	12.4	0.350	0.066	0.890
15	1.72	0.366		
20	0.425	0.344		
	Mean	0.396	.....	0.878

The computed death-rates are sufficiently constant to be used for the computation of the temperature coef-

ficient. We find this, according to the formula from p. 120, for *Bact. coli*

$$Q_{10} = \sqrt[3.8]{\left(\frac{0.878}{0.396}\right)^{10}} = 11.8$$

TABLE 97.—TEMPERATURE COEFFICIENTS OF DEATH BY HEAT FOR DIFFERENT SPECIES

Author	Organism	Number of experiments	Average Q_{10}	Temperature interval, °C.
Gage and van Stoughton (1906)	*Bact. coli*	3	27.9	50–55
		2	26.5	50–60
Chick (1910).....	*Bact. coli*	2	12	49–52
Chick (1910).....	*Bact. typhosum*	3	110–170	49–54
Chick (1910).....	*Micr. pyogenes*	1	29	49–53
Sattler (1928).....	*Micr. sulfureus*	4	5.3	63–80
	Micr. sulfureus	2	2.3	80–99
	Micr. pyogenes	4	2.6	63–80
	Micr. pyogenes	4	1.4	80–99
	Bact. aerogenes	4	2.5	63–80
	Bact. aerogenes	2	1.7	80–99
	Ps. fluorescens	4	2.0	63–80
	Pink *Torula*	2	1.2	63–80
(Temperature coefficient for spores)				
Ballner (1902)....	*B. anthracis*	8	10	90–105
Eijkman (1912)...	*B. anthracis*	1	40	80–90
Meyer (1906)....	22 different bacilli	1 of each	8.3	80–100
Bigelow (1922)....	15 different thermophiles (see p. 323, Table 99)	1 of each	8.77	100–130
Williams (1928)...	*B. subtilis*	1	5.8	95–105

In a similar way, Chick (1910) found the temperature coefficients of the death rates for other organisms; so

did various authors. The results are compiled in Table 97. We are dealing here with unusually high temperature coefficients, corresponding to those for the destruction of enzymes (p. 129).

The only exception to these high temperature coefficients are the results obtained by Sattler (1928) on death rates of bacteria in milk. He measured the following temperature coefficients:

TABLE 98.—TEMPERATURE COEFFICIENTS OF DEATH BY HEAT IN MILK

(Temperature interval)

	62–80°C.				Average	80–99°C.				Average
Micr. sulfureus	4.9	5.1	5.4	5.6	5.3	2.1	...	...	2.4	2.3
Micr. pyogenes aureus	2.1	2.3	2.6	3.4	2.6	1.2	1.3	1.4	1.6	1.4
Bact. aerogenes	2.1	2.3	2.7	3.0	2.5	1.7	1.7	...	...	1.7
Ps. fluorescens	1.4	1.7	2.1	2.4	2.0					
Pink *Torula*	1.2	1.2	...	...	1.2					

The reason for these low coefficients must be sought in the great range of temperatures. Skrabal (1916) has pointed out that the temperature coefficient is not constant, but must decrease with increasing rates of reaction. The change is slight with normal coefficients, but is great with abnormal coefficients such as those observed with death-rates. Skrabal computed from the results of Arthur Meyer (1906) and of Ballner (1902) the following temperature coefficients:

	Q_{10} at 0°C.	Q_{10} at 100°C.	
Spores of *B. subtilis*	19.6	4.9	(A. Meyer)
Spores of *B. robur*	30.8	6.3	(A. Meyer)
Spores of *B. anthracis*	69.7	9.7	(Ballner)

A striking experimental example is given by Henderson Smith (1923) for spores of *Botrytis cinerea:*

at 31.0 to 37.0°C.,	Q_{10} = 690
37.0 to 44.3	132
44.3 to 47.0	92.8
47.0 to 50.3	29.5

In Sattler's experiments, the temperature of 80°C. is so high above the maximum temperature that the temperature coefficient of the death rate must have fallen to nearly normal.

It is possible to compute temperature coefficients also from endpoint experiments provided that they all have the same initial number of bacteria. Under these circumstances, the initial number, as well as the final number, is the same, the only variable at different temperatures being the time required to reduce the cells to the same number. The formula for the death-rate can be applied to the two temperatures which, for simplicity's sake, shall be assumed to be 10°C. apart, and we obtain

$$0.434K_T = \frac{\log a - \log b}{t'}$$

$$0.434K_{T+10} = \frac{\log a - \log b}{t''}$$

where t' and t'' are the two disinfection times at the two temperatures. The temperature coefficient is the quotient of the two, and since $\log a$ and $\log b$ are the same in both equations,

$$Q_{10} = \frac{t''}{t'}$$

For temperature differences other than 10°C., the formula of p. 120 must be applied.

Since the temperature coefficient is an exponential function of the temperature, a straight line should be obtained if the logarithms of death-rates were plotted against temperature. The above formulae show that the death rates are proportional to the times required for killing, and, therefore, the logarithms of these times

plotted against temperature should be a straight line in case the coefficient is constant. This is shown to be true with the spores of thermophilic bacteria by Bigelow (1922).

In Table 99 are given the data as averaged by Bigelow for the fifteen most typical spore-formers. If we compute the temperature coefficients from these data, it becomes at once evident that they are not very constant, but fluctuate considerably.

Table 99.—Time Required to Kill All Spores of Thermophilic Bacteria

(Averages of 15 experiments by Bigelow)

Heated to, °C.	Killing times in minutes	Average	Q_{10}			Q_{10} calculated from 5°C. intervals	
100	788 –834	811					
105	383 –405	394	6.8			4.24	
110	117 –122	119.5		9.4		10.87	
115	40 – 44	42	10.4			8.10	8.77
120	11 – 12	11.5		9.9		13.34	
125	3.9– 4.6	4.25	5.9			7.32	
130	1.7– 2.2	1.95		5.3		(4.75)	
135	0.7– 0.9	0.80	2.6			(5.94)	
140	0.6– 0.9	0.75				(1.14)	

The killing times beyond 125°C. are so short that the time required for the heat penetration must necessarily mean a considerable part of the total observed. Hence, probably all these data are too high, and the actual time required for killing must be much shorter. The average of all temperature coefficients from 100–125°C. is 8.77.

These data are plotted logarithmically in Fig. 36. In plotting curves from data by the endpoint method, the definite points are changed to lines, and the correct point lies somewhere on this line. This adds to the inaccuracy of plotting. Owing to this and to the fact that logarithms make deviations appear much smaller, the curves of Fig. 36 are very straight, showing hardly any fluctuations, while the temperature coefficients computed from the same data fluctuate widely.

In the same figure are included some data by Weiss (1921) on the death of spores of *Clostridium botulinum* and also the data of Ballner's

(1902) on the heat sterilization of spores of *B. anthracis*. In one corner of the figure, the theoretical curves for different values of Q_{10} are shown. The angle with the abscissa determines the value of Q.

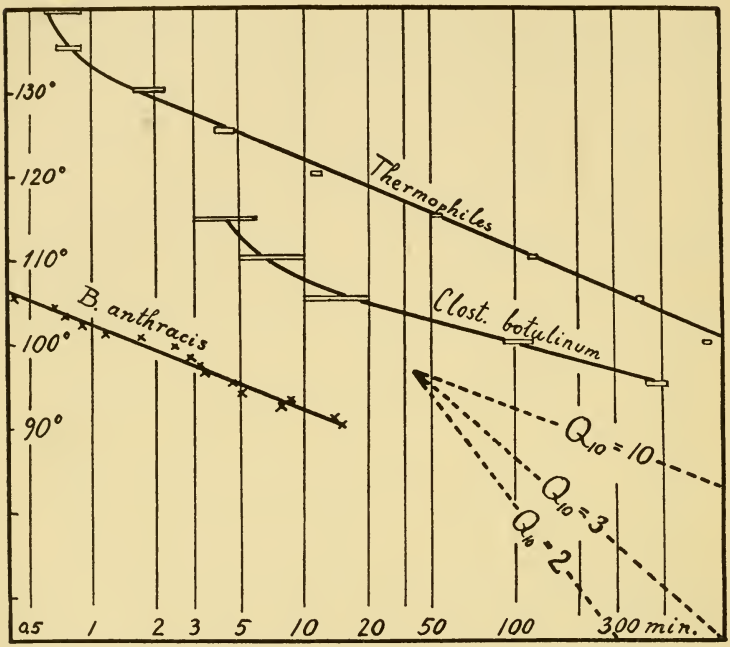


FIG. 36.—Relation between temperature and thermal death times of spores, illustrated by 15 thermophilic organisms, *Clostridium botulinum*, and *B. anthracis*. The slope indicates the temperature coefficient, the distance from the base the relative resistance to heat.

If this curve were not plotted against logarithms of time, but against time directly, the lines would not run straight, but in parallel logarithmic curves. This type of curve is well known to the dairy bacteriologist from the picture of Fig. 37 by North, which can be found in many textbooks on milk pasteurization. The killing times are exponential functions of the decreasing temperature. Assuming $Q_{10} = 11$ to hold for all bacteria, the time required to kill a bacterium at 70°C. would be eleven times as long as at 80°C., and if the pasteurizing temperature is lowered another 10°C., the killing time would have to be multiplied again by eleven.

The close parallelism of the lines indicates that the bacteria concerned as well as enzymes and albumin are all supposed to have practically the same temperature coefficient. This is not correct

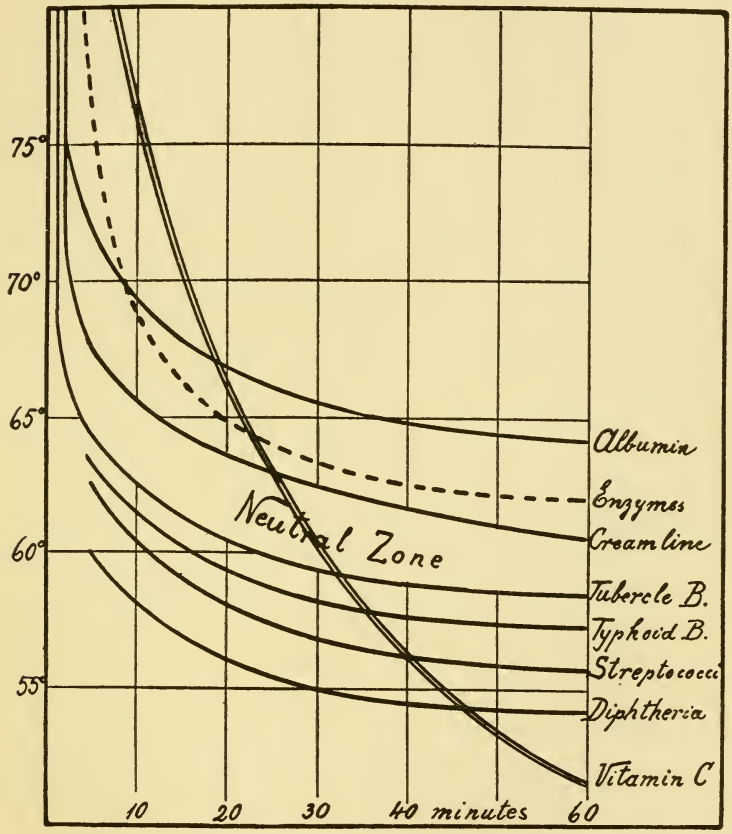


FIG. 37.—Lines of equal destruction by heat and time, for bacteria and compounds of milk. All temperature coefficients are extremely high (approximately $Q_{10} = 11$) except for Vitamin C which is normal.

(see Table 97). Otherwise, this well-known graph represents the behavior of bacteria towards heat quite correctly.

Vitamin C has probably a normal temperature coefficient (Rahn, 1925) and its curve crosses all others. Prolonged holding at fairly low temperatures destroys more of this vitamin than short exposing

to high temperature. This is quite different from the behavior of bacteria, or enzymes, or coagulating proteins.

(c) THE CAUSE OF DEATH BY HEAT

In trying to find the actual chemical or physical changes in the cell causing death by heat, it will be necessary to keep in mind the very high temperature coefficient, and also the necessity of moisture. It has been shown that death by dry heat is primarily an oxidation process, with a temperature coefficient not much above normal. This makes it certain that oxidation cannot be the cause of death by moist heat.

There are three groups of reactions which show high temperature coefficients: the inactivation of enzymes, the inactivation of toxins, and the coagulation of proteins. Let us take the coagulation of hemoglobin which was studied by Martin and Chick (1910) as an example. Table 100 is a summary of their data and shows that the coagulation of hemoglobin by heat is a time process of measurable rate, but with a very high temperature coefficient. The coagulation is a monomolecular process; this suggests either a hydrolysis, or a splitting off of water. The temperature coefficient is 1.3 for 1°C. increase, or 13.8 for 10°C. increase. The same authors determined the temperature coefficient for the coagulation of egg albumin (which is not a simple monomolecular process, however) to 1.91 for 1°C. or 646.0 for 10°C. increase.

Dried ovalbumin, heated to 120° for five hours, remained perfectly soluble; the presence of water seems to be essential for coagulation, and this again suggests a reaction with water.

The monomolecular process of the heat coagulation of albumin has a striking similarity with the death by

TABLE 100.—THE HEAT COAGULATION OF HEMOGLOBIN

Temperature °C.	Time of heating in minutes												
	2	3	4	6	7.5	9	10	20	30	45	70	90	
	Percentage of unchanged hemoglobin												
60.0	.....	.....	.....	.....	.....	.....	.....	.....	54.0	....	....	13.5	
62.6	.....	.....	.....	.....	.....	.....	.....	42.0	....	12.0	8.0		
65.6	.....	.....	.....	.....	.....	.....	35.5	11.0	5.0				
67.7	.....	61.4	.....	34.8	.....	24.9							
70.4	52.5	.....	25.3	14.1	7.6								
	1,000 × rate of hemoglobin coagulation												Average rates
60.0	.....	.....	.....	.....	.....	.....	.....	.....	21	....	....	22	21.5×10^{-3}
62.6	.....	.....	.....	.....	.....	.....	.....	44	....	46	44	....	45×10^{-3}
65.6	.....	.....	.....	.....	.....	.....	104	111	99	....	....	....	105×10^{-3}
67.7	.....	164	.....	175	.....	154	.....	.....	....	....	....	....	164×10^{-3}
70.4	370	.....	350	350	350	.....	.....	.....	....	....	....	....	350×10^{-3}

heat of bacteria. The same range of temperatures is involved in both; the temperature coefficients are very high in both cases, well above 10 for a 10°C. increase.

The resemblance is also borne out by the circumstance that dry bacteria are much more resistant to heat than moist bacteria. A further similarity is the very great influence of very small amounts of acid upon protein coagulation and upon the death rate of bacteria. The theory of Chick that "death through heat is due to the action of water upon some one protein which is essential for the life of the bacterium and that the character of this reaction is conditioned by the chemical action of water upon its constituent proteins," seems quite well founded.

Temperature coefficients of ordinary chemical reactions are considered fairly constant. High temperature coefficients according to Skrabal (p. 321) should increase with decreasing rate of reaction, i.e., with lower temperatures. This explains why we ordinarily speak of definite coagulation temperatures. A drop of a few degrees makes the reaction almost immeasurably slow.

In the discussion of maximal temperatures, (p. 215), it has been shown that growth and fermentation fail at this point because the rate of regeneration is slower than the rate of deterioration. We were dealing there with cell constituents such as enzymes which can be produced by the cell, and which are present in the cell in a fairly large number of molecules. Temperatures just above the maximum may lead to death by endogenous catabolism if prolonged.

Death by heat which follows the logarithmic order is probably a different chemical process; it seems to be due to the inactivation of at least one most sensitive gene. It seems reasonable to compare this inactivation with the coagulation of proteins.

We know nothing about the borderline between death through the destruction of genes and death through the destruction of enzymes or similar catalysts. The observation that the logarithmic order is not always followed when the rate of death is exceedingly slow, may be due to this change from one cause of death to another.

Perhaps, with some organisms, the most sensitive gene is inactivated before all enzyme in the cell is destroyed.

The great resistance of spores to heat is accounted for by some authors by the assumption of a very concentrated cell content. According to Almquist (1898), spores have a specific gravity of 1.35–1.40; this would indicate a very low moisture content. The data had been obtained by centrifugalizing the organisms in salt solutions of known density.

In favor of a low moisture content speaks the comparatively low temperature coefficient of the death-rate of spores, as contrasted with vegetative cells. Data on the death-rates of vegetative cells and spores of the same organism are not known to the author, but the temperature coefficient of spores seems to be fairly uniformly about 10, while that of non-sporulating bacteria is usually higher.

It is not necessary, however, to resort to this explanation. Thermophilic bacteria are known to grow at temperatures which kill most vegetative forms; they multiply rapidly in milk during pasteurization. Actively growing cells cannot possess concentrated cell contents. Thermophilic bacteria seem to have a protoplasm which is less sensitive to temperatures between 40 and 70°C. The temperature coefficient of the death rate may not differ from that of other bacteria, but the death-rate itself may be much lower, and a higher temperature would then be necessary to bring about inactivation of the growth mechanism.

The resistance of spores may be due to this same cause, i.e. to a smaller death-rate. The evidence, so far, seems to be in favor of the assumption of concentrated cell contents. The experiments of Williams (1929) suggest

another possibility, namely a lower ash content of the spores which would decrease the rate of coagulation of protein.

(*d*) DEATH BY HEAT IN CONCENTRATED SOLUTIONS

That partial dehydration protects the cells against the influence of heat, can be shown in some (unpublished) data which, in 1911, Mrs. Coffeen made upon the author's suggestion. Bacteria were suspended in broth;

TABLE 101.—RETARDATION OF DEATH IN SUGAR SOLUTIONS
(Number of cells per c.c. of broth or broth + 50% sucrose)

Saccharomyces ellipsoideus

Temperature	50°C.				52.5°C.			55°C.		
Minutes of exposure	0	5	10	20	5	10	20	5	10	20
Broth	741	5	0	0	0	1	0	0	1	0
Broth + 50% sugar	328	402	120	7	367	158	9	275	62	1

Streptococcus lactis

Temperature	55°C.				60°C.			65°C.		
Minutes of exposure	0	5	10	20	5	10	20	5	10	20
Broth	198	180	11	0	0	0	0	0	0	0
Broth and sugar	152	140	47	0	39	8	1	0	0	0

Bacterium prodigiosum

Temperature	65°C.				67.5°C.			70°C.		
Minutes of exposure	0	5	10	20	5	10	20	5	10	20
Broth	29,000	184	30	4	73	0	0	135	81	4
Broth and sugar	32,000	8,000	236	13	1,063	25	7	158	8	4

in broth + 50% sucrose; and in broth saturated with salt, and were exposed to different temperatures for different lengths of time. Tables 101 and 102 give the results.

