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THE IMPORTANCE OF PHYSICAL-CHEMICAL FACTORS (pH AND rH_2) FOR THE LIFE ACTIVITY OF MICROORGANISMS

by

IRINA L. RABOTNOVA

[Following is the translation of a book, originally written in Russian with the title "The Importance of Physical-Chemical Factors (pH and rH_2) for the Life Activity of Microorganisms" by Irina L. Rabotnova, Professor of Microbiology in the Lomonossov University of Moscow, translated into German by H. Garisch Culmberger, revised and abridged by a group in the Institute of Microbiology (Institut fuer Mikrobiologie) of the Ernst Moritz-Arndt University, Greifswald: Dipl. Biol. Gottfried Bartsch (Chap. 4), Dipl. Chem. Lothar Gatzsche (Chap. 9), Dr. Johannes Gumpert (Chaps. 3 and 7), Dipl. Biol. Helmut Jaschhof (Chaps. 5 and 6), Dr. Manfred Koehler (Chap. 8), Dr. Brunhilde Marchlewitz (Introduction and Chap. 1), Dipl. Biol. Reinhard Suckow (Chap. 2), edited by Irina L. Rabotnova and W. Schwartz, with the authoress assuming the responsibility for the accuracy of the bibliographic entries, published in Jena by VEB Gustav Fischer Verlag, 1963, 226 pages.]

AUTHOR'S FOREWORD TO THE GERMAN EDITION

The present book, now in German translation, is the result of a combination of lectures, practical experience and research work as Professor of Microbiology in the Lomonossov University, Moscow, during the last fifteen years. It was published in the USSR in 1957. For the German edition, the text was provided with references to recent bibliography and supplemented with the results of recent research and was edited jointly by W. Schwartz and by the author.

IRINA L. RABOTNOVA.

FOREWORD TO THE GERMAN TRANSLATION

It is not simple to translate a scientific work, written in a foreign language and in the characteristic style of that language, so that it is also easily readable in its new style. This is especially true for translations from Russian. We worked hard, together with the author, for a good solution to this problem. If we have not always succeeded, we beg the reader's indulgence.

We have followed the last edition of Bergey's Manual, 1957, for type designation of bacteria and actinomycetes. We attempted to identify the types not found there by using the 1959 German edition of Krassilnikov's Diagnosis of Bacteria and Actinomycetes. Types whose designation is not ascertainable either from the Manual or in Krassilnikov, are indicated in the footnotes.

Sincerest appreciation is expressed to the science collaborators and technical assistants, who participated in one way or another in the work of translation, as well as to the VEB Gustav Fischer Verlag, Jena.

W. SCHWARTZ.

Greifswald, August 1962.

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INTRODUCTION

Two kinds of metabolism can be distinguished in microorganisms as in all other forms of life:constructive metabolism by which body substances are synthesized, and destructive metabolism by means of which energy is provided for maintaining the structure of the living cells and for the synthesis of new body substances.

Originally it was believed that the two kinds of metabolism were quite independent of each other. According to this point of view the release of energy consumed for predominantly endothermic constructive processes depends only on the energy processes.

Tausson (1950) observed that there is a relationship between dissimilatory and assimilatory metabolism. The products of the incomplete oxidation of the substrate are, therefore, not only valuable for the organism, because a certain amount of energy is released by their formation, but they are also important as building material for the construction of the body substances. Thus, for example, they act as intermediate products in the formation of sugar molecule compounds that serve as starting material for the synthesis of amino acids. The complete oxidation of the substrate is not necessary for the utilization of the greatest possible amount of energy. The products of incomplete oxidation can serve synthesis as "semi-manufactured" items for whose assimilation slighter amounts of energy are required.

Clifton (1946, 1952) also sees, in biological synthesis, a series of reactions in which intermediate products from the catabolism of energy producing compounds participate. According to Clifton, the provision of compounds for constructive metabolism is more important than the total amount of released energy in the catabolism of organic compounds in respiration or fermentation metabolism. The acetyl group is one of the most important building materials of this origin. Synthetic and catabolic processes are, consequently, related to each other not only according to the balance of energy, but also according to stoichiometric relationships. The released energy is only a "by-product," similar to water and carbon dioxide. Veselov and his collaborators (1954, 1955) developed the ideas of Tausson, Clifton and others further.

Synthesis does not always occur with utilization of the energy content of organic compounds, as is the case with carbon heterotrophs. In carbon autotrophic microorganisms the reduction of CO_2, with which assimilation is started, is tied to the supply of energy. Photoautotrophic organisms are able to utilize light energy for this purpose. Chemoautotrophic microorganisms use up the energy released by the oxidation of simple organic or inorganic compounds (for example, ammonia, hydrogen sulfide, molecular hydrogen,methane).

Schaposchnikov (1944, 1955) referred to the fact that also in ferment organisms proteins, as very important body substances, are more strongly reduced than the most utilized resulting substrate carbohydrate. Here also, as with carbon autotrophes, the same principle

obtains: the energy converted into destructive metabolism is utilised for the partial reduction of waste matter.

The relationship between energy producing dissimiliatory metabolism and systhesizing assimilatory metabolism became more evident with the discovery of energy-rich organic phosphoric acid compounds. The energy obtained by the respiration or the fermentation processes is primarily stored up in adenosine triphosphate (ATP). This substance and some others are a reservoir of energy for the various energy producing processes, from which the organism draws according to its needs (Engelhardt, 1945). ([Note:] Adenosine triphosphate (ATP) results from the diphosphate (ADP) by taking on a third molecule of phosphoric acid. The reaction requires a great expenditure of energy that is supplied from the exothermic processes of metabolism. In enzymatic hydrolysis 12,000 cal per Mol of released energy can be used directly by the organism.). The same holds also for carbon autotrophes (Vogler and others, 1941, 1943; cf. Rabotnova, 1946).

A second group of this kind of substances are the polyphosphates. They are present in the cells partly in a free state, and partly bound to organic substances (nucleic acid) (Beloserski and others, 1957).

The close relationship between constructive and destructive metabolism also results from the experiments of Schaposchnikov (1955) on the course of fermentation processes. If the behavior of the population is related to the fermentation process, two phases, clearly demarcated from each other, can be distinguished. The first phase, that extends up to the end of the "log phase" of the growth curve, is characterized by the intensive multiplication of the cells. In the second phase the number of cells no longer increases, and it can even fall off, although the fermentation process continues.

In the first phase sugar, that must be reduced, is consumed by the synthesis of cell albumin; consequently, fermentation products that are more strongly oxidized than sugar result. When cell multiplication decreases in the second phase, relative strongly reduced compounds also appear among the fermentation products. Schaposchnikow first of all established the two phases by means of acetone-butanol fermentation; later, also by means of heterofermentative lactic acid fermentation, propionic acid, butyric acid and acetone-ethanol fermentation. Obviously it is a question of a general phenomenon with fermentation processes.

The usual procedure to date for the production of energy supplying metabolic reactions in the form of chemical equations with a statement of the amount of calories is, therefore, one-sided. Metabolic reactions, rather, should be described so that the relationships between synthesizing and energy providing processes are evident from them. There already are experiments of this type, as, for example, in the monographs on the physiology of bacteria by Oginsky and Umbreit (1954) and by Clifton (1957).

The interrelations of anabolism and catabolism in aerobes give an idea of "oxidation assimilation". This description was first applied by Barker (1936) in studying the metabolism of the colorless algae Prototheca. An equation that includes both the

oxidative (energy providing) and the synthesizing (constructive) process can be drawn up from the results of quantitative determinations of the consumed sugar or of another substrate, of the consumed O_2 and of the CO_2 given off, as well as of the newly-formed cell substance. Barker gives the following equation for the utilization of acetic acid by means of Prototheca:

$$CH_3COOH + O_2 \rightarrow CO_2 + H_2O + \underset{\text{body substance}}{(CH_2O)}$$

Prototheca is especially suitable for this type of experiments, because it forms its body substance extremely economically; that is to say, a considerable part (50% to 80%) of the substrate utilized (alcohol, sugar, organic acids or glycerin) is used for forming the body substance.

Similar relationships were discovered also later by Clifton (1952) in a series of other microorganisms -- in Bac. subtilis, Esch. coli, Pseudomonas and in yeasts -- on various substrates.

Three types can be distinguished in microorganisms, according to Schaposchnikov (1944):

Simple oxidation of the substrate without decay of the carbon cycle of the utilizedd substance.

Anaerobic desmolysis; the carbon cycle of the substrate is broken down in connected redox processes into reduced and oxidized portions.

Aerobic (oxidative) desmolysis; break-down of the carbon cycle of the substrate connected with oxidation of the products of desmolysis by means of air oxygen.

The individual types of microorganisms may carry out one or several of these processes. The facility with which the modification is performed depending on the culture conditions is typical of many microorganisms.

Simple oxidations are caused by obligate aerobes. Acetic acid bacteria, for example, belong to this group. Their typical reaction lies in the oxidation of the alcohol or carbonyl group into the acid- or carboxyl group; in addition, gluconic acid bacteria from Pseudomonas fermentation oxidize glucose into gluconic acid. ([Note:] Here also metabolic processes are possible, that correspond to another type. Acetic acid bacteria, for example, may oxidize acetic acid into CO_2 under certain conditions.)

The chemoautotrophic bacteria must also be included here. They utilize energy from the direct oxidation of a series of mostly inorganic substances (NH_3, Fe^{++}, H_2, H_2S) for the reduction of carbonic acid that is sufficient as the only source of carbon for the formation of organic body substance.

As already mentioned (Vogler, cf. Rabotnova, 1946), energy-rich phosphorus compounds are also present in the oxidation process. They can be used as needed by the cells as a source of energy, al-

though it is doubtful that they can support life, if oxidizable substances are lacking. ([Note:] Boemeke (1939) established an endogenous respiration in nitrifying bacteria with the utilization of organic reserve material. The conditions, therefore, are similar to the ones found in green plants that change organic compounds (reserve material) in catabolism, whereas light energy can only be used in anabolism as chemical energy.)

There are comparatively few microorganisms that are exclusively capable of a connected oxidation-reduction reaction without additional hydrogen acceptors. Several groups of obligate anaerobic bacteria must be mentioned here; for example, the large group of butyric acid bacteria to which pectin and cellulose enzymes belong, and the anaerobes of albumin decomposition (Stephenson, 1952). The homofermentative lactic acid bacteria likewise belong here; in some cases they are able to develop also as facultative aerobes.

Obligate aerobic desmolysis is characteristic of bacteria of the Azotobacter group that oxidizes a great number of various organic compounds, among others sugar, alcohols, acids, into CO_2 and H_2O. Almost no intermediary products are collected in the culture medium. This type of metabolism corresponds to respiration in animals and plants. Aerobic decomposition bacteria, by which albumins are oxidized, and many Actinomyces behave in like manner.

Most heterotrophic microorganisms are capable of performing various types of metabolism. With access to air they produce oxidation processes that are different, in part, from typical respiration; when deprived of air, they can cause fermentations. Bacteria of the coli-aerogenes type display this behavior. Depending on the culture conditions, they can cause purely aerobic processes, as well as fermentation processes.

Yeasts and some other plants, that support a real oxidation metabolism with a good supply of air, pass over to alcoholic fermentation when deprived of air. When oxygen is included citric acid fermentation occurs, for example.

Denitrifying bacteria live as well under anaerobic as under aerobic conditions. In the first case the oxygen in the nitrates serves as a hydrogen acceptor in the oxidation of organic carbon compounds. Therefore, it would be more accurate to classify the denitrifiers as oxidative microorganism, for they also produce no fermentation with anaerobiosis.

Hydrogen bacteria also oxidize molecular hydrogen, in addition to organic substances. According to Belyayeva (1954), they make use, depending on the conditions under which they live, of O_2, NO_3, S, CO and CO_2 as hydrogen acceptors.

CO_2 can be used by microorganisms as a hydrogen acceptor in the oxidation of organic substances. Thus, for example, some methane bacteria are able to oxidize organic acids with the help of CO_2 as a hydrogen acceptor releasing CH_4 (Baker, 1949).

In many cases, we have succeeded in engaging experimentally

during the course of the fermentation processes in research that has contributed substantially to the explanation of the biochemical relationships. Let us only recall the work of Neuberg to whose importance Omelyanski already called attention in 1926.

The addition of bisulfite to the culture medium has been successful in controlling the alcoholic fermentation of yeast so that glycerin is accumulated.

Acetone-butanol bacteria oxidize the medium primarily by means of fermentation. Predominantly neutral fermentation products develop concurrently: Ethanol, butanol and acetone. When the medium is neutralized with chalk, on the other hand, acid formation predominates.

Therefore, it is possible to modify the course of the metabolic processes by changing the environment.

There are many environment factors. Most important of all are the quantity and quality of the source of energy, the supply of fermentation agents with carbon, nitrogen, mineral salts, vitamins, trace elements, etc. For each type of microbe there are different requirements that have to be determined separately for each case.

On the other hand, there are environment conditions of a general type: Physical-chemical nature of the culture medium, especially the pH value and the redox potential, in addition to temperature, light conditions, osmotic value, etc. They influence the life activity of all microorganisms and are to be taken into account if we want to interfere with the metabolic processes.

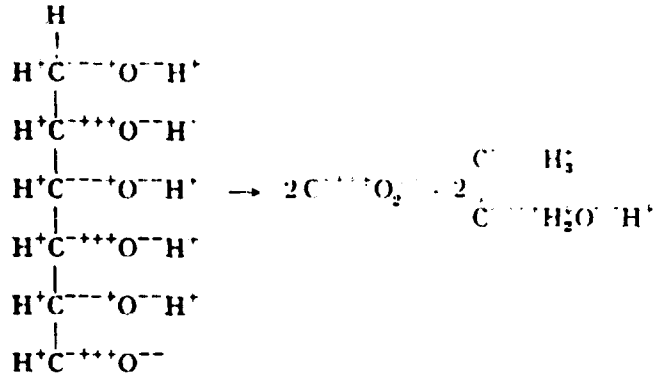
The experimental influence and control of the redox conditions in the medium deserve special attention, for they are related to the oxidative and reductive reactions of metabolism.

The relationships become clear if the reactions of metabolism are considered from the point of view of the electron theory to whose importance for biochemical processes Uspenski, for example, has referred. ([Note:] Unpublished lectures in the Moscow State University, 1933.) In completely oxidized anhydride carbonic acid all the valency electrons of the carbon are linked to oxygen. The carbon atom has become carrier of four positive charges, whereas oxygen that has taken on the carbon electrons has a negative charge: $C^{++++}(O^{--})_2$.

In completely reduced methane, carbon not only retains its electrons, but also takes on the electrons from four hydrogen atoms and thus is converted into a carrier of four negative charges: $C^{----}(H^{+})_4$.

Part of the carbon atom in sugar is more strongly oxidized, part more weakly. Some atoms may be completely oxidized, which leads to a complete reduction of other atoms and to the release of a certain amount of energy that can be used in fermentation.

Alcoholic fermentation can, for example, be represented as follows:



At the same time, the transfer of electrons leads to the formation of potentials on electrodes that are immersed in the solution. Consequently, there are close relationships between the oxidation-reductions in the culture medium and the potential of the electrodes that we measure as the redox potential of the medium.

However, in order to be able to understand the operation of any given factor, quantitative data are necessary. We can express acidity or alkalinity quantitatively by means of the pH value. Concerning the redox potential, there are still more differences in opinion. We are usually satisfied with the basically inaccurate distinction between aerobic and anaerobic conditions. Aerobic conditions are present with free access to air. However, if reducing substances are present in the medium, even when the medium is in contact with air, the conditions are so little aerobic that anaerobes can also live. On the other hand, if oxidizing substances (hydrogen acceptors) are present in the medium, aerobes can also live without access to air.

The redox potential is the measuring scale for the redox conditions. Usually the electrical tension serves as a measure; it is measured on the electrodes immersed in the experimental fluid. The rH_2 index by means of which the oxygen-hydrogen ratio is characterized is analogous to the pH value that indicates the proportion of H^+ and OH^-. Since the H_2/O_2 ratio is related to the presence of all other oxidizing and reducing substances in the culture medium, rH_2 characterizes the entire redox condition of the medium, that is, the direction of the electron transfer

and the oxidative or reductive condition of the medium. γH_2 gives, in a scale from 0 to 40, all degress of reduction or oxidation of the medium from saturation with hydrogen to saturation with oxygen.

The concept of the redox potential has existed in physical chemistry for a longer time. In the 1920's its application to biological subjects was resorted to in numerous experiments on the redox conditions in the culture medium and in living cells.

It is shown in that way that carbohydrates are broken down into energy supplying processes according to the conditions present in the medium. Under strongly oxidative conditions glucose, for example, supplies, in the presence of free oxygen, the maximum energy contained in it. Under reductive conditions without access to air that portion of energy is released that can be obtained by the transfer of electrons within the molecule. These conditions are expressed quantitatively by means of the size of the γH_2 value.

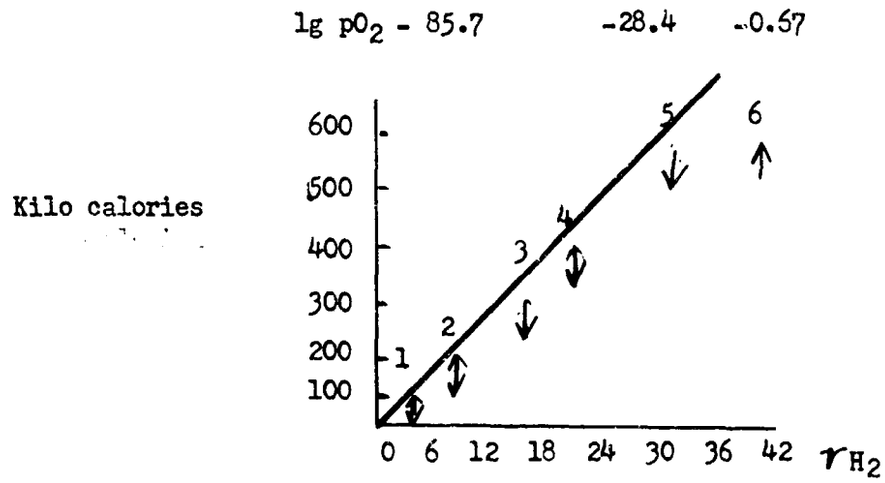


Fig. 1. The γH_2 dependency on the energy released by the decomposition of glucose (according to Wurmser, 1926). [In all numerical values, substitute decimal point for comma.)

1. Bacteria suspension. 2. Intracellular conditions with anaerobes. 3. Intracellular conditions in plant cells. 4. The same in animal cells. 5. Steam. 6. Air.

The connections become clear if the dependency of the heat of combustion of the glucose on the γH_2 factor is represented graphically (Fig. 1). While by the oxidation (combustion) of glucose in air 675 calories are released per gram-molecule of glucose, with $\gamma H_2 = 17$ (the value that we find inside of cells) not 674 but rather only 240 calories are available. Under these conditions it is more economical for microbes that have suitable catalyzers available not to oxidize the glucose but rather to ferment it.

Uspenski (1936) successfully conducted research on controlling metabolic processes with the aid of the γH_2 factor (Kusznezov, 1932, cf. Chap. 7).

The hydrogen ion concentration, expressed in the pH value, is the second most important factor for the operation of the metabolistic processes. Fermentation activity and dissociation of metabolic products depend on the pH value, for example. This will be gone into more in detail later.

The remaining general factors of the environment -- temperature, rays of different wavelengths, osmotic pressure, and others -- have not yet been sufficiently investigated in order to be able, at present, to give a somewhat exhaustive description of their manner of operation. However, here also there are examples of the fact that the influence of these factors is important for the biochemical activity of microorganisms.

Thus Manteyfel and Antischeva (1935) showed with the example of acetone-butanol fermentation that the formation of fermentation products is influenced by changes in temperature: the lower the temperature, the more butanol is formed. At 20° to 25°C, 12 to 13 grams per liter are obtained; at 40° C., only 7 grams per liter, while alcohol and acetone are produced always in equal amounts. We have observed similar phenomena. Disarrangements in the percentage portion of the individual fermentation products appeared already with from 5° to 6°C. changes in temperature. On the other hand, the speed of fermentation was scarcely affected.

Streptoc. lactis forms lactic acid principally at 30° to 37° C; at 15° to 20° C. by-products, among others acetyl-methyl carbinol (Bang, 1949), develop in considerable amounts.

The physiological effect of rays of various wave-lengths is known. The bactericide, fungicide and mutational effect of short-wave radiation has been investigated in numerous studies. As with other factors, it may be assumed that by decreasing the dose to the extent that the multiplication of cells is not affected, other effects are evident with this or that life function, especially if the dose is chosen so low that the cells undergo a lasting effect.

Visible light is indeed important for the metabolism of microorganisms, also if the effect of the UV section is disregarded. A number of components of the cells absorbs in the visible part of the spectrum: cytochromes are an example of this.

As is well-known, light is not an unimportant factor for moulds (Tatarenkov, 1954; Foster, 1949). It affects conidiospore formation and growth of mycelia. The effect can be inhibitive or stimulating, according to intensity, wavelengths and type of fungus (Hawker, 1950).

Experiments on the influence of light on the fermentation processes appear to be lacking up until now. It is possible that here also relationships can be established.

The following chapters will attempt to summarize all that has been learned about the two culture medium factors, pH and rH_2 that have been investigated in particular detail, about their control and about their effect on certain metabolic processes.

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CHAPTER 1

DETERMINATION OF THE HYDROGEN ION CONCENTRATION

Research work in the field of metabolic and trophic physiology is unthinkable without considering the hydrogen ion concentration.

Soerensen (1909) proposed characterizing acidity and alkalinity by means of the pH value; that is to say, by means of the negative logarithm of the H^+ concentration. In this way any given reaction — from very acid solutions, normal solutions of strong mineral acids ($pH \approx 0$) to strongly alkaline solutions of normal NaOH or KOH (pH 14) — can be described.

In practice, there are two common methods for pH determination: the colorimetric and the electrometric (Kordatzki, 1949; Mislovitzer, 1928).

I. Colorimetric Determination of the Hydrogen Ion Concentration.

Indicators are used for the colorimetric determination of the pH value; that is to say, substances that react to changes in the pH value within definite ranges with a change in color or with decoloration.

Indicators are organic compounds with characteristics of weak acids or weak bases. As ions they have another color than in the undissociated state. The degree of dissociation of the indicator depends on the pH value. If, for example, the indicator is a weak acid, then it shows up as a free acid in an acid medium; its solution has the color of the undissociated molecule. In an alkaline medium, it appears as a dissociated salt and gives the solution the color of the ion. In between there is a transition area whose color is contingent on the juxtaposition of dissociated and non-dissociated molecules. The pH ranges in which the individual indicators change differ, depending on their dissociation constants. In order to cover the entire pH scale from 0 to 14, series of indicators are, therefore, necessary; for example, the two-colored indicators of Clark and Lubs (Table 1) and Michaelis's one-colored indicator (Table 2).

With proteins, whose decomposition products or salts are present in the material being examined, the change range of the indicators is modified to a small extent: lack of albumin or salt causes it. Thus, for example, the pH value of bromcresol purple in a 3% NaCl solution is about 0.25 pH units higher with a color change than in pure water. On the other hand, the color change with thymol blue is about 0.17, with phenolphthalein about 0.17 and with methyl orange about 0.02 pH units lower than in pure water; likewise with neutral red in a 20% salt solution about 0.05 pH units.

TABLE 1

Two-colored indicators (according to Clark and Lubs)

Indicator	Color change	Change range pH	Indicator solution in ml. 0.1N NaOH*
Thymol blue	red-yellow	1.2 - 2.8	2.1
Bromphenol blue	yellow-blue	3.0 - 4.6	1.5
Bromcresol green	yellow-blue	3.8 - 5.4	1.4
Methyl red	red-yellow	4.4 - 6.0	(alcoholic solution)
Bromcresol purple	yellow-purple	5.2 - 6.8	1.8
Bromthymol blue	yellow-blue	6.0 - 7.6	1.6
Phenol red	yellow-red	6.8 - 8.4	2.8
Cresol red	yellow-red	7.2 - 8.8	2.6
Thymol blue	yellow-blue	8.0 - 9.6	2.1
Cresolphthalein	colorless - red	8.2 - 9.8	(alcoholic solution)

*In making up the indicator solution 0.1 g of indicator is pulverized in a mortar with the specified amount of N/10 NaOH and is dissolved in 250 ml of distilled water. A 0.04% alcoholic solution of the indicators may also be used.

TABLE 2

One-colored indicators (according to Michaelis, 1929)

Indicator	Color change	Change range pH	Solution
α - dinitrolphenol	colorless-yellow	2.2 - 4.0	0.1 g + 300 ml water
β - dinitrophenol	colorless-yellow	2.8 - 4.5	0.1 g + 200 ml water
γ - dinitrophenol	colorless-yellow	4.0 - 5.5	0.1 g + 200 ml water
p - nitrophenol	colorless-yellow	5.2 - 7.0	0.1 g + 100 ml water
m - nitrophenol	colorless-yellow	6.7 - 8.4	0.3 g + 100 ml water
Phenolphthalein	colorless-red	8.5 - 10.5	0.04 g in 30 ml alcohol + 70 ml water
Alizarin yellow GG (salicyl yellow)	colorless-yellow	10.0 - 12.0	0.05 g in 5 ml alcohol + 50 ml water

Among the commonly used indicators, the following should be mentioned: methyl violet whose range of change is between pH 0.1 and 3.2; methyl orange between pH 3.1 and 4.4; neutral red between pH 6.8 and 8.0. They are used in a 0.04% aqueous solution.

Buffer solutions are standard solutions with a known hydrogen ion content. Weak acids are weakly dissociated in an aqueous solution, whereas alkali salts completely dissociate these acids. If solutions of weak acids and their alkali salts are mixed, the dissociation of the acids changes depending on the salt concentration. With an increasing salt concentration, acid dissociation decreases. The H^+ concentration in such a mixture is determined by means of the following equation:

$$[H^+] = K \frac{[acid]}{[salt]} \qquad (1)$$

in which K is the dissociation constant of the acid.