A similar experiment with yeast at different sugar concentrations and at different pH was carried out by Rahn (1928). There seems little doubt that the retardation of death is due to partial dehydration. Yet, the

TABLE 102.—RETARDATION OF DEATH IN SATURATED SALT SOLUTIONS
(Cells per c.c. of broth and salt-saturated broth)

Saccharomyces ellipsoideus

Temperature	50°C.				52.5°C.			55°C.		
Minutes of exposure	0	5	10	20	5	10	20	5	10	20
Broth	960	61	6	0	3	0	0	5	2	0
Salt broth	770	89	43	4	20	6	2	7	3	0

Streptococcus lactis

Temperature	55°C.				57.5°C.			60°C.		
Minutes of exposure	0	5	10	20	5	10	20	5	10	20
Broth	44	27	27	4	18	6	1	9	0	0
Salt broth	50	17	19	8	27	30	3	9	6	1

Bact. prodigiosum

Temperature	67.5°C.				70°C.			72.5°C.		
Minutes of exposure	0	5	10	20	5	10	20	5	10	20
Broth	19,620	29	30	12	62	1	1	9	3	1
Salt broth	19,200	0	0	66	199	33	7	133	44	15

cause of death is not that of dry bacteria, it is not oxidation. It is some reaction with water, only the rate is decreased because the amount of available water has

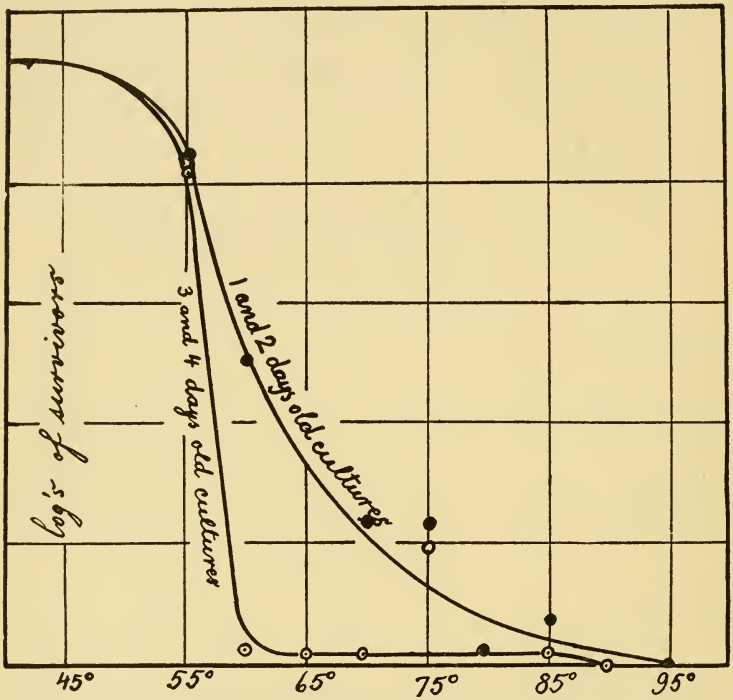


FIG. 38.—Survivors of young and old cultures of *Bacterium coli* heated for 5 minutes to different temperatures.

been reduced to a point where it can no more be considered as practically unlimited.

(*e*) THERMAL DEATH POINTS

The above discussions show very plainly that the old definition of the thermal death point as "the lowest temperature at which an organism would be killed in ten minutes" is without any real meaning because the killing time increases with the number of cells.

The thermal death-point is based on the same wrong conception as the coagulation temperature of the proteins. In reality, we have a reaction of measurable speed, but the very high temperature coefficient obscured this fact for a long time.

On the other hand, the high temperature coefficient prevents the probable error in thermal death-points from being very large. If, at a certain temperature, it takes ten minutes to reduce 1,000,000 cells to 1 cell, it will take 11.67 minutes to kill 10,000,000 bacteria, and 13.33 minutes to kill 100,000,000, and 15 minutes to kill 1,000,000,000 cells. Figuring with a temperature coefficient of 20, it would require an increase in temperature of 0.51°C., 0.96°C., or 1.35°C. respectively if the larger numbers of cells were to be killed in exactly ten minutes. These increases are quite within the limits of error of the ordinary technique of determining thermal death points.

The determination of thermal death-points becomes rather uncertain by the observation of Gage and Stoughton (1906) that in old cultures of *Bact. coli*, the last survivors are very hard to kill; they behaved very much like spores. Figure 38 gives an illustration of the result. While the great majority of cells (more than 99.99%) is killed at 60°C. in five minutes, a few will remain alive even after five minutes' heating to 85°C. The younger cultures are more uniform in their reaction with heat, and the smoother curve of their survivors indicates a more constant temperature coefficient.

This has led to the distinction of a "majority thermal death-point." Ayers and Johnson (1914) found streptococci with a low "majority thermal death-point," among which a few cells are able to survive the pasteurization temperature. "This ability . . . may be due to certain resistant characteristics peculiar to a few cells, or may be due to some protective influence of the milk."

The limitation of the thermal death-point to one experimental time of ten minutes is not very satisfactory for applied bacteriology. For the needs of food bac-

teriology, especially for milk pasteurization and for canning of vegetables and meat, this conception has been broadened to the "thermal death time," i.e., the time required to kill all cells at a certain temperature, as determined by the endpoint method.

The thermal death time is dependent upon all the factors mentioned above. It is not more accurate than the thermal death-point, but it is more useful in applied bacteriology. A detailed discussion has been given by Esty (1928).

(*f*) SUMMARY OF FACTS

In the death of bacteria by heat, the logarithmic order is well established.

Dead cells give to living cells a certain protection against heat which is too pronounced to be attributed to a decreased rate of heat transmission.

At higher temperatures, the bacteria die much more rapidly. There is considerable disagreement in the temperature coefficients of death of vegetative cells. For bacterial spores, the temperature coefficient of death is approximately 10, and is constant over a temperature range of about 30°C.

In concentrated solutions, death by heat is retarded.

SUMMARY OF THEORIES

Death by heat shows such close analogies to the heat coagulation of proteins, in temperature range as well as in the high temperature coefficients, that the general explanation of death by heat being due to coagulation of some parts of the protoplasm seems well founded. The greater resistance of dry cells corresponds well with the absence of coagulation of proteins when heated

in dry condition; dry bacteria at high temperatures do not die from coagulation, but from oxidation.

The greater resistance to heat of thermophilic bacteria may be due to a protoplasm of a higher average coagulation temperature. The rate of coagulation must be considerably lower than with most cell proteins, while the general rule of the high temperature coefficient probably holds true.

The greater resistance of spores may be due to the same cause, or it may be due to a high concentration of the spore contents; this would necessitate a low concentration of water which seems essential in the coagulation process; it may also be due to a very low ash content.

The slower death rates of bacteria in concentrated solutions of sugar, or of salt, coincide well with the assumption of death as a coagulation process, and with the necessity of water for this reaction.

There is no real thermal death-point, as there is no real coagulation temperature. The thermal death-point varies with the initial number of cells. But owing to the high temperature coefficients usually found in death by heat, the variation is so slight as to be within the very wide limits of error of this method. A much greater error enters from the medium in which the bacteria are tested. Water has a greatly varying effect, and a nutrient medium would be a much better test medium than water for the thermal death point.

VII. DEATH BY POISONS

(*a*) METHODS

The Medium for Testing.—In the Chapter on Death by Heat, it has been shown that accurate results can be expected only if the bacteria are exposed to the harmful

agent in a medium suitable for growth. The same applies, in principle, to death by chemical agents, but it is frequently hard to comply with, since many of the ordinary disinfectants will react chemically with the components of the medium. This reduces the concentration of the acting poison [Krönig and Paul, (1897); Chick and Martin, 1908].

Heavy metal salts will react with proteins and form insoluble precipitates. Formaldehyde also combines chemically with proteins. Acids and alkalis will be decreased in their efficiency through the buffer action of the nutrient medium. While it may be possible to find a good synthetic medium for each kind of disinfectant to be tried, it would be necessary to adapt the media to each bacterium, as well as to each disinfectant. This complicates matters to such an extent that for the sake of uniformity, a suspension of bacteria in water seems preferable for studying the general laws of chemical disinfection.

Removal of Poison after Action.—Experiments on the influence of chemical compounds may be carried out for different purposes. The food industry, in testing the efficiency of benzoate of soda, or of vinegar and spices, is not really interested in the killing of bacteria. Their purpose is fulfilled if the agents under test prevent growth and fermentation. In the disinfection of stools and of sputum, and of animal carcasses, actual killing is intended, and is accomplished without removing the disinfectant from the bacteria. For other purposes, the disinfectant is removed after action, e.g. in the disinfection of hands, instruments, tools, furniture and rooms, and fomites generally.

There is a great deal of difference in the efficiency of the various methods. The first way, the suppression of growth, shall not be discussed here to any extent, because it is nothing biologically definite, but a mixture

of growth and death problems difficult to analyze. There are, in the general technique of disinfection experiments, two distinct methods of procedure which give different results; the one is to remove the bacteria from the poisonous solution, usually together with a small part of the solution, and put them into broth or agar without any further treatment; the other is to remove by chemical or physical means any traces of the poison from the outside and inside of the cells as far as that is possible, i.e., as far as the poison has not formed irreversible compounds with the cell contents.

The first method is rather unsatisfactory inasmuch as the amount of poison transferred to the medium for cultivation of the survivors is not always the same. While the amount is not sufficient to prevent growth of healthy cells, it might prevent recovery of injured bacteria. A method of merely physical removal is sometimes used by transferring the cells from the disinfectant into water, centrifugalizing them off and eventually repeating the washing before placing them into the test medium. In this way, all soluble poison is removed from the outside and the inside of the cell, but chemical combinations of the poison with cell contents remain unchanged.

If the viability tests on culture media are supposed to correspond with the viability in the animal, the poison must be removed completely by chemical means. The tissue around the wound in which the treated bacteria are placed has a tendency to remove the last traces of poison from within the bacterial cell, and at the same time, it is a very good medium for growth. This is shown very clearly in some experiments of Rodewald (1923). The bacterium of fowl cholera was placed in 0.1% $HgCl_2$, removed after certain time intervals, and washed four times in succession with 40 c.c. of water. After seven minutes' treatment with $HgCl_2$, the washed bacteria showed no more growth on sterile culture media, but when injected into mice, killed them; even those which had been in the bichloride for three hours, still caused the disease. Similar results were obtained with phenol.

The action of the living tissues is perhaps partly due to their being an excellent food, but quite likely, their adsorptive properties

are also rather essential. Süpfle and Müller (1920) showed that with $HgCl_2$, it is possible to remove the last traces by physical adsorption. Bacteria were washed once or twice after treatment with $HgCl_2$, and then were mixed with freshly heated blood charcoal. This charcoal adsorbs the bacteria, and removes from them the last traces of soluble mercuric salts. If broth is added to this mixture of charcoal and organisms directly, bacteria will grow if alive. By this method, it could be shown that *Micrococci* were still alive after two hours' treatment with 0.1% $HgCl_2$, and the spores of *B. anthracis* survived treatment with 1% $HgCl_2$ for forty days, with 2% $HgCl_2$ for forty days, with 3% $HgCl_2$ for thirty-eight days, and with 5% $HgCl_2$ for eleven days. These data were confirmed later by Alfred Müller (1920) who removed the mercury salt from the washed bacteria by ammonium sulfide. Data on the difference of viability of washed and chemically treated bacteria, by Gegenbauer (1921), are given extensively on p. 359:

As antidotes in disinfection experiments, the following reagents are commonly used:

Against $HgCl_2$	Ammonium sulfide
Against Cu salts	Ammonium sulfide
Against chlorine	Na_2SO_3
Against formaldehyde	Ammonia
Against peroxide	Permanganate
Against permanganate	Sulfides
Against phenol, lysol, etc	Washing with dilute ammonia (no very efficient antidote is known)

In the chapter on Death by Heat, experiments by Lange (1922) were mentioned demonstrating that the addition of large numbers of dead bacteria protected the living bacteria against heat. The same is true with disinfectants; addition of large numbers of heat-killed cells decreases the toxic effect of a disinfecting solution.

(*b*) THE ORDER OF DEATH

The logarithmic order of death is the most common form in chemical poisoning of bacteria if the disinfectant is removed at the end of the experiment. The table below shows the action of phenol upon the spores of *B. anthracis* as determined by Chick (1908).

TABLE 103.—DEATH OF SPORES OF *B. anthracis* IN 5% PHENOL AT 20.2°C.

Time, hours	Survivors	$K = \frac{1}{t} \ln \frac{a}{b}$
0	434	
0.5	410	0.049
1.5	351	0.061
2.7	331	0.044
3.92	299	0.041
5.95	241	0.043
25.60	28	0.046
Mean.....		0.047

A second example shows the very slow death of *Bact. typhosum* in buffer solutions of different pH, according to Cohen (1922).

At pH 5 and 6.4 where the death rate is very low, it decreases while it is quite constant at pH 7.1 and 8.7. This might be expected if the decrease of the death rate is explained by variation in resistance. In very weak disinfectants, the exceedingly slow rate of death gives the most resistant bacteria a better chance to work against the destructive agent.

The logarithmic order requires a special justification here because *two* components are reacting with each other, namely, the bacterial cells (or some essential part of their protoplasm) and the poison. A logarithmic

order can be established only if one of the two reacting agents is in such great excess that its concentration

TABLE 104.—DEATH OF *Bact. typhosum* IN *m*/500 BUFFER SOLUTIONS OF DIFFERENT HYDROGEN ION CONCENTRATIONS

pH	3.8		5.0		6.4	
Exposure in hours	Survivors	*K*	Survivors	*K*	Survivors	*K*
0	231,000	.....	543,000	.....	1,000,000	.....
3	200	1,021	443,000	0.0295	620,000	0.0346
6	sterile	.....	365,000	0.0288		
12	.....	.....	277,000	0.0244	568,000	0.0205
24	.....	.....	253,000	0.0138	378,000	0.0176
48	.....	.....	160,000	0.0111	205,000	0.0143

pH	7.1		8.7	
Exposure in hours	Survivors	*K*	Survivors	*K*
0	705,000	.....	391,000	
3	93,300	0.146	102,000	0.194
6	.....	.....	30,400	0.185
12	46,000	0.099	2,000	0.191
24	875	0.121	12	0.188
48	1	0.122	sterile	

remains practically constant despite the reaction. Falk and Winslow (1926) come to the conclusion that the bacterial cells are the agent whose concentration remains constant:

"If it be assumed that the fundamental reaction of disinfection is unimolecular, we might consider that of the two reacting agents, (1) disinfectant and (2) living cells, one or the other is present in excess and that its concentration is not changing significantly during the

course of the reaction. Inasmuch as the constant K obviously changes with a change in the salt concentration, it must be assumed that the reacting substances of the bacterial cell (whether those cells are dead or alive) must always be present in excess if anything approaching the monomolecular curve is to be attained."

The author holds the opposite view. The monomolecular type of reaction is based on the decreasing numbers of living cells. A decrease of the concentration of poison has never been observed, nor does it enter the mathematical equation at all.

The assumption that the reagent whose decrease was observed, i.e., the living cells, is, in reality, constant, and that the other reagent about whose concentration nothing is known, is the decreasing compound, seems unjustified. The argument of Falk and Winslow that the rate of reaction changes with the concentration of poison, is no proof for their contention.

The change in the concentration of poison molecules can be but slight. To prove this, let us consider the action of 0.01% of $HgCl_2$ upon a suspension of *Bact. coli* with 1,000,000 cells per c.c. The amount of $HgCl_2$ per 100 c.c. is 10 mg; the weight of the bacteria (Table 132, p. 397) in 100 c.c. is $100 \times 10^6 \times 8 \times 10^{-10} = 0.08$ mg. Of this weight, 70% is moisture, the dry weight being only 0.024 mg. The ratio of $HgCl_2$ to bacteria bodies is 10:0.024. In order to compare molecules with molecules, these two weights must be divided by their molecular weight. That of $HgCl_2$ is 271.52; that of the bacteria cannot be stated; the weight given includes cell wall and cell contents; only parts of the latter will react with the bichloride. But assuming the entire weight to consist of protoplasm with a molecular weight of 10,000, we find the ratio of the numbers of acting molecules to be 0.037:0.000,0024, or 15,000 molecules of $HgCl_2$ for each molecule of bacterial protoplasm. Even if 4 or 10 or 100 of these 15,000 poison molecules should react with one of protoplasm, their concentration would remain practically unchanged.

(c) CHEMICAL CONSIDERATIONS REGARDING THE ACTION OF DISINFECTANTS

The study of disinfection on a biochemical basis dates back to the investigation of Krönig and Paul (1897) which might well be called a classic paper on disinfection. This paper shows that the disinfectant power of salts or acids depends upon their ionization. This was proved by exposing spores of *B. anthracis* to various mercury salts and counting the number of colonies developing after the action of the salts had been interrupted by washing with a 3% solution of ammonium sulfide. In the following table, the salts are arranged in order of decreasing ionization. The survivors increase with decreasing ionization.

TABLE 105.—ACTION OF MERCURY SALTS UPON SPORES OF *B. anthracis*

Salt solution, molar	Percentage of salts, %	Survivors after	
		20 minutes	85 minutes
$HgCl_2$ 1/16	1.68	(16 colonies after 6 minutes)	0
$HgCl_2$ 1/64	0.42	7	0
$HgBr_2$ 1/64	0.56	34	0
$Hg(CN)_2$ 1/16	1.57	∞	33

The same importance of ionization was also shown by the decrease of efficiency of these solutions whenever their mercury ion concentration was reduced by addition of other salts with the same anion.

In a similar way, the disinfection by salts of silver, gold, copper has been tested, and efforts were made to determine the disinfectant value of the metal salt anions.

Further investigations concerned the acids and alkalies. The next table shows that neither molar nor normal

solutions of different acids have the same disinfectant value, because their hydrogen ion concentrations differ. But the $\frac{1}{16}$ normal solutions of the stronger acids are completely, and therefore, equally dissociated, their

TABLE 106.—DECREASE OF DISINFECTANT POWER WITH DECREASE OF IONIZATION

(All solutions $\frac{1}{16}$ molar)

Salt mixture	Survivors after 8 minutes
$HgCl_2$	8
$HgCl_2$ + NaCl	32
$HgCl_2$ + 2NaCl	124
$HgCl_2$ + 3NaCl	282
$HgCl_2$ + 4NaCl	382
$HgCl_2$ + 4.6NaCl	410
$HgCl_2$ + 6NaCl	803
$HgCl_2$ + 10NaCl	1,087

TABLE 107.—DISINFECTANT POWER OF ACIDS TESTED ON SPORES OF *B. anthracis*

Acid	Concentration	Survivors after				Concentration	Survivors after 2.3 days
		20 min.	120 min.	5.42 hrs.	8.25 hrs.		
HCl	normal	...	385	38	5	$\frac{1}{16}$ normal	160
$HClO_4$	normal	...	123	1	.....	$\frac{1}{16}$ normal	283
HNO_3	normal	191	0	0	0	$\frac{1}{16}$ normal	54
$CHCl_2CO_2H$	.....	...	...	.....	.....	$\frac{1}{8}$ normal	396
CCl_3CO_2H	normal	243	0	0	0	$\frac{1}{16}$ normal	382
HF	normal	139	0	0	0		
HBr	normal	...	217	4	1		
H_2SO_4	normal	...	...	491	405		
H_2SO_4	molar	...	...	424	285		
H_3PO_4	molar	...	...	.....	1,090		
HCO_2H	normal	...	...	2,500	2,280		
CH_3CO_2H	normal	...	...	.....	2,780		
$(CO_2H)_2$	normal	...	...	97	34		

action is fairly alike, and equal that of an n/8 dichloracetic acid which has the same hydrogen ion concentration.

This table shows also that the anions of hydrofluoric acid, nitric acid and trichloracetic acid have a specific toxic action, which becomes less conspicuous upon dilution as compared with the hydrogen ion effect.

Further experiments indicate that the alkalies disinfect in proportion to their hydroxyl ions. A comparison of the data shows that the hydroxyl ions do not act as strongly as the hydrogen ions; this is especially true for Micrococci.

The same authors (Krönig and Paul) studied, further, the effect of oxidants such as chlorine, bromine, iodine, permanganate, chlorate, bichromate, persulfate, and hydrogen peroxide, also of organic disinfectants like phenol and its derivatives. The effect of alcohol upon the disinfectant value has been studied quite extensively. It is not the object of this book to go into these details. Krönig and Paul's paper, now over 30 years old, started a new era in disinfection experiments, and is still a model of carefully planned and executed technique. It was the necessary prerequisite for the discovery of the logarithmic order of death which followed ten years later.

After this discovery, various disinfectants were tried on a number of microorganisms in a quantitative way, but the test organisms in all these experiments were limited to a few species, and the kinds of disinfectants used also were few. The final outcome of all these investigations, which were very numerous in the years 1908–1912, has not fulfilled the expectations; they have had very little influence upon practical disinfection.

The main reason for this lies in the fact that ordinarily, no result can be duplicated exactly, not even by the same author. While we

have learned to eliminate some errors, such as using distilled water of unknown pH, or producing heat shocks and cold shocks, the variation of parallel experiments on different days is still very great. The only certain way to get uniform data is to prepare a large number of bacteria in a uniform way, and preserve them for later experimentation. This was done by Paul (1910) in drying a *Micrococcus* suspension on garnets, and by Myers (1929) in keeping a suspension of bacterial spores frozen. Both these authors obtained comparable data over long periods of time.

The old type of disinfection experiments by the endpoint method suffers naturally from the same irregularities, but since the results are not as precise as in plate count experiments, the variation is not so obvious. It is not logical, therefore, to reject data and findings obtained by the newer methods, because their more accurate measurements reveals plainly a great variability of the microorganisms while the cruder endpoint method does not show this so distinctly.

Very little attention has been paid so far to the specificity of the different species. It is to be expected that among unicellular organisms, differences in resistance will be much greater than among multicellular organisms. This is evidenced by bacteria capable of feeding on formaldehyde (Kaserer, 1906), xylol (Soehngen, 1906), metabolizing arsenic (*Penicillium brevicaule*), or living at pH 1 (Waksman's sulfur bacteria, 1922).

A first step in this direction is the observation of Cooper and Mason (1927, 28) that the *Pseudomonas* group (*Ps. fluorescens, Ps. non-liquefaciens* and *Ps. pyocyanea*) is quite sensitive to "physico-chemical" reagents causing precipitates of proteins, such as alcohol or phenol, while *Bact. coli* is less sensitive to these compounds, but reacts strongly with "chemical" disinfectants such as quinones.

The *Pseudomonas* group which is sensitive to precipitants is also much more sensitive to heat; this suggests that the colloidal state of their protoplasm is different from that of the colon group.

The analogy between the action of hot water and that of alcohols and phenols is borne out by experiments with thermophilic bacteria. These organisms are somewhat less sensitive to phenol than *Bact. coli*, but more susceptible to p.-nitrosodimethyl-aniline.

The consequence of this specificity is that the phenol coefficient (p. 353) which indicates how many times more toxic a certain disinfectant is than phenol, must be expected to vary considerably with different organisms. This has been observed by several authors. For example, the following phenol coefficients of sodium hypochlorite were obtained with different bacteria by Zoller and Eaton (1923).

Mycob. tuberculosis	42.8
Bact. alcaligenes	100.0
B. anthracis	160
Bact. neapolitanum	200
Gonococcus	280
Proteus vulgare	330

(*d*) EFFECT OF CONCENTRATION OF POISON UPON DEATH RATE

The influence of the concentration of a poison upon the death rate has been formulated according to physicochemical principles first by Watson (1908), after Ikeda (1897) and Chick (1908) had given empirical formulae. Later investigators came independently to the same equations, namely Paul, Birstein and Reuss (1910b) and Phelps (1911).

Assuming that n molecules of the disinfectant must react with one cell before the cell loses the power of reproduction, the reaction constant, or death rate, must be proportional to the nth power of the concentration.

$$K = kc^n$$

If the K-values for two different concentrations are known, we have

$$K_1 = kc_1^n$$
$$K_2 = kc_2^n$$

Dividing one equation by the other, we obtain

$$\frac{K_1}{K_2} = \left(\frac{c_1}{c_2}\right)^n$$

and consequently

$$n = \frac{\log \frac{K_1}{K_2}}{\log \frac{c_1}{c_2}} = \frac{\log K_1 - \log K_2}{\log c_1 - \log c_2}$$

The equation for the logarithmic order of death, which was

$$K \cdot t = \log \frac{a}{b}$$

for any one concentration of a poison, is then changed to

$$K \cdot t \cdot c^n = \log \frac{a}{b}$$

when different concentrations are concerned. Applied to the endpoint method where $\frac{a}{b}$, and consequently $\log \frac{a}{b}$ is constant, we must find the relation

$$t \cdot c^n = \text{constant, or } t_1 c_1^n = t_2 c_2^n, \text{ or } n = \frac{\log t_2 - \log t_1}{\log c_1 - \log c_2}$$

This computation of the concentration exponent is made under the assumption that the poison combines chemically with some molecules of the cell to a definite

compound, and that this is the cause of death. The next sub-chapter (p. 361) will show that this has not yet been proved.

The reaction of one molecule of the cell constituents with n molecules of poison does not contradict the analogy with monomolecular reactions. It is assumed that the concentration of the poison molecules remains practically constant during the reaction (see p. 341) because they are in great excess over the active molecules of the cell contents.

If each cell contained only one "reacting molecule" as the theory of Rahn (see p. 284) assumes, n must be an integer, providing that the cause of death is due to a compound of this molecule with the poison. If several molecules of the cell must be affected in order to cause death, the value of n need not be an integer (Watson, 1908).

A very instructive study of the effect of concentration of different acids was published by Paul, Birstein and Reuss (1910b). The death rate of *Micr. pyogenes*

Table 108.—Death Rate and Concentration of Acid

Acid	Normality	H-ions per liter, mg.	Death rate	n	
				From normality	From H-ions
Hydrochloric	1:150	0.7	0.035		
	1:100	1.0	0.042	0.45	0.45
	1:75	1.3	0.049	0.54	0.54
	1:50	2.0	0.062	0.58	0.58
	1:25	4.0	0.087	0.49	0.49
Acetic	1:31.17	0.75	0.018	1.12	2.21
	1:17.65	1.00	0.034	0.93	1.89
	1:2	3.00	0.234	0.89	1.75
Butyric	1:26.0	0.75	0.018	2.04	4.00
	1:14.7	1.00	0.057	2.03	4.03
	1:66	1.50	0.294	2.04	4.04

aureus was measured with different concentrations of hydrochloric, acetic and butyric acid. The data are summarized in Table 108.

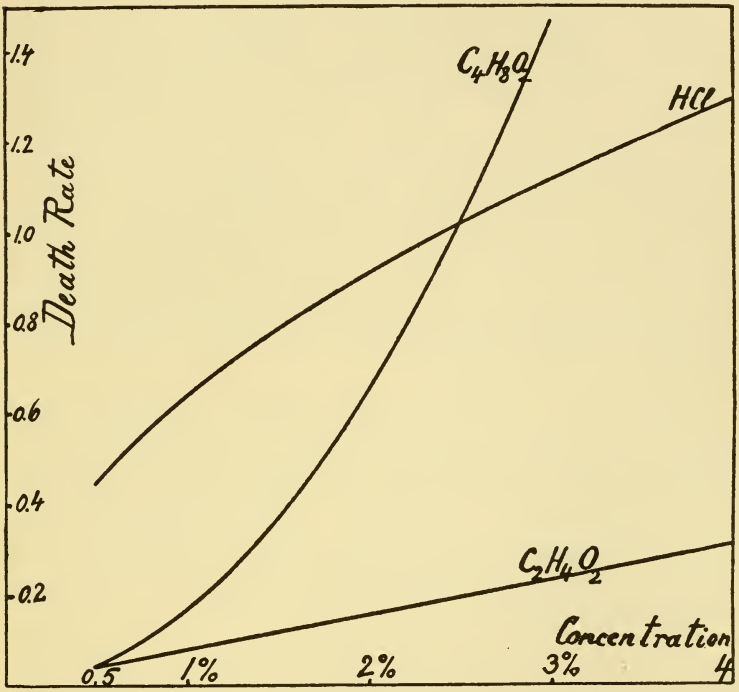


FIG. 39.—The death rates of *Micrococcus pyogenes* by increasing concentrations of 3 different acids.

An important conclusion is evident from these data, namely, that two disinfectants may, at one concentration, have the same disinfecting power while at other concentrations, they may differ widely, owing to a different concentration exponent n. The extrapolation of the data of Table 108 gives the curves shown in Fig. 39 where the ordinates indicate death rates, i.e., the intensity of the acid as a disinfectant. The concentra-

tion is given by weight, and not by hydrogen ions. The death rate by acetic acid, (with $n = 1$) is proportional to concentration, and therefore a straight line.

As has been shown before, the death rate in acid solutions depends primarily upon the hydrogen ion concentration. From Table 108, it is evident that in hydrochloric acid, the death rate is proportional to the square root of the hydrogen ions ($n = 0.5$) while with acetic acid, it is proportional to the square ($n = 2$) and with butyric acid, proportional to the 4th power of the hydrogen ion concentration ($n = 4$). This is hard to explain, even if we assume a special effect of the kations or the undissociated molecules. Neither these results nor the investigations of Winslow and Lochridge (1906) and of Norton and Hsu (1916) are based on direct pH measurements. Since the development of quicker methods of direct pH measurement, two papers have given extensive data on this point, those of Cohen (1922) and of Myers (1929).

Table 104 p. 340 by Cohen covers a wide range of pH. If the death-rate were proportional to the H^+ or OH^- concentration, then the rate divided by this concentration should give a constant. This is not the case; but a constancy over a wider range is obtained if we divide the rate by the square root of H^+ or OH^- concentration ($n = \frac{1}{2}$).

The same is true with Myers' experiments on the death of spores of a bacillus similar to *B. cereus*, in buffered solutions of highly alkaline washing powders. All of Myers' results are expressed by the time required to kill 99% of the bacteria, since the increasing death rate (see p. 296) made the determination of an average death rate impractical.

Excluding the one apparently wrong value of experiment No. 10, there is a good consistency of results. Myers found the products of the killing-time and OH^- concentration to be constant for pH 12 to 13. This means that the death-rate is proportional to the OH^- con-

TABLE 109.—DISINFECTION OF BACTERIAL SPORES BY STRONG ALKALIES AT 60°C.

Solution Nr	Solution	Normality	pH	OH-ion concentration, mg. per liter	Time for killing 99 % of spores	Killing time × (OH^-) concentration in gm. per liter		Killing time × square root of (OH^-) concentration	
1	NaOH	0.5	13.36	230	7.1 min.	1.6		0.34	
2	NaOH	0.5	13.10	126	16.2	2.5		0.58	
3	NaOH	0.25	13.12	132	24.0	3.1	2.2	0.88	0.57
4	$NaOH-Na_2CO_3$	0.5	13.11	129	16.3	2.1		0.59	
5	$NaOH-Na_3PO_4$	0.5	13.17	148	12.0	1.7		0.46	
6	Na_3PO_4	0.5	12.36	22.9	79	1.7		12.0	
7	$NaOH-Na_2CO_3$	0.5	12.27	18.6	184	3.4		25.1	
8	$NaOH-Na_3PO_4$	0.5	12.22	16.6	110	1.8	2.45	14.2	18.7 25.9
9	NaOH	0.5	12.22	16.6	182	2.9	(3.8)	23.5	
10	NaOH	0.04	12.45	28.2	326	(9.2)		(54.8)	
11	Na_2CO_3	0.5	11.39	2.46	444	0.9		22.0	
12	Na_3PO_4	0.5	11.35	2.24	300	0.6		14.2	
13	$NaOH-Na_2CO_3$	0.5	11.30	2.00	540	0.9	0.90	24.2	22.5
14	$NaOH-Na_3PO_4$	0.5	11.28	1.91	480	0.9		21.0	
15	NaOH	0.5	11.40	2.51	618	1.2		31.0	
16	Na_3PO_4	0.5	9.1	0.0126	4,500	0.057		16.0	
17	NaCl	0.1455	7.5	0.00092	5,650	0.000052		3.2	
18	NaCl	0.5	7.5	0.00092	5,000	0.000046		3.1	

centration. This proportionality does not hold for the lower pH range. Here, a much better agreement is obtained with the square root of the OH^- concentration. From pH 9.1 — 12.4, it is satisfactory. This is a wider range of pH than we had before, and even the values for pH 7.5 are not entirely out of range. At pH 13, the agreement does not hold at all. This proportionality to the square root of the OH^- concentration agrees with the just mentioned data of Cohen's.

Theoretically, n must increase at very low concentrations. There is a limit for any poison below which it will not harm the cell; it might even stimulate. In other words, K becomes infinitely small when C is still measurable. The value for n is computed from the equation:

$$n = \frac{\log \frac{k_1}{k_2}}{\log \frac{c_1}{c_2}}$$

If c_2 becomes so small that there is no death, the death rate K_2 becomes O, and $\frac{k_1}{k_2} = \infty$, and therefore $n = \infty$. The change of n from a definite number to infinite is not abrupt but gradual, as will be seen from Fig. 41, p. 358, and from the calculations of n on p. 361. This must be kept in mind when the concentration exponent is determined; it will be constant only beyond a certain limit. Reichel (see p. 356) called this limit a and did his calculations by assuming $K = K_0(c - a)^n$.

A number of concentration exponents have been determined, and they are compiled in Table 110. They are mostly computed from the concentrations by weight though it would be more correct probably, in most cases, to consider ionization. In some instances, both ions will have a deleterious effect, while other compounds are not ionized at all. The computation has

been limited therefore, to the simplest way of measuring concentrations. It seems that different n-values can be obtained for the same disinfectant with different bacteria. A comprehensive study of the concentration exponents would seem quite desirable. The table seems to indicate that aside from the value 0.5, the n-values are very nearly integers.

Whatever may be the theoretical explanation of these n-values, their existence is beyond doubt. The great differences for various disinfectants and bacteria make comparisons almost impossible. Chick (1908) and Phelps (1911) have both called attention to the fact that the customary method of stating the disinfectant power by the "phenol coefficient" or "carbolic acid coefficient" cannot possibly give results which allow a general application.

TABLE 110.—CONCENTRATION EXPONENTS FOR THE POTENCY OF VARIOUS DISINFECTANTS

$HgCl_2$ (anthrax spores)	n = 0.25	Alfred Müller (1920) p. 362
Oxygen (dry bacteria)	0.5	Paul (1910) p. 305
HCl (Micrococci)	0.5	Paul, Birstein and Reuss (1910b) p. 348
H_2O_2	0.5	Reichel, 1908
$HgCl_2$ (Micrococci)	0.5	Gegenbauer (1921) p. 360
$AgNO_3$	0.86	Chick (1908)
Acetic acid	1.0	Paul, Birstein and Reuss (1910b) p. 348
Formaldehyde	1.0	Gegenbauer (1922) p. 362
HCl (anthrax spores)	1.5	Gegenbauer and Reichel (1913)
Butyric acid	2.04	Paul, Birstein and Reuss (1910b) p. 348
$HgCl_2$ (*Bact. typhosum*)	3.8	Chick (1908)
$HgCl_2$ (anthrax spores)	4.9	Chick (1908)
Phenol (*Bact. typhosum*)	4.0	Reichel (1909) p. 357
Phenol (Micrococci)	4.0	Reichel (1909) p. 357
Phenol (*Bact. paratyphosum*)	5.5	Chick (1908)
Phenol (anthrax spores)	5.5	Chick (1908)
Phenol (*Bact. typhosum*)	6.0	Lee and Gilbert (1918)

The method originally suggested by Rideal and Walker (1903) was to determine the minimum time required to kill a bacterial suspension by a certain concentration of the disinfectant under test, then to determine the phenol concentration required to kill the same organism in the same time. Dividing the one concentration by the other, the "phenol coefficient" was obtained which stated how many times

TABLE 111.—DETERMINATION OF THE PHENOL COEFFICIENT

	Dilution	2.5	5	7.5	10	12.5	15 minutes
Phenol	1:90	+	0	0	0	0	0
	1:100	+	+	+	0	0	0
Unknown disinfectant	1:500	+	+	0	0	0	0
	1:550	+	+	+	0	0	0
	1:600	+	+	+	+	0	0

550:100 = 5.5 is the Phenol coefficient.

TABLE 112.—PHENOL COEFFICIENTS OF $HgCl_2$ AND $AgNO_3$

Disinfectant	Time of disinfection, min.	Concentration per cents	Phenol coefficient
$HgCl_2$	2.5	0.088	13.6
C_6H_5OH	2.5	1.20	
$HgCl_2$	10	0.006	173
C_6H_5OH	10	1.04	
$HgCl_2$	30	0.0018	550
C_6H_5OH	30	0.990	
$AgNO_3$	2.5	0.0066	176
C_6H_5OH	2.5	1.2	
$AgNO_3$	10	0.0015	693
C_6H_5OH	10	1.04	
$AgNO_3$	50	0.0009	
C_6H_5OH	50	0.83	922

more or less efficient the disinfectant under test was than phenol (see Table 111).

This comparison was inaccurate. On account of the differences in the concentration exponent n, two disinfectants having equal power at a certain concentration may vary widely if they are both diluted at the same ratio (see Fig. 39). Chick (1908) brought out this point very strongly by showing that the phenol coefficient was a function of the killing time, as is shown in the data of Table 112.

As a result of these investigations, all modern methods of comparing disinfectants including the Rideal-Walker test have standardized the time in which the organisms must be killed. This is of limited value only, however, since the phenol coefficient holds true only for the time set in the experiment, e.g. ten minutes with *Bact. coli*, and gives not even an approximate conception of the concentration required to kill in thirty minutes or in five minutes. The choice of phenol as the standard was especially unlucky since it has the highest concentration coefficient of all disinfectants.

If we realize further, that the selection properties of bacteria interfere, and that we obtain different phenol coefficients with different bacteria (see p. 346), there seems to be little left to rely upon in the Rideal-Walker test. But no better or more practical method has so far been suggested for standardization. About the various kinds of phenol coefficients, their history, their virtues and objections, and the factors influencing their accuracy, see Tanner and Wallace (1929).

(*e*) THE MECHANISM OF POISONING

A very thorough attempt to obtain a knowledge of the mechanism of poisonous action was made by Reichel (1909). He measured the distribution of phenol between oil and water, and the influence of salt upon this partition (Fig. 40), demonstrating that addition of salt increases the phenol concentration in the oil. The same experiment was then repeated with coagulated albumin instead of oil, and the same relation was found to take place. The ratio of phenol in the solid albumin phase and in the watery phase was plainly that of a partition by physical solubilities, and not of adsorption or chemical

combination. Bacterial bodies showed this same relation. In all cases, salt changed the ratio, but not the principle of distribution.