If different amounts of salt and acid are mixed, the H^+ concentration changes:

$$K = \frac{[anion] \cdot [H^+]}{[non\text{-}dissociated\ part]} \qquad (2)$$

With an equal concentration of acid and salt the concentration of the hydrogen ion of the acid dissociation constant is numerically equal:

$$[H^+] = K \qquad (3)$$

while the pH value corresponds to the negative logarithm of the dissociation constant:

$$pH = \log K \qquad (4)$$

The dissociation constant of acetic acid behaves like that at 25° C. $1.86 \cdot 10^{-5}$; its negative logarithm is 4.73. In this way a solution with a pH value of 4.73 results by mixing equimolar amounts of acetic acid and sodium acetate. Since not all ions are found in an "active" state in solutions of strong electrolytes, the actual concentration must be multiplied by the activity factor α in order to arrive at the concentration of the active ions. The factor is approximately 1, in strongly diluted solutions (for example 0.01N) exactly 1. Taking this fact into account equation (1) reads:

$$[H^+] = K \frac{[acid]}{[salt] \cdot \alpha}$$

For an 0.1N acetate solution $\alpha = 0.79$ and the pH value of the acetate mixture then is not 4.73 but 4.62. ([Note:] Preparation of the standard acetate solution according to Michaelis: mix 50 ml of normal NaOH, 100 ml of normal acetic acid and 350 ml of distilled water.) If more acid than salt is

present, the pH value becomes lower than 4.62. If less acid is present the pH value is higher. A buffer mixture includes only a small part of the pH scale, specifically about 1.5 units under and over that pH value resulting from the mixture of equal amounts of acid and salt. In order to cover the entire scale of pH values, some batches of buffer solutions with different dissociation constants of the acids must be used (Tables 3 and 4).

The concentration of the hydrogen ions becomes substantially smaller, as is to be expected theoretically, only by diluting one thousand times or more. By diluting one hundred times an N/10 or N/100 solution the pH value is changed only about 0.1. Therefore, only the correlations of equal normality are given in Table 3, in which case can be any convenient amount within broad limits.

Buffer solutions exhibit two peculiarities: the H^+ concentration does not change when the buffer mixture is diluted with water, because the pH value depends only on the correlations of the acid and the salt and not on their absolute quantity. Acid dissociation increases with dilution and the H^+ concentration is held at the same level.

TABLE 3

pH values of various buffer solutions

Mixture of equimolar solutions of two buffer substances in a proportion of	Tartaric acid + sodium tartrate	Acetic acid + sodium tartrate	KH_2PO_4 + $Na_2HPO_4 \cdot 7\ H_2O$	NH_4Cl + NH_4OH
32: 1	1.4	3.2	5.3	8.0
16: 1	1.7	3.5	5.6	8.3
8: 1	2.0	3.8	5.9	8.6
4: 1	2.4	4.1	6.2	8.9
2: 1	2.7	4.4	6.5	9.2
1: 1	3.0	4.7	6.8	9.5
1: 2	3.3	5.0	7.1	9.8
1: 4	3.6	5.3	7.4	10.1
1: 8	3.8	5.6	7.7	10.4
1:16	4.2	5.9	8.0	10.7
1:32	4.5	6.2	8.3	11.0

The second peculiarity of buffer solutions is expressed by adding acids or alkalis. For example, an acetate mixture contains sodium ions, CH_3COO ions and a known amount of hydrogen ions, that can be controlled by dissociation of the acetic acid. If hydrochloric acid is added, H^+ and Cl^- are also present in the solution. The acetate ions of the completely dissociated sodium acetate that encounter the hydrogen ions of the strong acid combine immediately and form acetic acid. Only the hydrogen that is caused by the dissociation constants of the acetic acid remains in the form of ions.

The strongly dissociated hydrochloric acid restrains the dissociation of the acetic acid and the pH value is not changed as

greatly as the correlation of the acetic acid and the salt is changed. If we add 1/100 mol of hydrochloric acid to an M/10 solution of an acetate buffer mixture, the pH value only changes from 4.62 to 4.55. However, if we put the same amount of HCl in water, the pH value drops to 2.02. The buffer action depends, therefore, on the CH_3COO ions contained in the solution; that is to say, on the amount of salt. In practice a buffer solution acts only until half or at the most 2/3 of the anions are released. The proportions are similar if alkali, for example NaOH, is added. The change in pH is, moreover, much smaller than with the addition of the same amount of alkali to water.

In microbiological practice buffer solutions are used for the following purposes: as standard solutions for the colorimetric determination of the H^+ concentration and for the maintenance of a definite pH level in cultures of microorganisms. In the first case solutions diluted from M/10 to M/20 are used; in the second case stronger, M/3, solutions are used.

If the pH value of the solution being examined is completely unknown, an approximate pH determination is undertaken with the aid of an indicator substance or with indicator paper.

TABLE 4

Dissociation constants of acids frequently used in microbiology (according to Kordatzki 1949)

acid	K	pK	T
Boric acid	$6 \cdot 10^{-10}$	9.2o	25°
Carbonic acid			
1st dissociation	$3 \cdot 10^{-7}$	6.51	18°
2nd dissociation	$6 \cdot 10^{-11}$	10.20	18°
Phosphoric acid			
1st dissociation	$1 \cdot 10^{-2}$	2.0	25°
2nd dissociation	$2.9 \cdot 10^{-7}$	6.71	25°
3rd dissociation	$3.6 \cdot 10^{-13}$	12.44	25°
Sulfuric acid			
2nd dissociation	$1.6 \cdot 10^{-2}$	1.8	25°
Sulfurous acid			
1st dissociation	$1.7 \cdot 10^{-2}$	1.8	25°
2nd dissociation	$5 \cdot 10^{-6}$	5.3	25°
Hydrogen sulfide	$6.3 \cdot 10^{-8}$	7.20	18°
Formic acid	$2 \cdot 10^{-4}$	3.70	18°
Acetic acid	$1.8 \cdot 10^{-5}$	4.75	25°

Lactic acid	$1.4 \cdot 10^{-4}$	3.85	25°
Oxalic acid			
1st dissociation	$4.0 \cdot 10^{-2}$	1.40	25°
2nd dissociation	$4.1 \cdot 10^{-5}$	4.39	25°
Phenol	$1.2 \cdot 10^{-10}$	9.91	25°
Picric acid	$1.6 \cdot 10^{-1}$	0.80	25°
Racemic acid	$1 \cdot 10^{-3}$	3.00	25°
Trichloroacetic acid	$1.3 \cdot 10^{-1}$	0.90	18°
Tartaric acid			
1st dissociation	$1 \cdot 10^{-3}$	3.0	25°
2nd dissociation	$5 \cdot 10^{-5}$	4.3	18°
Citric acid			
1st dissociation	$8.3 \cdot 10^{-4}$	3.08	25°
2nd dissociation	$4.1 \cdot 10^{-5}$	4.39	25°
3rd dissociation	$3.2 \cdot 10^{-6}$	5.49	25°

If the pH value has to be determined exactly to 0.1, a standard buffer solution must be used:

M/10 buffer solutions with pH values that are in the range of the pH zone of the test solution are put in test tubes with 5 ml markers. A small tube is to be filled with 5 ml of the material to be tested. Four to five drops of the appropriate indicator are added to each tube. Solutions with a scale of transition colors that are compared with the colors of the test solution in a comparator are kept.

Gillespie's method without buffer solutions. The principle of this method is based on the combination of acid and alkaline standard solutions with different amounts of indicator. These solutions form a color scale. Each standard consists of two test tubes; one contains a definite amount of indicator with an excess of acid, the other one with an excess of alkali. These two test tubes are observed in the comparator against the light (Fig. 2). Usually the two-colored indicators of Clark and Lubs are used. A definite pH value corresponds to each tint. The values are given in Table 5. The method is accurate up to 0.1 pH.

The figures in Table 5 are the result of the following calculation:

$$pH = pK + \lg \frac{a}{10-a}$$

in which a is the number of the indicator drops in the acid and pK the dissociation constant of the indicator.

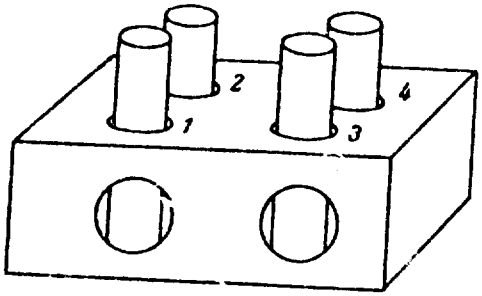

Figure 2. Comparator for the colorimetric determination of the pH value.

1. Standard solution with indicator.
2. Test solution, turbid or colored.
3. Test solution with indicator.
4. Water.

The stability of the standard solutions is very slight in Gillespie's method due to carbonic acid absorption.

Thymol blue has two change ranges. In an acid medium with pH 1.2 to 2.8 from red to yellow and in an alkaline medium with pH 8.00 to 9.6 from yellow to blue. Therefore, by using thymol blue in the acid series, not acid but rather a 1% KH_2PO_4 solution or distilled water is added.

TABLE 5

pH values of standard solutions (according to Gillespie)

No. of pairs of test tubes	Number of indicator drops alkaline	acid	Bromo phenol blue	Methyl red	Bromo-cresol purple	Bromo thymol blue	Phenol red	Cresol red	Thymol blue
1	1	9	3.1	4.05	5.3	6.3	6.7	7.15	7.85
2	2	8	3.5	4.4	5.7	6.5	7.1	7.5	8.2
3	3	7	3.7	4.6	5.9	6.7	7.3	7.7	8.4
4	4	6	3.9	4.8	6.1	6.9	7.5	7.9	8.6
5	5	5	4.1	5.0	6.3	7.1	7.7	8.1	8.8
6	6	4	4.3	5.2	6.5	7.3	7.9	8.3	9.0
7	7	3	4.5	5.4	6.7	7.5	8.1	8.5	9.2
8	8	2	4.7	5.6	6.9	7.7	8.3	8.7	9.4
9	9	1	5.0	5.95	7.2	8.0	8.65	9.05	9.75

II. Electrometric Determination of the Hydrogen Ion Concentration

Electrodes are used for the electrometric determination of the pH value. The theory of the determination of the H^+ concentration can be briefly described as follows: If a metal rod is immersed in water, part of the metal goes in solution in the form of positive ions. The metal rod retains a negative charge and is able to attract cations so that there is a difference of potential between the metal and the solution. The size of the potential difference depends on two factors: on the solution pressure of the metal, therefore, on th tendency of the metal to go into solution, and on the osmotic pressure of the solution. If both factors are equal in size, there is no potential difference. As the size of the difference between both factors increases, the electrical potential goes up. If, for example, two solutions of a salt are used in different concentration, the metal electrodes immersed in both solutions have different potentials. The potential difference, measureable according to Nernst, has the following relation to the salt concentration:

$$E = \frac{RT}{nF} \ln \frac{C_2}{C_2}$$

in which E is the measurable potential difference; C_1 and C_2 are the concentrations and RT/nF is the Nernst constant. Finally it includes the gas constant (R), the absolute temperature (T), the valence of the ions (n) and the electrochemical equivalent (F).

By calculating the Nernst constant for 18° C. and transcribing the natural logarithm in the decimal system, we get the quantity 0.0577.

The following dependency of the Nernst constants on the temperature was determined:

16° C. 0.0573	24° C. 0.0589	32° C. 0.0604
18° C. 0.0577	26° C. 0.0593	34° C. 0.0608
20° C. 0.0581	28° C. 0.0597	36° C. 0.0612
22° C. 0.0585	30° C. 0.0600	38° C. 0.0616
		40° C. 0.0620

If the potential difference has been measured and a concentration is known, the others can be calculated. This method is often used in electrochemistry. It is also used for determining the H^+ concentration.

Since hydrogen is close to metals in its characteristics, we assume an electrode composed of hydrogen. If this electrode is immersed in a solution containing hydrogen ions, the hydrogen ion concentration can be determined with the method outlined above. An electrode that acts like a hydrogen electrode can be made by saturating a platinum or gold disk with hydrogen. As precious metals platinum and gold behave indifferently, if they are immersed in the solution; that is to say, they give up no ions in the solution, but on the contrary they adsorb hydrogen easily. An

electrode saturated with hydrogen behaves like a pure hydrogen electrode. In order that the platinum may adsorb a great amount of hydrogen, it is covered with a coating of platinum black that enlarges the area of the electrode considerably.

If this electrode is immersed in a solution containing a gram equivalent of hydrogen ions per liter, a so-called hydrogen electrode is obtained for comparative purposes. The normal hydrogen electrode has a potential of 0.276 volts in relation to the zero electrode. For the sake of simplicity its potential is assumed to equal zero.

If the difference in potential is measured, the unknown hydrogen ion concentration is calculated in accordance with Nernst's equation:

$$E = 0.0577 \lg \frac{2}{[H^+]} \qquad pH = -\lg [H^+]$$

$$pH = \frac{E}{0.0577} \qquad \text{(for 18° C.)}$$

The normal hydrogen electrode finds no application in practice. An electrode with a stable potential is selected in place of it, one that differs from the normal hydrogen electrode by a definite amount and that permits more convenient operations, for example a saturated calomel electrode (Fig. 3).

The electrolyte contains a specific amount of mercury ions given off from the weakly water-soluble calomel, Hg_2Cl_2. In addition to Hg_2Cl_2 there is a second electrolyte, KCl, in the solution, affecting the dissociation of the calomel:

$$K = \frac{[Hg^+] \cdot [Cl^-]}{[HgCl]}$$

If the number of chlorine ions in the calomel solution is increased by adding KCl, the number of mercury ions decreases and the negative charge of the electrode becomes smaller. The size of the potential on such an electrode depends on the KCl concentration. If KCl is chosen in a specific concentration, a specific potential of the calomel electrode is obtained. Saturated KCl solutions are used in practice.

The potential is transferred from the metallic mercury to the platinum electrode immersed in the mercury and is connected with the rest of the apparatus.

The calomel electrode is connected with the experimental electrode by means of a saturated KCl solution.

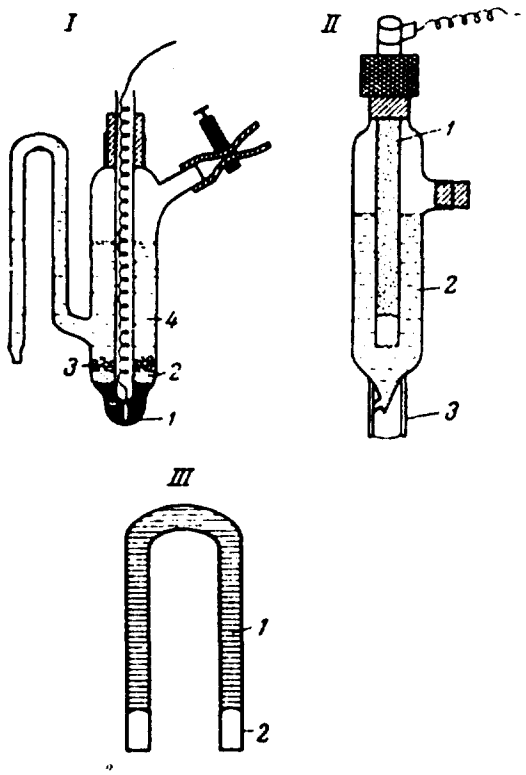

Figure 3. Calomel electrodes and a U-tube with KCl-agar.

I. Calomel electrode:
1. Mercury.
2. Calomel layer.
3. KCl crystals.
4. Saturated KCl solution

II. Sketch of a calomel electrode used for P-5 and LP-4 potentiometers:

1. Calomel paste with immersed electrode.
2. Saturated KCl solution.
3. Connection with the solution being examined.

III. U-tube with agar:
1. Agar with KCl.
2. Filter paper stoppers.

The potential of the calomel electrode changes slightly depending on the temperature. With varying temperatures it diverges from the normal hydrogen electrode by the following values:

°C.	volts	°C.	volts
15	0.2525	22	0.2475
16	0.2517	23	0.2468
17	0.2509	24	0.2463
18	0.2503	25	0.2458
19	0.2495	37	0.2355
20	0.2488	38	0.2350
21	0.2482		

Quinhydrone electrodes are mostly used in practice in place of the hydrogen electrode as experimental electrodes. Quinhydrone breaks down in an aqueous solution into quinone ($C_6H_4O_2$) and hydroquinone ($C_6H_4O_2H_2$), finally into quinone and hydrogen:

$$C_6H_4O_2H_2 \rightleftharpoons C_6H_4O_2 + H_2$$

There is in the solution a very slight but constant concentration of hydrogen at 10^{-12} atmospheres. Thanks to this stability the hydrogen of the hydroquinone can be used in place of a saturation of the liquid with hydrogen, by adding an excess of quinhydrone to the solution.

It is possible even to conceive of a quinhydrone electrode that is oxidative-reductive. Quinone and hydroquinone are an oxidation-reduction pair that forms a redox potential on an indifferent electrode. The correlation of quinone to hydroquinone that provides the quinhydrone molecule is always 1:1. Therefore, the redox potential remains always the same in quinhydrone solutions and depends only on the pH value. The potential of the quinhydrone electrode at pH 0 is about 0.044 volts more positive than the potential of the normal hydrogen electrode used at pH 0.

In microbiological practice the electrode pair for the electrometric determination of the pH value usually consists of a calomel electrode (in place of the normal hydrogen electrode as an emitting electrode) and of a quinhydrone electrode (in place of the hydrogen experimental electrode).

The potential difference must be determined in order to be able to compute the pH value by using Nernst's formula. Here the potential difference cannot be determined with a voltmeter, because the current that is supplied through the element might generate chemical changes as well as a polarization of the electrodes and the electromotive force might be changed during the measurement operation.

The potential difference is determined by the compensation method with an accuracy of up to 1 mv. At this point the system composed of the calomel electrode and of a quinhydrone electrode is almost dead.

The two-volt battery that provides the current for the large circuit does not yield an accurate, stable potential difference. Since the voltage of the battery changes slightly, the Weston normal cell that always produces one and the same well known potential is used for determining the potential.

The following figures show how its potential (in volts) depends on the temperature:

10° C.	15° C.	20° C.	25° C.	30° C.
1.0189	1.0188	1.0186	1.0184	1.0181

The experimental cell can be plugged into the small circuit in place of the Weston cell or be connected in series with the Weston cell (Fig. 4).

With a parallel circuit $Ex = 1.0186 \frac{b}{a}$.

In the above equation a is the zero point for the Weston cell and b for the experimental circuit.

With a series circuit $Ex = 1.0186 \frac{a - b}{a}$.

In the above equation a is the zero point for the Weston cell and b for the Weston cell plus the experimental circuit.

After the electromotive force has been determined the pH value is computed by means of Nernst's formula. It must be borne in mind that here a calomel electrode is used in place of the normal hydrogen electrode and a quinhydrone electrode instead of the experimental electrode. The Nernst formula reads:

$$pH = \frac{0.4541 - E}{0.0577} \quad (18^\circ \text{ C.})$$

in which the magnitude 0.4541 = E of the quinhydrone electrode - E of the calomel electrode is (0.7044 to 0.2503). If the calomel-hydrogen series is being worked with, the following only must be subtracted from the calomel electrode:

$$pH = \frac{E - 0.2503}{0.0577} \quad (18^\circ \text{ C.})$$

If quinhydrone is added to a more strongly alkaline medium, the pH value of an alkaline liquid cannot, consequently, be determined with the quinhydrone electrode. The quinhydrone electrode in practice is usable only for pH values that do not exceed 8.7.

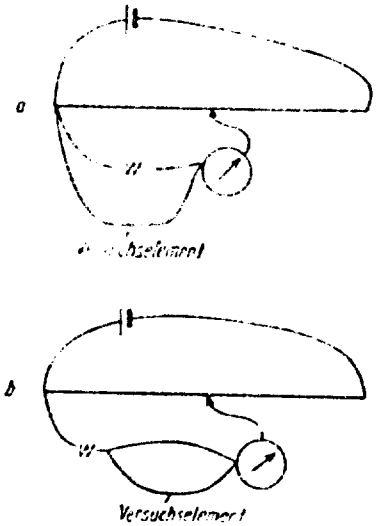

Figure 4.
Circuit diagram of
the experimental cell:

a. Parallel circuit
b. Series circuit
W = Weston cell

<u>Versuchselement =</u>
experimental cell

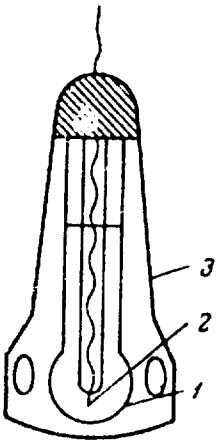

Figure 5. Glass electrode

1. Thin-walled glass globe filled with solution
2. Platinum wire
3. Glass cap as a guard for the electrode.

The entire pH scale can be covered by using glass electrodes. The principle of the glass electrode (Fig. 5) is as follows: If two solutions with a different pH value are separated by a very thin glass partition, a measurable electromotive force develops on this partition. It depends on the size of the pH value on either side of the partition. If we have on one side an acid or salt solution and on the other side solutions with a different pH value, the electromotive force depends on the pH value of the second solution.

Glass electrodes must always be gauged against known buffer solutions. They are customarily used in conjunction with calomel electrodes on a KCl bridge.

Since the glass partition impedes the passage of the current, it must be very thin. Therefore, a determination on Whatstone's bridge is not possible with an ordinary galvanometer (sensitivity A^{-6}). In working with glass electrodes either a more sensitive galvanometer (A^{-9}) is used, therefore, or the current is amplified with vacuum tubes. For that purpose a vacuum tube potentiometer is used. In laboratory practice we usually do not work with Wheatstone's bridge, but rather with a potentiometer (same construction principle). The potential (in volts) is found directly on a special scale and the corresponding pH values on a special table belonging to it. By this means the lengthy calculations involved in the use of the vacuum tube potentiometer are eliminated.

In addition to ordinary potentiometers, vacuum tube potentiometers, suitable for working with glass electrodes are made. The scale is arranged so that the pH value can be read off directly. In the GDR, instruments of the Kuestner Firm, Dresden (Clamann and Grahnert) are used.

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CHAPTER 2

REDOX POTENTIAL AND METHODS FOR ITS DETERMINATION IN MICROBIOLOGY

I. Theoretical Fundamentals

The physical-chemical factors of the environment are especially important for the life activity of microbes. In addition to acidity the oxidizing or reducing characteristics of the medium especially belong here.

In the simplest case oxidation means an oxygen intake. Wieland took the opposite point of view that hydrogen also plays an important part in oxidation processes. In oxidation processes hydrogen is given off and in reduction it is taken on. Finally, Clark and his collaborators pointed out that the transfer of electrons is alone sufficient for transition from one to the other state.

Oxidation can also mean:

Addition of oxygen:

$$KNO_2 \rightarrow KNO_3$$

Removal of hydrogen:

$$\begin{matrix} CH_2COOH & & CHCOOH \\ & \rightarrow & \\ CH_2COOH & & CHCOOH \end{matrix}$$

succinic acid fumaric acid

Removal of electrons:

$$Fe^{++} - e \rightarrow Fe^{+++}$$

The measurement of the oxidizing or reducing characteristics of a solution is possible by chemical means by titration with oxidation or reduction substances. Thus in order to determine the reduction ability of a solution it can be titrated with an oxidation substance, for example $KMnO_4$. The oxidation ability of the solution can be ascertained with the aid of a reducing substance, for example $Na_2S_2O_3$. In this way the total oxidation or reduction power of the solution is determined. The active oxidation-reduction ability of the medium cannot be ascertained by titration. It can be determined only with the aid of the redox potential.

If an indifferent electrode is immersed in a solution containing a mixture of oxidation and reduction substances, that is, sub-

stances that possess the capability of giving off or taking on electrons, then on this electrode there is a potential whose size depends on the concentration of oxidation and reduction substances. Therefore, oxidation and reduction substances must be in a state of a reversible chemical equilibrium. If for example Fe^{+++} is present in the solution, then it can be reduced to Fe^{++} and it acts as an oxidizer since it is itself reduced. The reverse is the case with Fe^{++}. The potential present on the platinum electrode that is in the solution of the Fe^{+++} and Fe^{++} mixture depends on the proportion of the Fe^{+++} and Fe^{++} concentrations. The build-up of potential results either from the flow of electrons in the direction of the electrode or vice versa.

Nernst investigated the relationship between the size of this potential and the concentration of oxidation and reduction substance and established the following relation:

$$E = E_0 + \frac{RT}{nF} \ln \frac{\text{oxidation substance conc.}}{\text{reduction substance conc.}}$$

in which E_0 is the potential with an equal concentration of the oxidizing and the reducing substance and RT is the Nernst constant (see Chap. 1, I).

This potential characterizes the redox condition of the medium, the redox voltage. The greater the concentration of the oxidation substance, the higher the potential. If a solution with a lower potential is put in one with a higher potential, the first one is oxidized by the second one. If there are several oxidation pairs in a solution, then an equilibrium appears between them:

$$K_1 \frac{Fe^{+++}}{Fe^{++}} = K_2 \frac{Mn^{++++}}{Mn} = K_3 \frac{\text{fumaric acid}}{\text{succinic acid}} = K_4 \frac{[H^+]^2}{[H_2]} = \text{etc.}$$

A given pair can be selected with reference to the redox conditions in a compound mixture and its potential determined. An especially well suited pair is hydrogen in an oxidized and reduced state. Since molecular hydrogen consists of two atoms of hydrogen, the following relation results:

$$\frac{[H^+]^2}{[H_2]}$$

The redox potential with reference to hydrogen is designated as eH. It is impossible to determine the potential of a single electrode. It is only possible to measure the potential difference of two electrodes, with one of them serving as a comparing electrode whose potential is assumed to be zero. In this way it is possible to measure the potential difference between the experimental and the comparing electrodes. Then eH is the redox potential, with reference to the normal hydrogen electrode that serves as emitting electrode:

$$eH = E_0 + \frac{RT}{2F} \ln \frac{[H^+]^2}{[H_2]}$$

The normal hydrogen electrode is a platinum electrode immersed in an N/1 solution of hydrogen ions under a hydrogen gas pressure of one atmosphere. The concentration of the oxidized and of the reduced form of hydrogen equals one under these conditions.