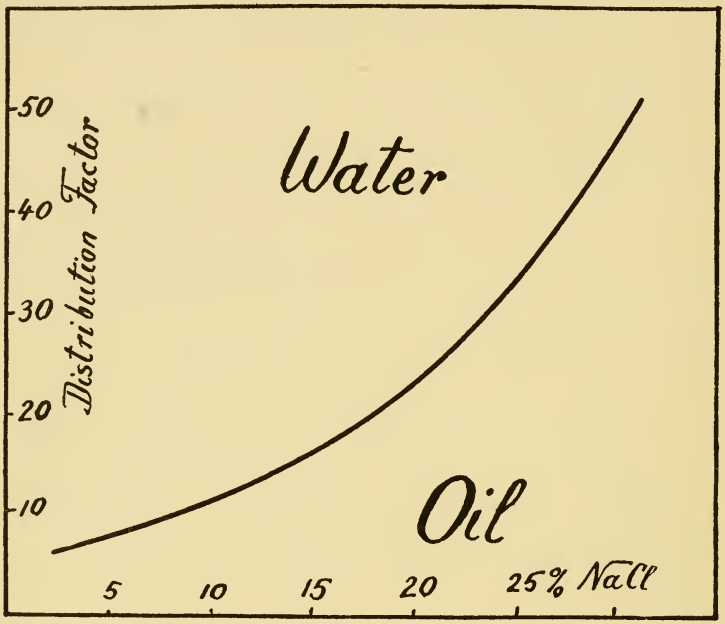


FIG. 40.—Distribution of phenol between oil and salt solutions of different concentration.

Then, the disinfecting value of phenol solutions with and without NaCl was tried by the endpoint method. The results show a good agreement with the partition of phenol between water and albumin, especially when the killing time is at least one hour.

The relation of the killing times to the phenol concentration is given by the equation

$$\text{time} \times (c - a)^n = K$$

where c is the phenol concentration, a is an initial minimum concentration of phenol showing any effect

at all, and n the concentration exponent. The exponent n is found by the equation

$$n = \frac{\log t_2 - \log t_1}{\log (c_2 - a) - \log (c_1 - a)}$$

which corresponds to the formula used for the endpoint method of disinfection (p. 347) except that a was not considered there. Reichel found for *Bact. typhosum*

$$\text{time} \cdot (c - 0.0023)^4 = 4.6 \times 10^{-9}$$

and for *Micr. pyogenes aureus*

$$\text{time} \cdot (c - 0.0045)^4 = 45.5 \times 10^{-9}$$

He came to the conclusion that the bacteria die when a certain phenol concentration is reached inside of the cell. He did not try to really find the fundamental chemical reaction.

In 1921, Gegenbauer investigated the fundamental reaction of poisoning by bichloride of mercury. His work started as a duplication of Reichel's experiments, but the results soon proved to be quite different. There was a true partition of the bichloride between coagulated serum albumin and water. $HgCl_2$ is soluble as such in the solidified albumin. This is not an adsorption process. But besides this, part of the mercury salt reacts with the protein, forming an insoluble mercury-proteinate which cannot be washed out. The amount of Hg bound by the albumin is independent of the concentration; this proves it to be a real chemical compound.

Addition of H_2S to a piece of albumin which contains dissolved $HgCl_2$ will precipitate HgS at once. But even in albumin which has been washed for eight to ten days after contact with $HgCl_2$, and which contains no more

dissolved $HgCl_2$, hydrogen sulfide will precipitate HgS from the protein compound, and the protein is liberated again.

These experiments were repeated with yeast and gave the same general results.

Then, Gegenbauer made disinfection experiments with *Micr. pyogenes* in different concentrations of $HgCl_2$,

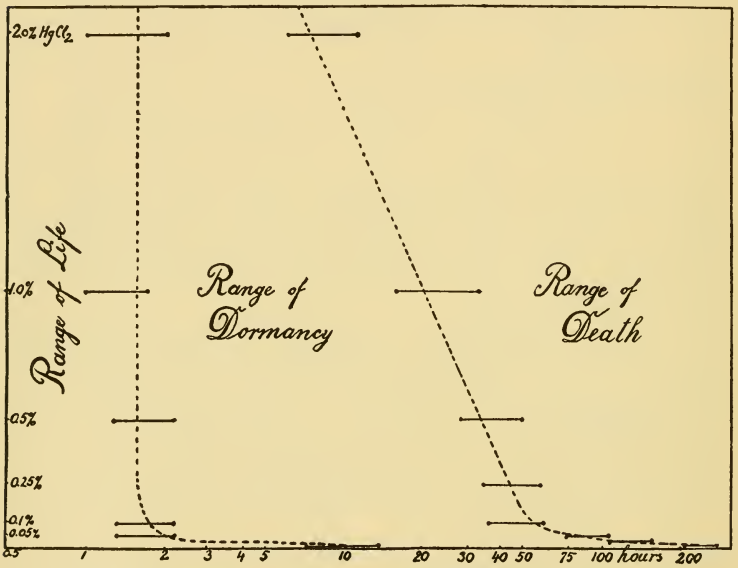


FIG. 41.—Influence of the concentration of $HgCl_2$ upon the length of time required to prevent growth of *Micrococcus pyogenes* when the poison is removed by washing (left curve) and when it is removed by an antidote (right curve).

in one series washing the bacteria after disinfection repeatedly with water, in the other treating them with H_2S to remove or counteract the poison chemically. The results are summarized in Table 113. There are nine series with chemical treatment, and three series with washing only. Parentheses in the table indicate

that the value given is not an average, but is the only datum available.

TABLE 113.—DISINFECTION OF *Micr. pyogenes aureus* WITH $HgCl_2$

Concentration of $HgCl_2$, %	Times required for complete disinfection			
	If cells are washed with water		If cells are treated with H_2S	
	Growth, hours	No growth, hours	Growth, hours	No growth, hours
0.01	7	13	208	265
0.025	.....	.....	108	149
0.050	1.3	2.3	72	101
0.100	1.3	2.3	36	59
0.250	(2)	(3)	34	56
0.500	1.3	2.3	28	48
1.000	1.0	1.7	16	33
2.000	(1.0)	(2.0)	6	11
3.000	.....	.....	2.3	3.7

Figure 41 gives these data in graphic form. The logarithms of time were chosen for plotting to make the relations more outstanding. The two limiting times for each concentration are connected by lines. The correct point must be somewhere between these limits. This graph resembles very much that of Fig. 36, and the influence of Q_{10} is here substituted by that of n. The slope of the curve is an indicator of the magnitude of n.

In solutions above 0.05% $HgCl_2$, the resistance of the bacteria was not affected by the concentration of the poison, unless the bacteria were treated with an antidote This indicates the formation of a chemical combination of the protoplasm with the mercury salt which prevents

reproduction. The independence of the formation of this compound from the concentration of the poison suggests an analogy with the compound observed in serum albumin and yeast. The amount of this compound was also independent of the bichloride concentration provided that enough time was allowed for the formation. The minimum time for *Micr. pyogenes* to lose the power of reproduction under the conditions of this experiment was about one hour.

This insoluble compound of some part of the protoplasm with mercury did not kill the cell, however. When the compound was broken up chemically by the addition of H_2S, the mercury was precipitated as HgS, and the protein was set free again, ready to take up its normal function. The bacteria were not really dead after one or two hours' contact with $HgCl_2$, but reproduced after treatment with H_2S, if administered within a certain time. Only when the dormant state was quite prolonged, death would set in, and the cells could no longer recover after chemical treatment.

Thus, the action of $HgCl_2$ is two-fold. First, there is the "stunning effect"; a dormant stage is produced; the cell ceases to function. It might be compared with a stone in the cogwheels of the cell machinery. The removal of this "stone" by chemical means proves that the cell is still alive. At the same time, a slower chemical process takes place which kills the cells. This process is independent of the first.

The nature of this second process is not clear. It depends upon the concentration of the bichloride. Above 0.1% $HgCl_2$, this relation has been expressed by Gegenbauer in the equation

$$\text{killing time} \times [HgCl_2]^{0.5} = 1.5$$

The concentration exponent varied considerably, between 0.36 and 1.23%, and averaged 0.65. Four experiments between 0.025 and 0.1% averaged 0.78, and three experiments between 0.01 and 1% averaged 0.88. The cause of this increase of n at very low concentrations of poison has been explained on p. 352.

Death is not caused by the formation of the mercury-protein compound. Neither is it caused by the liberation of HCl from $HgCl_2$. It is not the mere interruption of the cell activity by the "stone in the cogwheels," for this would make the killing time independent of the concentration. Any of the above explanations would fit the case of the anthrax spores in Table 114a, but not the *Micrococcus*.

Spores of *B. anthracis* were tested with $HgCl_2$ by Gegenbauer with the same technique, and the death time proved to be independent of the concentration, with and without antidote:

TABLE 114a.—DISINFECTION OF SPORES OF *B. anthracis* BY $HgCl_2$ (END POINT METHOD)

Concentration of $HgCl_2$	0.01%	0.05%	0.1%	0.5%	1.0%	2.0%	3.0%
Growth after.....	110	95	95	95	95	95	95 days
No growth after.....	115	100	100	100	100	100	100 days

Müller's data (1920) do not agree with those obtained by Gegenbauer. Only one table of Müller's can be used for this comparison. The critical times for anthrax spores at 37°C., were:

TABLE 114b

Concentration of $HgCl_2$.....	0.1%	1.0%	2.0%	3.0%	4.0%	5.0%
Growth after.....	9 (15)	7	7	6	6	5 days
No growth after.....	12	9	9	7	7	6 days

This table resembles that of Gegenbauer's for the *Micrococci*. Its concentration exponent is $n = 0.25$, between 1.0% and 5.0%, or $n = 0.23$ between 0.1% and 5%.

This picture of disinfection is completed by an experiment with cyanide of mercury. The action of this salt upon coagulated albumin and yeast is quite different. In yeast, no definite compound of mercury with protein had been formed in ten days, and with serum albumin, very little was found after fifteen days. The distribution factor for the dissolved salt between protein and liquid was smaller.

The disinfection experiments agree with this behavior. Since no insoluble protein compound is formed promptly, chemical removal of the mercury salt has no effect upon the killing time. Death, except for the very lowest concentrations, is practically independent of the concentration of the poison. The "fundamental reaction" must be of a different nature here than in the case of the bichloride.

Gegenbauer (1922) studied further the mechanism of formaldehyde disinfection. He found a true physical partition of formaldehyde between water and oil, as in the cases of phenol and $HgCl_2$ mentioned before; with yeast, however, there was no physical solution at all in the solid phase, but a well-defined chemical compound was formed. The amount of formaldehyde bound by yeast was independent of the concentration of aldehyde. However, for shorter times, less than six hours, the amount of formaldehyde bound by the yeast protoplasm was proportional to the formaldehyde concentration.

This is shown in the disinfection experiments. Both with Micrococci and with anthrax spores, the rate of disinfection was quite nearly proportional to the formaldehyde concentration, which means $n = 1$.

Washing with water or counteraction of the poison with ammonia did not alter the death rate materially. This is to be expected, since there is no dissolved formaldehyde in the protoplasm; however, the chemical compound between formaldehyde and protoplasm is so stable and irreversible that it is not changed back by water or ammonia.

In summary, it has been shown in this chapter that some very interesting efforts have been made to obtain a

clearer conception of the fundamental reaction between poison and cell, but they did not lead to the end. In Reichel's experiments, death occurred as soon as a certain concentration of phenol was present in the cells. However, since phenol formed no chemical compound with the cell, we still do not know the real cause of death.

In Gegenbauer's research with $HgCl_2$, a chemical compound was formed with protoplasm, and this compound prevented multiplication; it could be broken up again by H_2S, however, and the cell might be still alive, or might be dead, the one or the other depending upon the time of action and the concentration of disinfectant. The real cause of death is still unknown.

(*f*) THE TEMPERATURE COEFFICIENT OF POISONING

The influence of temperature upon the rate of chemical disinfection was first studied by Madsen and Nyman (1907) and by Chick (1908). Most of the latter's data were obtained by the endpoint method. Some

TABLE 115.—TEMPERATURE COEFFICIENTS OF DISINFECTION WITH *Bacterium paratyphosum*

$HgCl_2$:0.1%		$HgCl_2$:0.01%		$AgNO_3$:0.017%		$AgNO_3$:0.0017%	
	Q_{10}		Q_{10}		Q_{10}		Q_{10}
25°C.		40°C.		40°C.		40°C.	
			3.3		3.3		
20°C.	3.3	30°C.		30°C.		35°C.	3.9
			3.4		2.9		
15°C.		20°C.		20°C.		30°C.	
	2.6		2.8		2.3		2.6
10°C.		10°C.		10°C.		25°C.	
	4.0		2.7				2.4
5°C.		0°C.				20°C.	
	5.8						3.0
0°C.						15°C.	

of the data are shown in Table 115. Usually, the temperature coefficients were but slightly above normal, but occasionally, they went much higher.

Chick made the very remarkable observation that the temperature coefficient increased with an increase in the number of cells used for the test.

TABLE 116.—DEPENDENCE OF THE TEMPERATURE COEFFICIENT UPON THE CONCENTRATION OF BACTERIA

1% phenol			0.8% phenol			0.6% phenol		
Cells per unit volume	Temp. range, °C.	Q_{10}	Cells per unit volume	Temp. range, °C.	Q_{10}	Cells per unit volume	Temp. range, °C.	Q_{10}
1,000	21–11	3.3	440,000	21–11	6.0	6,600	31–21	4.3
750,000	21–11	6.0	76,000,000	21–11	>8.2	750,000	31–21	10.4
			187,000	21–11	5.5	110,000	31–21	5.5
			56,000,000	21–11	12.2	16,000,000	31–21	10.3

Young cultures show a higher temperature coefficient than old ones. This leads to the consequence that at very low temperatures, the death rates of young and old cells must be nearly alike while ordinarily young cells are much more sensitive (see p. 289).

These still unexplained complications of the temperature effect upon chemical disinfection should caution us against hasty conclusions.

Several very extensive experiments on the influence of temperature have been published by Gegenbauer and Reichel (1913) concerning the action of hydrochloric acid on spores of *B. anthracis*, including the change of acid concentration, and the addition of NaCl. The temperature coefficient is found to be independent of the temperature, averaging 4.6, but it decreases as the concentration of the acid increases.

Paul, Birstein and Reuss (1910c) also give a large number of temperature coefficients for the death of *Micrococcus* by hydrochloric acid.

Recently, this problem has been treated with a different technique by Cooper and Haines (1929), and these authors thought they had proved that three different groups of disinfectants existed, one showing no temperature coefficient at all, one with a normal one, and one, very high, ranging from 8 to 20 for an increase of temperature from 20°C. to 37°C. Their technique was practically that of the phenol coefficient, for they measured the concentrations just sufficient to kill the bacteria in thirty minutes. The temperature coefficient was obtained by dividing one concentration by the other.

This method of computing the temperature coefficient is not permissable, because it implies that the disinfectant action is proportional to the concentration while we have seen in the preceding chapter that this is by no means always the case. The error of these authors can be shown easily by going back to our standard equation for the death rate, on p. 347

$$\text{At } 37°\text{C:} \qquad K_1 C_1{}^n t = \log \frac{a}{b}$$

$$\text{At } 20°\text{C:} \qquad K_2 C_2{}^n t = \log \frac{a}{b}$$

In the endpoint method, $\log \frac{a}{b}$ is constant, t was experimentally equalized to thirty minutes, therefore

$$K_1 C_1{}^n = K_2 C_2{}^n$$

The relation between the two death rates is given by the formula (p. 127).

$$K_1 = K_2 Q_{17}$$

where Q_{17} is the temperature coefficient for the increase in temperature from 20°C. to 37°C.

$$K_2 Q_{17} C_1{}^n = K_2 C_2{}^n$$

$$Q_{17} = \left(\frac{C_2}{C_1}\right)^n$$

Only if $n = 1$, we have $Q_{17} = \frac{C_2}{C_1}$ as Cooper and Haines assumed; but for n larger or smaller, this ratio varies.

Applying to the experimental data by Cooper and Haines the values $n = 4$ for phenol and $n = 0.5$ for H_2O_2, (Table 110) and assuming arbitrarily $n = 0.5$ for toluquinone, the following temperature coefficients are obtained:

for phenol, $Q_{10} = 4.2$ instead of 1.43 by Cooper and Haines
for H_2O_2, 1.16 instead of 1.30
for toluquinone, 2.58 instead of 6.6

For the first two compounds, similar values had been claimed which are probably not similar, and the high coefficients for substances like toluquinone might be explained by a small value of n. The claims of Cooper and Haines are improbable, and cannot be considered proved until all n-values have been ascertained.

(*g*) OTHER THEORIES OF CHEMICAL DISINFECTION

In 1919, Traube made a very general statement that all toxic action is brought about by surface reactions. He also extended this theory to disinfection: "If we disregard the oxidizing disinfectants like ozone, peroxide, and hypochloric acid, it may well be claimed that the action of the other disinfectants is based exclusively on physical processes. Salts of heavy metals, like mercury or copper, cause death by irreversible precipitates, while most of the acids, bases, and organic disinfectants, largely by means of their very strong swelling and solving properties, produce such changes in the bacterial cells that they must perish."

The two papers are limited to the discussion of a few series of disinfectants where a proportionality between surface tension depression and disinfecting power exists, but even in these selected examples, exceptions occur. While it cannot be denied that depression of surface tension might be a cause of

death (see next chapter), the theory of Traube seems far too generalized to be accepted as such. It is already partly disproved by the researches of Reichel and Gegenbauer (p. 363). The same criticism applies to a similar theory by Davis (1927).

A different explanation of toxic action upon cells was given by Rahn (1915) who believed that chemical poisons are catalysts for certain destructive reactions in the cell. He used the well-known parallelism between heat effects and poison effects, (see p. 137) assuming that the poison accelerates the constructive processes as well as the destructive ones, but that the latter are affected more strongly.

However, it seems most probable that death i.e. loss of power of reproduction by chemical poisoning is caused by the reaction of at least one gene with the poison. Fermentation and similar life processes might still continue after the cell has lost the power to reproduce. This is the only cause of death likely to give a logarithmic order of death.

Possibly, some chemicals may not affect the chromosomes, but inactivate enzymes, destroy membranes or produce other damage. In this case, many molecules must have doubtless reacted before the cell is dead, and this must result in a period of no deaths, a bulging survivor curve and an increasing death rate.

Gaidukov (1910) defined death as a change from a hydrosol to a hydrogel. He considered the dying of the protoplasm to be a coagulation process. Twenty years later, Bancroft and Richter (1931) came to the same conclusion. They demonstrated the coagulation in the dark field, but failed to prove that the cells showing this symptom were dead. Probably, they had been dead for a considerable time, and coagulation of the

protoplasm is perhaps one of the later stages of the death process, and not one of the causes.

(*h*) SUMMARY OF FACTS

Bacteria die from chemical poisons in the same general order that is found with death by heat, or by drying.

Bacteria, which, by chemical poisoning, have lost the power to multiply, may regain it if an antidote is used. This complicates the definition of death.

The same disinfectant does not act equally strong upon all organisms. Certain genera and species are more resistant to a certain poison than others. Well known examples of special resistance are that of tubercle bacteria against alkali, of aciduric bacteria against acid, of many molds against formaldehyde.

The toxicity of a disinfecting solution increases with its concentration, but not always in direct proportion. Different disinfectants behave quite differently, and two different disinfecting solutions of equal toxicity may vary widely if they are both diluted with the same amount of water.

The disinfecting power of a solution is increased by an increase of temperature.

SUMMARY OF THEORIES

The fundamental reaction that causes death when a chemical poison acts upon a living cell is still unknown, despite several promising attempts to determine it. Several theories exist; we may assume a chemical combination of the poison with essential molecules, or changes of interface tension which bring about a colloidal state of protoplasm, or a catalysis of some normal catabolic cell process by the poison.

With $HgCl_2$, the formation of mercury-proteinate does not seem to be the cause of death, but only of dormancy, because the administration of H_2S as antidote brings the cells back to life unless the dormancy has been very prolonged.

The death rate is sometimes proportional to the concentration, but more commonly, it is proportional to the *n*th power of the concentration where *n* is known to vary from 0.5 to 4 or even 6. This means that the relative strength of two different disinfectants cannot be expressed by a simple factor unless the comparison is limited to a definite range of concentration, or, as in the case of the Rideal-Walker method, to only one definite killing time. No better method has as yet been found to compare disinfectants.

For low concentrations, the exponent becomes larger. At very low concentrations, the toxic effect disappears entirely, and a stimulation is frequently observed.

The temperature coefficient of disinfection shows some queer anomalies. It increases with an increasing concentration of bacteria, and it is higher with young cells than with old ones, but as a rule, is normal, or only slightly above normal.

VIII. DEATH BY SURFACE TENSION DEPRESSION

The theory that all death by chemical poisoning is caused by surface tension depression has already been discussed on p. 366 and found to be an unsupported generalization. However, in solutions of very low surface tension, death is probably brought about primarily by physical rather than chemical causes.

The first experiments of Ayers, Rupp and Johnson (1923) showed no parallelism between *surface* tension and death rate. These authors, therefore, considered

the chemical effect of the surface tension depressant upon the cell to be the real cause of death. However the measurements refer to the tension between medium and air, and this need not be proportional to that between cell and liquid. Pizarro (1927) also believes that chemical reaction is the real cause of death.

Some light has been thrown on this question by the experiments of Frobisher (1927) who studied the efficiency of disinfectants in the presence of chemically inert surface tension depressants (soaps). He found that 0.1% of sodium oleate had no effect on phenol disinfection, but 0.25% increased the poisonous effect, and 0.5% prevented it. The latter is explained by the law of Gibbs and Thompson that surface tension depressants are strongly adsorbed on the surfaces. The layer of adsorbed soap around the cell may become so thick that phenol cannot readily penetrate to the cell. An excess of sodium oleate also decreased the action of hexyl resorcinol. Hansen (1922) found also that saponin or soap had frequently but little or no effect, and sometimes even counteracted the efficiency of disinfectants.

Leonard and Feirer (1927) experimented with hexyl resorcinol which was the most powerful depressant they could find, reducing the surface tension of water from 73 to 34 dynes, but reducing that of glycerol only from 72.5 to 67. In pure glycerol, this substance did not kill bacteria readily while in water, a 0.1% solution killed bacteria in fifteen seconds. The authors claim the slower action in glycerol to be a proof that surface tension depression is really the killing agent. With more knowledge concerning the protective action of concentrated solutions against other causes of death (see p. 330), the lower death rate of bacteria in glycerol + hexyl resorcinol might be accounted for on a different basis. But the proportionality between surface tension and disinfecting power of the different substituted resorcinols is a good indication that here we are dealing with a physical rather than a chemical cause of death.

For this reason, the order of death by depressed surface tension would be very enlightening, but no data about this are known to the author.

IX. DEATH BY LIGHT

It is an old experience that bacteria are killed by light and also by rays of shorter wave lengths. The order of death is usually logarithmic. Table 117 gives the data obtained by Lee and Gilbert (1918) on the death of *Bact. coli* by the light of a 100 Watt lamp. The death rate is a decreasing one and indicates an heterogeneous culture.

Meader (1926) calls attention to the fact that only certain wave lengths of the sunlight will kill bacteria, and that the action of sunlight is different at different times of the day.

Two causes of death are possible: either the rays act upon the cell, or they act upon the medium, producing in it harmful substances, as, e.g., H_2O_2. In this latter

TABLE 117.—DEATH OF *Bact. coli* BY THE LIGHT FROM A 100 WATT LAMP

Time of exposure, minutes	Survivors	*K*
0	107	
2	92	0.021
3	86	0.031
5	81	0.024
10	74	0.016
15	65	0.014
20	56	0.014
25	49	0.014
30	43	0.013
40	33	0.013
50	27	0.012
60	16	0.013

case, irradiated water should be toxic. This has been found not to be true (Browning and Russ, 1917, Coblentz and Fulton, 1924). Consequently, the light acts upon the cells directly. The observation of a very low temperature coefficient, and the fact that the pH is of practically no influence upon the death rate by ultraviolet light (Bayne-Jones and van der Lingen, 1923, Gates, 1929) point in the same direction.

A large number of experiments have been carried out with ultra-violet light. The results by Gates (1929) are among the few derived from monochromatic light. The test organism (usually *Micr. pyogenes*) was spread on agar surfaces, exposed to the rays, and the survivors counted by the colonies developing. With *Micrococcus*, the logarithmic order was not established (see p. 296).

Exposure for thirty minutes to wave lengths of 3340 and 3660 Å.u. did not decrease the number of living cells noticeably, and even 3130 Å.u. seemed to have little effect. Wave lengths of 2500 to 3000 Å.u. were quite efficient, and even at 2250 Å.u., bactericidal action was observed, though the intensity of this wave length was very low with the lamp used by Gates.

The temperature coefficient is very nearly 1, indicating that death is primarily due to a photochemical reaction.

Another group of rays has been frequently proved to be harmful to bacteria, namely the X-rays. Holweck (1929) and Lacassagne (1929) computed from their experiments with *Ps. pyocyanea* that this organism must have a sensitive zone of about 0.5μ diameter. If this zone is hit by one "quantum," the cell dies. If the cell is hit outside of this zone, it does not die. Wyckoff (1930) made computations for *Bact. coli* from experiments with wave lengths from 0.56 to 3.98 Å.u., and came to the conclusion that the measured zone is

TABLE 118.—DEATH OF *Bact. coli* BY X-RAYS OF VARIOUS WAVE LENGTHS

Intensity of light measured by ionization of air.....	73.1	94.1	173.1	274.5	133.8 e.s.u.
Wave length, Å.u...	0.564	0.710	1.537	2.29	3.98
Line of light used...	*K*	*K*	*K*	*K*	*L*
Target metal.....	Ag	Mo	Cu	Cr	Ag
Seconds	Percentage of survivors				
5	.....	.....	71.4		
10	.....	86.3	68.7	62.9	
20	67.6	69.3	51.2	39.2	68.9
30	.....	61.2	27.7	29.3	
40	44.3	49.1	22.0	18.5	51.8
50	.....	.....	.....	12.8	
60	33.4	38.0	14.9	10.0	32.2
80	.....	30.0			
90	19.2	.....	.....	.....	26.3
120	12.4	.....	.....	.....	17.3
Seconds	Death rates (0.434*K*)				
5	.....	.....	0.0292		
10	.....	0.00640	0.0163	0.0201	
20	0.00850	0.00796	0.0145	0.0203	0.00809
30	.....	0.00711	0.0186	0.0178	
40	0.00884	0.00771	0.0164	0.0183	0.00714
50	.....	.....	.....	0.0178	
60	0.00794	0.00700	0.0138	0.0167	0.00820
80	.....	0.00653			
90	0.00796	.....	.....	.....	0.00644
120	0.00755	.....	.....	.....	0.00635

really the sphere of action of the hitting quantum. This sphere decreases with the wave length and proves the sensitive zone to be quite small. The zone as such, is not more than one-hundredth of the volume of the

cell, perhaps much smaller. For a coccus of 1μ diameter, the sensitive zone would correspond to a sphere of 0.215μ diameter.

The logarithmic order is well established, as may be seen from Table 118. The same is true for cathode rays, as observed by Wyckoff and Rivers (1930) (see Fig. 35, p. 297).

If death is caused by the hit of a single quantum, the death rate by a uniform source of X-rays should be the same for all species of bacteria having the same sensitive zone. Wyckoff found it to be identical for the two species he tried, i.e., *Bact. coli* and *Bact. aertryke.*

SUMMARY

The exposure of bacteria to light will cause them to die if the intensity of the light is strong enough. Ultraviolet light is very efficient in wave lengths between 2500 and 3000 Å.u.

The effect of light is not brought about by a chemical change of the medium through the light; the fundamental reaction which causes the death is produced inside of the cell by the light.

The temperature coefficient is very low, indicating a physical or photochemical reaction as the cause of death.

Death by X-rays and cathode rays is caused by the bombardment of the bacteria with electrons which will kill the cell if they hit its sensitive zone. This sensitive zone has a volume about one-hundredth of the cell.

The order of death by rays is logarithmic.

X. DEATH THROUGH AGE

In the chapter on the endpoint of growth, the cause of death by old age of bacterial cultures has already been

discussed, and has been shown to be due to one of three possible causes.

Whether the cause of death is starvation, injurious action by fermentation products, or by some specific, thermolabile compounds, the order of death should be logarithmic. As a matter of fact, it is very nearly logarithmic.

In Table 130, p. 390, two experiments on the death of *Ps. fluorescens* by suffocation are given. The two checks, with air, show a normal growth until a maximum

TABLE 119.—DEATH OF *Strept. lactis* IN MILK CULTURE

Age of culture, days	Plate count per c.c.	Lactic acid in medium, %	Death rate
1	1,137,000,000	0.625	
2	905,000,000	0.900	0.099
4	572,000,000	0.972	0.099
7	129,000,000	1.026	0.159
10	2,360,000	0.990	0.298
13	3,600,000	.....	0.208

is reached; this is followed by a decrease, which is death by old age. The successive death rates, without omission, after passing the maximum number, are:

Exp. I.....	0.0051	0.0052	0.0058	0.0045	0.0050
	0.0040	0.0036	0.0038	0.0029	0.0026
	0.0022	0.0022			
Exp. II.....	0.0115	0.0042	0.0060	0.0054	0.0047
	0.0048	0.0043	0.0031	0.0039	0.0034
	0.0031	0.0026	0.0026	0.0024	0.0024

The order of death allows for no conclusion regarding its cause, but the very poor medium, together with the very low maximum number of only seventy millions per

cubic centimeter (compared with about one billion in broth) makes starvation the most probable cause.

A case where fermentation products are the most likely cause of the decrease in living cells has been mentioned by Rahn (1911) for a culture of a lactic streptococcus in milk. The death rates in this experiment (Table 119) increase, but this is probably due to an increase of lactic acid. Even after the maximum number has been passed and the cells already have begun to decrease, the acid still increases, and this increase must necessarily increase the death rate.

Another set of data we owe to Chesney (1916) who studied the death of pneumococci in old broth cultures. The death rate in this case is quite constant. Probably, death is brought about by chemical action of the products of metabolism.

Buchanan and Fulmer (1928) distinguished between a period of accelerated death rate, and a period of constant death rate. The first-named phase must necessarily exist. Bacteria, after having grown to a maximum number, will not die suddenly at a constant rate. There is a gradual increase of the products beyond the tolerable concentration, and the increase in death rate under these conditions has been shown in Table 119. The period of accelerated death is just an intermediate stage between maintenance and death, and involves no new biologic principle.

SUMMARY

Death of bacteria in old cultures may be due to one of the following three causes: starvation, fermentation products, or specific thermolabile metabolic products. The existence of the third cause has been questioned for most species, but is not disproved.

The order of death gives no clue as to which of the possible causes is acting.

The cause of death by old age does not seem to lie in the cell, but in the unfavorable environment.

XI. DEATH BY STARVATION

(a) STARVATION IN WATER

Death of bacteria by starvation has received considerable attention on account of its importance in water bacteriology, and in the self-purification of lakes and rivers. Many data have been accumulated for this reason, but the interpretation is very difficult, and in many cases impossible. One difficulty lies in the fact that bacteria, like all other organisms, will be able to live for a certain time on their reserve substances, without any food; death by starvation will start only after these stored food substances are used up. This "period of no deaths" will vary considerably. Another difficulty is founded in the fact that the change from a culture medium to distilled water, or to a balanced salt solution, might injure or kill many cells for reasons other than starvation, e.g., by plasmolysis, leaching out of important body substances, change of acidity, change of interface tension, traces of toxic compounds in water.

Theoretically, the order of death from starvation is not logarithmic; there should be a period of no deaths before the cells begin to die. Cohen (1922) calls this the induction period. This initial period of no death or slow death is characterized by an increase of the death rate.

A large set of data on the death of *Bact. coli* and *Bact. typhosum* is given by Hinds (1916). The death rates decreased quite decidedly. Very probably, this was caused by "cold shock."

To show this more clearly, the death rates have been computed for each time interval, averaged, and recomputed on the basis that this average is 100. The result is shown in Table 120, which represents

the average of 5 to 6 experiments. The bacteria had been grown at 37° for twelve hours before being placed in the water. At 8°C., the death rate was very high during the first six hours, at 20°C., not quite so high, and only for two hours, while there is nothing abnormal at all at 37°C. The numbers in brackets are not averages; each represents but one single datum. The very high death rate for the first few hours, which was observed only at low temperatures, is very probably due to cold-shock. Those cells which survived the cold-shock died more slowly from starvation.

TABLE 120.—RELATIVE DEATH RATES OF *Bact. coli* AND *Bact. typhosum* IN WATER
(Standardized to the average = 100)

Death rate during, hours	*Bact. coli*			*Bact. typhosum*		
	8°C.	20°C.	37°C.	8°C.	20°C.	37°C.
0– 2	211	187	105	(194)	154	126
2– 6	180	130	70	140	160	84
6– 12	128	115	100	117	105	90
12– 24	72	70	105	100	92	
24– 36	57	80	(136)	84	78	
36– 48	54	90	(154)	85	80	
48– 72	45	97	.....	69	73	
72– 96	41	47	.....	52	66	
96–144	(32)	(59)				
Average.....	100	100	100	100	100	100

A typical starvation experiment is the following by Rogers (1918) which shows a rapid decline of bacteria in sewage if dialized in running water, after the first day. Here, we see plainly the short induction period due to reserve food materials in the cells, and the increasing death rate.

Quite extensive experiments were carried out by Cohen (1922) who studied the death of *Bact. typhosum* and of

Bact. coli in buffered mineral solutions of different pH, as well as in tap water and distilled water. The death rate for *Bact. coli* is lowest between pH 5 and 6.4, and increases if the pH is increased or decreased beyond these limits (see p. 340).

TABLE 121.—COLON COUNTS IN DILUTE SEWAGE HELD IN PARCHMENT BAG IN RUNNING WATER

Days	Colon cells	*K*
After 0	190,000	
1	130,000	0.165
2	19,000	0.500
3	9,000	0.441
4	20	0.994
7	30	0.543
		0.5

The death rates of Cohen's experiments show very clearly the effect of reserve food material, which causes a period of very slow death, as indicated by an increase of the death rate. At 0°C., this is overshadowed by a cold-shock effect, which even at 10°C. is still noticeable.

TABLE 122.—TEMPERATURE COEFFICIENTS OF STARVATION

°C.	*Bact. typhosum*		*Bact. coli*	
	K	Q_{10}	*K*	Q_{10}
0	1.186	....	0.0176	
	.....	1.62	.....	2.12
10	1.910	....	0.0373	
	.....	1.53	.....	4.36
20	2.928	....	0.1654	
	.....	1.77	.....	3.76
30	5.176	....	0.6214	

Cohen also computed the temperature coefficients of starvation. These coefficients are too low because at lower temperatures, the cold-shock is superposed, which increased the death rate. It is interesting to note that Hinds as well as Cohen found that *Bact. typhosum* had a lower temperature coefficient than *Bact. coli.* (1.71 against 4.30 between 20°C. and 37°C.)

(*b*) STARVATION IN SALT SOLUTIONS

While the above data relate to bacteria with practically no food whatever, other data are available where at least the minerals were supplied.

Zeug (1920) found that standard physiological salt solution caused some bacteria to die faster than they did with distilled water. He then tried salt solutions which would be less toxic to bacteria, and found that solutions of one salt were quite unsatisfactory, but mixtures of several salts were capable of keeping bacteria alive for a few days without much loss of viability. Most sensitive against 0.85% NaCl were *Micrococcus pyogenes, Bact. vulgare, Vibrio Metchnikovi,* while *Bact. acidi lactici, Bact. septicemiae hemorrhagicae* and *Ps. pyocyanea* decreased but little in three days and *Bact. typhi murium* even increased. Zeug then set out to find suitable solutions for the three sensitive organisms.

The result of this study was that different species required different salt mixtures for long viability. The solution best fitted for one was not satisfactory for the other. This is shown in Table 123, where the optimum mixture for *Micr. pyogenes* is seen to cause a fairly rapid death of *Bact. vulgare,* while the solution best suited for *Bact. vulgare* is not very satisfactory for *Micr. pyogenes.* Some of the best combinations are shown in Table 124.

Zeug did not succeed in finding a suitable mineral solution for *Vibrio Metchnikovi.* Without calcium lac-

TABLE 123.—DEATH OF *Bact. vulgare* AND *Micr. pyogenes* IN DIFFERENT SALT SOLUTIONS

Solution	Test organism	Survivors after				
		Start	1 day	2 days	3 days	4 days
Suited for Micr. pyogenes.	*Micr. pyogenes.*	235,000	210,000	210,000	200,000	200,000
	Bact. vulgare ..	145,000	100,000	30,000	8,000	0
Suited for Bact. vulgare.	*Micr. pyogenes.*	145,000	150,000	110,000	18,000	0
	Bact. vulgare ..	80,000	76,000	83,000	78,000	75,000

TABLE 124

	For *Vibrio Metchnikovi*, per cent	For *Micr. pyogenes*					For *Bact. vulgare*
		a	*b*	*c*	*d*	*e*	
NaCl	0.5	0.5	0.5	0.5	0.5	0.5	0.5
KCl	0.5	0.5	0.5	...	...	0.075	0.1
$CaCl_2$	...	0.1	...	...	...	.....	0.5
$MgCl_2$	0.1	0.5	0.5	0.4	...	0.4	0.5
$MgSO_4$	...	...	...	...	0.1	0.1	
Ca-Lactate	0.5	...	0.5	0.1	0.1	0.1	

tate, this species died fairly fast. This presence of organic compounds is experimentally objectionable because lactate might be food for the *Vibrio;* it was sufficient to produce multiplication with *Bact. vulgare.* Zeug emphasizes that there are many different mixtures equally well suited for keeping cells alive.

The best mineral medium would be the ideal medium for starvation experiments. Table 123 shows that

there is scarcely any death for four days if cells are kept in a well balanced salt solution. This makes it very clear that in most of the experiments mentioned before, death was not really caused by starvation.