If the potential of the normal hydrogen electrode is now set to equal zero, Nernst's equation assumes the following appearance:

$$eH = 0.029 \log \frac{[H^+]^2}{[H_2]}$$

$$\left(\frac{RT}{nF} \text{ at } 18^\circ \text{ C.} = 0.029\right)$$

By analogy with the pH the negative logarithm of the pressure of the molecular hydrogen is designated by rH_2.

$$eH = 0.029 \left(\log [H^+]^2 - \log [H_2]\right)$$
$$eH = 0.029 \left(-2pH - (-rH_2)\right)$$
$$eH = 0.029 \left(rH_2 - 2pH\right)$$

$$rH_2 = \frac{eH}{0.029} + 2pH$$

At a hydrogen gas pressure of one atmosphere and equilibrium between atmosphere and dissolved hydrogen $rH_2 = \log 1 = 0$. With decreasing pressure the rH_2 values rise.

If the oxygen pressure reaches one atmosphere, the hydrogen pressure drops to $10^{-42.6}$ atmospheres. This results from the dissociation of water vapor into hydrogen and oxygen.

$$[H_2]^2 \cdot [O_2] = K = 10^{-85.2}$$

$$2rH_2 + rO = 85.2 \text{ (rO at one atmosphere gas pressure = 0)}$$

$$rH_2 = 42.6$$

rH_2 values from 0 to 42.6 characterize, therefore, all degrees of saturation of aqueous solutions of hydrogen and oxygen. They characterize completely the redox conditions, since the pH is also included. The eH values alone can be affected by the pH. If we are successful in stabilizing (H_2), then eH depends only on the pH. The determination of the pH value with a hydrogen and a quinhydrone electrode is based on this.

The term rH_2 that goes back to Clark (1919-1920), has been only little used to date. In later studies Clark did not recommend its use. Hewitt (1950) also objects to its use. We shall return to this later.

II. Some Peculiarities of the Redox Potential in Biology

It must be borne in mind that rH_2 is by no means always characterized by means of the hydrogen pressure. Under aerobic conditions at rH_2 25-30 hydrogen can no longer exist in the medium. In this case other redox systems appear in place of the hydrogen taking over its function. Whereas in chemistry we are concerned with quickly reacting reversible oxidation-reduction pairs, in biology it is mostly a question of complicated, organic substances that react only slowly with each other. By adding an inactive enzyme, in an oxidative-reductive relationship, as an acceleration substance the potential is strongly modified, since the enzyme forces the slowly reacting pair to react.

A series of biologically important oxidation and reduction substances act irreversibly. They include, for example, compounds with SH groups that easily give up their hydrogen. Cysteine can easily be oxidized into cystine with which any desired substance is reduced.

$2HS \cdot CH_2 \cdot CH(NH_2) \cdot COOH$
cysteine

$$HOOC \cdot CH(NH_2) \cdot CH_2 \cdot S{-}{-}S \cdot CH_2 \cdot CH(NH_2) \cdot COOH + 2H$$
cystine

Dipeptide glutathione and a series of other compounds react exactly like this. These irreversible substances also form a potential on the electrode.

Nekrasov (1937) attempted to describe the physical significance of potentials of this type.

If a platinum electrode or another indifferent electrode is inserted in a solution containing a reduction substance, then the electrons of the reduction substance flow to the electrode until this process comes to a stop due to the opposite process. The electrode then has a constant, negative potential whose size depends on the state of equilibrium of the oxidation-reduction pair.

In biological systems the relationships are considerably more complicated. While one substance gives off electrons on the electrode, another one that has no relationship with the electron emitter brings this process to a stop. By way of example, cysteine lets a negative potential develop on the electrode, while oxygen interrupts this process. In most cases the molecular oxygen takes over the function of the oxidation substance.

The speed of both processes, the accumulation and the emission of electrons, affects the size of the potential. Therefore, the corresponding speeds $\frac{K_1[\text{Red.}]}{K_2(0)}$ must be included in Nernst's formula.

$$eH = \frac{RT}{F} \ln \frac{K_2 [O_2]}{K_1 [\text{Red.}]} + \frac{RT}{F} \ln [H^+] + E_0.$$

The reaction speed is affected by catalyzers. An increase in the oxidation activity is equivalent to an increase in the activity of the oxygen. Dehydrogenases show the same ac on as reduction substances. This can be verified by means of experimental research.

Although the conditions in biological mediums are extremely complicated, the potentials and consequently also the rH_2 values characterize quantitatively the redox condition of the medium. The condition depends on the concentration of the oxidation and reduction substances, on the speed of their reaction, on the influence of the reversible pairs and on the influence of the atmospheric oxygen. The rH_2 characterizes all these factors summarily and it is a quantitative measure of the redox conditions.

III. History of the Study of the Redox Potential in Microbiology

Long before the idea of the redox potential was current in biology statements were made on the reduction characteristics of bacterial cultures.

The reducing characteristics of bacteria cultures had been known already since 1883. Helmholtz observed the decoloration of litmus in a medium containing decomposing albumin. He already recognized the reductive nature of this phenomenon and remarked that by shaking, that is, by contact with air, the original color may reappear. Similar effects were known with some other dye substances that may be used on this basis as indicators for reduction conditions.

Potter (1911) measured the redox potential in bacteria cultures with the aid of electrodes. He used a culture medium that was divided into two halves by a porous diaphragm. One half was inoculated with yeast or bacteria, the other half remained sterile. A platinum electrode was immersed in both halves. The potential on the electrode in the inoculated culture medium was about 0.2 to 0.4 volts lower than the one on the electrode in the sterile medium.

The work of Gillespie (1920) signifies a further step forward. He borrowed the notion of the redox potential from physical chemistry. He already used the normal hydrogen electrode as an emitting system. Gillespie discovered that a strongly reductive potential is present in bacterial cultures, just as in a hydrogen atmosphere, and that with the admission of air the potential rises again. Various types of bacteria change the potential differently. The concept of the redox potential attained its definitive form with Clark's experiments. He started from the fact that dyes are decolorized under reduction conditions and that electrometric measurements can also characterize these conditions. The result is that there are various methods for determining redox conditions.

Both the colorimetric and the electrometric method were drawn upon for measuring the potential. They both complement each other (Clark, 1923-1925). In the subsequent years numerous studies appeared on redox conditions in animal and plant cells as well as in suspensions and cultures of microorganisms. In the

years following 1935 the number of publications on the redox potential declined, and in the following ten years only a few individual studies appeared. This is related primarily to the fact that so much data had been accumulated that the evaluation of the statistical data was, therefore, very difficult. The physical importance of the observed phenomena frequently remained vague. That was caused primarily by the fact that the entire theory of the redox potential in physical chemistry was constructed on the reversible redox equilibrium systems. However, most of the biological redox systems are not in a state of equilibrium; they are irreversible and act slowly.

In the year 1937, then, Nekrasov conducted basic research on redox potentials. He attempted to clarify the physical importance of the potentials in the complicated, irreversibly acting systems, and he also came to the conclusion that the rH_2 values characterize the redox state of a medium.

Therefore, the redox potential is of interest here from two points of view. It is used in chemistry for computing the energy released by reactions. This field of application in biology has only limited importance.

The redox conditions in biology are an extremely important factor in physiology and ecology.

IV. Methods for Determining the Redox Potential.

1. Colorimetric determination of the redox potential.

Some dyes change their color with a change in the redox conditions. In a reduced state they are usually colorless; in an oxidized condition they are predominantly colored blue.

Clark was the first to suggest a set of indicators that covers approximately the rH_2 range (Table 6). Since each indicator can be used only for a very limited Eh range of 120 mv, or two rH_2 units, a considerable number of dyes must be combined. E_0' gives the potential of the dye reduced to 50% at pH 7.

TABLE 6

Redox indicators (according to Clark)

rH_2 indicator	Color of the oxidized form	E_0' at pH 7 and T=30°C. in mv.	rH_2 of the indicator reduced app. 50%
neutral red	red	-340 (325)*	3.0
rose indulin 2G	red	-281	---
Janus green	rose	-258	5.5
phenosafranine	red	-242 (252)	5.9

cresyl violet	red	-167	8.0
indigo monosulfonate	blue	-160	8.7
Nile blue	blue	-142	9.3
indigo disulfonate	blue	-125	10.0
indigo trisulfonate	blue	- 81	11.2
indigo trisulfonate	blue	- 46	12.3
indigo tetrasulfonate	blue-green	- 35	12.3
toluidine blue	blue	- 11	----
methylene blue	blue	11	14.5
brilliant cresyl blue	blue	32 (47)	15.7
thionine	blue-violet	62 (63)	16.1
toluylene blue	blue	115	17.8
1-naphthol-2-sulfonate-indo-2,6-dichlorophenol	blue	119	18.0
1-naphthol-2-sulfonate-indophenol	blue	123	18.1
thymol indophenol	blue	174	19.8
2,6-dichlorophenol indocresol	blue	181	20.1
o-cresol indophenol	blue	191	20.4
m-cresol indophenol	blue	208	21.0
2,6-dichlorophenol indophenol	blue	217	21.5
phenol indophenol	blue	227	21.5
o-bromophenol indophenol	blue	230	21.7
o-chlorophenol indophenol	blue	233	21.8
phenol m-sulfonate indo 2,6-dibromophenol	blue	273	23.0

*Numerals in parentheses: E_0' according to Hewitt (potential of dye reduced to 50% at pH 7.)

Substances whose color is changed independently of the pH value were chosen as much as possible as redox indicators. Unfortunately this did not work out in every case. Thus neutral red is red up to pH 7 and becomes orange-yellow at pH 7-8. In a reduced state this dye is also yellow, but it has another tint. It fluoresces in reflected light in a bright yellow-green. Thymol indophenol is blue at pH 9, below it is red. 2,6-dichlorophenol is blue at a pH over 6, reddish under 6. Two color changes are to be observed with Janus green: from blue-green to red at rH_2 12.3 (irreversible action) and from red to colorless at rH_2 5.4. Above rH_2 23.1 there are to date no indicators.

Indicators for the lower rH_2 values were proposed later by Wurmser (1935). In practice, however, they have been little used up to now (Table 7).

In recent years 2, 3, 5 triphenyl tetrazoliumchloride has been used in every increasing amounts; in an oxidized state it is colorless and with reduction it forms a red, unoxidizable precipitate. With the aid of the amount of precipitate it is possible to determine the amount of reduced indicator according to the weight or colorimetrically.

If it is feasible to determine the amount of reduced dye substance, then a relationship to the eH value can be derived from it. This is possible, for example, with o-dichlorophenol indophenol, thymol indophenol, 1-naphthol 2-sulfonate indophenol, thionine, methylene blue and indigo sulfonates. With a change in the degree of oxidation from 98% to 2% the eH changes about 100 mv with these dyes (Table 8)

$$eH = E_0 + K(30^o)$$

TABLE 7

Indicators for lower rH_2 values (according to Wurmser, 1935)

rH_2 indicator	E_0' at pH 7 in mv
benzyl viologene	-359 (400)
neutral violet	-340
indulin scarlach	-300
safranine T	-289
tetramethyl phenosafranine	-273
dimethyl phenosafranine	-260
tetraethyl phenosafranine	-264
β-anthraquinone sulfate	-250
1,5-anthraquinone sulfate	-200
neutral blue	-192
alizarin brilliant blue	-173
gallophenin	-142
ethyl capri blue (nitrate)	- 72
methyl capri blue	- 61 (60)
azure I	11
gallocyanin	21
2,6-dibromophenol indoguaiacol	159
m-bromophenol indophenol	248
phenol m-sulfonate indophenol	250
quinone	271

In one experiment, for example, indigo tetrasulfonate was reduced to 75% at pH 7.8 (determined colorimetrically). $E_0' = -77$ mv at pH 7.8. It is evident from Table 8 that with an approximately 75% reduction eH works out equal to -14 mv; consequently the eH of the medium is -77-14 = -91 mv.

Not only the oxidized, dyed form ofthe indicator can be used for colorimetric determination, but also the colorless, reduced form (leukobase). There are many methods for producing leukobases.

1. The dye solution is reduced by adding platinum asbestos in the hydrogen stream (Fig. 6). The completed leukobase is kept in a firmly airtight pipette. It is added to the experimental solution in hydrogen atmosphere.

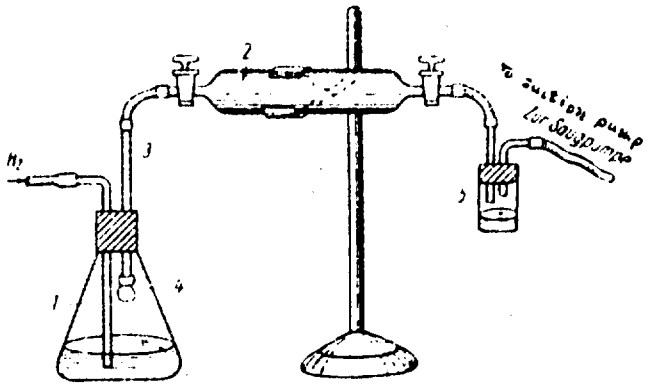

Figure 6. Experiment set-up for producing leukobases for rH_2 indicators.

1. Flask with dye solution and platinum asbestos.

2. Receptacle for the leukobase.

3. Glass tube with cloth filter (4) for absorbing the completed leukobase.

5. Receptacle for the solution being examined.

2. Reduction by means of thiosulfate (corresponds to hyposulfite in the original Russian text) in an acid medium (Semenov, 1934). Two to five milliliters of 0.1N $Na_2S_2O_3$ are added to 100 ml of a 0.01% dye solution at a pH of 3.5 (2 to 6 ml of 0.1N HCl per 100 ml). After two hours the reduction is finished.

3. A knife-tip's full of dithionite (corresponds to hydrosulfite in the original Russian text) dyes reduced instantaneously at pH 5.4, with which, however, some dyes (indophenols) are destroyed.

4. If 0.05N NaOH (pH 9-8.5) is mixed with a 0.015% methylene blue solution containing 5% glucose, the dye is reduced in heat.

Some difficulties result in the use of redox dyes in microbiology.

In eH measuring we cannot proceed in the same way as with pH indicators, because experimental solutions are oxidized in contact with air and they change their potential. Therefore, we proceed as follows:

Indicator solutions are added sterilely to the inoculated culture mediums. In order to eliminate as much as possible the oxidizing effect of air, substrates containing agar are used primarily. If 0.2% of agar is added to the culture solution, anaerobes develop a few millimeters under the surface of the medium. It can be inferred from the change in color of the indicator how far the rH_2 of the medium has dropped due to the growing culture.

TABLE 8

Complementary values of eH indicators, depending on the degree of oxidation (according to Hewitt, 1950)

Percentage of oxidation	K(mV)	Coloring
99	+60	completely colored
98	+51	
95	+38	
90	+29	
85	+23	
80	+18	
75	+14	
70	+11	
65	+ 8	
60	+ 5	
55	+ 3	
50	0	half decolored
45	- 3	
40	- 5	
35	- 8	
30	-11	
25	-14	
20	-18	
15	-23	
10	-29	
5	-38	
2	-51	colorless
1	-60	

Redox indicators are used primarily, then, if one wants to verify the results obtained on the electrodes. This is particularly important in cases in which mediums with H_2S or other substances that disturb the formation of potentials are being examined. If various

amounts of indicator are added to the medium being examined and the speed of reduction is observed, then it is possible to form an idea of the "capacity" of reducing systems. On the other hand electrometric measurements characterize only the "intensity" or the level of reduction.

The colorimetric method has the following fundamental defects: It is possible only rarely to observe an unmistakable color change except by utilizing colorless mediums that are relatively seldom used in microbiology. Usually we must be content with ascertaining whether or not reduction occurs; however, the degree of reduction cannot be determined. The values obtained with the colorimetric method are, therefore, to be considered only as approximate values. Indeed, the addition of the indicator to the experimental medium alters its redox condition. If the color change indicates reduction, other components of the medium must be oxidized. If the leukobase is oxidized, then a component of the medium is reduced simultaneously. Therefore, redox indicators are not at all neutral substances. They can oxidize or reduce the experimental medium which occasionally leads to fundamental errors. If a large amount of an oxidizing indicator is added to a reduced medium in which it must be decolored, the reduction substances of the medium with slight redox buffering are unable to reduce the entire added dye. A part of the dye remains oxidized and leads to the false argument that the eH value of the medium is higher than the E_0' of the added indicator. This possibility must also be considered in measuring intracellular eH values. Therefore, it is necessary to add the indicators to the medium only in such minimal amounts that coloring and color change can be ascertained accurately. It must be observed moreover that an equilibrium appears only slowly in the medium and that the dye acts actively on the experimental systems and may alter the redox condition.

Many dyes act as cell poison, and, therefore, they cannot be used for research on living subjects without a preliminary check.

2. Electrometric Determination of the Redox Potential.

a. Methods for Redox Determination.

Electrodes. eH determination can be made with polished platinum electrodes, with platinized platinum electrodes or more usually with gold and iridium electrodes. Nekrasov and Parfenova (1938) recommend platinized electrodes. However, there are not yet any general rules for the choice of appropriate electrodes.

Lepper and Martin (1931) compared the values of a gold and of an iridium electrode in a phosphate buffer solution through which they transmitted N_2, O_2 and H_2. They ascertained that the iridium electrode reacts equally well to hydrogen and to oxygen. The gold electrode, on the contrary, reacted almost hardly at all to a hydrogen saturation of the medium and only weakly to an oxygen saturation. The gold electrode showed good results only after the addition of a redox indicator with a low E_0' (safranine).

The same observations were able to be made in bacteria cultures that eliminate hydrogen (Clostr. welchii on beef peptone bouillon).

A low eH was indicated only by the iridium electrode. The potential of the gold electrode dropped first with the addition of safranine, not as much, however, as the potential of the iridium electrode.

Michaelis (1932) points out that platinized platinum electrodes are sensitive with respect to the oxygen and hydrogen gas present in the culture medium, whereas this is not the case to such a strong degree with polished platinum electrodes. Electrodes made of different precious metals can, therefore, show different behaviors.

We chose appropriate electrodes on the basis of preliminary experiments. Since platinum electrodes are extremely common in laboratories, and gold and iridium electrodes, on the other hand, are mostly lacking, we started with platinum electrodes.

Polished electrodes. The shape and size of the electrodes have particular importance in work with polished electrodes in microbian cultures. A small, needle-shaped electrode, 2 to 3 mm. long, yields higher values than a 2 X 4 mm. plate electrode. The difference amounts up to 100 mv when measuring with simple and vacuum-tube potentiometers. With a simple potentiometer polarization phenomena cause great difficulties. If current is delivered by the experimental cell, the potential of the polished electrode is changed considerably. The size of the potential of the polished electrode changes about 20 mv and more, if a current with a tension of 100 mv is delivered for a fraction of a second which is frequently the case in testing the compensation point. It is not possible to ascertain the compensation point precisely. The spread range within which the potentiometer is dead can be extended over a range of up to 100 mv. These difficulties are slighter by using vacuum-tube potentiometers.

The rH_2 and eH values, measured with polished electrodes in multiplying cultures, are too high, according to our observations, in comparison with the data of the rH_2 indicators. (Experiments with yeasts in malt and Esch. coli in beef peptone bouillon with glucose with the addition of 0.3% agar.) The difference can amount to 100 to 200 mv or 10 rH_2 units and more. Polished electrodes are apparently not sufficiently sensitive with respect to reducing substances and hydrogen that may develop during the growth.

The values obtained with polished electrodes coincide with the data of the rH_2 indicators only in fermentation processes with turbulent hydrogen formation, as for example during acetone-butanol fermentation (if the medium is supersaturated with hydrogen).

Polished electrodes in sterile mediums (beef peptone bouillon, beer malt, corn mash) show about 4 to 5 units lower rH_2 values than platinized electrodes. The same can also be observed with rH_2 measurements in old cultures that are saturated with atmospheric oxygen. Polished electrodes, therefore, seem to ve less sensitive with respect to the dissolved oxygen in the medium than platinized electrodes.

Platinized electrodes. Values are obtained with these electrodes that are independent of the size and shape of the electrode. Variations with simple and vacuum-tube potentiometers amount to few mv. With them

polarization is not so pronounced.

The data from platinized electrodes in multiplying cultures agree very well with the data from rH_2 indicators. In old cultures saturated with atmospheric oxygen and in sterile mediums the values are higher than with polished electrodes.

Platinized electrodes display a higher sensitivity both with respect to dissolved gases (oxygen and hydrogen) and with respect to substances that are eliminated by microorganisms during growth. There is a disadvantage in the fact that platinum black occasionally acts as a catalyzer that affects the course of biochemical processes in cultures. Thus, for example, it is known that platinum black catalyzes the decomposition of formic acid into H_2 and CO_2. In addition it adsorbs hydrogen so that its concentration on the electrodes can be higher than in the medium. If the rH_2 decreases in microbian cultures that were colored with indicators, in many cases decoloration begins in the vicinity of the platinized electrodes. Nevertheless these disadvantages are unimportant for the majority of experiments.

Preparation of the electrodes. The electrode is a platinum plate 0.25 X 0.25 cm., fastened to a platinum wire 0.3 mm. thick and 10-15 mm. long. The wire is coiled in a glass tube in such a way that part of the plate is held by it and its end remains free within the tube and can serve as a contact with the measuring apparatus. Contact can be produced with mercury poured into the glass tubes, or a copper wire is previously soldered on the platinum wire (Fig. 7).

Platinization results electrolytically in a platinum chloride solution on the cathode of a four volt battery. After platinization the electrode is washed carefully and polarized cathodically in 10% sulfuric acid.

Cathode polarization cleans the spongy surface of the electrode of the remains of the platinizing liquid and facilitates besides the build-up of the potential (Sacharyevski, 1940; Nekrasov, 1927). Our experiments were able to verify this.

Measurement of the potential. The potential on the electrode is measured in the simplest case by means of the compensation method on the Wheatstone bridge, just as in pH determination (Fig. 8).

When the compensation point has been determined, the magnitude of the potential of the platinum electrode in comparison with the calomel electrode is computed in accordance with Poggendorf's formula. However, since the calomel electrode, in comparison with the normal hydrogen electrode, indicates a value of +250 mv (at 20°C.), we must add 250 mv to the measured potential independently of the sign. This can best be explained by means of a diagram. The positive potentials lie to the right of the zero point and the negative ones to the left (Fig. 9). The zero point is the potential of the normal hydrogen electrode; K is the potential of the calomel electrode (+250 mv); x is the measured positive and y the measured negative potential.

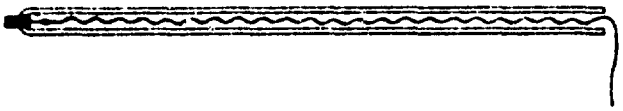

Figure 7: Electrode for determining the redox potential.

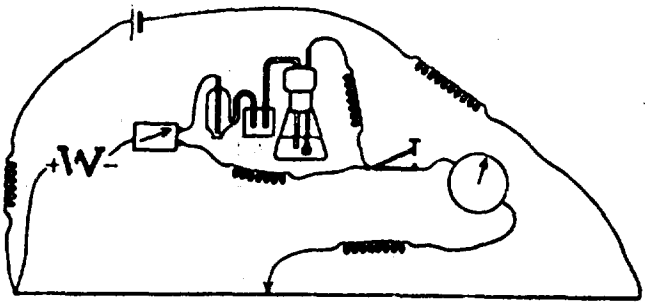

Figure 8: Lay-out diagram for determining the redox potential in microorganism cultures, using the Wheatstone bridge.

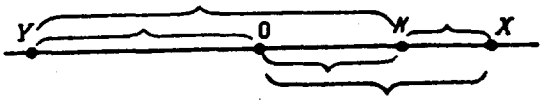

Figure 9: Potentials of the calomel and the experimental electrodes (explained in the text).

Then eH = Kx + 250 or eH = -yK + 250.

The measurements may also be taken with simple potentiometers or with vacuum-tube potentiometers.

Polarization of the electrodes. During the determination of the compensation point a weak current flows constantly in the experimental circuit, causing a deflection of the galvanometer pointer. A polarization of the electrode can already be produced with a current of several tenths of a milliampere, which disturbs the measurement of the potential and completely distorts its magnitude. The setting must be found on the potentiometer at which no current flows and the pointer does not deflect. Measurements are considerably simpler to make with the aid of vacuum-tube potentiometers. In this case the experimental circuit draws no current, and a polarization of the electrodes does not occur. The potential merely produces a static charge on the vacuum-tube grid. The rH_2 can be measured accurately only with this kind of vacuum-tube potentiometers or vacuum-tube amplification. When working with a simple potentiometer there is always a certain degree of inaccuracy. In order to avoid errors resulting from polarization of the electrodes, the following method is often used: If an electrode has become polarized, the switch lever must immediately be brought into the position that produces an opposite current and, consequently, an opposite polarization of equal strength. In this way the one polarization is compensated to a certain degree by the other one. In order to attain still greater accuracy, not only one but two to three electrodes are used. One electrode serves for approximate determination and always produces some errors due to polarization. The second electrode can then be put already closer to the compensation point and the error becomes significantly smaller. In this way the exact compensation point is found in the shortest time.

Determination of rH_2 in individually extracted samples and in microbian cultures. The measurement of rH_2 in a sample extracted from the experiment receptacle runs into difficulties, since the reduced substances present in the medium may be oxidized in contact with atmospheric oxygen. If the culture is in the stage of intensive multiplication, the eH is usually very low. The taking of the specimen is combined with a thorough mixing of the medium and increase in the potential. The result is an active growth of aerobes and an inhibition of anaerobic germs. This in turn signifies a new decrease in the eH values. Therefore, the measurements do not produce a perfect picture of the redox conditions prevailing in the culture.

In cultures that have already reached the stationary phase the conditions are somewhat more favorable. The eH goes up only slightly as a result of enrichment with oxygen, and the original value soon reappears. Samples can also be taken from cultures that have been killed by adding thymol after standing for about twelve hours for measurement, since thymol does not affect either the eH or the pH values.

The question arises in this type of measurements as to how quickly the potential is built-up on the electrode and how quickly the test fluid returns to an equilibrium with air.