It should be emphasized here that Zeug always made parallel experiments in plain distilled water and in doubly distilled water; viability was greatly prolonged by the second distillation.

Here also should be mentioned the experiments of Wilson (1922) on the viability of *Bact. suipestifer* in Ringers solution (600 mg. NaCl, 75 mg. KCl, 100 mg. $CaCl_2$, 100 mg. K_2CO, 1 liter H_2O). There was little

Table 125.—Percentage of Survivors of Bacteria Washed and Centrifuged in Water and in Broth

	B. cereus		B. megatherium		Bact. prodigiosum		Bact. coli	
	Broth	Water	Broth	Water	Broth	Water	Broth	Water
Before centrifuging.....	100	100	100	100	100	100	100	100
After centrifuging.....	88	0	197	0	44	0	70	280
One hour later.....	43	0	300	0	37	0	70	131

difference between the death rates in the original solution and in different dilutions, as far as 1 part Ringer solution + 15 parts of water.

Winslow and Brooke (1927) verified the results of Zeug's studies. Different bacteria were washed from agar slants into distilled water, centrifuged, resuspended in fresh distilled water, and recentrifuged.

Check experiments were made with broth. Table 125 gives the numbers of survivors, in percentages.

It is quite evident that *Bact. coli* is the only one of the organisms tested which can survive in distilled water for any length of time. It should be borne in mind, therefore, that results on starvation with *Bact. coli* are exceptional cases, and must not be generalized.

These authors then determined the limits of dilution which would keep cells of *B. cereus* alive for one hour after centrifugation, and found that broth diluted 1:100 protected the bacteria perfectly, broth diluted 1:1,000 only for a short time. Ringer's solution, and sugar solutions of different concentration offered no protection. A peptone concentration of 0.005% or a meat extract concentration of 0.003% kept the cells alive, but one-tenth of these concentrations caused a rapid reduction of living cells.

Death in the last experiments is so rapid that it cannot very well be explained as being caused by starvation. Since Ringer's solution and isotonic sugar solution offered no relief while colloids in very low concentrations sufficed to save the life of the cells, it seems quite possible that some toxic compounds were contained in the distilled water which were absorbed or chemically bound by the colloids. That bacteria will die from starvation within one hour is quite improbable.

(c) STARVATION FROM LACK OF NITROGENOUS FOOD

Chemical changes taking place in starving cells have already been discussed in Chapter I, on pp. 10 to 18. Those experiments refer mostly to yeast kept in a sucrose solution without nitrogenous compounds. These are the only experiments known to the author as referring to death in the presence of fermentable material, or energy food, but in the absence of nitrogenous material. They correspond to experiments on protein-free diet with animals.

Two experiments by Rubner (1913) are sufficiently detailed to give data for the computation of successive death rates. In both experiments, the yeast remained for twenty-four hours in a 10% sugar solution, was then

separated from this solution by centrifugation, and was resuspended in a new sugar solution. This was repeated after each twenty-four hour period.

The death rates increase in both experiments for the first five days. This is in accordance with our expecta-

TABLE 126.—YEAST IN DAILY RENEWED SUGAR SOLUTION WITHOUT NITROGENOUS FOOD

	Plate counts		Death rates		Rate of nitrogen loss	
	I	II	I	II	I	II
Start	20,355	38,700				
After 1 day	20,898	24,800	0.000	0.193	0.074	0.038
After 2 days	13,728	12,300	0.086	0.249	0.074	0.058
After 3 days	824	2,300	0.464	0.409	0.075	0.062
After 4 days	218	187	0.492	0.579	0.074	0.070
After 5 days	8.2	80	0.679	0.537	0.075	0.073
After 6 days	6.2	.....	0.586	.....	0.087	0.074

tion. The rate of nitrogen loss of the yeast cells also increases slightly in one experiment while it is constant in the other. There is no strict parallelism between loss of reproductive power and loss of nitrogen.

(*d*) THE FUNDAMENTAL REACTION IN STARVATION

Leaving aside all the possible reactions which might cause death in starvation experiments without being essential to starvation, such as cold-shock, traces of toxic compounds in water, diffusion of electrolytes, etc., two possible causes present themselves: it may be either a hydrolysis, or an oxidation of some essential molecules in the cell. Both of these reactions have been already

shown to occur, oxidation in the case of dry cells, hydrolysis (or some similar reaction with water) in death by heat.

It seems easy to decide whether oxidation is a cause of death; in this case, starvation should not kill the cells so rapidly in the absence of oxygen. Such experiments (p. 388) have shown *Bact. coli* to die more slowly in the absence of oxygen, while starving *Bact. typhosum* died more readily without oxygen. If these experiments can be considered representative, oxidation may be the main death cause of some bacteria, and not of others. This agrees with our experience that some bacteria are killed by oxygen even in the presence of food, namely, the obligate anaerobes (see also p. 203).

Oxidation might then be the cause of death of starving cells of *Bact. coli.* Something is oxidized in the cells which, in normally nourished cells, would be reduced again. Reductions require energy; energy requires food.

TABLE 127.—RATE OF DEATH OF *Micr. pyogenes* AT DIFFERENT TEMPERATURES

Death by		49°C.	40°C.	30°C.	20°C.	10°C.	0°C.
Oxidation....	High	0.76	0.247	0.078	0.0247	0.0078	0.0024
	Low	0.055	0.017	0.0055	0.0017	0.0005	0.00017
Hydrolysis...	High	41.4	1.60	0.055	0.0019	0.000067	0.0000023
	Low	7.5	0.285	0.0099	0.00035	0.000012	0.0000005

Typhoid bacteria die faster without oxygen. An hydrolysis is probably the cause, which, in air, can be counteracted by the cells for a while, but not in the absence of air. Possibly, *Bact. typhosum* has some reserve substance which can be utilized only with oxygen, as e.g., fat.

Since the death rate by oxidation is known for dry Micrococci (p. 308) and the death rate by heat has also been determined, (p. 320), the rates from the two causes can be calculated for all temperatures, assuming the temperature coefficient for oxidation to be 3.16, and that of hydrolysis to be 28.9. Table 127 gives these.

While these calculations have no sound basis at all, and are entirely speculative, they illustrate the possibility that a bacterium in the same medium might die at 20°C. from oxidation (death by oxidation being ten times as rapid as by hydrolysis), while at 40°C., it may die from hydrolysis (death from hydrolysis here being six to twenty times as rapid as that by oxidation).

(*e*) SUMMARY OF FACTS

Bacteria and yeasts die if food is lacking. The decrease of living cells is more rapid in water than in balanced salt solutions. In well balanced solutions, which are specific for each species, the bacteria may show hardly any decrease of viable cells for three to four days. If death is accelerated in water, it seems safe to assume that they do not die from starvation, but from other causes. One of these has been found to be cold-shock. The greater viability, if the water used for making salt solutions is distilled twice, suggests the presence of traces of toxic substances in the distilled water. These are counteracted by peptone or meat extract, but not by sugar or balanced salt solutions.

Most of the experiments supposed to be starvation experiments really present death by other causes. The true death by starvation must show a survivor curve starting parallel to the abscissa, and must show an increasing death rate.

In the absence of oxygen, starving cells of *Bact. coli* die more slowly, while starving cells of *Bact. typhosum* die more rapidly.

Bact. coli can survive in water very much longer than most other bacteria.

SUMMARY OF THEORIES

Death by starvation does not show the logarithmic order of death. The survivor curve bulges above the straight line.

In balanced salt solutions, the death rate by starvation is independent of the salt concentration, within fairly wide limits.

The death of yeast cells on a protein-free diet shows the typical bulging starvation curve. The death rate is not proportional to the rate of loss of nitrogen from the cells.

The cause of death by starvation may be either an oxidation or a hydrolysis of some cell compound which cannot be counteracted by the cell if food is lacking. With *Bact. coli,* oxidation seems to be the main cause, while with *Bact. typhosum,* oxidation is certainly not the main cause.

The temperature coefficient of death by starvation is not known accurately. All experiments have such an evident effect of cold-shock at the lower temperature that the true rate of death by starvation at low temperatures is not known. All such temperature coefficients mentioned in literature are very probably too low.

XII. DEATH BY SUFFOCATION

Suffocation shall be defined here as death by the absence of oxygen. All animals die ultimately in the absence of oxygen, though some lower animals, like the intestinal parasites, can live for a considerable period without it, and thrive better at a low oxygen tension.

With bacteria, death from this cause is not so well known. Some experiments by Whipple and Mayer (1906) show that *Bact. typhosum* in tap water dies more

rapidly if air is excluded (see Table 128). *Bact. coli* was tested by the same authors in nitrogen atmosphere, and displayed no appreciable difference in the death rate.

TABLE 128.—DEATH OF *Bact. typhosum* IN TAP WATER, WITH VARIOUS GASES

Days	Air		Hydrogen	
	Cells per c.c.	Death rate	Cells per c.c.	Death rate
0	600,000	.....	600,000	
2	455,000	0.138	2,400	2.76
4	190,000	0.300	25	2.54
8	120,000	0.200	0	
12	67,000	0.185	0	
18	25,000	0.176	0	
26	9,250	0.161	0	
33	2,150	0.171	0	
40	132	0.210	0	
47	6	0.223	0	
54	0	.....	0	
		0.196		2.65

	Air		Carbon dioxide	
Days	Cells per c.c.	Death rate	Cells per c.c.	Death rate
0	400,000	.....	400,000	
2	265,000	0.206	110,000	0.645 } 2.148
4	.....	.....	1,500	1.400 }
8	50,000	0.260	0	

This result was verified by Hinds (1916). He studied the effect of nitrogen and hydrogen upon starving bacteria, and the average death rates found are given in Table 129. The rates are then computed for the death rate in air as standard of 100; we find that *Bact. typhosum*

dies more rapidly when the air is replaced by other gases, while *Bact. coli* dies more slowly.

This might be caused by different types of reserve compounds; glycogen could be used as a source of energy in the absence of oxygen, while fat could not. However, it might also be that the cell contents of *Bact. coli* are more readily oxidized than *Bact. typhosum*. The complication of suffocation with starvation gives no good data, and more information could probably be obtained with bacteria under normal growing conditions.

TABLE 129.—DEATH RATES OF STARVING CELLS OF *Bact. coli* AND *Bact. typhosum* IN VARIOUS GASES

	Bact. coli			*Bact. typhosum*		
	Air	Nitrogen	Hydrogen	Air	Nitrogen	Hydrogen
Series I.....	0.336	0.285	0.238	0.100	0.118	0.100
Series II	0.359	0.153	0.318	0.365	0.817	0.607
Series III	0.359	0.064	0.207			
	Relative death rates					
Series I.....	100	85	71	100	117	99
Series II	100	43	89	100	224	166
Series III	100	18	88			
Average.....	100	49	83	100	171	133
Whipple & Mayer.....	100	94	...	100	...	1,350

Some excellent experiments on death by suffocation of nourished cells are given by Müller (1912) who grew *Ps. fluorescens* and *Bact. coli* in a medium containing only 40 mg. of asparagin and 60 mg. of ammonium lactate per liter, besides minerals. This medium was

TABLE 130.—DEVELOPMENT AND OXYGEN CONSUMPTION OF *Ps. fluorescens*

Age of culture hours	Plate count per mm.³		Dissolved oxygen per liter, mg.	Oxygen consumed per hour by 10^9 cells	Age of culture hours	Plate count per mm.³	
	Open flask	Closed bottle				Open flask	Closed bottle
0	4	4	8.825		46½	64,700	80
3½	5	5	8.825		52	72,823	75
8	9	12	8.819	0.13 mg.	58½	69,742	
11½	19	23	8.800	0.30	74½	56,859	85
14½	53	61	8.695	0.93	98½	42,574	27
18	187	349	8.277	0.81	122½	29,409	23
21	618	521	7.699	0.45	170½	21,429	15
23	1,654	2,200	6.214	0.69	194½	14,583	68
25	3,920	3,596	3.867	0.41	218½	15,833	12
26	.....	4,855	1.759	0.50	242½	15,167	
27	.....	9,991	0.000	(0.25)	266½	11,204	
28	11,436	14,333			290½	14,565	
33	45,655	2,654			362½	11,428	
37	68,342	396			386½	12,604	
42	67,782	260			410½	12,772	
			Second experiment				
0	69	69	8.833		45½	78,146	38
2½	86	104	8.821	0.059	51	67,221	50
7	109	250	8.660	0.22	57½	69,182	
10½	255	507	8.221	0.35	73½	53,217	29
13½	432	1,008	7.315	0.42	97½	40,613	3
17	1,690	3,884	3.355	0.57	121½	34,171	1.6
17½	.....	5,600	1.969	0.59	169½	19,326	
18½	.....	9,847	0.000	(0.26)	193½	17,926	
20	9,250	13,167			217½	23,247	0.9
22	29,129	8,500			241½	13,583	0.1
24	.....	3,452			265½	13,892	0.2
27	58,259	1,003			289½	13,780	
32	64,701	570			361½	11,428	
36	64,420	405			385½	12,268	
41	77,306	23			409½	11,204	

so poor that even *Bact. coli* could not grow on it in the absence of oxygen. Müller distributed the inoculated

medium over a large number of glass-stoppered bottles which were filled completely so as to leave no air space. The bacteria multiplied readily until the dissolved oxygen in the stoppered bottles was used up. This was determined by chemical analysis. The growth is shown in Table 130 for *Ps. fluorescens*, and in Table 46, p. 187 for *Bact. coli*. In both cases, growth continues for about one hour after the oxygen supply is exhausted, and then the bacteria begin to die. The death rates were found to decrease decidedly.

Death of *Bact. coli* by suffocation is very slow as compared with that of *Ps. fluorescens*, the death-rate being only about one-tenth. The data suggest a logarithmic order of death, with considerable variability of resistance of the individual cells. There is an indica-

TABLE 131.—SUCCESSIVE DEATH RATES OF SUFFOCATING CELLS

Ps. fluorescens.								
I.....	0.146	0.173	0.124	0.122	0.095	0.048	0.039	0.029
	0.021	0.016						
II.....	0.095	0.145	0.160	0.135	0.095	0.131	0.100	0.078
	0.050	0.047	0.039	0.021	0.023	0.020		
Bact. coli.								
I.....	0.0156	0.0110	0.0132	0.0032	0.0011	0.0003	0.0005	0.0012
	0.0015	0.0019						
II.....	0.0050	0.0121	0.0075	0.0033	0.0018	0.0013		

tion of a short initial increase in the death rate; this may be just the period of changing over from no death to the final death rate. The continuation of multiplication for about one hour after the oxygen is completely used up suggests storage of oxygen, either physically or chemically, and deserves attention.

Possibly, the absence of oxygen produces a compound in the cell which hinders further cell activity. These experiments offer no opportunity to decide whether this is the case. The fundamental reaction of death by suffocation is still unknown.

A possible line of attack of this problem is suggested by the experiments of Meyerhof (1916a) who found that a culture of Nitrobacter after having starved for nitrite for twenty hours at 35°C., will oxidize as rapidly as before starvation began; the same culture, after having been without oxygen for the same time, had lost half of its oxidizing capacity. The cell mechanism had evidently been hurt by the long absence of oxygen, but not by the absence of nitrite.

SUMMARY

Certain bacteria will die in the absence of food more rapidly when oxygen is removed. This does not hold true for all bacteria.

If bacteria are cultivated in a medium which cannot be used by them without presence of oxygen, they will die from suffocation soon after the oxygen supply is exhausted.

XIII. GRAND SUMMARY OF THEORIES OF DEATH

It can be considered quite well established that dying is a chemical process. On the curve indicating the gradual changes in the cell under adverse conditions, death is not a definite point. Death is a matter of definition. The bacteriologist's definition that death is the loss of reproductive power of the cell is satisfactory for bacteriological purposes; it probably is satisfactory for other purposes as well, as long as the reproductive power of each cell, and not of each organism, is considered. If different definitions of death are chosen, different results might be expected.

In most cases, bacteria die in a definite mathematical way approaching the logarithmic order closely enough

to allow of the computation of a death rate. This order becomes the more evident the more nearly the bacteria are of uniform age and resistance. The computation of this death rate, as such, does not involve any definite conception concerning the mechanism of the reaction. The utilization of death rates is not different in principle from the utilization of the times required to kill all bacteria of a given suspension, and makes no more assumptions. It is more accurate, and more likely to guard the investigator against errors since there are several points to be considered and coordinated, instead only the one datum, i.e., the killing time. If the death rate is not approximately constant, it cannot be used for drawing conclusions.

The fundamental reaction, i.e., the reaction in the cell which causes death, is known only in very few instances. The death of dry bacteria is known to be an oxidation process. Death by heat is probably due to the coagulation (or hydrolysis) of a certain compound in the cell. Good progress has been made in the search for the fundamental reactions in chemical poisoning. It would seem possible that most of the causes of death will ultimately be shown to result in the same chemical process, or in the reaction upon the same molecules, but if that should be the case, we are far from having proved it.

Of the enormous number of experiments on chemical disinfection, an extremely small fraction only is fit to throw light on the underlying causes of death. On account of the importance of the subject, an enormous number of new experiments is to be expected, and it is hoped that the technique of these future experiments will live up to the standards set by Krönig and Paul more than thirty years ago.

The causes of death by old age can be identified, partly at least, with well-known causes. The reactions leading to death by interface tension depression are quite unknown.

Death by light and by rays of shorter wave-length has been shown to be due to a photochemical (or physical) process. The reaction itself is unknown.

Death by freezing is ordinarily a complication of several factors, which cannot, as a rule, be separated. Experiments on starvation also usually involves several factors, only one of them being starvation proper, while cold-shock and, perhaps, traces of poison, or leaching out of cell constituents, may have a stronger deleterious effect than starvation. Death by suffocation is nothing but a special case of starvation.

APPENDIX

I. THE SIZE OF MICROORGANISMS

One of the common sources of error in studying physiology of bacteria is a wrong conception of the amounts or quantities of living matter or living protoplasm involved. A feeding experiment with pigs, recording accurately the quantities of food used and the amounts of CO_2, urea, excreta, etc., given off, but without a record of the weight of the pigs before and after the experiment, would appear very unscientific. Yet, that is the customary way of describing fermentation experiments with bacteria. For this reason a computation of the size, weight, surface, and composition of an average representative bacterium cell is given here. *Bact. coli* has been chosen because it is probably the best known micro-organism, and because it is of average size.

The size of *Bact. coli* varies considerably with age, and also with the kind and amount of food. But we can consider a length of 1.5μ and a diameter of 0.8μ as representing the average size of cells in a well grown broth culture. For the simplicity of calculation, we shall suppose the cell to be a cylinder. Its volume is

$$V = 0.4 \times 0.4 \times \pi \times 1.5\mu^3 = 0.75\mu^3$$

The specific gravity is larger than 1, but only slightly so, for a very high speed of the supercentrifuge is necessary to precipitate the cells. If we assume 1.07 as density of the cell (twice that of skim milk, which contains half as many solids as bacteria), the weight of one cell would be

$$W = 8.0 \times 10^{-10} \text{ mg.}$$

The surface of the cells is important because all metabolism takes place through this surface only. It is

$$S = 2 \times 0.4 \times \pi \times 1.5 + 2 \times 0.4 \times 0.4 \times \pi = 4.77\mu^2$$

These figures mean little, unless we compare them with things we can really conceive. We cannot imagine the size of μ^3 or μ^2. One c.c. of a full grown culture of *Bact. coli* contains about one billion cells. The space occupied by these is

$$0.75 \times 10^{-9} \times 10^9 \text{ mm.}^3 = 0.75 \text{ mm.}^3$$

One billion bacteria occupy a space smaller than 1 mm.^{3}, or less than 0.1% of the volume of the liquid in which they grew. The weight of these is 0.8 mg. or, in other words, 1 mg. of moist bacteria contains about 1,250,000,000 cells.