It turns out that the rapidity of potential build-up depends to a great degree on the condition of the electrode (previous utilization, etc.). The cause of this behavior, however, is still unknown.

Usually when the electrode is introduced into the test liquid the potential is too high at first; then it decreases and remains at the same level. A constant potential is occasionally attained after 5-10 minutes, sometimes only after 30-60 minutes. In each it must be taken into consideration that the eH values in measurements under aerobic conditions and in an oxygen-free atmosphere (nitrogen, vacuum) may be very different. If potentials that are conditioned only by the redox systems present in solution are to be determined, they must always be measured with oxygen excluded. The best method is to feed in nitrogen that must be completely free from oxygen (Raynaud and Viscontini, 1945).

In this way, for example, in completely air-free beef peptone bouillon the eH is -125 mv; after the admission of air it is -40 mv, and after supplying ordinary, commercial-grade nitrogen (oxygen content about 0.6%) it is -90 mv.

Good results are obtained by measuring in a vacuum. According to Utevskaya and Yefimov (1937) at 50 mm. of vacuum the eH in blood and in other biological fluids drops about 40 to 45 mv, compared with the measurements obtained without any particular exclusion of air. Vaseline is an inadequate protection from oxygen, since gases can pass through without difficulty.

The direct rH_2 measurement in the microbe culture itself is to be preferred. It is not very practical to insert the electrodes in the experimental receptacle since a certain amount of time must always first elapse for the potential to appear. The electrodes, rather, remain constantly in the culture receptacle during the experiment. A curve of the time lapse of the eH changes is thus obtained.

We measured with two and three electrodes (Fig. 10), in order to avoid inaccuracies and accidents. The experimental medium is sterilized in a flask or in another receptacle. The electrodes and a KCl-agar tube are immersed in the culture solution, held by the wadding stopper. The tube produces the connection to the calomel electrode. The 3% agar does not contain saturated but rather only a 10% KCl solution, so that not too much KCl will pass over to the medium. The diffusion potential present at the boundary between the agar bridge and the calomel electrode is disregarded (it is not greater than 1 to 2 mv).

The agar bridge is made as follows: The end of a glass tube, 8 to 10 mm. in diameter, is sealed up about 2-3 mm. by melting over a flame. Then a bundle of asbestos fibers about 1 cm. long is firmly melted down in the still remaining opening, so that a leakage of the agar is prevented even when sterilizing in an autoclave. The completed tube can be used for a great number of experiments. The agar is covered over with a layer of a saturated KCl solution, in order to produce a connection with the calomel electrode.

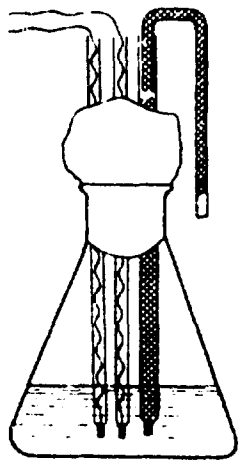

Figure 10. Apparatus for determining the eH in microbian cultures.

b. Difficulties in Measuring the Redox Potential in Cultures and Suspensions of Microorganisms.

There frequently are difficulties in measuring the potentials, in spite of attention to technical details.

Aubel and his collaborators (1946) had considerable difficulties in determining the eH values with polished platinum electrodes in cultures of Clostr. sporogenes. The cause probably was the action of metabolic products on the electrodes.

Kluyver and Hooyerheide (1936) used polished gold electrodes. They found that the potential is occasionally built-up very badly on the electrode. Apparently no equilibrium occurred between the redox systems of the cells and the electrode. In these cases the addition of a redox system, for example a redox indicator, is recommended. The potential then appears only when the dye is partially reduced; that is, an oxydation-reduction pair forms that is in equilibrium with the medium. If the redox systems of the cells cause this kind of condition, the potential can easily be measured.

The eH interval, in which a dye is partially reduced, is very small -- altogether about 100 mv. Therefore, it is preferable to use not only one dye but a mixture of dyes covering a greater eH range. With strongly reductive conditions the following mixture, for

example, is used: Nile blue, brilliant alizarin, Janus green, phenosafranine and neutral red. 0.001% of the mixture is added to the experimental medium. The following come into question for higher eH ranges: Gallocyanin, thionine, brilliant cresyl blue and methylene blue. A well measurable potential is built-up on gold electrodes in the presence of such "potential transmitters."

The method of Kluyver and his collaborators has, however, a disadvantage in that the added redox systems may alter the potential of the experimental medium, if the redox system of the medium is only weakly buffered.

Moreover, the range of the eH changes in the culture may be so great that the entire set of indicators must be added in order to cover it. The method breaks down with high rH_2 values, since we have no rH_2 indicators available over rH_2 23.

Other substances, besides rH_2 indicators, are occasionally used. Ward (1938) used 0.00031 molar K_3 $[Fe(CN_6)]$ in a synthetic medium in experiments with Esch. coli.

In order to obtain more accurate values, it is occasionally recommended to polarize the cathodes of the electrodes before using them (Nekrasov, 1927, Nekrasov and Parfenova, 1938). The same result is obtained, if the electrode is treated with hydrogen (Sacharyevski, 1940). Platinum is released by this means from oxidized substances that are a disturbing factor in the determination of the potential.

Our experience indicated that it is absolutely necessary to clean the electrodes from time to time in this manner.

Measurement errors can be avoided and better results obtained, if differently treated electrodes yield measurement values that agree. Nekrasov and Parfenova polarized oppositely two electrodes. After depolarization the measurement values with both electrodes agreed very well in the final result. In this way potentials of complicated biological mediums, like hay decoction and Esch. coli cultures, are determined. The potential of the electrodes in hay decoction, after polarization, was constant after one to two hours (Fig. 11).

In a culture of Esch. coli (Fig. 12) the potential is built-up unusually quickly. That is proof that redox substances with strong buffer characteristics were present in the medium. This method is particularly important in cases in which the redox condition of the medium is to be characterized. It is not usable if it is desired to trace the dynamics of the redox changes.

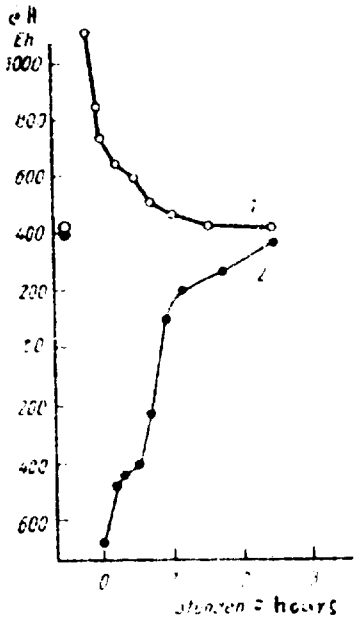

Figure 11. Depolarization curve in a sterile hay decoction (according to Nekrasov and Parfenova, 1938)

1. anode polarization

2. cathode polarization

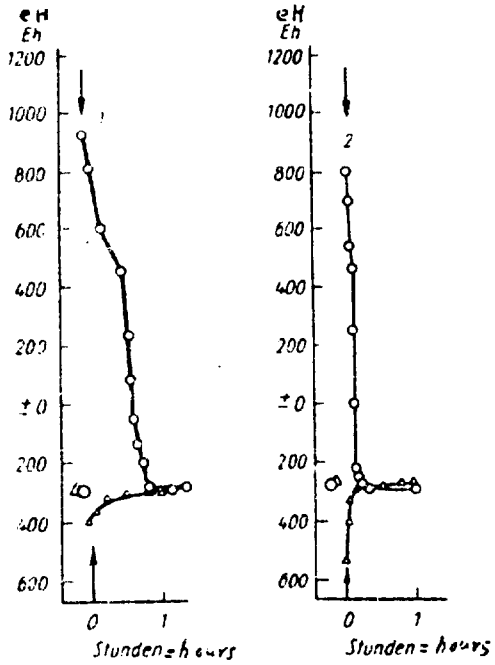

Figure 12: Depolarization curve in a culture of Esch. coli, (according to Nekrasov and Parfenova, 1938)

1. 2 days after commencing experiment
2. 3 days after commencing experiment

3. Characterization of the Redox Potential by means of eH and pH or by means of rH_2.

The size of the redox potential is given in volts or millivolts. It is the potential of the experimental electrode compared with the potential of the normal hydrogen electrode.

In addition the redox potential can be given in rH_2 values, that is, as a negative logarithm of the partial pressure of gaseous hydrogen. This term, as has already been mentioned, was proposed by Clark. In order to characterize a redox potential completely, it is absolutely necessary to take the pH value into account, since it affects the size of the eH. If it is desired to compare the eH of two different culture mediums, it must always be taken into consideration at which pH the eH measurement occurred. Therefore, only the eH and the pH characterize completely the redox condition of the medium.

The rH_2 includes within itself the size of the pH at which the measurement was taken:

$$rH_2 = \frac{eH}{0.030} + 2pH \text{ or } rH_2 = \frac{eH+0.06pH}{0.03}$$

The importance of the redox potential in biology lies primarily in the fact that it represents a quantitative measure of the redox condition of the medium. The rH_2 characterizes the saturation of the culture medium with oxygen or hydrogen and gives simultaneously the proportion of oxidation and reduction substances in the medium.

Clark's formula for computing the rH_2 assumes that there is a definite dependency of the eH on the pH: The eH changes about 60 mv per pH unit. There are cases, however, that display another dependency relationship.

Clark's formula retains its validity only for those cases in which the ratio $\frac{ox}{red}$ is $red^- \rightleftarrows ox^+$. If the case occurs, for example, that red is a dibasic acid (Kollath and Stadler, 1939) that dissociates in the following manner:

$$\text{red (not dissociated)} \rightarrow red^- + H^+ \rightarrow red^{--} + H^+ + H^+$$

then the ox/red ratio varies depending on the degree of dissociation of the acid. If two hydrogen atoms are formed by the dissociation, the change in eH amounts to 0.06 volts per pH unit. If one hydrogen atom is formed, the change amounts to 0.03 volts.

According to Michaelis the change in eH may amount in some cases to 0.120 volts per pH unit.

Consequently, opinions to date differ on whether to use the rH_2 for characterizing the redox potential or merely the eH values. While they work primarily with eH values in England and in America, both are in use in the Soviet Union and in France.

In order to clarify the question whether rH_2 may be used as an indicator of the redox condition of the medium or whether redox systems actually appear in microbiological research, in which the formula developed by Clark has no validity, special research was conducted on the dependency of the eH on pH (Tables 9 and 10).

It becomes evident that the various indicators change the size of the eH depending on the pH. This occurs with some dyes in accordance with Clark's formula. By computing rH_2 from the pH and eH data one and the same amount results with any given pH value with neutral red, Janus green, indigo monosulfonate, indigo trisulfonate, indigo tetrasulfonate (with the last three only at not too high pH), toluylene blue, 1-naphthol 2-sulfonate indophenol, indo 2,6-dichlorophenol 1-naphthol 2-sulfonate, thymol indophenol, o-cresol indophenol, 2,6-dichlorophenol indocresol, 2,6-dichlorophenol indophenol, o-bromophenol indophenol, phenol indophenol, o-chlorophenol indophenol.

As can be learned from Tables 9 and 10, the rH_2 values do not completely agree at the various pH values. If the differences do not amount to more than 1-1.5 rH_2 units, they may be disregarded, because usually no greater accuracy can be attained in biological experiments. However, with some indicators, like phenosafranine, cresyl violet, brilliant cresyl blue, methylene blue and thionine, the deviations are more considerable. With these dyes the rH_2 values are considerably higher in an alkaline environment than in an acid one.

With some biologically important redox systems, for example with ascorbic acid, Clark's formula retains its validity at pH values of 2 to 4 (Table 11). However, the dependency relationship is changed in an alkaline environment.

In addition we attempted to ascertain how mixtures of organic substances behave in this respect.

The conditions in culture mediums and culture liquids are of particular interest.

We found, in bibliography on the subject, some data on the dependency of the eH on the pH for culture mediums from which the rH_2 values can be computed.

Knight (1930) investigated the eH of a sterile, air-exhausted and buffered bouillon at various pH (Table 12). The eH was determined electrometrically and colorimetrically. In a beef peptone bouillon the rH_2 changed slightly at different pH values.

Stuart and James (1938) obtained completely other results in a medium of peptone (1%), gelatin (2%) and yeast-water (20%) to which various amounts of NaCl were added for cultivating halophile microbes (Table 13). ([Note:] The rH_2 values were computed by us in measuring eH in an N_2 atmosphere at 28° to 30° C.)

In contrast with Knight's observations the rH_2 in this medium was higher the more the medium was alkaline. The differences reached 4 rH_2 units in a pH range of 4 to 8. Here rH_2 characterizes the redox con-

TABLE 9

Dependency of E_0 on the pH at 30° C. (according to Clark) (The upper figure indicates the eH in mv, the lower one, the rH_2)

pH	neutral red	Janus green red-colorless	phenosafranine	cresyl violet	indigo monosulfonate	Nile blue	indigo disulfonate	indigo trisulfonate	indigo tetrasulfonate	Janus green blue-red (irreversible)	brilliant cresyl blue	methylene blue
1	-	-	-	-	210 9.0	-	240 10.0	280 11.3	310 12.3	-	-	-
2	-	-	-	-	135 8.5	-	170 9.8	210 11.0	250 12.3	-	-	-
3	-	-	-	-	80 8.7	-	120 10.0	160 11.3	190 12.3	-	-	-
4	-	-	-	-	25 8.8	-	65 10.1	100 11.3	130 12.3	130 12.3	-	170 13.7
5	-205 2.9	-111 6.3	-	-80 7.3	-50 8.3	-20 9.3	-10 9.7	32 11.1	65 12.2	60 12.0	136 14.5	101 13.4
6	-279 2.7	-183 5.9	-211 5.0	-123 7.9	-100 8.7	-85 9.2	-69 9.7	-28 11.1	6 12.2	24 12.8	78 14.6	47 13.6
7	-340 2.7	-258 5.4	-242 5.9	-167 8.4	-160 8.7	-142 9.3	-125 9.8	-81 11.3	-46 12.5	-35 12.3	32 15.7	11 14.6
8	-392 3.0	-330 5.0	-273 6.9	-190 9.6	-210 9.0	-	-167 10.4	-121 12.0	-83 13.2	-	0 15.0	-20 15.3
9	-440 3.3	-379 5.4	-304 7.9	-215 10.8	-240 10.0	-	-199 11.4	-152 12.9	-114 14.5	-	-33 17.8	-50 16.3
10	-	-406 6.5	-335 8.0	-	-270 11.0	-	-	-190 13.4	-147 15.0	-	-	-82 17.3

rH_2 average value	3.0	5.5	Is strongly changed	7.0	8.7	9.3	10.0	11.2	12.3	12.3	Is strongly changed	14.5

TABLE 10

Dependency of E_o on the pH at 30° C. (according to Clark)

pH	thionine	toluylene blue	1-naphthol 2-sulfonate indophenol	indo 2,6-dicholorophenol 1-naphthol 2-sulfonate	thymol indophenol	o-cresol indophenol	2,6-dichlorophenol indocresol	m-cresol indophenol	2,6-dichlorophenol indophenol	o-bromophenol indophenol	phenol indophenol	o-chlorophenol indophenol	phenol m-sulfonate 2,6-dibromophenol
5	138 14.6	221 17.7	-	262 18.7	-	-	335 21.2	-	366 22.2	-	-	-	390 23.0
6	94 15.1	162 17.4	183 18.1	196 18.5	253 19.8	256 20.5	261 20.7	272 21.1	295 21.8	296 21.9	287 21.6	301 22.0	330 23.0
7	62 16.1	115 17.8	123 18.1	119 18.0	174 19.8	191 20.4	181 20.1	208 21.0	217 21.2	230 21.7	227 21.6	233 21.8	273 23.1
8	30 17.0	82 18.7	62 18.1	46 17.5	110 19.7	130 20.3	112 19.7	148 21.0	150 21.0	152 21.1	155 21.2	155 21.2	218 23.3
9	1 18.0	51 19.7	3 18.1	-12 17.6	41 19.4	57 19.9	50 19.7	76 20.5	89 21.0	79 20.6	83 20.8	82 20.7	158 23.6
rH_2 average value	Is strongly changed	17.8	18.1	18.0	19.8	20.4	20.5	21.0	21.5	21.7	21.5	21.8	23.0

ditions of the medium only approximately. Furthermore, it is worthy of note that a strong increase in the salt concentration (up to 17.5% and 27.5%) leads to a lowering of the rH_2. According to Stuart and James the optimum for the growth of the halophile bacteria being investigated with increasing salt concentration was at higher pH values. Consequently the optimum growth seems to be linked constantly to the same rH_2 value. In a salt-free medium an rH_2 of 19 occurred at pH 3.93; in a medium with 27.5% NaCl the same rH_2 value first appeared at pH 5.37.

Zobell (1946) measured the redox potential of marine sediments with polished electrodes and a vacuum-tube potentiometer. He ascertained that the eH values can be changed about 50 to 138 mv per pH unit.

We also examined various organic mediums, such as, for example, beef peptone bouillon, malt and other culture liquids. The measurements were performed both in sterile, non-inoculated culture solutions and also in culture solutions in which the development of microbes had concluded ([Note:] 10% of the total volume of the culture solution consisted of 1/3 M phosphate buffer with pH values of 5 to 8. We used platinized electrodes for the measurements). In a pH range of 5 to 8 the rH_2 values changed a maximum of about 3 units, usually, however, only about one unit. The same was the case in old cultures of Bac. Subtilis, Bac. megaterium (beef peptone bouillon and yeast (malt).

In microbiological research we are constantly concerned with very strong variations that attain 500 to 1000 mv. On the other hand the pH changes in a culture seldom exceed two units. In such cases we can use the rH_2 value unhesitatingly. This is all the more justifiable since we can not only compute rH_2 but also measure it directly, even though the methods have not yet been tested in microbiology.

Vles and Dex (1943) proposed a bimetal element that indicates, after immersion in the experimental solution, a potential that agrees completely with the rH_2. It consists of an ordinary antimony electrode and a platinum electrode. The experimental solutions with various eH and pH values contained dithionite, hypochlorite, ferro- and ferricyanide.

An analogous principle forms the basis of the use of another pair of electrodes, consisting of a glass and a platinum electrode (Kordatzki, 1953). The rH_2 can be computed from the amount of the electromotive force with the aid of a formula. It is easier to compute the rH_2, if the nomogram proposed by Usov and Pogodayev (1956) is used.

V. Summary.

The redox potential makes possible a quantitative characteristic of the redox condition of biological culture mediums. It is formed by the presence of reversible oxidation-reduction pairs, of irreversible reducers and by the action of the free oxygen and hydrogen in these mediums.

The redox potential is characterized completely by means of the symbol rH_2 with a single numeral. Since the eH is changed in different ways in the various redox systems due to modification of the pH, the rH_2 values computed from the measured pH and eH values are subject to

TABLE 11

E_0' and rH_2 of some biologically important substances at various pH values (according to Hewitt, 1950)

ascorbic acid			catechol			adrenalin		
pH	E_0'	rH_2	pH	E_0'	rH_2	pH	E_0'	rH_2
2.16	260	13.3	4.07	547	26.9	4.07	556	27.3
3.04	209	13.3	6.08	427	26.9	6.08	430	27.0
4.00	154	13.3	7.66	333	26.8	7.66	345	27.2
5.19	115	14.4	9.28	253	27.2	-	-	-
6.32	78	15.3	-	-	-	-	-	-
7.24	51	16.2	-	-	-	-	-	-
8.57	-12	16.6	-	-	-	-	-	-

TABLE 12

Dependency of the eH and rH_2 on the pH in bouillon made air-free with and without rH_2 indicators (according to Knight, 1930)

ph	without dye		with dye			colorimetric determination		
	eH	rH_2	dye	eH	rH_2	pH	eH	rH_2
5.96	+5	12.1	indigo tetrasulfonate	-9	11.7	5.35	+2	11.4
6.06	+15	12.6	indigo tetrasulfonate	-13	11.7	6.60	-45	11.3
6.42	-25	12.0	indigo tetrasulfonate	-45	11.3	9.09	-207	9.1
6.82	-55	11.7	indigo trisulfonate	-67	11.3	9.16	-225	9.4
6.92	-71	11.4	indigo trisulfonate	-65	11.5	-	-	-
7.31	-92	11.4	indigo disulfonate	-111	10.8	-	-	-
7.62	-130	10.7	indigo disulfonate	-171	9.4	-	-	-
8.45	-195	10.6	indigo disulfonate	-238	8.7	-	-	-
8.57	-181	10.9	indigo disulfonate	-245	8.6	-	-	-

TABLE 13

Dependency of the eH and rH_2 on the pH in a culture medium with the addition of various amounts of NaCl (according to Stuart and James, 1938)

pH	without NaCl		7.5% NaCl		17.5% NaCl		27.5% NaCl	
	eH	rH_2	eH	rH_2	eH	rH_2	eH	rH_2
3.93	319	19.0	329	19.1	290	17.8	290	17.8
4.66	323	20.4	282	19.0	282	18.7	261	18.3
4.85	317	20.6	277	20.2	279	19.3	276	19.2
4.91	310	20.5	279	19.4	268	19.0	258	18.2
5.37	292	20.4	282	20.4	243	19.1	241	19.0
5.64	292	21.4	277	20.8	235	21.5	239	19.5
5.79	290	20.8	263	20.6	228	19.2	234	19.7
6.14	266	21.5	259	21.2	216	19.7	228	20.2
6.42	249	21.4	268	22.0	205	19.9	226	20.6
7.04	229	22.1	236	22.2	203	21.1	195	20.9
7.37	227	22.9	232	22.7	200	21.6	194	21.4
8.00	214	23.4	230	23.9	193	22.6	188	22.5

a slight error of one to two rH_2 units.

The eH can be determined colorimetrically with a set of indicator dyes. Colorimetric determination is used in microbiological practice principally only for orientation, since it is difficult to ascertain the degree of reduction when atmospheric oxygen is admitted. We can usually only determine whether or not the dye in question is reduced. Some other sources of error are added to this. Therefore the colorimetric determination method for the rH_2 is suitable for the approximate determination of the pH by means of a set of indicators. Platinum electrodes are to be preferred for the electrometric measurement of the eH.

The pH must also be measured in addition to the eH, in order to be able to calculate the rH_2. The speed of the potential build-up is usually without significance in measuring the eH in growing cultures. Even if the electrode does not directly indicate the changes in the medium, it makes scarcely any difference at all to the characteristic picture of the potential curve. The time that elapses until the appearance of the potential is important only in cases in which a sample is taken out of the culture receptacle and is measured.

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CHAPTER 3

Active Acid Content and Redox Conditions in Living Cells.

I. Intracellular Hydrogen Ion Concentration.

After working out the electrometric and colorimetric methods of determining the hydrogen ion concentration the question of the pH within the living cell arose. The first studies appeared in the years 1914 to 1916 (Reiss, 1926.)

1. Hydrogen Ion Concentration in Plant Cells.

Experiments on the pH value in cells of higher plants were conducted with colorimetric and electrometric methods, for example on pressed juices and on tissue sections.

At first the colorimetric method seemed to be the most promising. It sufficed to immerse the cells in a dye solution and to observe how the various components of the living cells are dyed. However, further questions arose on the penetration of the dyes in the individual parts of the cells and on the storage of dyes.

Since the majority of living plant cells contains predominantly cell sap and the cytoplasm lies close to the cell wall only as a thin layer, usually only a coloring of the cell sap and not of the cytoplasm is observed.

Concentrated indicator solutions should not be used for live coloring, since they may have a toxic action. On the other hand, by using strongly diluted solutions and thin subjects the coloring cannot be detected under the microscope. It only becomes visible, then, if the dye, compared with the concentration in the surrounding solution, has become considerably enriched in any part of the cell. The enrichment usually occurs in the cell sap and not in the cytoplasm. For this reason results obtained from plant subjects by the colorimetric method usually apply only to the cell sap and in no case to the protoplasm.

The first observations showed that the pH values of juices pressed from leaves, roots, sprout stems and storage tissues of numerous plants lie between pH 4.6 and 7.7, on the average between pH 5.0 and 6.5 (cf. Reiss, 1926).

The experiments of Small and his collaborators (Small, 1927; Rea and Small, 1927; Martin, 1927 a,b) should be mentioned among the later studies. The authors used thin tissue sections, eliminated the destroyed cell residue by washing in neutral water and put the sections over night in a pH indicator solution prepared according to Clark. After still more washings they were then examined under the microscope.

It was assumed that the cells were dyed uniformly and that differences between nucleus, cytoplasm and cell sap were present only in the

intensity of the coloration but not in the tinting. The pH value of individual cell components was not taken into consideration in these studies. In general the pH values of various tissues from 165 kinds of plants ranged between pH 4 and 6. The tissues of the sunflower were the favorite subject. The epidermis hair had the highest value, with pH 9, the epidermis cells and root hairs had the lowest, with pH 4.0 to 4.4. The other tissues (phloem, pericycle and root tissue) gave pH values at 5.2 to 6.0.

Sabinin (1921-1923) arrived at similar results by dying sections of Sedum Telephium and Impatiens noli tangere with neutral red.

Martin (1927 b) assumed that differences in the pH value of various tissues were caused by a weak buffering of the cells. After pressing out the cell sap and clearing it of albumin he found a phosphate content of 0.005-0.007 mol. The buffering power was proportional to the corresponding phosphate concentration, and a slight buffering of the cell sap corresponded to a slight phosphate concentration. In agreement with this the pH value of the cell sap could easily be changed by means of CO_2: with a 5% CO_2 content in the atmosphere it changed from pH 5.6 to pH 5.4 and with 90% CO_2 to pH 3.8-4.0.

The pH value of a plant tissue can be determined on the whole with electrometric methods (Uspenskaya, 1934). The subject is incised with a piece of broken glass. Quinhydrone is put in the notch and an electrode inserted. The connection with the emitting electrode (calomel electrode) is accomplished over a U-tube filled with agar stuck in near the measuring electrode. By means of this method the following pH values were given by various plants: in the parenchyma of the sprout stems of Begonia rex, 2.0; Begonia semperflorens hybrida, 1.8; Bryophyllum calycinum, 3.0; Tradescantia virginiana, 4.6; Tradescantia zebrina, 3.8; in the watery tissues of Euphorbia candelabrum, 4.5; Euphorbia splendens, 4.2; Phyllocactus Boothii, 3.7; Cotyledon agavoides, 4.5; Cereus speciosissimus, 4.0; Echinocactus tephracanthus, 5.0; Aloe Patu, 3.8; Cotyledon Pringlea, 3.7.