The solids of *Bact. coli* have been determined to 26.65%, of which 17.2% is protein. The weight of one dry cell would then be 2.2×10^{-10} mg., and 1 mg. of dry cells would contain about 4,500,000,000 cells. MacNeal found 5,500,000,000 dry cells of *Bact. coli* to weigh 1 mg.

The bacteria solids of 1 liter of a full grown broth culture amount to 220 mg. Moyer (1929) found for *Bact. aerogenes* in the average of 8 experiments, 308 mg. of dry bacteria per liter if fed with glycerol, and 562 mg. if fed with glucose. It is easy to see how near this comes to a catalyst, considering the rapid rate of decomposition brought about by only 0.3 gm. of substance per liter.

This rate of decomposition is explainable only by the enormous surface. The surface of the bacteria in this one liter of broth culture amounts to $4.77 \times 10^{12}\mu^2 = 4.7m^2$ = about fifty square feet, which is enormous considering the 800 mgs. of bacteria bodies. The same ratio of surface to weight with man would amount to 0.47 square kilometers or 130 acres.

There is one group of organisms still smaller than bacteria, i.e. the bacteriophage. Their size has been estimated to only 20mμ, or 0.02μ. Assuming them to be spheres, their volume would be $0.01 \times 0.01 \times 0.01 \times \pi \times \frac{4}{3}\mu^3 = 4.2 \times 10^{-6}\mu^3 = 4.2 \times 10^{-15}$ mm.^{3}. Since one cell of *Bact. coli* had the volume of $0.75\mu^3$, it is 180,000 times as large as a bacteriophage. The surface of a bacteriophage is $4 \times \pi \times 0.01 \times 0.01 = 12.5 \times 10^{-4}\mu^2$.

One mg. of bacteriophage contains 225,000,000,000,000 cells, and their surface is 0.28 m.^{2}.

TABLE 132.—DIMENSIONS OF MICROORGANISMS

	Sacch. cerevisiae	*Bact. coli*	Bacterio-phage
Length	7.2μ	1.5μ	$20m\mu$
Diameter	5.6μ	0.8μ	$20m\mu$
Volume of 1 cell	$118\mu^3$	$0.75\mu^3$	$4.2 \cdot 10^{-6}\mu^3$
Weight of 1 cell	$130 \cdot 10^{-9}$ mg.	$8 \cdot 10^{-10}$ mg.	
Surface of 1 cell	$118\mu^2$	$4.77\mu^2$	$1.25 \cdot 10^{-4}\mu^2$
Moisture content	70%	73.35%	
1 mg. of dry cells contains	$26 \cdot 10^6$ cells	$4.5 \cdot 10^9$	
1 mg. of moist cells contains	$7.7 \cdot 10^6$ cells	$1.25 \cdot 10^9$	$225 \cdot 10^{12}$ cells
Surface of 1 mg. of moist cells	30 cm.^{2}	60 cm.^{2}	2,800 cm.^{2}
Number of cells per c.c. of full grown culture	$50 \cdot 10^6$	10^9	10^{11}
Weight of cells in one liter of full grown culture (moist)	6.5 gm.	800 mg.	0.42 mg.
Weight of cells in one liter of full grown culture (dry)	1.95 gm.	220 mg.	
Surface of cells per liter of full grown culture	5.9 m.^{2} (60 square feet)	4.7 m.^{2} (50 square feet)	0.125 m.^{2} (1 square foot)

Their number in a good medium has been estimated to 10^{11} per c.c. (d'Herelle p. 98). This means $4.2 \times 10^{-15} \times 10^{11} = 4.2 \times 10^{-4}$ or 0.004 mm.^{3} of bacteriophage per c.c. of a full grown culture. Even in a liter of medium, their total moist weight would be only half a milligram, their number totalling about 10^{14}.

The yeasts are much larger than bacteria. An average-sized bread-yeast cell has the dimensions of about $7.2 \times 5.6\mu$, according to Henneberg, the variation with age being very great.

The volume of one such cell would be that of an ellipsoid

$$\tfrac{4}{3} \times \pi \times 3.6 \times 2.8 \times 2.8 = 118\mu^3$$

Volume of 50,000,000 cells in 1 c.c. of liquid = 6 mm.^{3} = 0.6% of volume of medium.

All these dimensions are summarized in Table 132 which shows plainly that we cannot generalize from yeasts to bacteria, or from bacteria to bacteriophage.

The weights, volumina and surfaces of mold mycelium are again on a different order of magnitude, but the variation is so great and their mode of growth so different that no general statements can be made.

II. MULTIPLICATION OF BACTERIA

Geometrical Progression: Bacteria divide by fission into two equal cells; the successive generations, 2; 4; 8; 16; etc., can be described by the geometrical progression 2^1; 2^2; 2^3; 2^4; etc.

If one cell is placed in a nutrient solution and multiplies, all cells deriving from this one cell will be of the same age, for there is no old cell left when the two young cells have been formed. Each new set of cells is called a new generation.

If a cells are transferred to a new medium, the multiplication follows the order a; $2a$; 2^2a; 2^3a; . . . 2^na;

This rule holds true only for organisms which divide by fission; it is not really accurate with yeasts. There, we have a budding process with the old cell plainly distinguishable from the daughter cells, even if the budding has gone through several generations. Thus, the yeast cells in a fermenting liquid are not all of the same age, and old cells may not bud as freely as the younger ones. Ordinarily, the multiplication will, even in yeasts, follow nearly the progression 2^x; but it is never safe to rely upon it.

Generation time: The time required by one cell to divide completely in two new cells is called generation

time. It may be observed directly (p. 267, Table 74), or may be computed from microscopic or plate counts of the numbers of cells in a liquid at the beginning and the end of an experiment, or by the weight of the cells, e.g. in yeasts. The initial number of cells be a, and the number at the end of the experiment, after the time t, be b. Then, we find

$b = a2^n$, where n is the number of generations in the time t. Since a and b are known, we find n from the equation

$$n = \log^2 \frac{b}{a}$$

or transformed into decimal logarithms,

$$n = \frac{\log b - \log a}{\log 2}$$

The experiment lasted for the time t; in this time, the cells divided n times; therefore, the time for one generation must be

$$g = \frac{t}{n}$$

$$= \frac{t \log 2}{\log b - \log a}$$

This is the standard formula for the generation time which was given in a somewhat different form as early as 1876 by Pedersen.

The generation time is not constant during the growth of a culture; it is, *a priori*, impossible that it can remain constant, because growth cannot continue in a culture for an indefinite time. The multiplication within a limited space soon comes to point where it is retarded more and more, until it ceases altogether, and the generation time becomes infinite.

TABLE 133.—DEVELOPMENT OF TWO MILK CULTURES OF *Strept. lactis*

Age, hours	Culture I						
	Plate counts per c.c.	C.c. $n/10$ NaOH per 10 c.c. milk	% of lactic acid	Generation time, minutes	Fermenting capacity times 10^{10}		
					Formula	Average	Corrected
0	38						
	38	1.65	0				
				79.0			
3	160						
	205	1.65	0				
				55.0			
6	1,720						
	1,780	1.65	0				
				52.7			
9	21,700						
	24,800	1.65	0				
				48.5			
12	300,000						
	302,000	1.65	0				
				45.1	(103.4)	(65.3)	
15	4,700,000	1.70					
	4,900,000	1.70	0.005				
				56.8	13.3	9.7	9.6
18	43,000,000	1.75					
	43,000,000	1.85	0.011				
				88.5	5.6	4.9	
21	172,000,000	1.95					
	181,000,000	1.95	0.027				
				92.2	9.7	8.1	
24	650,000,000	3.20					
	720,000,000	3.25	0.132				
				210.3	7.2	7.1	
27	1,230,000,000	5.35					
	1,240,000,000	5.45	0.338				
				620.	4.7	4.6	
30	1,470,000,000	7.00					
	1,560,000,000	7.15	0.489				
				∞			
33	1,280,000,000	7.45					
	1,500,000,000	7.55	0.527		0.43	0.43	
				∞			
36	1,670,000,000	7.50					
	1,830,000,000	7.60	0.531				

TABLE 133.—DEVELOPMENT OF TWO MILK CULTURES OF *Strept. lactis*.—(*Continued*)

Age, hours	Culture II						
	Plate counts per c.c.	C.c. $n/10$ NaOH per 10 c.c. milk	% of lactic acid	Generation time, minutes	Fermenting capacity times 10^{10}		
					Formula	Average	Corrected
0	38,000	1.65	0				
	38,000						
				74.8			
3	184,000	1.65	0				
	216,000						
				58.1			
6	1,600,000	1.65					
	1,820,000	1.75	0				
				50.8	(31.9)	(21.8)	10.6
9	18,700,000	1.70					
	20,500,000	1.75	0.007				
				70.9	(6.9)	(5.5)	
12	112,000,000	1.85					
	114,000,000	1.85	0.018				
				92.2	9.5	8.3	
15	430,000,000	2.60					
	440,000,000	2.60	0.086				
				130.	8.1	7.5	
18	1,000,000,000	4.55					
	1,270,000,000	4.60	0.263				
				663.	4.6	4.5	
21	1,250,000,000	6.35					
	1,490,000,000	6.60	0.434				
				1010.	1.03	1.03	
24	1,490,000,000	6.95					
	1,610,000,000	7.00	0.479				
				∞		0.53	
27	1,300,000,000	7.15					
	1,390,000,000	7.30	0.502				
				∞		0.55	
30	1,170,000,000	7.35					
	1,420,000,000	7.60	0.524		0.31		0.31
				∞			
33	1,380,000,000	7.30					
	1,500,000,000	7.55	0.520				
				∞		0.21	
36	1,550,000,000	7.55					
	1,660,000,000	7.70	0.538				

An example of the changes of the generation time is given in Table 133 representing the growth of *Streptococcus lactis* in sterile milk at a constant temperature of about 20°C. In the first three hour period, seventy-five to eighty minutes are needed for the cells to double, but soon the minimum time of forty-five to fifty minutes is reached, and the cells divide in less than one hour. This period of most rapid growth lasts only six to twelve hours, and then growth slackens, the generation time increases progressively, and soon reaches infinite which means that the culture has ceased growing altogether.

Amount of Inoculum: It is not unusual to find statements in literature where it is anticipated that by doubling the amount of inoculum, the time for completion of the fermentation can be reduced to one-half. In thinking of the geometrical progression of bacterial multiplication, it is evident that this is an error; the time saved by doubling the inoculum is just the time which is required for the inoculum to double, i.e. the time of one generation which is usually not much more then one hour. By using one thousand times the inoculum, the amount of time saved is the time of ten generations, since $2^{10} = 1{,}024$.

We can test this in Table 133 where the two parallel cultures differ only inasmuch as the first was inoculated with one thousand times as many cells as the other. The average generation time for the first few hours is about sixty minutes. It should therefore require ten times sixty minutes or ten hours for the first culture to come to the point where the second culture started, and from then on, there should be no difference between the two. This is actually the case. After nine hours, culture I has 23,000 cells per c.c. which, after ten hours, would have increased to about 50,000. The time saved in the first experiment by using 1,000 times as many bacteria for seeding the culture was about nine and a half hours.

The generation time is the reciprocal of the growth-rate, and is least when the growth rate is greatest. In order to have a direct measure of the growth rate, Slator (1916) suggested the "growth-constant." He computed this by starting from the assumption that the growth at any time must be proportional to the number of cells present. This leads to the equation

$$\frac{db}{dt} = kb$$

$$\frac{db}{b} = kdt$$

Upon integration, this changes to

$$\ln b = kt + C$$

For $t = O$, we have $kt = O$ and $b = a$

$$\ln a = O + C$$

subtracting this equation from the preceding, we eliminate C:

$$\ln b - \ln a = kt$$

$$k = \frac{1}{t}(\ln b - \ln a) = \frac{1}{t}\frac{(\log b - \log a)}{0.434}$$

This value of k is called the growth-constant. Its meaning and its relation to the generation time is explained on p. 190.

The formula for the generation time is accurate only in the exponential branch of the growth curve, i.e., only as long as the rate of growth does not decrease. If this is the case, the formula is based on the false assumption of an exponential increase. Since we are without accurate knowledge of the cause of the decreasing growth rate, we cannot very well put it into a correct mathematical equation. The assumption that growth proceeds in a straight line will be more accurate at this stage than the above formulas, be it the one for generation time, or that of Slator.

To show graphically the meaning of this, Fig. 42 has been used which represents the growth of *Ps. fluorescens* after data obtained by Müller (1903). The dotted lines in this curve illustrate the wrong assumption made in the computation of the generation time at this

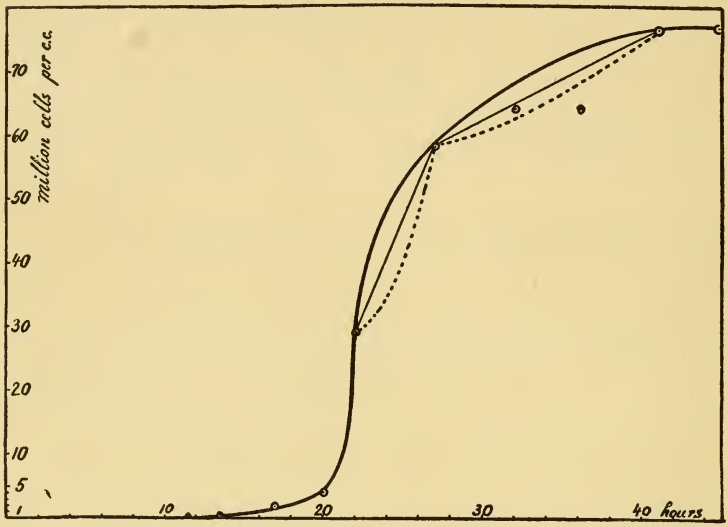


FIG. 42.—Growth curve of *Pseudomonas fluorescens*. For the explanation of the dotted and thin lines, see text.

stage from the customary formula. It is impossible, however, to compute generation times if we assume an arithmetical increase as shown by the thin straight lines, which represent the arithmetical averages.

III. THE FERMENTING CAPACITY OF THE SINGLE CELL

The various means of obtaining experimentally fermentation without growth have been mentioned on p. 102. It is also possible, however, to eliminate the disturbing factor of the multiplication of cells during

fermentation by a mathematical calculation, provided that the increase in cells is known.

After an attempt by Burchard (1899) which was based on a wrong mathematical principle, Rahn (1911) developed a formula in the following way:

If we assume that the single cell up to the time where it is completely divided produces the amount of products s, then the amount of products in the successive generations will be

$$as \quad 2as \quad 4as \quad 8as \quad . \quad . \quad . \quad a2^n s$$

It is arbitrary to state which shall be the last member of this progression, whether 2^n or 2^{n-1}. Rahn decided that 2^{n-1} would be more accurate to assume. The total amount of the products formed, S is the sum of all these amounts.

$$S = as + 2as + 4as + 2^3as + \cdot \cdot \cdot + 2^{n-1}as$$

Applying the sum formula of geometrical progressions, we get

$$S = as(2^n - 1) = s(a2^n - a)$$

and since $2^n a = b$, we get

$$S = s(b - a)$$

or

$$s = \frac{S}{b - a}$$

In this formula, s is the amount of products formed by one cell in one generation, i.e., in the time g. Ordinarily, the fermenting capacity per hour will be more

interesting. If we call the amount of products formed per cell per hour, x, we find

$$x = \frac{s}{g}$$

Substituting in this equation the formula for the generation time from p. 399, we obtain the final expression for the fermenting capacity of a cell per hour to be

$$x = \frac{S(\log b - \log a)}{t(b - a)\log 2}$$

We had made the arbitrary assumption here that the last member of the progression to form products was $a2^{n-1}$; perhaps, we might as well have assumed that it was 2^n. The more correct way to solve the problem is the application of calculus. This was done by Slator (1913) and independently, also by Buchanan and Fulmer (1918). The rate with which the products are formed is proportional to the number of bacteria. Using the same designations as before, the cells increase to the progression $a2^n$, and the products increase accordingly in the ratio $sa2^n$. The sum of all products formed between the numbers a and $a2^{n'}$ is given by the integral of the rate of increase

$$S = \int_0^{n'} sa2^n dn$$

between the limits $n = 0$ and $n = n'$

The solution of the integral gives

$$S = \frac{sa(2^{n'} - 2^0)}{\ln 2} = \frac{s(a2^n - a)}{\ln 2}$$

$$= \frac{s(b - a)}{\ln 2} = \frac{0.434s(b - a)}{\log 2}$$

Computing from this equation the fermenting capacity per hour by substituting $s = \frac{x}{g}$ we get

$$S = \frac{x \cdot t \cdot 0.434(b - a) \log 2}{\log 2(\log b - \log a)}$$

or

$$x = \frac{S \cdot (\log b - \log a)}{0.434 \cdot t \cdot (b - a)}$$

For the short period Δt, with the increase in products ΔS, we obtain

$$x = \frac{2.301 \cdot \Delta S \cdot (\log a - \log b)}{\Delta t \cdot (a - b)}$$

This formula has been applied in this book wherever the fermenting capacity per cell has been computed. It holds fairly true as long as the bacteria multiply rapidly. After the curve has passed the point of inflexion, however, the products increase no more according to geometrical progression. At this stage, the computation of the fermenting capacity is more accurate if the increase in products is simply divided by the arithmetical average of the number of cells at the beginning and the end of the period. This corresponds to the principle demonstrated in Fig. 42.

Table 133 shows some computations by the two methods, and it is evident that the difference is not great.

In this table, the initial values obtained for the fermenting capacity are far too high. This is due to the great probable error in the determination of the extremely small amounts of acid formed. For instance, if we assume for culture II that at nine hours, not 0.007%, but 0.002% of acid had been formed, the fermenting capacity would have been for the period from six to nine hours 9.1 and for the period

from nine to twelve hours 9.4×10^{-10} mg. This difference corresponds to only 0.05 c.c. of alkali in titration, and is well within the limits of error. As soon as the quantities of acid become much larger than the experimental error, the fermenting capacity per cell becomes quite stable. This stabilization can also be accomplished by computation over larger periods, as is shown in Table 133 in the column marked "corrected."

For the first value of the fermenting capacity in these cultures, different zero-points are possible. They gave very similar results. For culture II, the fermenting capacity was for the period 0 to twelve hours: 10.6, for three to twelve hours, 11.25, and for six to twelve hours, 11.3 10^{-10}. This agreement is a good proof for the accuracy of the formula.