Tissues from fruits and storage organs were also examined in this manner (Table 14).

TABLE 14

The pH value in fruits, storage roots and tubers (according to Uspenskaya, 1936)

subject	pH values of different growth stages		
	Cell division	Cell Growth	end of growth
Frangaria grandifolia	4.4-4.6	3.7-3.6	5.6-5.8
Frangaria vesca	4.4-4.7	3.6-3.8	6.0-6.1
Rubus idaeus	4.3-4.5	3.7-3.9	4.6-5.8
Pyrus malus (Aport. Apfel)	4.8	3.1-3.3	5.1-6.7
Pyrus malus (Antonovka)	4.6-5.0	2.8-2.9	5.1-5.2
Cucumis sativus	6.5-6.7	6.	6.5-6.8
Daucus carota	6.4-6.7	5.6-5.7	6.5-6.7
Beta vulgaris	6.0-6.2	5.7-5.8	6.6-6.7
Solanum tuberosum	7.0-7.1	6.7-6.8	6.6-6.7

Rabotnova (1936) found the following pH values in small tubers and rhizothamnidium:

Galega officinalis	pH 5.8-5.9
Pisum sativum	pH 6.6-6.9
Vicia cracca	pH 7.1
Lupinus polyphyllus	pH 6.8-7.0
Alnus incana	pH 5.6-5.75
Elaeagnus argenteus	pH 6.0-6.3

Uspenskaya (1939) put algae cells in buffered dye solutions (pH 5 to 7.6; M/90 phosphate) with 0.01% dye. The use of various pH substances was necessary because of the different permeability of the dyes. The viability of the cells after coloring was verified in flagellate types by observing their motility; in other types by determining the limit concentration at which neither growth nor multiplication results. The pH values lay in a broad range from pH 4.5-6.9. Spirogyra inflata, Spirogyra neglecta, Eudorina elegans, Scenedesmus quadricauda and Pediastrum duplex had an acid cell sap, Asterionella formosa, Fragillaria crotenensis, Melosira varians, Synedra ulna, Aphanizomenon flos aquae, Anabaena spiroides crassa and Microcystis aeruginosa had an almost neutral cell sap.

Brooks (1926) experimented with the large sea-algae Valonia belonging to the Siphonales. The cell sap of masked and pressed cells gave colorimetrically and electrometrically (with a glass electrode) a pH value of 6.4.

Here also the reaction of the cell sap could be changed by immersing the cells in acids and bases without death of the cells. After a fifteen minute action period of a 0.0025 mol NH_4Cl solution the pH value of the cell sap amounted to 9. When CO_2 was introduced into the water in which the algae were, the pH value dropped to 5.2

Since many dye solutions permeate badly, Rapkine and Wurmser (1926 b) used a method of microinjection. The filamentous alga Spirogyra was the subject of experiments. With bromcresol purple, methyl red and bromothymol blue they maintained a pH value of the cell contents at pH 6.0 ± 0.2.

Mahdihassan (1930) combined immersion in the dye solution with microinjection. With the aid of a Peterfi micromanipulator needle (2.5 μ diameter) he injured the cell membrane and then dyed the cells in solutions of phenol red, bromothymol blue, bromcresol purple, diethyl red and p-nitrophenol.

With Sacch. cerevisiae Strain XII an intracellular pH value of 5.9 to 6.0 resulted, and with Fusarium lini the values were around pH 6.0-6.1. Here also there was no difference between the pH of the vacuoles and of the cytoplasm. In general the majority of the pH values found in plant subjects must be applied to the cell sap. Data on the color difference of the cytoplasm and of the cell sap are found in no study. However, it can scarcely be concluded from this that in all cases there is no difference between them with regard to the pH value. As the experiments cited show, the cell sap may display quite different pH values, from pH 2 to pH 7. It is difficult to conceive that the hydrogen

ion concentration of the cytoplasm is likewise low in cells that form acid and whose cell sap has a low pH value. The protoplasm of animal cells is extraordinarily buffered and is scarcely altered by varying reaction conditions in the exterior medium. The cell sap of plant cells on the other hand is only weakly buffered. It probably acts as a reservoir that collects the excess acids formed by the cytoplasm and thereby avoids strong pH variations. Unfortunately there are still no detailed experiments on the pH value of the cytoplasm in plants whose cell sap is very acid.

2. Hydrogen Ion Concentration in Animal Cells.

Cells of animal origin consist primarily of cytoplasm. The vacuoles are usually small or are lacking, and pH indicators are not enriched in the cytoplasm. The plasma granules remain colorless even by crushing, for example, an ameba (Chambers and Pollack, 1927). The pH indicator penetrates exclusively in vacuoles and granules and dyes them. For this reason the intracellular pH value is more difficult to determine with the colorimetric method in animal cells than in plant cells.

Pantin (1923) put sea amebae in a neutral red solution and compared the coloring with the colors of buffer solutions. The values were: in the endoplasm, at pH 7.6; in the ectoplasm, at pH 7.2; and in the pseudopodium, at pH 6.8. Sea water with pH 8 always had a higher pH value than the cells.

In order to eliminate the permeability question, the cells were injured here also. This occurred in the simplest way by crushing. Vles (1924) determined the intracellular pH value in the eggs of echinoderms up to 150μ in size. He put them in a drop of dye solution between a slide and a coverglass by pressing on the coverglass to burst them, so that the coloring of various cell components could be observed under the microscope. According to Reiss (1926) this method is usable only if the observation is made very quickly, that is immediately after crushing the cells.

According to Reiss, much importance must be placed on carbonic acid, which, together with bicarbonate, conditions the weakly acid reaction of the medium in normally breathing cells. By crushing cells to a thin cell mash or extract, carbonic acid is released after cessation of respiration, the cell environment becomes alkaline, and too high pH values result. In order to avoid this error in the pH measurement of crushed cells, Reiss used a refrigerated mortar in which the crushing could be performed simultaneously with freezing. He found that the pH value in the cells of various tissues from invertebrates and in muscles, lungs and liver of vertebrates fluctuated between pH 5.5 and pH 6.0.

Schmidtmann (1924) inserted with the aid of a Peterfi micromanipulator a microscopically small crystal of the pH indicator, attached with gelatine to the end of a fine needle, into muscle cells of mice. The coloration of the cells was compared with the color of minute droplets of buffer solution that were applied on the cover glass alongside of the cells being examined. The result was a pH value of 6.7 for the muscle cells.

The microinjection method was used with still greater precision by Needham and Needham (1925, 1926 a). Their experimental subjects were primarily protozoa and the eggs of various marine echinoderms. They used a modified Chambers micromanipulator. They used as indicators Clark and Lubs dyes with a small albumin error: phenol red (0.5%), bromothymol blue (0.8%) and neutral red (0.2%). The dyes were injected into the cells with a 2-4 μ diameter micropipette whereby the amount of dye did not exceed 1/5 of the volumen of the cells.

With Amoeba proteus 81 of 90 injections yielded pH values around 7.6. This must have corresponded to the pH of the living cells, since the amebae still remained mobile at least 20 minutes after the injection and they stretched out their pseudopods.

In the eggs of marine echinoderms and mussels the pH value amounted to 6.6; this value remained unchanged even after fertilization and during division up to the 16-cell stage, and it dropped to 4 to 5 only after death of the cell due to cytolysis.

The number of subjects examined has become very great in the course of time. Reiss (1926) discussed the results of earlier studies. Numerous experiments were performed by Chambers and his collaborators (Chambers and Pollack, 1927; Chambers, 1928; Resnikoff and Pollack, 1928; Pollack, 1928). Sea-urchin eggs were also used for experiments. The pH indicators methyl red, bromcresol purple, bromothymol blue, phenol red, cresol red and neutral red in acid and alkaline solution were injected into the eggs of Asterias forbessii. The result was a pH value of 6.7 $\pm$ 0.1. Chambers also injected the indicator into the cell nucleus and found a pH value of 7.5 $\pm$ 0.1.

Rapkine and Wurmser (1926 a) obtained different results in their research on the salivary glands of Chironomus and the eggs of Paracentrotus lividus and Asterias rubens. They found, with the same method that Needham also used, a consistent pH value of 7.2 in the cytoplasm and nucleus of all subjects.

All the authors who investigated the pH value of the cytoplasm of animal cells ascertained a strong buffering. A change of the pH value from 6 to 8 in the external medium, for example, had no effect on the dyeing of an ameba with neutral red.

Chambers injected pH indicators in cells of Amoeba dubia and in unfertilized eggs of Asterias forbessii and Echinorachnius parma and put them in a humid chamber through which air enriched with CO_2 and NH_3 was supplied. The nucleus and cytoplasm did not change their color. The inclusions (vacuoles and granules) on the other hand became acid in air enriched with CO_2 and alkaline in air containing NH_3. When a small amount of hydrochloric acid at pH 2 was injected into a cell of Amoeba dubia, the pH value indeed became lower at the place of injection, but it immediately went up again to its original value. If a larger amount of acid was injected, no change back to the original value occurred in the respective section of the cytoplasm. The acidified section died and was eliminated from the cell. The same result was obtained by injecting a phosphate buffer solution at pH 5.6-8.0.

Moreover, according to Beck (1933), cytoplasm has a great buf-

fering power. He particularly emphasized the importance of cell permeability. Thus NH_3, CO_2, and acetic acid penetrate rapidly into starfish eggs; NaOH and HCl on the other hand penetrate very slowly. If cells previously treated with permeable acids or bases are put in solutions of neutral red and methyl red, these indicators are enriched in granules and vacuoles, an indication that these cell components are acidified or become alkaline due to permeating acids or bases. The pH value of the cytoplasm is apparently not changed, which, however, cannot be stated with complete certainty, since the cytoplasm is not dyed by means of pH indicators.

According to Reiss (1926) the nucleus is less strongly buffered than the cytoplasm. By means of pH changes in the external environment of the cell it can become more acid or more alkaline.

The intracellular pH value of animal cells, that is of the cytoplasm with an average value at pH 6, is, therefore, affected to a remarkably small degree by the external conditions. The buffering is probably to be ascribed to the proteins and to the carbonate system.

The consistency of the pH value in the cell plays a significant part. All the conditions and properties that are important for the life of the cell, such as osmotic pressure, origin, surface tension and other surface phenomena, viscosity of the albumin etc., are dependent on the hydrogen ion concentration.

3. Hydrogen Ion Concentration in Bacteria Cells.

The measurement of the pH value in bacteria cells is difficult because of the small size of the cells. A coloration of the individual living cells, even if it has penetrated, cannot usually be differentiated from the coloration of the surrounding medium. The microinjection method is not practicable.

Balint (1924) suspended staphylococcus cells in litmus and rosolic acid solutions, separated them by centrifuging the solutions, and examined them under the microscope. He found that the cells were dyed, blue with litmus and orange-red with rosolic acid, and concluded that the bacteria cell has a pH value between 7 and 8. The attempt to alter the intracellular pH value of the bacteria by means of pre-culture in beef bouillon with various buffer solutions turned out negative. Here also, therefore, it appears that the cell consisting primarily of cytoplasm has an effective buffer system.

Therefore, a coloration of the cells can be detected under the microscope only if groups and clumps of cells lie together, as is the case for example with staphylococci.

Gutstein (1932) determined the pH value in Esch. coli, staphylococci, streptococci, Klebsiella pneumoniae and yeasts with a method that also depends on the coloration of cell clusters. Mediums containing specifically a total of 17 pH indicators were used for cultivation. The growing colonies were dyed, in which case their coloration was often strongly different from the color of the medium. The cells, therefore, have an individual cell pH value that is different from the one of the medium; that is, it cannot be forced on the cells by the

medium. This method is also usable with other colony-forming microorganisms to the degree that the colonies have no coloring of their own. The coloration of individual cells can also be observed in yeast cells under the microscope.

There is a disadvantage in that, because it cannot be ascertained whether the surface of the cell, the slime capsule, the cell wall or the cell interior are colored.

Gutstein found in this manner a pH value of 6.1-6.3 in yeasts, staphylococci and streptococci; a pH value of 7.2-7.6 in Esch. coli and Klebsiella pneumoniae. The pH value went up with the age of the colony. Differences in the pH value of the medium between 6.8 and 7.4 were without appreciable effect on the coloration of the colonies.

Rabotnova (1936) determined the intracellular pH value in tuberous bacteria by using Gutstein's method. Cultivation occurred on mannitol yeast agar (pH 6.9) to which indicators were added after sterilization. After two to four days well-colored colonies had developed. The color may also be changed around the colonies in the previously uniformly colored medium. ([Note:] Rhizob. japonicum turns a medium with mannitol somewhat alkaline. Glucose mediums were acidified by all bacteria that were examined.)

After spreading the colony on a porcelain plate and comparing it with the pH indicators the following values resulted: Rhizob. leguminosarum pH 6.0-6.4, Rhizob. lupini pH 7.2-7.8 and Rhizob. japonicum pH 7.4-8.0. No change in coloration could be detected in six to eight day old colonies.

If it is assumed that with this method not only the membrane but also the interior of the cell is dyed, and if it is considered that bacteria cells consist for the most part of cytoplasm, then the pH values found are, therefore, probably the values of the cytoplasm. That probably means that different types of bacteria are different, due to their intracellular pH values, in contrast with animal cells. The difference may amount to up to 2 pH degrees. This is understandable in the light of the much more diverse metabolism of microorganisms in comparison with animal cells and tissues.

Although the problem of the intracellular pH value and its relationship with the metabolic characteristic has not been investigated very much to date, several principles can be determined nevertheless. In animal cells the basic energy producing process is respiration with carbonic acid as one of the final products. The carbonate system, as already mentioned, is very important for the maintenance of a neutral or weakly acid pH range. Other acid products play no part in animal cells under normal conditions and in this connection, because either they are not formed in the respiration process or they are quickly disposed of again by the cells.

It is different in the case of cells of the higher plants. They also breathe, but under determined conditions they can store organic acids. These acids probably strongly acidify the protoplasm, if they are not eliminated in some way. Since the lack of a circulatory system and the firm cell membrane prevent elimination, a reservoir must be

provided in the cell for the detrimental metabolic products. It is a vacuole with cell sap. By means of it there is a likely explanation for the fact that plant cells always show large vacuoles, as we never observe in animal cells. Acid metabolic products are found very frequently in bacteria cells. They can, however, be easily eliminated into the medium. For this reason bacteria only rarely have vacuoles, and the cell is usually filled with cytoplasm.

II. Intracellular Redox Potential.

The intracellular redox potential can only be determined colorimetrically. To date there has been nothing written on microelectrodes that are inserted in a cell for this purpose. Only the rH_2 of tissues can be measured electrometrically.

1. The Redox Potential of Plant Cells.

Uspenskaya (1939) conducted research on the rH_2 in one-celled fresh-water algae with the following indicators: methylene blue, thionine, toluylene blue, o-cresol indophenol and 2,6-dichlorophenol indophenol. The cells were suspended in buffer solutions; the dyes were added in an oxidized form and as leukobases in 0.001% concentrations.

The buffer solutions had pH values from 5.0 to 7.6 when the oxidized dyes were added. The leukobases were prepared with thiosulfate and dithionite or by means of reduction in the hydrogen stream in the presence of platinized asbestos (cf. p. 38-39). By using thiosulfate the pH value was at pH 3.5, with dithionite at pH 5.5 and after treatment in the hydrogen stream at pH 5.0-6.4

Care must be taken that the dye really permeates in the cell. If it is present in the cell in an oxidized form, this is detected by the coloration of the cell contents. On the other hand the reduced form must be oxidized first. $K_3[Fe(CN)_6]$ is ordinarily used for this purpose. It permeates easily into the cell and oxidizes all dyes whose rH_2 is lower than 22-23.

Some algae, for examples diatoms, can be dyed well. With others, on the contrary, vital coloration is not possible. In such cases the following method can be employed:

Cells of Pediastrum are suspended at pH 5.4-6.0 in a methylene blue solution, in which case only the membrane is dyed. Then they are transferred to an alkaline buffer solution at pH 7.3. After five minutes a gradual decoloration of the membrane and the penetration of the dye into the interior of the cell are observed. Cell colonies of Eudorina retain their motility ever with repeated use of this procedure.

Uspenskaya arrived at the following results: If the intracellular pH value is almost neutral and the rH_2 relatively high, an intensive coloration results with all basic dyes, both in an alkaline and in an acid medium. If the intracellular pH value is acid and the rH_2 comparatively low, the basic dyes color the cell content only at a pH value above 6.2-6.9 and acid dyes only at a pH value under 5.0-5.8.

Some algae (protococci and Volvox) could only be dyed after the cell content had been made alkaline with ammonia (2%), that is, after a change in the original pH value. Obviously its cell content is strongly acid.

Uspenskaya determined the lowest rH_2 in Spirogyra inflata at 16.4. In Chalmydomonas and Eudorina elegans the rH_2 lay between 16 and 18. For Spirogyra neglecta it amounted to 17.5, and in Fragillaria crotonensis, Synedra ulna and Cladophora fracta the values lay between 18 and 20.5. More strongly reduced protoplasts are also acid at the same time.

By attacking aquatic flowers with copper vitriol it turned out that various algae behaved differently in the presence of the poison. While blue algae and diatoms stopped their development already with 0.5-0.75 mg of $CuSO_4/l$, algae of the order of the Protococcales showed an intensive multiplication with these concentrations. The question arose, therefore, whether there was a relationship somewhere between heavy metal tolerance and intracellular pH value and rH_2. Uspenskaya's experiments demonstrated that cells are more easily permeable in the presence of heavy metals if they show a high redox potential and an alkaline reaction.

Brooks (1926) investigated the intracellular rH_2 of various kinds of the sea-alga genus Valonia, after the pH value of the cell sap had been brought from pH 6.4 to 5.2 or 9.0 by treating the cells with CO_2 and NH_3. In buffer solutions the cells had various pH values. The following redox indicators were used: 2,6-dibromophenol indophenol, methylene blue, indigo tetrasulfonate, indigo disulfonate, 1-naphthol 2-sulfonate indophenol, o-chlorophenol indophenol and o-cresol indophenol in concentrations of 0.00035 mol. The dyes were not stored in the cell sap. Their concentration was always less than in the external medium. 2,6-dibromophenol indophenol permeated into the cell only in a reduced state, and indeed the more acid the medium was the greater the amount permeated. On the other hand methylene blue permeated only in an oxidized state in which case the amount of permeating dye was independent of the pH value of the cell sap and of the medium. Indigo disulfonate and 1-naphthol 2-sulfonate indophenol generally did not penetrate into the cell sap; indigo tetrasulfonate only in the yellow modification. o-chlorophenol indophenol and o-cresol indophenol permeated all the better the more the medium was acid.

By comparing the results, Brooks came to the conclusion that the rH_2 lies between 16 and 18 in the cell sap. A pH value between 4 and 8 and an aH between +210 and +480 mv were taken as approximate values for the cytoplasm.

Rapkine and Wurmser (1926 b) injected rH_2 indicators in Spirogyra cells. The leukobase from methylene blue was oxidized in the cell. The oxidized form remained colored. On the other hand, 1-naphthol 2-sulfonate indophenol and 2,6-dibromophenol indophenol were reduced. Accordingly, the rH_2 could probably lie between 14.4 and 17.6 in the algae cells.

Experiments on plant tissues were conducted primarily with the electrometric method. The measurements were taken with air excluded, in order to avoid oxidation by atmospheric oxygen. The following goes somewhat more into detail about the method (Uspenskaya, 1934; Rabotnova, 1936).

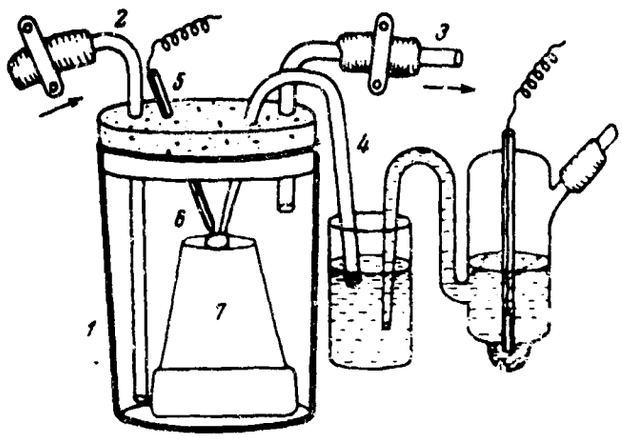

Figure 13. Set-up for measuring the eH in plant tissues with the electrometric method in a nitrogen atmosphere (explanation in the text).

The subject (6, Fig. 13) is placed on a cork base (7) in a glass receptacle. The receptacle (1) is closed with a cork through with a U-tube (4) with KCl agar that is drawn up on one end to a capillary, in addition a platinized electrode in the form of a tapering needle (5) and two glass tubes (2 and 3) for supplying N_2 gas lead into it. The glass receptacle is sealed with paraffin; the U-tube and the electrode are brought so close to the subject that they almost touch it. In order to displace the air, purified nitrogen is fed into the receptacle for a period of 15-20 minutes. For measuring, the electrode and the U-tube are lowered until they penetrate the tissue being examined. If it is a question of small subjects, for example thin roots or tubers of leguminous plants, they are put on a small sheet of KCl agar. The U-tube is stuck into the agar and the electrode is led into the subject.

Measurements of the rH_2 on potato sections and leguminous tubers under nitrogen and in the air show how important it is to perform the measurement in a nitrogen atmosphere. The eH of potato tubers amounted to 379mv under nitrogen and 400 mv in the air. Considerable differences also resulted with leguminous tubers (Table 15).

A hydrogen atmosphere should not be used, because H_2 lowers the eH considerably. Thus, for example, in a hydrogen atmosphere the rH_2 of a potato tuber is lowered from rH_2 25 to rH_2 6.4 within an hour.

TABLE 15

The rH_2 of plant tissues measured in a nitrogen atmosphere and in the air

(Rabotnova, 1936)

Subject	in nitrogen atmosphere	in the air
Pisum sativum	17.0-17.7	22.4
Galega officinalis	26.1	29.0
Vicia cracca	23.1	26.9
Lupinus polyphyllus	23.7	26.6
Elaeagnus argenteus	19.3	23.8
Alnus incana	18.7-19.1	20.4-21.6

In plants with a strongly acid cell sap of the Begonia type (pH 2) or Bryophyllum (pH 3) and Tradescantia (pH 4.6) Uspenskaya found an rH_2 between 16.6 and 23.3. Cultivated plants with a slight acid content (potato tubers) have an rH_2 of 24-25.

In similar organs and tissues the rH_2 values changed with the stage of development (Table 16).

During cell multiplication and embryonal growth the values for eH, rH_2 and pH are relatively high (eH 200-360 mv; rH_2 20-21; pH 4.4-7.1), which indicates normal respiration. During the period of cell division the values drop (eH 194-282 mv; rH_2 13.6-18.6; pH 2.8-6.0). This indicates anaerobic processes that lead to acid-storing and a drop in the pH value. The mature stage is again characterized by high values (eH 270-300 mv, rH_2 22-25, pH 5.1-6.7), which means that the oxidation processes predominate.

Uspenskaya (1939) obtained a low rH_2 in growing tissues (Table 17). In potato tubers of the Silesia species the rH_2 of the growing tubers was lower than the rH_2 of the mature ones. The buds (eyes) have an rH_2 that is lower than in the parenchyma of the tuber especially in the spring at the beginning of germination.

The germination of tubers and the budding of shoots can be accelerated by means of a series of methods. Uspenskaya (1934) succeeded in producing germination in dormant potato tubers in late autumn and in winter by putting them for an hour in a 2% solution of NH_4CNS and for 48 hours in a hydrogen atmosphere, or both combined. As a

result of this treatment the rH_2 of the tubers dropped from 23 to 16-17.

TABLE 16

Change in the Redox Condition (rH_2) of Cells of Fruits and Storage Organs in various Stages of Development (according to Uspenskaya, 1936). Legend: I = Cell Multiplication and Embryonal Growth; II = Cell Division; III = Mature Stage.

Subject	Development Stages		
	I	II	III
Fragaria grandifolia	20.5-21.1	15.0-16.0	23.9-24.5
Fragaria vesca	20.9-21.0	16.7-17.0	23.6-24.7
Rubus idaeus	20.7-21.3	14.8-15.0	22.1-23.2
Vaccinium vitis-idaea	---	13.6-13.5	22.3
Pyrus malus (Antonovka)	20.6-21.6	14.2-14.0	24.5-25.2
Pyrus malus (Aport-Apfel)	21.4-21.8	14.9-15.6	24.0-24.4
Cucumis sativus	20.9-21.0	18.4-18.6	23.0-23.6
Daucus carota	19.5-20.1	17.8-18.0	23.5-24.0
Beta vulgaris	20.9-21.3	18.1-18.0	23.7-23.8
Solanum tuberosum	21.3-21.6	19.4-19.9	22.4-23.8*

*rH_2 24-25 after longer storage.

TABLE 17

eH, rH_2 and pH in potato tubers during various periods of development (according to Uspenskaya, 1939)

Date	Condition of the tuber	period	tuber parenchyma			tissues in the area of the buds		
			eH	rH_2	pH	eH	rH_2	pH
15 AUG	growing	about one month before harvest	220	20.7	6.7	201	18.7	6.0
3 SEP			229	21.6	7.0	215	19.1	6.0
20 SEP	fully developed	during harvest	258	22.6	7.0	248	20.8	6.3
20 OCT		after one month's storage	316	23.3	6.4	303	22.0	6.0
20 MAR		after six month's storage	340	23.7	6.2	329	22.1	5.6
1 APR		early spring	375	24.5	6.0	300	20.8	5.4

If these data are compared with the above-mentioned rH_2 determinations in plants with a special ability for regeneration (Begonia, Tradescantia) whose rH_2 amounts likewise to 16-17, it appears that cell multiplication and growth are preceded by a drop in the intracellular rH_2.