AUTHOR INDEX

PAGE

BAKER, J. C., J. D. BREW and H. J. CONN, 1919, Relation between lactic acid production and bacterial growth in the souring of milk. New York (Geneva) Agr. Exp. Station Tech. Bull 74..... 106, 112

BALLNER, 1902, Experimentelle Studien über die Desinfektionskraft gesättigter Wasserdämpfe bei verschiedenen Siedetemperaturen. Sitz. Ber. Akad. d. Wissensch. Wien, math.-nat. Klasse **111**, 97..... 320

BALLS, A. K., and J. B. BROWN, 1925, Studies in yeast metabolism, I and II. J. Biol. Chem. **62**, 789 and 823..... 174, 178, 210

BALDWIN, I. L., see Allyn

BANCROFT, W. D., and G. H. RICHTER, 1931, The chemistry of disinfection. J. Physic. Chem. **35**, 511..... 367

BARBER, M. A., 1908, The rate of multiplication of *Bacterium coli* at different temperatures. J. Infect. Diseases **5**, 379.... 196, 219, 222

BARON, M. A., 1928, Bakterien als Quellen mitogenetischer (ultravioletter) Strahlung. Centr. f. Bakt. II Abt. **73**, 373..... 34

BARTLETT, C. J., and F. B. Kinne, 1913, Resistance of microorganisms in glycerine or oil to the sterilizing action of heat. Science n.s. **38**, 372..... 312

BAYLISS, W. M., 1924, Principles of General Physiology. 4th ed. London; Longmans, Green and Co..... 2, 15, 16, 18

BAYNE-JONES, S., and J. S. VAN DER LINGEN, 1923 a, The bactericidal action of ultra-violet light. Johns Hopkins Hospital Bull. **34**, 11 372

——— and H. S. RHEES, 1929, Bacterial Calorimetry, II. J. Bact. **17**, 123..... 31

BERGHAUS, W. H., 1907, Ueber die Wirkung der Kohlensäure, des Sauerstoffs und des Wasserstoffs auf Bakterien bei verschiedenen Druckhöhen. Archiv f. Hygiene **62**, 172..... 19

BERMAN, N., and L. F. RETTGER, 1918 a, Further studies on the utilization of protein and non-protein nitrogen. J. Bact. **3**, 367 56, 203

———, ———, 1918 b, The influence of carbohydrate on the nitrogen metabolism of bacteria. J. Bact. **3**, 389..... 159

———, see also Rettger

BI KERT, F. W., 1930, Zur Differentialfärbung toter und lebender Bakterien. Centr. f. Bakt. I Abt. Orig. **117**, 548..... 274

BIGELOW, W. D., 1922, The logarithmic nature of thermal deathpoint curves. J. Infect. Diseases **29**, 528..... 320, 323

BIOLETTI, F. T., 1908, Improved methods of wine making. California Agr. Exp. Station Bull. 197..... 156

BIRSTEIN, see Paul

BOYSEN, H. H., 1928, unpublished data..... 209

BRANHAM, S. E., 1929, The effects of certain chemical compounds upon the course of gas production by baker's yeast. J. Bact. **18**, 247..... 140

PAGE

——— and C. J. MARTIN, 1908, The principles involved in the standardization of disinfectants, and the influence of organic matter upon the germicidal value. J. Hygiene **8,** 654..... 336

———, ———, 1910, On the heat coagulation of proteins. J. Physiol. **40,** 404..... 326

CHRISTENSEN, L. M., see Fulmer

CLAASSEN, H., 1927, Stoffwechselvorgänge und Stoffbilanz bei der Vergärung der Melasse nach dem Lufthefeverfahren. Z.d. Vereins Deutsche Zuckerindustrie **77,** 607..... 174, 176

———, 1928, Versuche über den Ersatz der Malzkeime in der Lufthefefabrikation durch Ammoniakverbindungen. Z. angewandte Chemie **41,** 1161..... 243

———, 1929, unpublished data..... 237–243

CLARK, H. W. and S. DE M. GAGE, 1903, On the value of tests for bacteria of specific types as an index of pollution. 34th Ann. Report, State Board of Health, Mass. 245..... 295

CLARK, W. M., B. COHEN and associates, 1928, Studies on oxidation-reduction. U. S. Public Health Service, Hygienic Lab. Bull. 151 86, 88, 90, 92, 94

———, see also Rogers

COBLENTZ, W. W., and H. R. FULTON, 1924, A radiometric investigation of the germicidal action of ultra-violet radiation. Scientific Papers, Bureau of Standards **19,** 641..... 372

COHEN, B., 1922, Disinfection studies. J. Bact. **7,** 183..339, 350, 352, 377

———, 1931, The bacterial culture as an electric half-cell. J. Bact. **21,** 18..... 96

———, see also Clark

COFFEEN, MRS. V. A., 1911, unpublished data..... 330, 331

CONN, H. F., see Baker

CONRADI, H., and O. KURPJUWEIT, 1905, Ueber spontane Wachstumshemmung der Bakterien infolge Selbstvergiftung. Münch. med. Wochenschr. **34,** 1761..... 234

COOPER, E. A., and R. B. HAINES, 1928, The influence of temperature on bactericidal action. J. Hygiene **28,** 163..... 365

——— and J. MASON, 1927, Studies of selective bacterial action. J. Hygiene **26,** 118..... 345

———, ———, 1928, Further investigations on selective bacterial action. J. Path. Bact. **31,** 343..... 345

COPPING, A. M., 1929, The effect of "bios" on the growth and metabolism of certain yeasts. Biochem. J. **23,** 1050..... 203

CROZIER, W. J., 1924, On biological oxidations as a function of temperature. J. Gen. Physiol. **7,** 189..... 135

COULTER, C. B., 1929, Reduction potentials of sterile culture bouillon. J. Gen. Physiol. **12,** 139..... 97

PAGE

HENRICI, A. T., 1928, Morphologic variation and the rate of growth of bacteria. Springfield; C. C. Thomas..... 162, 198, 201, 234

D'HERELLE, F., 1926, The Bacteriophage and Its Behavior. Baltimore; Williams and Wilkins..... 397

HERZOG, R. O., 1903, Ueber Milchsäuregärung. Z.f. physiol. Chem. **37,** 381..... 37

HEWITT, L. F., 1930, Oxidation-reduction potentials of haemolytic streptococci. Biochem. J. **24,** 513 and 669 and 676 and 1551 86

HEWLETT, R. T., 1909, Disinfection and disinfectants. Lancet, **1909,** I, 741..... 278

HILDESHEIMER, A., see Neuberg

HILLIARD, C. M., and M. A. DAVIS, 1918, The germicidal action of freezing temperatures upon bacteria. J. Bact. **3,** 423..... 314

———, C. TOROSSIAN and R. P. STONE, 1915, Notes on factors involved in the germicidal effects of freezing and of low temperatures. Science **42,** 770..... 314

HINDS, M. E., 1916, Factors which influence longevity of *B. coli* and *B. typhosus* in waters. Univ. of Illinois Bull.; Water Survey Series **13,** 225..... 377, 380, 388

HOFMANN, PAUL, 1922, Ueber die Gültigkeit des Arndt-Schulz'schen Grundgesetzes bei der Wirkung von Bakteriengiften. Dissertation, tierärztl. Fakultät, München..... 255, 272

HOLWECK, F., 1929, Production de rayons X monochromatiques de grande longueur d'onde. Action quantitative sur les microbes. Compt. rend. Acad. Paris. **188,** 197..... 372

HOLWERDA, B. J., 1921, Ueber den Einfluss der Milchsäure auf die Milchsäuregärung. Biochem. Z. **128,** 465..... 144

HOPKINS, F. G., 1921, On the auto-oxidizable constituents of the cell. Biochem. J. **15,** 286..... 97

HSU, P. H., see Norton

HUCKER, G. J., 1928 a, Production of carbon dioxide by the streptococci. New York (Geneva) Agr. Exp. Station, Techn. Bull. 142 146, 174

———, 1928 b, Certain biochemical reactions produced by streptococci. New York (Geneva) Agr. Exp. Station Techn. Bull. 143 52

———, see also Carpenter

HÜNE, 1909, Die begünstigende Reizwirkung kleinster Mengen von Bakteriengiften auf die Bakterienvermehrung. Centr. f. Bakt. I Abt. Orig. **48,** 135..... 228

HUNTER, O. W., 1923, Stimulating the growth of Azotobacter by aeration. J. Agr. Research **23,** 665..... 185, 209

HUTCHINSON, H. B., 1907, Ueber Form und Bau der Kolonien niederer Pilze. Centr. f. Bakt. II Abt, **17,** 602..... 22

PAGE

KRÖNIG, B., und TH. PAUL, 1897, Die chemischen Grundlagen der Lehre von der Giftwirkung und Desinfektion. Z.f. Hygiene **25,** 1 ..... 336, 342, 344, 393

KRUSE, WALTER, 1910, Allgemeine Mikrobiologie. Leipzig; Vogel. 196, 202

KURPJUWEIT, O., see Conradi

LACASSAGNE, A., 1929, Action des rayons X de grande longueur d'onde sur les microbes. Compt. rend. Acad., Paris, **188,** 200 260, 372

LAFAR, F., 1904–1914, Handbuch der technischon Mykologie. Jena, Gustav Fischer 246, 274

LANGE, B., 1922, Keimmenge und Desinfektionserfolg. Z.f. Hygiene **96,** 92 ..... 318, 338

LEDINGHAM, J. C. G., and W. J. PENFOLD, 1914, Mathematical analysis of the lag-phase in bacterial growth. J. Hygiene **14,** 242.. 196

LEE, R. E., and C. A. GILBERT, 1918, Application of the mass law to the process of disinfection. J. Physical Chem. **22,** 348 295, 353, 371

LEONARD, V., and W. A. FEIRER, 1927, The bactericidal activity of hexyl resorcinol in glycerine. Bull. Johns Hopkins Hospital, **41,** 21 370

LINDNER, P., 1905, Die Assimilierbarkeit der Selbstverdauungsprodukte der Bierhefe durch verschiedene Heferassen und Pilze Wochenschrift f. Brauerei **22,** 528 and **23,** 519; see also Chemikerztg **34,** 1144 ..... 14, 169, 170

———, 1919, Zur Verflüchtigung des Biosbegriffs. Z.f. technische Biologie **7,** 79 203

———, und T. UNGER, 1919, Die Fettbildung in Hefen auf festen Nährböden. Z.f. technische Biologie **7,** 68 179

———, see also Euler

LINGEN, F. S. VAN DER, see Bayne Jones

LINHART, G., 1920, The free energy of biological processes. J. Gen. Physiol. **2,** 247 ..... 183, 185

LIPMAN, C. B., 1931, Living microorganisms in ancient rocks. J. Bact. **22,** 183 311

LOCHRIDGE, E. E., see Winslow

LOEB, J., and J. H. NORTHROP, 1917, On the influence of food and temperature on the duration of life. J. Biol. Chem. **32,** 103..275, 293

MADSEN, T., and M. NYMAN, 1907, Zur Theorie der Desinfektion. Z.f. Hygiene **57,** 388 ..... 278, 295, 363

MAGOON, C. A., 1926, Studies upon bacterial spores: I. Thermal resistance as affected by age and environment. J. Bact. **11,** 253 299

PAGE

MÜLLER, MAX, 1895, Ueber den Einfluss von Fiebertemperaturen auf die Wachstumsgeschwindigkeit und die Virulenz des Typhus-Bacillus. Z.f. Hygiene **20**, 245 ..... 193, 196

———, 1903, Ueber das Wachstum und die Lebenstätigkeit von Bakterien bei niederer Temperatur. Archiv f. Hygiene **47**, 127 ..... 192, 219, 404

MÜLLER-THURGAU, 1885, Verhandlungen der X. Generalvers. d. Deutschen Weinbauvereins Geisenheim, quoted from Lafar, Handbuch der Technischen Mykologie Bd. V, 435 151

MUSCULUS, 1876, Sur le ferment de l'urée. Compt. rend. Acad. Paris, **82**, 333 36

MYERS, R. P., 1929, The germicidal properties of alkaline washing powders. J. Agric. Research **38**, 521 ..... 295, 297, 299, 345, 350

NAEGELI und SCHWENDENER, 1877, Das Microscop, 2nd ed. p. 640; Quoted from Max Müller, 1903 191

NELSON, J. M., and W. C. VOSBURGH, 1917, Kinetics of invertase action. J. Amer. Chem. Soc., **39**, 790 116

NEUBERG, C., und A. HILDESHEIMER, 1911, Ueber zuckerfreie Hefegärungen. Biochem. Z. **31**, 170 44

NIEL, C. B. VAN, 1928, The Propionic Acid Bacteria. Haarlem, J. W. Boissevain 52, 62

NILSSON, R., 1930, Studien über den enzymatischen Kohlehydratabbau. Arkiv för Kemi, **10** A No. 7 39

NORRIS, D., see Penfold

NORTH, C. E., and AL., 1925, Publ. Health Bull. 147, U. S. Publ. Health Service 324

NORTHROP, J. H., 1923, The kinetics of trypsin digestion; II. J. Gen. Physiol. **6**, 423 116

———, see also Loeb

NORTON, J. F., and P. H. HSU, 1916, Physical chemistry of disinfection. J. Infect. Diseases **18**, 180 350

NYMAN, M., see Madsen

ORLA-JENSEN, S., 1919, Lactic Acid Bacteria. Copenhagen; Fred Host and Son 142, 152

PARSONS, L. B., and W. S. STURGES, 1927, Quantitative aspects of the metabolism of anaerobes. J. Bact. **14**, 181 60

———, see also Sturges

PARKS, G. S., see Baas-Becking

PASTEUR, L., 1860, Note relative au *Penicillium glaucum* et à la dissymétrie moléculaire des produits organiques naturels. Compt. rend. Acad. Paris **51**, 298 159

———, 1876, Etude sur la biére. Paris 5, 24

PAGE

———, 1913, Die Ernährungsphysiologie der Hefezelle bei der alkoholischen Gärung. Archiv f. Physiol. Supplement 1913, 1.....10, 12, 15, 40, 105, 109, 133, 383

RUPP, P., see Ayers

RUSS, S., see Browning

SAND, H. J. S., see Slator

SARKARIA, R. S., and B. W. HAMMER, 1928, Influence of temperature on the changes produced in milk by certain bacteria. J. Dairy Science **11**, 89..... 156

SATTLER, W., 1928, Untersuchungen über die Absterbegeschwindigkeit einiger für den Molkereibetrieb wichtiger Bakterien. Milchw. Forschungen **7**, 100..... 295, 320

SCHIERBECK, N. P., 1900, Ueber die Variabilität der Milchsäurebakterien in Bezug auf die Gärungsfähigkeit. Archiv f. Hygiene **38**, 294..... 151

SCHULTZ, J. H., und H. RITZ, 1910, Die Thermoresistenz junger und alter Coli-Bakterien. Centr. f. Bakt. I Abt, Orig. **54**, 283.. 289

SCHÜTZENBERGER, P., 1876, Les Fermentations. Paris, Librairie Germer Bailliére..... 12

SCHWENDENER, see Naegeli

SEYDEL, S., see Koch

SHERMAN, J. M., and W. R. ALBUS, 1923, Physiological youth of bacteria. J. Bact. **8**, 127..... 289

SKRABAL, A., 1916, Reaktionsgeschwindigkeit—Temperatur—Studien. Sitz. Ber. Akad. d. Wissensch. Wien, Math. naturw. Klasse II b, **125**, 259..... 321, 328

SLATOR, A., 1906, The chemical dynamics of alcoholic fermentation by yeast. J. Chem. Soc. **89**, 128..... 103, 114, 116, 122, 133, 135

———, 1913, The rate of fermentation by growing yeast cells. Biochem. J. **7**, 197..... 406

———, 1916, The rate of growth of bacteria. Trans. Chem. Soc. **109**, 2..... 190, 403

——— and H. J. S. SAND, 1910, The role of diffusion in fermentation by yeast cells. J. Chem. Soc. **97**, 922..... 107, 116

SMITH, J. H., 1921, The killing of Botrytis spores by phenol. Ann. Applied Biol. **8**, 27..... 284

———, 1923, The killing of Botrytis cinerea by heat. Ann. Applied Biol. **10**, 335..... 321

SMITH, THEOBALD, 1896, Reduktionserscheinungen bei Bakterien und ihre Beziehungen zur Bakterienzelle. Centr. f. Bakt. I Abt. **19**, 181..... 97

SEPTHENSON, MARJORY, 1930, Bacterial Metabolism. London; Longmans, Green and Co..... 57

PAGE

THÉNARD, L. J., 1803, Memoire sur la fermentation vineuse. Ann. Chim. Phys. **46,** 294 12

THIELE, 1896, Die Temperaturgrenzen der Schimmelpilze. Dissertation, Leipzig 157

THORNTON, H. R., and E. G. HASTINGS, 1929, Studies on the oxidation-reduction in milk. J. Bact. **18,** 293 and 319.. 86, 94, 98, 199

TOROSSIAN, C., see Hilliard

TRAUBE, J. 1919, Die physikalische Theorie der Arzneimittel- und Giftwirkung. Biochem. Z. **98,** 177 and 197 366

TRAUNER, R., see Rieger

TREECE, E. L., 1928, Gas production from commercial peptones by *B. aerogenes* and *B. coli.* J. Infect. Diseases **42,** 495 76

UNGER, T., see Lindner

UTKINA-LJUBOWZOWA, X., and O. STEPPUHN, 1930, Ueber die Identität der Zellproteasen verschiedensten Ursprungs. Biochem. Z. **220,** 41 66

VALLEY, G., and L. F. RETTGER, 1927, The influence of carbon dioxide on bacteria. J. Bact. **14,** 101 ..... 168, 203

VAN and VON, see corresponding names.

VOGEL, J. see Gerlach

VOSBURGH, W. C., see Nelson

WAKSMAN, S. A., and J. S. JOFFE, 1922, *Thiobacillus thiooxidans,* a new sulfur-oxidizing organism from soil. J. Bact. **7,** 239 345

WALDSCHMIDT-LEITZ, E., 1929, Enzyme actions and properties. New York, J. Wiley and Sons 116

WALKER, J. T. A., see Rideal

WALLACE, G. J., see Tanner

WARBURG, OTTO, 1912, Untersuchungen über die Oxydationsprozesse in Zellen, III. Münch. med. Wochenschr. **47,** 2550 33

———, 1921, Physikalische Chemie der Zellatmung. Biochem Z. **119,** 134 97

WARD, H. M., 1895, On the biology of *Bacillus ramosus* (Fränkel), a schizomycete of the river Thames. Proc. Royal Soc. London. **58,** 265 ..... 216, 219, 251

WATSON, H. E., 1908, A note on the variation of the rate of disinfection with change of the concentration of the disinfectant. J. Hygiene **8,** 536 ..... 346, 348

WATTERSON, ADA, 1904, The effect of chemical irritation on the respiration of fungi. Bull. Torrey Bot. Club **31,** 291 138

WEBER, H., see Meyerhof

WEBSTER, L. T., 1925, Biology of *Bact. lepisepticum.* J. Exper. Med. **41,** 571 202

WEHMER, C., 1913, Selbstvergiftung in Penicillium-Kulturen. Ber. d. Deutsch. Bot. Ges. **31,** 210 170

SUBJECT INDEX

D

E

F