Krasinski (1936) determined the redox potential in pressed plant juices electrometrically. Among others, sugar-beets, potato tubers and peas were subjects of experiments. The pressed juice was covered over with a 1cm thick layer of toluol, in order to avoid contact with air. Measurement was performed with non-platinized electrodes. In the potato the rH_2 amounted to 15.5 (lower, therefore, than in Uspenskaya's experiments), in the peas 20.9, and in various other plants an average of 17. The pH value in most cases was somewhere around 6. However, such a method is not perfect, because the pressed juice comes in contact with air when it is extracted and may be oxidized. A layer of toluol, one centimeter thick, is hardly a reliable protection from atmospheric oxygen, since it can even diffuse through viscous vaseline.

It is worthy of note that the rH_2 values of green, chlorophyll-bearing cells and cells having no chlorophyll do not differ substantially from each other. Low rH_2 values, up to 13-14, were observed in all subjects, although it could be assumed that green cells have higher rH_2 values due to oxygen elimination during photosynthesis.

Obviously the heterogeneity of the cell contents is extremely important. The elimination of molecular oxygen through the chloroplasts is superimposed on the cytoplasm by reduction processes. Cytoplasm and cell sap are protected from the oxidizing effect of oxygen. What we measure are average values with possibly considerably differences in various areas, even within the cytoplasm.

Chloroplasts, by which the oxygen is eliminated, are active only under reductive conditions. Isolated chloroplasts dried on leaves of white clover adsorbed CO_2 and eliminated O_2 under strongly reductive conditions (rH_2 about 5) (with 0.1% fructose at pH 8.2) Boytschenko, 1943, 1944).

In this way the chloroplasts themselves remain in a reduced state, whereas the oxygen eliminated by them oxidized only the solution over the chloroplasts.

All rH_2 values of the plant cells found in the above-cited studies are valid for normal life-conditions.

Apparently there are no data on the experimental shiftability of the redox circumstances in plant cells due to a change in the redox conditions in the surrounding medium.

2. The Intracellular Redox Potential of Animal Cells.

The cytoplasm of animal cells also is not dyed by rH_2 indicators, so that the microinjection method must be used for determining the intracellular rH_2.

The first experiments were conducted by Needham and Needham (1925, 1926 a, b, 1927). They injected rH_2 indicators in an oxidized form or

as leukobases, produced with the aid of zinc powder at 85° C. in the nitrogen stream, into the cell of Amoeba proteus. The amount of the 1% dye solution that was injected did not exceed one fourth of the cell volume. By comparing the coloration of the cell contents with the various rH_2 indicators rH_2 values of the cytoplasm between 17 and 19 were obtained.

Under aerobic and anaerobic conditions in oxygen-, nitrogen-, or hydrogen-atmosphere the rH_2 values remained unchanged for 20 minutes. Here also, apparently, a strong buffering of the intracellular redox potential is present in the living cell.

In the living anaerobic protozoa Nyctotherus cordiformis (intestinal parasite) and Opalina ranarum the intracellular rH_2 was 19-20 in the same experiment under aerobic conditions, therefore, approximately the same value as in the living aerobic ameba, while under anaerobic conditions it dropped to 9.5-10.5.

In contrast with the obligate aerobes the rH_2 of the protplast of facultative aerobes follows the changes in the oxidation conditions in the external medium.

Dying cells in the process of autolysis reduce dyes with a very low E_0', which indicates that in dying, reducing substances are released.

Experiments on the eggs of echinoderms, mussels and polychaeta show that the rH_2 of 19-22 is not changed in these cells even under anaerobic conditions. Even after fertilization it stays at the same level until the 8-cell stage.

Analogous experiments were performed simultaneously by Rapkine and Wurmser (1926, a, c) and Rapkine (1927). They injected pH and rH_2 indicators into animal cells in accordance with Chambers's method and they also used Janus green as an rH_2 indicators.

Salivary glands of Chironomus, larvae of Calliphora and oocytes of Paracentrotus lividus and Asterias rubens. The pH indicators were injected into the nucleus and cytoplasm as 2% solutions and the rH_2 indicators as 1% solutions. In both cases the same pH and rH_2 values were ascertained (pH 7.2: rH_2 19-20.4). Rapkine and Wurmser also assumed that reversible redox systems are present in the cells that keep the rH_2 constant.

The rH_2 remained likewise at the same level with the simultaneous injection of the indicator and oxidizable substances, like sugar, pyroracemic acid and succinic acid. In this case the large cells of the salivary glands of Chironomus were experimental subjects.

In the years 1928 to 1933 Chambers and his collaborators published a series of redox studies (Cohen, Chambers and Resnikoff, 1928; Chambers, Pollack and Cohen, 1929, 1931: Chambers, Cohen and Pollack, 1932). They experimented on Amoeba dubia and the eggs of marine echinoderms under aerobic and anaerobic conditions. The reversible reduction of the indicators was verified by the injection of a 1% ferricyanide solution. If the indicator was in a reduced state within the cell, it was colored after injection of the oxidation substance. The humid chamber

in which the subject was placed could be closed up airtight so that aerobic and anaerobic conditions could appear. We do not find this type of precautions in Needham's studies. The rH_2 indicators and leukobases were injected into the nucleus and cytoplasm. Special attention was paid to the amount of the indicators that were injected, since by injecting too large an amount the reduction ability of the cell is overburdened. This can lead to the fact that an indicator is not considered as reducible merely because its amount was too large.

In contrast to Needham, Chambers found under aerobic conditions an rH_2 value of 12.0 (eH -60 mv; pH 7) in aerobic cells, and under anaerobic conditions an rH_2 of 7.9 (eH -148 mv; pH 7). The presence of various geographic strains could not be the cause, for fresh-water amebas, Paracentrotus and Sabellaria, from European locations produced the same result. In contrast with Needham's experiments it must, therefore be assumed, according to Chambers, that the intracellular rH_2 is changed depending on the air supply conditions and that there is no stable rH_2 supported by strong buffer systems in the cells being examined.

Chambers, Beck and Green (1933) were able to produce a coloration of the vacuoles and granules in individual subjects. Several drops of a 1/10000 mol indicator solution were added to a 2 ml sterile suspension of starfish eggs. Some indicators were concentrated in the vacuoles and granules. It was tested with a 1% ferricyanide solution whether the dyes permeated the cell and were reduced. The eggs were examined alive and in a cytolytic condition. Under aerobic conditions the eH amounted to -60 mv in uninjured and autolyzed eggs at pH 6.8-7.0. Under anaerobic conditions in a Thunberg tube the eH also dropped below -167 mv in living, cytolysed cells. The corresponding rH_2 values are rH_2 11.9 under aerobic conditions and rH_2 8.4 under anaerobic conditions.

Beck (1933) investigated the intracellular eH of the same subjects, but he changed the pH value within the cell. Since a pH change is only possible within the vacuoles and granules, the results, therefore, are valid only for this part of the cell, but not for the cytoplasm. The eH drops with an alkalization of the granules and vacuoles; that is, dyes are reduced that normally are not reduced. The eH rises with an acidification of the cell contents.

Machlis and Green (1933) in one case put one drop of the indicator (1/2500 mol) in a suspension of starfish spermatozoa. They checked the reversibility of the reduction with K_3 $[Fe(CN)_6]$. The measurements were performed under aerobic (shaking in air for 10 minutes) and anaerobic conditions (Thunberg tubes). Under aerobic conditions the eH of the sperm cells lay between +77 and +150 mv (rH_2 15-17); under anaerobic conditions. between -228 and -197 mv (rH_2 5-6).

Machlis and Green assumed that the redox potential in the cell depends on the equilibrium between the dehydrogenase systems, that produce low potentials, and the positive potentials of the oxidases.

This opinion is also maintained in a later study (Green, Stickl and Tarr, 1934). After experiments with dehydrogenase preparations (succinic acid and glucose as substratum) the socalled anaerobic potential of the cells seems to be conditioned kinetically; that is to say that it depends on the speed of reduction of the indicator due to

a fermentation system and on its oxidation due to another system.

The eH and rH_2 of animal cells can, therefore, be measured in two ways: by the microinjection method and by the suspension method. In the second case only the granules and vacuoles are dyed, but not the cytoplasm, while the whole cell is dyed after microinjection.

The results obtained with the microinjection method contradict each other. According to Needham and Rapkine and Wurmser the rH_2 in the cells lies at a definite level (rH_2 19-20) and is maintained by means of a redox system. Chambers and others used the indicators in small concentrations, and found a significantly lower rH_2 under anaerobic conditions, that is to say indicators are reduced that, according to Needham's data, are not subject to reduction. However, in Needham's experiments the quantity of dye used was too large. The rH_2 is higher under aerobic conditions than under anaerobic ones. By using the suspension method, the rH_2 was also lower under anaerobic conditions than under aerobic ones. The results indicate that there are no redox systems in the cells that buffer the rH_2 to a level of 19-20, but rather that there are only reducing substances that are activated by dehydrogenases. They produce strongly reductive conditions within the cell under anaerobic conditions. A higher rH_2 results from contact of the cell with atmospheric oxygen; it is conditioned by the activation of the oxygen due to the oxidases.

According to Machlis and Green the potential of the cells is, therefore, built up from the low potential of the dehydrogenase systems and the positive potential of the oxidases. The fact that it depends on a state of equilibrium of these systems, therefore, gives it a dynamic characteristic. Consequently the aerobic intracellular potential also has no thermodynamic significance and cannot be used in computing the energies of the resultant reactions in the cell. The aerobic potential only indicates which more strongly or more weakly reduced systems are acting in the cell at a given moment.

3. The Intracellular Redox Potential of Bacteria and Yeasts.

The rH_2 measurement of the cell contents of bacteria is also difficult, because here vital coloration of the individual cells is impossible. Cell clusters can indeed be dyed with indicator dyes, which raises the question, however, of whether the values actually correspond to the intracellular condition or only to the conditions on the surface of the cells and their external environment. Vacuoles or chondriosomes in yeasts are dyed, but not the cytoplasm. There are only very few observations on the rH_2 in the cytoplasm of yeasts and bacteria.

Aubel and Genevois (1927) investigated the vital coloring of Esch. coli and of yeasts that grew on a culture medium dyed with rH_2 indicators. They assume that they have also determined the intracellular rH_2 with the cell coloration. Under anaerobic conditions they found in yeasts a value of approximately rH_2 7 that was somewhat lower in Esch. coli. Janus green and methyl red did not dye the cells. The rH_2 of the cells was higher under aerobic conditions. Esch. coli was dyed rose with Janus green, corresponding to an rH_2 ≈ 12. In facultative aerobes the intracellular rH_2 w[illegible] range of rH_2 5-19 agrees with the rH_2 of the environment.

According to Guillermond and Gautheret (1939) and Guillermond (1939-40) rH_2 indicators are not stored in the cytoplasm. They merely pass through it and can be enriched only in the vacuoles. By cultivating yeasts in an alkaline medium with Nile blue (rH_2 of the mid-degree value of the dye = 9) and cresyl blue (rH_2 15), on the other hand, a vital coloration of the cytoplasm occurred, if the cells came in contact with air. Without access to air the cells reduced the dye in the cytoplasm.

According to Meisel and Pomoshnikova (1952) neutral red is reduced in the cytoplasm of yeasts. Therefore, reductive conditions with an rH_2 under 3 must occur in living yeast cells. Reduction conditions of this kind are, however, not characteristic of living, functionating yeasts. Moreover the rH_2 in the external medium may drop strongly in yeasts: neutral red is partially reduced. Under aerobic condition the rH_2 is higher than 12.

Beck and Robin (1934) made use of the fact that yeasts have natural redox indicators in the cytochromes in their determination of the rH_2. The condition of the cytochrome can be ascertained by using the absorption spectrum. The spectra are different in an oxidized and in a reduced state, so that indications on the degree of activity of the oxygen in the cell are given. If we want to have an idea of the size of a very low potential, as can occur in yeasts, the cytochrome system does not suffice for that. It is already reduced at higher eH values than are obviously given in anaerobic cells. The eH in yeast cells drops to negative potential values, while the E_0' of the cytochrome has, according to Green, a value of +123 mv.

In order to measure the low rH_2 of yeasts, other indicators must be used. The method was the same as in the above-cited study by Machlis and Green. The yeast was suspended in an indicator solution and was examined on penetration of the dye and its reduction in the cell. Thionine and all indicators above it on Clark's scale were reduced under aerobic conditions with a pH value of the medium at 6-9. This corresponds to an rH_2 of 16. All indications except phenosafranine are reduced under anaerobic conditions in a Thunberg tube; the rH_2 amounts, therefore, to 5-8.

The reduction power of the cells, that is the reduction of various indicators by the cell, can be examined with specific inhibitors of the oxidases and dehydrogenases.

Because of this the result was a dependency of the eH on the activity of the oxidative system. The greater the activity the higher the eH was, and vice versa. An intensification of the dehydrogenase activity led to a decrease and an inhibition corresponding to a rise in the eH.

Baumberger (1939) measured the eH in a yeast suspension, and determined simultaneously the absorption spectrum of the cytochrome in the cells. The result was a clear connection between the condition of the cytochrome and the eH in the suspension. Therefore, he succeeded in establishing an agreement between the eH outside the cell and the state of the cytochrome redox system inside the cell. The cytochrome is oxidized by contact with air, and the eH rises to +150 mv. Reduction results under anaerobic conditions, and the eH drops to -150 mv. The high

eH value of +150 mv, achieved by the addition of rH_2 indicators with a high eH, for example 2,6-dichlorophenol indophenol, is quite detrimental to the yeasts.

The spectral lines characteristic of a reduced cytochrome appear in the range from +150 to +110 mv. That drops off with the E_0' of the cytochrome (+123 mv).

A decrease in the eH to very low values, as occurs under anaerobic conditions, is linked to the condition of other redox systems of the cell that have a lower E_0' than the cytochrome.

An argument analogous to the one arrived at by Machlis and Green in their research on animal cells, can be attained by examining yeasts. The "reduction power" under aerobic conditions, or, which is the same thing, the "intracellular potential", is determined by means of the equilibrium between the oxidative and reductive systems in the cell. The observations also agree with the opinions of Nekrasov on the nature of the redox potential in biological subjects (cf. Chap. 2). Obviously the cell has a mechanism available that keeps the extraction of electrons from the oxidizable substrate at the same level. If oxygen is present, it finally takes over the electrons; if oxygen is not present, they enter the redox systems of the cell, among others the cytochromes. In this connection it is worthy of note that the presence of reductive substances is recorded by an electrode that is outside the cell. The redox systems, therefore, also act outside the cell.

In Baumberger's experiments, not only free oxygen but also combined oxygen in the form of oxyhemoglobin acts as a hydrogen acceptor (electron acceptor).

Although the intracellular conditions in yeasts can be evaluated up to a certain degree according to the coloration of the cell, this is possible only with great difficulty in bacteria because of their small size. In measuring the potential in bacterial suspensions Korr (1935) was able to measure only the potential of the suspension on the whole. He experimented on suspensions of the photogenic bacterium Photobact. fischeri in beef peptone bouillon. The culture displayed under anaerobic conditions in an atmosphere of pure nitrogen a very low, reproducible potential. At 24° - 28° C. and pH 7 it amounted to -214 ± 3 mv. corresponding, therefore, to $rH_2 = 7$. Korr arrived, besides, at the result that the reduction activity of the suspension is not conditioned by autolysis or by the release of reducing substances from the cells, since a rise in the eH occurs with autolysis. He assumes that there is a great number of redox systems in the cells, whose potential depends on the proportion of the oxidized to the reduced form. They doubtlessly play a part in the formation of the cell potential; the total potential built up by them is, however, not a reversible redox potential. It is kinetic in nature and depends on the relative speed of two opposing processes: oxidation by means of oxygen and activation of hydrogen by means of dehydrogenases.

Pyrophosphate, arsenite and halogen acetates (monochloracetae, monobromacetate. monciadacetate) inhibit specifically indificual dehydrogenase systems and may change the entire system that conditions the

formation of the reduction potential. In their presence the anaerobic potential is higher. Increased temperature and narcotics inhibit all systems. The relative speeds of all reduction processes are likewise slowed down, and the size of the anaerobic potential has its normal value of -214 mv. The same principles that Machlis and Green observed in the sperm of echinoderms and Beck and Baumberger in yeasts appear, therefore, to be present in bacteria.

Judkin (1935) measured the eH in suspensions of facultative aerobes (Esch. coli, Bact. alcaligenes) and anaerobes (Clostr. sporogenes). By adding sugar or another usable substrate to the suspension a low eH is built up; to -400 mv in suspensions of Esch. coli and to -300 mv in suspensions of Bact. alcaligenes. Also if the electrode is isolated from the cells and is put in a collodion saccule, a low eH is produced. Effective substances must also diffuse in the medium. The redox systems of the cell accordingly appear outside both with bacteria and with yeasts. The nature of these substances in yeasts and bacteria is unknown to date.

Rabotnova (1936) attempted to dye tuber bacteria vitally with rH_2 indicators in accordance with the method used by Gutstein.

Rhizob. leguminosarum, Rhizob. lupini and Rhizob. japonicum were cultivated on mannitol yeast agar with the following indicators: methylene blue (1:10000), indigo tetrasulfonate (1:3000) and indigo disulfonate (1:5000). With methylene blue and indigo tetrasulfonate the colonies of all three types were pale blue, almost colorless. With indigo disulfonate, on the contrary, they were dyed an intense dark blue. A coloration of the cells was not detectable under the microscope. The reduction of indigo tetrasulfonate and methylene blue and the strong coloration with indigo disulfonate indicate a potential of rH_2 9.9 - 12.5. In Rhizobium suspensions that were adjusted to pH 6.86 with a phosphate buffer the rH_2 after one hour was 10.4 and the added methylene blue was decolorized. When the culture was agitated, the eH rose immediately, and a blue coloration appeared. In Rhizob. lupini the rH_2 dropped to 11.5 after one and one-half hours. Therefore, in tuber bacteria we find the same principles as in yeasts.

Up until now there have been no experiments on the intracellular conditions in anaerobic bacteria. All that is known is that the rH_2 drops down to zero in cultures and suspensions of anaerobes. It is not known whether intracellular and extracellular redox potentials are in agreement in anaerobes.

Wurmser (1935) summarized all known observations to date on the redox potential of cells: the intracellular potential is the potential of the electroactive substances contained in the cell, substances that can emit or receive electrons. These substances are designated as redox systems and also condition the reduction potential. Finally, however, it never reaches its limiting value which is very low (rH_2 6) under anaerobic conditions. It is in a kinetic equilibrium with the oxidation processes that are linked with oxygen or other hydrogen acceptors.

Sugar and compounds with SH groups, for example glutathione, are known cell reducing substances. No doubt there are others unknown to us at present. Wurmser and Rapkine attempted to "titrate" the reduct-

ive substances of the cell with dyes and ascertained that their effect is approximately ten times greater than the effect of glutathione.

The electroactive systems play the part of buffer substances with regard to the redox conditions similarly to the buffer systems that stabilize the pH value of the cell. Wurmser's views are still valid today, because after 1935 only little has been done about the problems of the redox potential. The few studies that have appeared after 1935 merely confirm his explanations. It is of course necessary to modify accordingly the concepts on redox buffering. Whereas by buffering the pH value the hydrogen ion concentration in the cell remains constant, the rH_2 is changed considerably by buffering conditioned by redox systems. All modifications of the redox conditions in the medium are taken over by the electroactive substances in the cell or the redox systems, whereby the basic oxidation processes are protected from an excess or deficiency of hydrogen acceptors and a constant course of the vital oxidation processes is guaranteed.

III. Summary

The bulk of the cell contents in living plant cells consists mostly of cell sap. Therefore, all data on the intracellular pH value and the rH_2 of plant cells apply predominantly to the cell sap. Its reaction is usually weakly acid, but in some plant tissues it is strongly acid. It is buffered slightly. Its pH value can easily be changed, if the cell comes in contact with carbonic acid or ammonia. The redox potential of plant cells under normal aerobic conditions is at approximately rH_2 20, although there are also more strongly reduced cells that as a rule are also acid.

It is noticeable that there are no differences in the rH_2 value between cells with and without chlorophyll, although the former eliminate oxygen by means of photosynthesis. Obviously the cell is protected from "over-oxidation.

Animal cells consist for the most part of protoplasm. The vacuoles represent only a small part of the cell contents. The intracellular pH value and the rH_2 can be determined by means of the microinjection method. The pH value of the cytoplasm lies around the neutral point. The cytoplasm is strongly buffered; its pH value can scarcely be altered in living cells. The pH value of the vacuoles and granules on the other hand can easily be changed like in plant cells.

The intracellular rH_2 of animal cells is not constant. Under aerobic conditions it is around 20; under anaerobic conditions it drops strongly. Its value depends on the degree of oxidation or reduction of the redox systems that are activated by fermentation. The same is true in yeasts.

Bacterial cells have been experimented on very little up until now with regard to their intracellular state. In all probability they behave like animal cells and yeast cells; that is, the redox conditions in them can be changed depending on their access to air or their lack of air.

In yeast and bacteria cells there is a close relationship between the condition of the redox systems in the cell and the measurable potential outside the cell in the surrounding medium.

The differences in the rH_2 in bacteria and yeast cells under aerobic and anaerobic conditions are very great and amount to 10-15 rH_2 units. They act accordingly as a buffer; that is, the oxidation processes can take place with a constant speed in spite of different conditions (aerobic or anaerobic).

Obviously all living cells have available a specific mechanism that guarantees a constant rate for the oxidation processes. If they come in contact with oxygen, this is the hydrogen acceptor. However, if the oxygen is insufficient, then the hydrogen of the oxidized substrate reduces a series of redox systems. The reduced substances also appear outside the cell. They produce a formation of a low rH_2 in cell suspensions from which air has been excluded.

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CHAPTER 4

LIFE ACTIVITY OF MICROORGANISMS AND HYDROGEN ION CONCENTRATION OF THE CULTURE MEDIUM

I. Influence of the Hydrogen Ion Concentration on Multiplication.

1. Direct Influence of H and OH Ions on Microorganisms.

Each microorganism is adapted to a definite pH range outside of which it cannot exist. The nature of the relationships is unknown in detail; undoubtedly the pH value affects the fermentation activity.

The limits of the pH range within which multiplication is possible are different for the individual types of microbes. Pasteur was of the opinion that generally bacteria were probably damaged by an acid reaction and the formation of mould was promoted, while a neutral or weakly alkaline reaction usually acted in the opposite manner. However, there are numerous exceptions to this rule. The adaptation of Thiobac. thiooxidans to a strongly acid medium is especially conspicuous, because it is unusual for bacteria (Fig. 14).

The autotrophic Thiobac. thiooxidans oxidizes sulfur and thiosulfate to sulfuric acid which accumulates in the medium. The optimum pH range is at 2.5 to 3.5. Growth becomes poorer with a decreasing pH; however, it is still possible down to pH 0.6. Moreover, growth slackens with an increasing pH(4 to 6); at pH values over 6 the cells are autolyzed (Starkey, 1925).

Many sulfur bacteria grow at pH 9 to 10 (van Niel, 1931). An aerobic bacillus, extremely adapted to an alkaline reaction, that still grows at pH 11 and even tolerates a saturated solution of $Ca(OH)_2$ was isolated by Mosevitch (1935) from a tanning vat. The activity of the decomposition agents to which the bacillus isolated by Mosevitch probably belongs ceases only when a pH value of 12.4 is reached in the vat. Many microorganisms (Diploc. pneumoniae, Vibrio comma) have a very narrow pH range (Fig. 15), others (for example moulds) on the other hand grow within very wide pH limits. Thus species of the genus Penicillium are viable from pH 1 to 10.

Dernby (1921) conducted the first systematic studies on the course of development at a different pH starting value. The growth of the microorganisms was determined visually on the basis of turbidity in beef bouillon mediums with pH values from 3.1 to 8.6 after 6 and 48 hours. At the first reading, growth had occurred in culture solutions with an optimum pH value; at the second reading it had also occurred in culture solutions with a less favorable pH. For staphylococci the optimum range was between pH 7 to 8; growth was possible between pH 4 to 8 (Fig. 16). Scheer (1922) concerned

himself with determining the pH limits for Esch. coli.

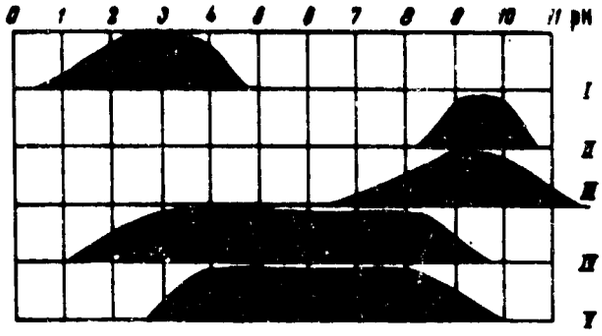


Figure 14. Growth curve of various microorganisms, depending on the pH value (according to data derived from studies on the subject).

I. Thiobac. thiooxidans
II. Chromatium spec.
III. Sporogenic decomposition agent from a tanning vat.
IV. Penicillium spec.
V. Sacch. cerevisiae

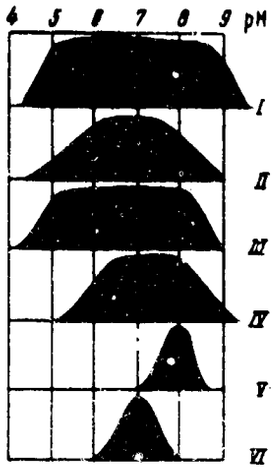


Figure 15. Growth range of some saprophytic and pathogenic bacteria, depending on the pH value (according to Dernby, 1921)

I. Esch. coli;
II. Proteus vulgaris;
III. Serratia marcenscens;
IV. Bac. subtilis;
V. Diploc. pneumoniae;
VI. Vibrio comma.

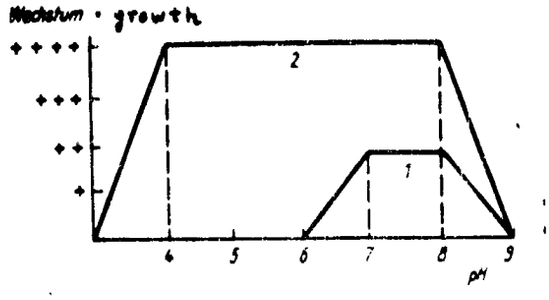


Figure 16. Dependency of staphylococci multiplication on the pH value of the medium (according to Dernby, 1921).

1. After 6 hours;
2. After 48 hours.

Following Dernby we experimented on types of Azotobacter. The strains were cultivated in Ashby's culture medium with a pH from 5 to 8, in intervals of 0.3 pH. ([Note:] In place of calcium carbonate, calcareous tap water was used.) The optimum for Azotob. chroococcum, agilis, beijerinckii (designation according to Vinogradskii) and vinelandii (idem) was at pH 6.5 to 6.7. At pH 5 and 8 still no growth was observed after one to two days. A multiplication at pH 5 and pH 7.7 could be detected only several days later. The number of bacteria was approximately the same in five to seven day-old cultures at various pH values. The initial pH value had shifted to alkaline in an acid medium and to the acid range in an alkaline medium. Special attention must, therefore, be paid to the pH regulating influence of the bacteria. It is possible to attain the optimum range only in the early growth stage, because the cells in unfavorable pH ranges shift the pH value of the medium toward the more favorable range for them. The erroneous finding of Fedorov (1952), who assumes a lack of sensitivity with regard to the pH values of the medium for Azotobacter in using two to four week-old cultures is obviously due to the fact that he disregarded this.

Cohen and Clark (1919) determined the generation period at various pH values. Specific types of differences resulted here also (Table 18).

TABLE 18

Number of generations during one hour of the logarithmic growth phase (according to Cohen and Clark, 1919)

Subject	pH range of constant periods	average number of generations
Esch. coli	5.0-8.1	2.5
Aerob. aerogenes	4.7-8.4	2.7
Shigella flexneri	5.6-8.3	1.6
Shigella dysenteriae	5.5-8.5	1.1
Proteus vulgaris	6.5-8.8	1.5
Alcaligenes faecalis	6.9-9.7	0.9

The observations of Cohen and Clark on Lactobac. bulgaricus are worthy of note. No growth was detected at pH 4.5; from pH 5.1 to 6.8, 1.6 generations per hour grew; at pH 7.5 multiplication was still possible. Although no growth could be observed at a starting pH of 4.5 in the medium, in old cultures the pH value was changed to 3.9. The authors concluded that in acid mediums a fermentation is quite possible, but at the most a neglible multiplication. Rabotnova made the same observations (unpublished) with acetic acid bacteria. Thus at an initial pH of 3.1 Acetob. schützenbachii ([Note:] This type of designation is not recognized either in Bergey or in Krassilnikov) cannot grow. In this kind of old cultures, to which alcohol is supplied continuously, the acid content, on the other hand, increases still more at pH 2.8.

The activity of old bacteria cultures at a pH value that does not allow a multiplication of young cultures, therefore, cannot be explained according to Cohen and Clark. If assimilatory metabolism is inhibited due to unfavorable pH conditions in the medium, the cells can scarcely carry on an active fermentation. It is more probable that some cells may have adapted themselves gradually to a higher degree of acidity during the development of the culture and the accompanied acidification of the medium. These cells can also develop under conditions under which unadapted cells are not viable. Yermakimski's (1932) experiments on acetic acid bacteria indicate this explanation: With Acetob. vini aceti ([Note:] This type of designation is not recognized either in Bergey or in Krassilnikov) the increase in acid also stops with the termination of cell multiplication. In computing the amount of acid formed by a cell it became evident that the cells were most productive in the period of energetic multiplication. In the quiet stage they were much less active. Cells that have completely lost their ability to multiply obviously cannot oxidize alcohol to acetic acid.

The dependency of cell development on the pH value of the medium cannot be considered separately from the rest of the culture conditions. Among other things the composition of the medium is

very important. Thus Esch. coli (Speyer, 1924) grows equally well in peptone-bearing beef bouillon at pH values of 4.7 to 9.0. Multiplication at pH 4.5 to 9.0 is also possible in a plain synthetic medium with ammonium lactate as a source of nitrogen and carbon, although minimal at the limiting values. Growth is especially difficult in this medium in the acid range in which it is possible only after previous adaptation of the cells at pH 5.4. In comparison with beef bouillon the optimum growth range for a synthetic medium is very narrow and lies around pH 7 (Fig. 17, a).

Paratyphus bacteria also have a broad optimum pH from 5 to 8.4 in beef bouillon. The optimum pH in a synthetic medium with an ammonium salt as source of nitrogen and acetate as source of carbon is at 7.1 to 8.4. A parasitic microorganism in cold-blooded animals has an optimum pH at 6.6 to 7.8 in beef bouillon (Kondo, 1925), and at pH 7.1 to 7.8 in a synthetic medium with acetate and an ammonium salt.

The wide pH range within which growth ensues on very richly nutritive culture mediums is probably conditioned by the fact that bacteria have more opportunities to control the pH on these mediums than on plain culture mediums. Growth slackening at the pH limiting range is particularly conspicuous in mediums containing toxic substances. Thiocystis, an autotrophic purple sulfur bacteria experimented on in detail by van Niel (1931), developed at pH 6.5 to 9.5, but only with low H_2S concentrations. Hydrogen sulfide is certainly oxidized; however, it acts toxically in higher concentrations. This applies especially to the acid range in which H_2S is present as a non-dissociated molecule, few toxic sulfides appear in the alkaline range. With an increase in the H_2S concentration in the medium the pH range, in which growth is possible, becomes more and more narrow especially on the acid side. Growth is still possible, finally, only in a pH range of 8.5 to 9.0 with 0.1% to 0.2% H_2S (cf. Fig. 17,c).

The ecological behavior of microbes is determined, among other ways, by their ability to adapt themselves to wider or narrower pH ranges. Saprophytic living bacteria, that are widespread in soil and water, can exist under very diverse conditions, among others in a different acid content of the substrate. In general they are adapted to wide pH ranges. That is true, according to Dernby (1921), for example, of Esch. coli (pH 4.6 to 9.6), Proteus vulgaris (pH 4.5 to 8.5), Serratia marcescens (pH 5 to 8), Bac. subtilis (pH 5.5 to 8.5), (cf. Fig. 15).

Microorganisms that are adapted to special locations and special ecological conditions, somewhat like pathogenic bacteria in animal bodies, have for the most part a narrow pH range, for example typhus bacteria at pH 6.5 to 7.5, pneumococci at 7.5 to 8.2, cholera vibrios at 6.5 to 7.5.

Types of the genus Mycobacterium grow under very diverse ecological conditions (Kondo, 1925). Some live in the soil and on the surface of plants as saprophytes; others live as parasites in cold-blooded animals. Mycobact. tuberculosis lives in humans and

some warm-blooded animals. If representatives of these three groups are cultivated in beef bouillon at various pH values, they behave in accordance with their ecological characteristics. The saprophytic types develop in a broad pH range from 6.0 to 8.4; the ones that are parasitic on cold-blooded animals grow in narrower pH limits from 6.5 to 7.8; Mycobact. tuberculosis grows only between 7.5 to 7.8 (cf. Fig. 17, b).

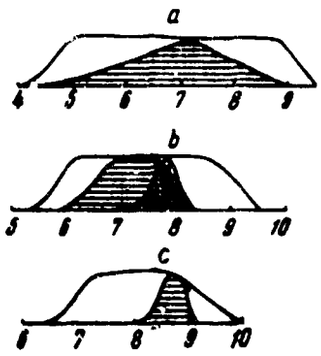


Figure 17

a. Growth curve of Esch. coli in a medium with ammonium lactate (cross-hatched) and in beef bouillon (according to Speyer, 1924).

b. Growth curve of three representatives of the genus Mycobacterium (according to Kondo, 1925).

c. Growth curve of Thiocystis spec. at various H_2S concentrations. Cross-hatched area: Growth at a high H_2S concentration (according to van Niel, 1931).

2. Indirect Influence of the Hydrogen Ion Concentration on Microorganisms.

The degree of dissociation of vitally necessary substances for the cell depends on the pH value of the culture medium. In an acid medium weak acids appear predominantly as molecules, in an alkaline reaction as ions, because the salts of weak acids are strongly dissociated and the acids themselves are only weakly dissociated. Each acid and each salt, therefore, has a critical pH range.

An indirect pH effect depending on the above was described by Uspenski (1927) in experiments on the importance of iron for the propagation of algae in bodies of water. Since the dissolved iron content depends on the pH value and is higher in an acid range than in an alkaline one, algae that are sensitive in the presence of higher iron concentrations prefer water with a weak alkaline reaction, and vice versa. Cladophora fracta is an alga that does not grow in water with pH values under 7.2. Uspenski ascertained from laboratory cultures that it still grows in synthetic mediums with a low iron concentration at pH 5.8.

Another case of indirect pH effect comes up when organic acids play a part in metabolism. The degree of dissociation of fermentations leading to the formation of neutral products is important for their persistence. Thus acetone and butanol result from the acetone-butanol fermentation of acetic and butyric acid. Acetylmethyl carbinol and butylene glycol are formed from ketopropionic acid by means of fermentation of the aerogenes type. ([Note:] It was formerly assumed that the acids themselves were not changed into neutral substances, but rather the compounds preceding them from which the neutral products are formed in an acid environment, the acids being formed by means of an alkaline reaction.)

In all these cases the formation of neutral products results only in an acid environment, if the acids, therefore, are present primarily as non-dissociated molecules. In neutral mediums, in which the acids appear in the form of strongly dissociated salts, no neutral products can be formed, but rather the acids are stored.

Similar observations were also made on other acid producing microorganisms. Brucella (Gerhardt and others, 1953) oxidizes a series of acids (ketoglutaric acid, succinic acid, fumaric acid, oxaloacetic acid, acetic acid and ketopropionic acid) at pH 5.5 and more slowly at pH 6.8. It is possible that undissociated molecules are more easily accessible also in this case. The authors associate this fact with the different permeability of the cell for ions and molecules.

As has already been mentioned, some purple sulfur bacteria prefer alkaline conditions, because hydrogen sulfide is toxic as H_2S in larger quantities in an acid environment, but is much less poisonous in an alkaline medium in an ionized state.

The same is also true of sulfate reducing bacteria that, according to Rubentschik (1947), do not grow, however, in acid

mediums. Their pH range in which growth results extends primarily over the alkaline range probably because of the toxicity of the non-dissociated H_2S molecules at pH 6.5 to 9.5.

With dyes also the toxic effect depends not only on the concentration but also on the degree of dissociation. Kobs and Robbins (1936) cultivated Rhizopus nigricans, Fusarium, Oxysporum and Gibberella on potato agar with sugar and on a synthetic medium at various pH values from 4 to 8 regulated with phosphoric acid and NaOH. Acid dyes (eosin, rose bengal) and a basic dye (dahlia) were added to the culture medium. The result was determined by weight in liquid, synthetic mediums, and by the size of the colonies in potato agar. A medium with the same pH value but without dye served as control. The basic dye had a toxic effect in a more alkaline medium, the acid dye in a stronger acid medium. Moreover, dyes, as free acids or bases, are more strongly toxic than dissociated salts.

The entire molecule is not always responsible for the toxic effect of acids. Occasionally the harmful action is associated only with the anion. Dervichian and Mousset (1949) have described such a case for lauric acid. They cultivated Esch. coli and Proteus vulgaris on a synthetic medium with sugar, ammonium nitrogen, nicotinic acid as an indispensable biocatalyzer and lauric acid which is non-toxic in concentrations of 0.04% to 0.4% at 7.5 and below, but which inhibits growth over 7.6. In relatively low concentrations of 0.04% to 0.15% it acts bacteriostatically, and bactericidally in concentrations over 0.15%. It is to be assumed that the toxicity of acid anions or of the entire acid molecule depends on their ability to permeate the cell.

Summary. Life activity of microorganisms is altogether possible in the very broad pH range from about 0.6 to 11.0. For the individual types the range is considerably more narrow. Basophilic and acidophilic microbes can be differentiated according to the pH demands.

Microorganisms that have become adapted to special living conditions usually grow only in narrow pH ranges, for example pathogenic bacteria. On the other hand, saprophytes that are diffused everywhere in nature can exist within wide pH ranges. The influence of the pH value on microorganisms can be direct (due to direct action of the H and OH ions on the cell) or indirect (due to the influence of the condition of the intermediary products formed in metabolism).

II. Influence of the Hydrogen Ion Concentration on Metabolism.

The effect of the pH value is seen quantitatively in a growth retardation particularly at the limits of the characteristic range for the respective type. The effect is of a qualitative nature within this range. Metabolic processes and the formation of the final products for example in the course of fermentation, are influenced by the pH value. Therefore, the pH value is one of the most important factors in the regulation of technically important fermentation processes. Along the way it influences the

course of the reaction by means of the formation and activation of enzymes. The degree of dissociation and the solubility of metabolites are modified by means of which their further production or storage is determined. The redox conditions are also dependent on the pH, in accordance with the following formula:

$$rH_2 = \frac{eH}{0.029} + 2pH$$

Under otherwise equal conditions the rH_2 is changed due to an alteration of the pH value. Oxidative conditions prevail in alkaline mediums, reductive conditions in acid mediums.

The studies made by Gale (1943, 1948; Gale and Epps, 1942, 1944) are important in this connection. In their research on the effect of the pH value on the biochemical activity of microorganisms he came to the conclusion that bacteria probably have a great number of ferments available. Depending on the external conditions, especially on the pH value, in different stages of development only part of the available ferments are present at any given time in an active state.

The research performed by Gale and Epps (1942) was made on Esch. coli and Microc. lysodeikticus ([Note:] This type of designation is not recognized either by Bergey or Krassilnikov.) A culture medium of casein hydrolysate obtained with the aid of trypsin was used. Esch. coli grows at pH 4.2 to 9.5 on a first-class medium like this. pH values from 4.5 to 6.0 were regulated with phthalates (M/60 concentration), from pH 6 to 8 with phosphates and from pH 8 to 9 with borates. The pH values could be kept constant within 0.5 pH units by using these buffer solutions. The activity of the various ferments was determined in washed cell suspensions after the addition of the substance being used as a substrate. The activity was determined with amino acid deaminases by measuring the forming ammonia, with decarboxylases by means of CO_2 formation, with dehydrogenases in accordance with Thunberg's method. It appeared that some ferments are formed and activated, independently of the pH values at which the microorganisms grew (group I). The dehyrogenases of formic acid and of alcohol, in addition to catalase, urease, fumarase, belong to these ferments that cause above all the destruction of toxic metabolic products. They are entirely active in a broad pH range from 5 to 8. ([Note:] According to Virtanen and Winter, 1928, catalase is formed at pH 4.8 to 8.5 in an equal amount.)

Other ferments (group II) on the other hand are formed depending on the pH value, like hydrogenase, succinic acid dehydrogenase, the zymase complex tryptophanase, alanine desaminase, glutamic acid desaminase, arginine decarboxylase, lysine decarboxylase, histidine decarboxylase.

The ferments that split the carboxyl group from amino acids and consequently reduce the excessive acid content are particularly active in an acid environment. They are formed by cultivation in a medium from pH 4.5 to 6.0. Alkaline compounds, the amines

$$RCHNH_2COOH \longrightarrow RCH_2NH_2 + CO_2$$

result from the amino acids after separation from the carboxyl groups.

They dissociate in the following manner:

$$RCH_2NH_3^+ + OH^-$$

Decarboxylases are produced only in small amounts when they are cultivated in an alkaline medium at pH 7.8. On the other hand the desaminases are active, so that ammonia and acids result from amines:

$$RCH_2NH_2 \longrightarrow RCOOH + NH_3$$

Ammonia is released and escapes due to deamination of the amino acids; the resulting acids cause a decrease of the pH value in the medium.

Gale's conclusions are not generally valid. In some cases the formation of the decarboxylases result with uniform intensity at various pH values, for example, according to Mardaschov and others (1949), in Clostr. cadaveris and Pseudobact. (designation according to Krassilnikov) spec., while the observations on Esch. coli agree with the results of Gale's research.

A further case of the effect of the pH on the process of metabolism is the influence of the state of metabolites, especially of acids, that appear free or as a salt depending on the pH value. They are present as undissociated molecules with a definite acidity, characteristic of each acid. They are neutralized in a weak acid medium and appear in the alkaline range only in the form of a strongly dissociated salt. (cf. Osburn and others, 1937, in this respect.)

Neuberg and Färber (1917) were the first to discover the dependency of the fermentation process on the acid and alkaline content of the medium in alcoholic fermentation. In the following years relationships of this type were established in many fermentations that occur on formation of neutral products.

The formation of other products (for example of acids by means of fungi and bacteria) is also dependent on the pH.

Alcoholic fermentation. The fermentation of living cells (Neuberg and Hirsch, 1919) and of cell sap of macerated cells (Neuberg and Färber, 1917) occurs both in an acid medium and in the presence of alkaline salts and bases with uniform intensity.

pH information was missing from Neuberg's first studies. However, it can be assumed from the salts used by him that he performed the fermentation at about pH 8, whereas normal fermen-

tation without any additive occurs at about pH 4.

A 10% sugar solution was completely fermented after the addition of 10% press-yeast (strain M) within 48 hours in the presence of the following salts and bases:

K_2CO_3 ..... 0.1 mol or 1.4%

K_2HPO_4 ..... 1.0 mol or 17.4%

MgO ..... 0.125 mol or 0.5%

Na_3PO_4 ..... 0.125 mol or 2.1%

$Na_2HPO_4 \cdot 12H_2O$ ..... 1.0 mol or 36.0%

$Zn(OH)_2$ ..... 0.5 mol or 5.0%

Considerable additives like these without a doubt kept the pH value of the medium in an alkaline range for 48 hours. On the other hand, in normal fermentation other fermentation products appeared, namely acetic acid, more glycerin, less alcohol.

According to Neuberg normal alcoholic fermentation occurs in the following manner:

$$C_6H_{12}O_6 \rightarrow 2\,CH_3COCHO + 2\,H_2O \qquad (1)$$

$$\begin{matrix} CH_3COCHO \\ CH_3COCHO \end{matrix} + \begin{matrix} H_2 + H_2O \\ | \\ O \end{matrix} \rightarrow \begin{matrix} CH_2OHCHOHCH_2OH \\ CH_3COCOOH \end{matrix} \qquad (2)$$

$$CH_3COCOOH \rightarrow CO_2 + CH_3CHO \qquad (3)$$

$$\begin{matrix} CH_3CHO \\ CH_3COCHO \end{matrix} + \begin{matrix} H_2 \\ | \\ O \end{matrix} \rightarrow \begin{matrix} CH_3CH_2OH \\ CH_3COCOOH \end{matrix} \qquad (4)$$

Under alkaline conditions the following reaction takes place instead of (4):

$$\begin{matrix} CH_3CHO \\ CH_3CHO \end{matrix} + \begin{matrix} H_2 \\ | \\ O \end{matrix} \rightarrow \begin{matrix} CH_3CH_2OH \\ CH_3COOH \end{matrix} \qquad (5)$$

Methylglyoxal serves simultaneously replaced by acetaldehyde, after reaction (2) as an additional source of glycerin and keto-propionic acid. Although nowadays methylglyoxal is no longer considered an intermediary product in fermentation, the representation of reaction (5) is none the less probable.

Peynaud (1940) demonstrated that yeasts form acetic acid at pH 7.5 up to 2 grams per liter, which disappears at pH 3.5 to 4.5 and is reduced to alcohol when it is added to the medium. Acetic acid, therefore, appears at the beginning of the yeast growth on neutral mediums and causes a drop in the pH value that is characteristic of alcoholic fermentation. No more acetic acid is formed after acidification of the medium. CO_2 and alcohol result as final products of the fermentation.

We are in a position, by means of experimental alteration of the pH value, to take advantage of the biochemical activity of yeasts for forming at times various products.

Butanol-isopropanol fermentation was thoroughly investigated by Werkman and colleagues. The Clostr. butylicum, isolated by Beijerinck and described by van der Lek, forms various neutral products by fermenting sugar: butanol, isopropanol, ethanol; in addition, acetic and butyric acid (besides these acids formic, lactic and keto-propionic acid could be established), and gases (CO_2 and H_2).

At first acetic and butyric acid result from the fermentation of sugar or of salts of keto-propionic acid. If the pH value of the medium drops below pH 6.3, the formation of acid ceases and butanol and other neutral products come into being. The critical pH value for butanol fermentation is, therefore, at pH 6.3. By adding a base (for example chalk) only acids are formed and increased in the medium. In this way it is possible to regulate the fermentation process so that either alcohol or acids result.

Acetone-butanol fermentation is used industrially to obtain butanol and acetone. The Clostr. acetobutylicum, so intimately connected with butyric acid bacteria, ferment starchy products to acids (acetic and butyric acid), to neutral products (acetone, butanol, ethanol) and to gases (H_2 and CO_2). In studying this fermentation Shaposhnikov (1939) was the first to express the thought that fermentation proceeds in two phases. According to numerous studies made by him a two-phase process is assumed for almost all bacterial fermentations.

During the first hours of an acetone-butanol fermentation acids (acetic and butyric acid) that decrease tge pH value in the medium come primarily into existence. In the ensuing second phase neutral products are formed predominantly (Fig. 18). The amount of the acid decreased due to conversion into neutral products. This fermentation process of mediums containing starch and sugar results under optimum nutrition and temperature conditions.

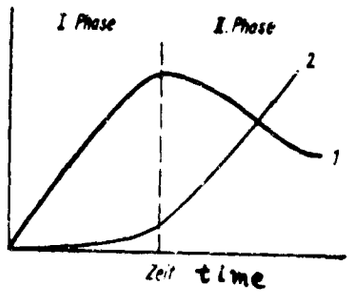


Figure 18. The two phases of acetone-butanol fermentation (according to Shaposhnikov, 1939).
1. Acids;
2. Neutral products.

A typical butyric acid fermentation can be produced by the influence of the reaction conditions. The addition of chalk is sufficient to hold the pH value in the neutral range and the fermentation in the first phase (Table 19).

TABLE 19

Fermentation products in mg per 50 ml of a 6% corn-mash in acetone-butanol fermentation

(according to Fernhauer and others, 1936)

Fermentation product	without chalk	with 2 g of chalk per 50 ml of culture solution
butyric acid	32.4	630
butanol	411.5	45.7
acetic acid	102.1	230.7
ethanol	44.5	22.2
acetone	222.3	13.2

The physiological age of the bacteria must also be considered. The bacteria can regulate the pH value particularly in the second phase of development by means of more or less intensive conversion of acids to neutral products. Since the different processes have a different pH optimum, specific processes predominate in the individual fermentation periods. First acids, then neutral products are formed. Therefore, the optimum pH of the fermentation is constantly shifting during the course of the fermentation. After four hours it is at 5.1 to 5.9; after 18 to 19 hours, at 4.6 to 5.3; after 28 hours, at 4.4 to 5.2

Acetone-ethanol fermentation has not been used to date industrially. However, it is possible that in time practical importance will be achieved. Bac. acetoethylicus ([Note:] This designation is not recognized either by Bergey or by Krassilnikov) is an agent that ferments starch or sugar by forming acetic acid, alcohol, acetone and gases (CO_2 and H_2).

According to Shaposhnikov, this process also is carried on in two phases. In the first phase Cannizzaro's reaction occurs under neutral conditions between two molecules of acetaldehyde. The resultant acetic acid shifts the pH value to the acid side. In the second phase an aldol condensation of two acetaldehyde molecules results from simultaneous oxidation at the expense of the acetic acid, in which case acetone results as a neutral product. Since this second process occurs without the formation of acid, the pH value remains unchanged.

Fermentation takes place within wide pH limits from about 4 to 10. Growth sets in within these limits, if the pH conditions are more favorable. The optimum initial pH value is at 6.5 to 7.5 (Saizev, 1939).

Arzberger and others (1920) studied the fermentation process of Bac. acetoethylicus (a type of designation not recognized either by Bergey or Krassilnikov) with different pH values in the medium. The pH value was around 5.8 in fermentation with chalk. Stronger alkaline conditions were produced by means of a daily addition of alkali to the fermenting medium with bromcresol purple (pH 6.2) or phenol red (pH 8) as an indicator. With an increase in alkalinity the amount of volatile acids (formic and acetic acid) increased, the quantity of neutral products (alcohol and acetone) decreased, although the intensity of the fermentation, judged according to the amount of the fermented sugar, remained approximately the same (Table 20).

Saizev (1939) made similar observations with the fermentation of potato-mash. The pH value was maintained specifically by the addition of HCl or NaOH. This was not possible with a phosphate buffer, because the regulated pH value dropped to 4.6 to 4.8 within 24 hours. The amount of acids formed increased with a rising pH value (Table 21).

TABLE 20

Influence of the pH value on the formation of some products in acetone-ethanol fermentation. Medium with 0.5% peptone 0.1% K_2HPO_4 and 2% carbohydrate. Duration of experiment: two weeks. Fermentation products in grams per liter (according to Arzberger and others, 1920).

fermentation substrate	pH	acetic acid	ethanol	acetone	unfermented portion
glucose	5.8	0.72	4.11	1.35	2.29
	6.2	3.27	3.12	0.68	3.49
	8.0	5.04	2.08	0.94	3.33
sucrose	5.8	2.55	4.75	1.06	4.86
	6.2	4.40	5.06	1.53	4.60
	8.0	5.40	3.46	1.20	5.07

TABLE 21

The influence of the pH value on the amount of fermentation products (in grams per liter) in the fermentation of acetone-ethanol (according to Saizev, 1939)

pH	ethanol (1)	acetone (2)	volatile acids*	proportion of (1):(2)
5.5-6.1	19-24	8-10	3.1	2.4
6.3-6.6	20-25	7.8-9.7	5.0-5.4	2.6
7.0-7.4	19.6-21.2	4.5-5.9	24-29	4.5

*Data in cm^3 of 0.1N solution per 10 cm^3

The proportion of the neutral fermentation products also changed, depending on the pH value. In each case more ethanol than acetone was formed. In the range of pH 5.5 to 7.5 the amount of alcohol was only slightly dependent on the pH. On the other hand the formation of acetone seemed to depend considerably more on the pH value. Double the amount of acetone was formed in the acid pH range than in the alkaline range.

The proportion of ethanol to acetone was, therefore, greater with an increasing pH value. Acetone was produced so abundantly in the acid pH range that the pH value went up; in the alkaline

range volatile acids were accumulated in a greater amount.

It is necessary for a technical application of obtaining acetone to know the pH range in which the acetone yield is greatest and the yield of the other products is smallest. The most favorable pH value of 5.5 to 6.1 for this is attained if the fermentation is performed with the addition of 1% to 2% of chalk.

With fermentations that occur with the formation of acetone the following general principles can be determined: The proportion of acids to neutral products can be influenced by means of the pH value. An alkaline environment excludes the formation of neutral products, especially of butanol and acetone. The formation of ethanol on the other hand is less strongly dependent on the pH. Up until now it has not been studied whether the pH value also influences the proportion of resultant acids, although this question can have practical importance, perhaps for obtaining butyric acid.

Butylene glycol fermentation appears with bacteria of the aerogenes group. Types and strains that form neutral products (butylene glycol and acetylmethylcarbinol) are also of interest from the point of view of technical microbiology, because the products formed by them find application in the chemical industry.

Mickelson and Werkman (1938) studied the dependency of the butylene glycol fermentation of Aerobacter indologenes on the pH value with washed suspensions and growing cultures. The critical pH value of this fermentation lies at 6.3, as it does with butanol-isopropanol fermentation. Outside of this value the fermentation takes place with variations (Table 22). The formation of neutral products (acetylmethylcarbinol and butylene glycol) occurs only in acid mediums. Acetate is converted to neutral fermentation products in a medium containing sugar. No formic acid results; in its place CO_2 and H_2 are formed as gases.

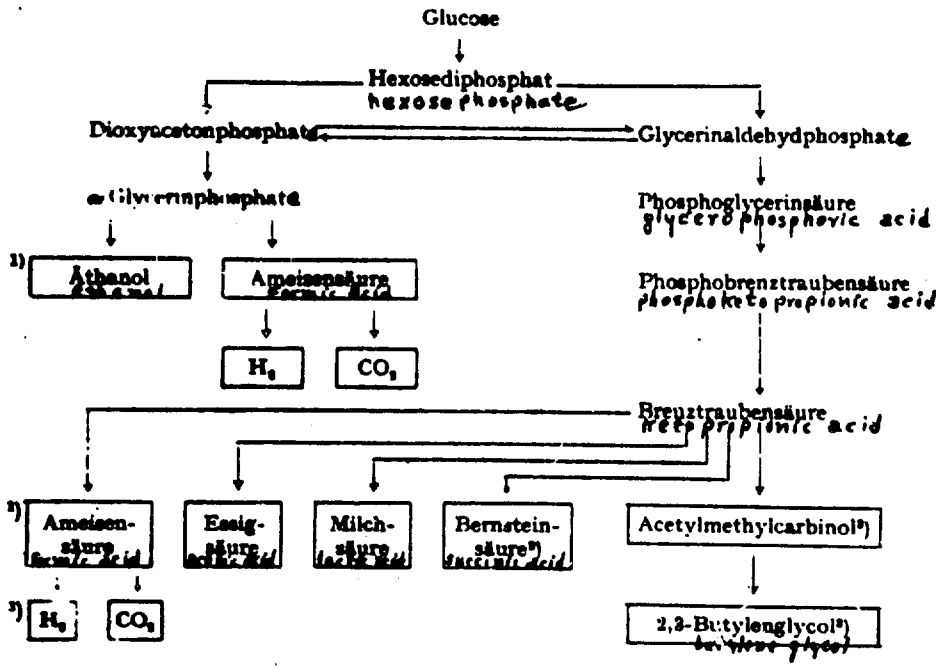
No acetylmethylcarbinol appears in a more alkaline environment in the course of the fermentation; butylene glycol is formed only in small amounts. Much formic acid but little gas is formed. Acetate added to the medium is not converted.

TABLE 22

Fermentation Products (in m mol per liter) of a culture of Aerobacter indologenes in a medium with glucose and acetate (according to Mickelson and Werkman 1938).

pH	amt. of fermented sugar	CO_2	H_2	formic acid	acetic acid	lactic acid	acetylmethylcarbinol	2,3-butylene glycol	ethanol	C in %
over 6.3	113	60	9	95	109	6.3	0	22.3	75	89
under 6.3	111	230	30	1	6	2.7	0.7	87	60	100

Gale (1943) formulated the course of coli-aerogenes fermentation as follows:



1) The boxed products are accumulated in the medium.

2) Succinic acid results from CO_2 and a C_3 compound.

3) Acetylmethylcarbinol and 2,3-butylene glycol are formed only by bacteria of the aerogenes group.

Part of the acetic acid is present in a free state (pK = 4.62) in acid mediums at pH values under 6.3; it is neutralized at pH 7. It can be assumed that acetic acid is further converted in the formation of neutral products if it is at least partially in a free state, which is the case at pH 5.6 to 6.0.

Another group of metabolic products that are subject to the effect of the pH are formic acid and the gases resulting from it, CO_2 and H_2. At pH 7 acid is increased in the medium, whereas in this case CO_2 and H_2 are formed only in traces. Formic acid is fermented to CO_2 and H_2 in the acid range.

Acetic and lactic acid appear as a third group of metabolic products. Lactic acid predominates in an acid medium; in an alkaline medium on the other hand acetic acid predominates. By fermenting a definite amount of sugar to lactic acid the medium is less acidified than in the case of acetic acid formation, because the lactic acid molecule consists of three atoms of carbon one carboxyl group acetic acid, however, consists of two carbon atoms one carboxyl group. The effect of a pH regulating mechanism is seen here. Gale and Epps (1942) studied the pH conditions in neutral products for the formation of the enzymes concerned by means of Esch. coli and Microc. lysodeikticus (this type of designation is not recognized either in Bergey or in Krassilnikov).

Silvermann and Werkman (1941) cultivated Aerobacter aerogenes in an acid and a neutral medium. The ferment that catalyzes the formation of acetylmethylcarbinol and butylene glycol in a cell-free extract of mechanically broken up cells can be identified only if the cells have been cultivated in an acid medium. In extracts of cells from a neutral medium the enzyme whose optimum activity was at pH 5.6 was not identifiable. The ability to form the carbinol-producing enzyme with Aerobacter aerogenes itself is not completely lost after numerous passages in a neutral medium. Under these conditions no neutral products are formed; fermentation occurs as with Esch. coli. However, acetylmethylcarbinol is formed again already in the first transfer to an acid medium.

Orlova's (1950) experiments on the fermentation process in growing cultures of Aerobacter aerogenes confirmed the data of Mickelson and Werkman (1938): The more the initial medium was alkaline, the more time was required to lower the pH value to 6.0-6.5. The formation of neutral products commenced only then. At a high alkaline initial pH value these conditions were achieved when a considerable part of the nutritive material in the substrate had already been used up, so that the neutral products were formed only in small amounts.

The proportion of the acids (acetic and formic acid) to each other also depends on the pH value. With an initial alkaline pH of 8.1, 64.5% of the total amount of acid was acetic acid; only acetic acid was formed at pH 4.98.

In addition to the bacteria of the Aerobacter aerogenes type a strain of Bac. subtilis (known as the Ford strain) produced

a similar fermentation. Neish and others (1945) investigated the influence of the pH value on the production of fermentation products, in order to make the industrial extraction of butylene glycol profitable. A medium containing sugar was used as substrate. In one case fermentation occurred without pH regulation under increasing acid conditions; in the second case the medium was kept constantly at a neutral reaction during the entire fermentation process by adding IN NaOH (Table 23).

TABLE 23

Fermentation products of Bac. subtilis (Ford strain) in m mol per 100 m mol of fermented glucose in an acid and a neutral medium (according to Neish and others, 1945).

fermentation products	pH 5.8-6.2	pH 6.8-7.6
2,3-butylene glycol	56.15	36.16
acetone	traces	traces
glycerin	26.28	16.39
ethanol	18.24	28.70
lactic acid	39.13	53.08
succinic acid	traces	5.05
formic acid	9.97	30.14
acetic acid	--	3.98
butyric acid	--	2.76
CO_2	130	101.01
glucose fermented in 4 days	73.7	68.2
C in %	100	97

The formation of butylene glycol depended on the pH in the same way as with bacteria of the aerogenes group: In an acid medium it was greatest; the same was true of glycerin. In contrast with the other neutral products ethanol was formed in large amounts in neutral mediums. Volatile acids were produced principally under neutral conditions; likewise lactic acid that is formed more intensively by bacteria of the coli-aerogenes group in acid mediums.

Neutral C_4 products appear predominantly at acid pH values in the course of butylene glycol fermentation. Here the formation of gas is also strongest. At a neutral pH value formic acid and acetic acid are formed, while lactic acid is produced at an acid pH value (except for Bac. subtilis). Ethanol is obtained with this fermentation, as it is with acetone fermentation, both in acid and in alkaline mediums.

TABLE 24

The fermentation of glucose by means of washed cell suspensions of Esch. coli in phosphate buffer at a different pH value. Fermentation products in % of fermented glucose in two parallel experiments (according to Tikka, 1935).

Fermentation products	pH 6.3 after 16 hrs.		pH 7.0 after 9 hrs.		pH 7.4 after 20 hrs.	
lactic acid	46.3	40.8	20.4	21.5	4.1	2.7
acetic acid	4.5	6.0	18.1	20.3	29.4	34.1
formic acid	2.8	3.7	16.2	12.0	20.2	26.8
alcohol	21.1	19.3	21.0	22.0	22.1	21.1
hydrogen in cm^3	181	146	45	46	29	37

Tikka (1935) studied, by means of the fermentation of Esch. coli the pH influence of the fermentation process on suspensions of cells washed in phosphate buffer (Table 24). Quite small changes in the pH range around 0.5 units produced considerable shifts particularly with respect to acid formation. Volatile acids (acetic and formic acid) were produced predominantly at pH 7.5, but only a little lactic acid. The proportions were reversed in a weakly acid medium at pH 6.3. Just as was the case with bacteria of the aerogenes group, CO_2 and H_2 were formed principally in an acid pH range. Ethanol was present in an approximately equal amount in weak alkaline and acid mediums.

Stokes (1949) obtained approximately the same results. The amount of the volatile acids merely grew somewhat more slowly with increasing alkalization; the alcohol yield remained the same at a pH range of 6.5 to 7.9 and decreased only under pH 6.5. The formation of succinic acid dropped with an acid reaction (Table 25).

TABLE 25

The influence of the pH value on the fermentation of Esch. coli. Fermentation products in mol per mol of fermented glucose (according to Stokes, 1949)

pH	resulting acids: lactic acid	succinic acid	volatile acid	alcohol
5.62	0.95	0.14	1.05	0.48
6.00	0.74	0.19	0.75	0.50
6.5	0.32	0.31	0.79	0.78
7.0	0.10	0.26	1.51	0.81
7.46	0.07	0.26	1.39	0.82
7.96	0.05	0.24	1.44	0.83

Cook and Alcock (1931) determined the activity of the ferments of Esch. coli at different pH conditions. They studied the oxidative ferments of the cells worked up with toluene under aerobic and anaerobic conditions with methylene blue as hydrogen acceptor. It turned out in these experiments that the dehydrogenases of formic acid, lactic acid and succinic acid were inactive in an acid medium at pH 4. At pH 5 to 6 the activity increased; it attained its maximum in the neutral range and with alkalization above all it remained at the same level. A decrease resulted only at pH 9.5. The results were approximately the same under aerobic and anaerobic conditions, with a slight shift of the optimum toward the acid range with aerobic conditions (Table 26).

TABLE 26

The optimum pH of the dehydrogenases of various acids with Esch. coli and the pK of the respective acids (according to Cook and Alcock, 1931)

Experimental conditions	acids		
	formic acid	lactic acid	succinic acid
Initial pH under anaerobic conditions with methylene blue	7.0	7.4	8.5
Initial pH under aerobic conditions	6.0	6.3	7.6
pK of the acids	3.68	3.86	5.28

From the above it follows that the dehydrogenases act only on neutralized, dissociated acids, but never on free acids. The resulting acids are oxidized only under alkaline conditions, while they accumulate under acid conditions. This holds true for lactic acid. On the other hand formic acid can be oxidized or -- in acid mediums -- fermented to H_2 and CO_2.

Moreover with Esch. coli fermentation is a complicated process with which the pH acts on a whole series of partial processes: on the formation of lactic acid which is produced primarily in an acid medium, and on the production of gases from formic acid which is also to be observed principally at an acid pH value. In addition some products -- ethanol and in part succinic acid -- are formed independently of the pH value.

Lactic acid fermentation, for example by means of Lactobac. delbrueckii, for the industrial extraction of lactic acid. The bacteria ferment maltose, in which case lactic acids results exclusively in amounts up to 98% of the fermented sugar. It is known from many years' experience that lactic acid fermentation accomplished by Lactobac. delbrueckii occurred independently of

whether or not the resulting acid is neutralized. There is, however, a substantial difference quantitatively. The fermentation quickly stops without the addition of chalk. The pH value drops so far that further multiplication becomes impossible (pH 3.9). In this case a maximum of 1.7% acid can be produced. Considerably greater amounts of sugar (up to 10%) are converted with neutralization: the reaction of the medium remains neutral.

Lactobac. delbrueckii apparently has only a limited number of ferments and can only ferment sugar to lactic acid. Since the acid is not converted further, its degree of dissociation is insignificant; the fermentation process is independent of the pH value of the medium.

Streptoc. faecalis var. liquefaciens can also supply up to 90% lactic acid from sugar, although it is not strictly homofermentative. The fermentation caused by it is susceptible to pH changes. Gunsalus and Niven (1942) found that the amount of lactic acid formed decreases with increasing alkalization of the medium, whereas the amount of volatile acids and of alcohol becomes greater (Table 27). The fermentation process, therefore, becomes heterofermentative under alkaline conditions.

TABLE 27

Fermentation products of Streptoc. faecalis var. liquefaciens in m mol with fermentation under various pH conditions (according to Gunsalus and Niven, 1942)

fermentation products	pH 5	pH 7	pH 9
lactic acid	87.0	73.0	61.0
acetic acid	6.1	9.4	15.6
formic acid	7.7	16.8	26.4
ethanol	3.5	7.3	11.2
fermented glucose	63.6	112.0	112.0
C in %	95.0	90.0	88.0

White and others (1955) obtained similar results with Streptoc. pyogenes. The shift of the fermentation in a heterofermentative direction was especially strong here under alkaline conditions (pH 7.8 to 8.2) in a medium with galactose, considerably weaker on the other hand with glucose.

Further research was conducted on Streptoc. faecalis (Gunsalus and Campbell, 1944). Citric acid was used as substratum, because it is more strongly oxidized than sugar. By this means the characteristic was affected substantially. In addition to lactic acid, CO_2 and acetic acid were also produced. Lactic acid, acetylmethylcarbinol and CO_2 resulted in an acid pH range in experiments in a nitrogen atmosphere with growing and dormant cells. At pH 8.5 the formation of lactic acid was less; CO_2, formic and acetic acid appeared in its place as fermentation

products.

According to Campbell and Gunsalus (1944) homofermentative types, like Lactobac. delbrueckii and Lactobac. casei, can also grow in mediums with citric acid as a source of energy. With it other fermentation products (acetic acid, CO_2) are also formed in addition to lactic acid. In this case the different degree of oxidation of the substratum seems to cause the conversion.

According to Shaposhnikov and Semenova (1949) the homofermentative bacteria (Lactobac. brevis) can not only produce lactic acid but also utilize it. Lactate is consumed at the beginning of growth at pH $\geqslant$ 6 in a peptone medium with sugar and calcium lactate. Lactic acid formation begins when the pH value drops to $\geqslant$ 6. On the other hand acetic acid results already at somewhat higher pH values (Table 28.)

TABLE 28

Change in the pH value and fermentation process in a growing culture of Lactobac. brevis (according to Shaposhnikov and Semenova, 1949)

duration of experiment in hours	pH	acids in m mol		alcohol in m mol	fermented substrate in m mol	
		lactic acid	acetic acid		glucose	lactic acid
0	7.6	--	--	--	--	--
4	6.6	0	9.6	4.6	4.5	4.4
8	6.3	0	9.9	6.4	6.4	6.2
24	6.1	4.2	13.2	5.1	11.7	0
120	5.2	18.0	17.0	17.5	30.0	0

Different types of lactic acid bacteria react differently, therefore, to pH changes in the medium. It holds true as a general rule that lactic acid is formed in acid mediums; however, at somewhat higher pH values volatile acids (acetic and formic acids) are formed.

The production of acids by fungi is frequently described under the heading "fungus fermentations" whose process is often difficult and unreliable to reproduce, presumably because the external conditions are not yet sufficiently known. First of all it is a question of the production of citric, gluconic and oxalic acid by aspergilli and of lactic, succinic and fumaric acid by mucoraceae.

Aspergillus niger has been studied most frequently. It produces citric, gluconic and oxalic acid as well as CO_2 when cultivated on mediums with a large amount of sugar. Individual strains of Asp. niger have been used for the industrial extraction of citric acid. Butkevitch (1924) made a detailed study of acid production by Asp.

niger. This fermentation occurs under strongly acid conditions that the fungus itself creates by means of acid production. At the same time its growth ceases with neutralization of the acids by means of chalk (Table 29).

TABLE 29

Fermentation products in g with Asp. niger, with and without the addition of chalk to the medium (according to Butkevitch, 1924)

Fermentation products	with $CaCO_3$	without $CaCO_3$
gluconic acid	0.840	0.280
citric acid	0.051	0.727
oxalic acid	0.474	0.0
fermented sugar	2.235	2.247
unfermented sugar	0.265	0.253

The growth of the fungus takes place equally well with and without calcium carbonate at a measured consumption of sugar. On the other hand the formation of metabolic products is different. The principal fermentation products of fermentation in an acid medium are citric acid and some gluconic acid. In a neutralized medium gluconic and oxalic acid were produced mainly, but only a little citric acid. Apparently gluconic acid is made in a medium without chalk at the beginning of fermentation before the pH value drops. Fermentation takes place in an acid medium when citric acid is produced. pH 2 is given as the optimum for Asp. niger. The optimum pH value with another citric acid producing fungus, Citromyces glaber is between 3 and 4 (Frey, 1931). Jacquot (1938) investigated in detail the dependency of oxalic acid formation on the pH value. Oxalic acid is produced only under alkaline conditions in which the optimum pH value is different for the individual types: For Asp. niger it is at pH 6-6.5; for Pen. solitum, at pH 7.5-7.8, and for Asp. oryzae at 8.0-8.5.

Here the self-regulating mechanism of the pH value by means of fungi is seen plainly. In Asp. niger with citric acid as a carbon source a weight-yield of 197 mg resulted, in which case no oxalic acid was produced. With potassium citrate as a source of carbon the medium became alkaline due to consumption of the citric acid, and 0.01 g of oxalic acid resulted, so that the alkalinity of the medium was compensated.

Summary. The hydrogen ion concentration of the culture medium has considerable influence on the course of the metabolic processes. It affects the production and the activity of the ferments, the type of intermediary products, their dissociation and solubility. Considerable alterations in the proportion of fermentation products can be attained by means of pH changes.

The following general principles apply: Fermentations in whose course neutral products are to be formed require an acid

reaction. Neutralization excludes the formation of neutral fermentation products. This applies to acetone, butanol, butylene glycol, acetylmethylcarbinol and ethanol, which is certainly formed in some cases both with an acid and an alkaline reaction.

The proportion of the acids formed by various microorganisms is also changed depending on the pH value. Thus formic acid is obtained in neutral and alkaline mediums, while it is broken up into CO_2 and H_2 with an acid reaction. Volatile acids are formed primarily in neutral mediums, and lactic acid is produced principally with an acid reaction. Fungi form mainly citric acid in an acid medium, oxalic and gluconic acid on the other hand in neutral and alkaline mediums.

By altering the pH value, therefore, the composition of the metabolic products is changed in a way that is advantageous for further growth. It is a question of an adaptive process that depends on the activity and the combined action of the individual enzymes.

III. Influence of Microorganism on the Hydrogen Ion Concentration of the Culture Medium.

Microorganisms are able to modify in the course of their development the pH value of the medium. If we start out with the nutritional requirements, three large groups of microbes can be distinguished: Fermentation microorganisms that use nitrogen-free substances as sources of carbon and energy; poly-fermentative microorganisms that utilize both nitrogen-bearing and nitrogen-free substrates as energy sources; saprogenous microbes that make use of albumin and its fission products as sources of carbon and energy.

Only acid and neutral products, but no alkaline products, are produced in the course of fermentation by the microbian conversion of nitrogen-free substances, primarily of carbohydrates. Consequently the medium is more or less acidified. Two types can be distinguished: microorganisms that form only acid and microorganisms that also produce neutral products in addition to acid.

Homofermentative lactic acid bacteria, butyric acid bacteria and acid-forming fungi are to be included in the first group. Homofermentative lactic acid bacteria and many acetic acid bacteria that oxidize alcohol to acetic acid cannot regulate or modify the fermentation process. Therefore, the process takes place as long as the acids support the life activity of the agents. Fungi and some of the acetic acid bacteria have a fermentation metabolism that can be regulated. They are highly resistant to low pH values. Even if the supply of fermentable carbohydrates and other substances is exhausted, they do not stop their development, but rather they utilize the acids formed by them and they usually oxidize them to CO_2 and H_2O. By that means the pH value goes up. Autolysis sets in only after utilization of their own metabolic products. It is tied in with an alkalization of the medium, because ammonia and organic bases are released from the native albumin.

The production of acetic acid from alcohol by acetic acid bacteria is characteristic of over-oxidation. The acetic acid bacteria probably are not always able to do this (Dratvina, 1937). They perform an over-oxidation when the pH value has not dropped too much after exhaustion of the alcohol supply and the life activity has not completely stopped. Then the types that are resistent to a low pH value commence over-oxidation and in this way shift the pH value from acid to the more favorable neutral range.

The acetone-butanol bacteria, the butanol bacteria, the acetone-ethanol bacteria and the types of the aerogenes group belong to the second group. The formation of neutral products is a process for preventing an excessive production of acid and for regulating the pH in the medium. In the growth of acetone-butanol bacteria on carbohydrates acetic and butyric acid are formed at first. When the hydrogen ion concentration has reached a certain value (pH 4.5 to 5.0), the process begins that prevents further acidification: the acids or their initial stages are converted to acetone and butanol. The fermentation of bacteria of the aerogenes group, that have a further ability to regulate the pH, takes place in a similar manner: Formic acid is formed in alkaline mediums, with the result that the pH value drops. Formic acid is broken down into CO_2 and H_2 under acid conditions. Since carbonic acid acidifies the medium less strongly than formic acid, the pH value is regulated in this way.

Saprogenous bacteria and polyfermentative microorganisms utilize albumin, peptone or other amino acids as material for energy metabolism. The carbon chain can be oxidized to CO_2 by using amino acids. The amino group is supplied to anabolism for the formation of native albumins. Since the amount of nitrogen necessary for anabolism is usually smaller than the amount included in the amino acids, excess ammonia nitrogen is released in the medium. The pH value in the medium goes up more or less; therefore, in general an alkalization occurs with cultivation on albumin mediums (Kopaczewski 1931). Ammonia is dissolved in the water as NH_4OH, in which case the pH value goes up to 8 or 9. In the presence of alkalization the individual microorganisms behave differently. Proteus vulgaris and Alcaligenes faecalis belong to the types that tolerate a strong alkaline reaction. Other types prevent the accumulation of ammonia, since they form urea. Thus Ivanov and Smirnova (1927) established that Bac. megaterium, Bac. tumescens (designation according to Krassilnikov), Bac. mesentericus (designation according to Krassilnikov), Bac. subtilis and Bac. cereus var. mycoides form 12.8 to 15.5 mg of urea on 10 ml of peptone-bearing gelatin. Urea is not increased in acid mediums, because urease is active in the acid range. The relationships are reversed in alkaline mediums. Therefore, a detrimental alkalization is prevented by means of the production of urea. This process is especially important on albumin mediums.

An excessive alkalization can also be inhibited by the formation of acid. Butkevitch (1903) made known the first information on this subject. Asp. niger, as a polyfermentative fungus, is able to grow as well on carbohydrate mediums as on peptone. Ammonia is enriched in the medium by cultivation on peptone; however, since Asp. niger forms large amounts of oxalic acid from peptone,

ammonia is neutralized. Pen. glaucum and Mucor do not have such a mechanism available. However, they form only small amounts of ammonia on peptone mediums, and they alkalize the medium only slightly. However, they decompose peptone only incompletely in catabolism and not all the way down to CO_2, H_2O and NH_3; acid amides are formed, causing only a weak alkalization.

Esch. coli has available a pH regulating mechanism similar to the one described by Butkevitch for fungi (Sierakowski, 1924). The pH value approaches the neutral point in each case with cultivation in beef peptone bouillon at pH values of 6.3 to 9.5; in old cultures, however, the medium becomes increasingly alkaline. The amino acids are extremely decomposed in an alkaline medium; CO_2 is formed, neutralizing the excessive alkali content. The more a medium is alkaline, the more CO_2 is produced and retained in the medium. If a substrate is acid, the resulting CO_2 is not retained but given off into the atmosphere, so that a neutral reaction gradually sets in as a result of the increasing ammonia content. In this way originally acid and alkaline mediums slowly become neutral. Then as the culture ages the amount of ammonia, produced simultaneously with CO_2, becomes so great that not enough CO_2 is present for neutralization. The subsequent alkalization finally reaches, regardless of the initial pH, values of pH 9.0 to 9.5 that stop all life activity.

Gale and Epps (1942) also called attention to the self-regulation of the pH value in coli cultures. Various ferments are formed by cultivating on albumin mediums under acid and alkaline conditions. The decarboxylases that separate CO_2 from the carboxyl groups of the amino acids are very active in an acid medium. On the other hand deaminases are produced by cultivation in alkaline mediums, so that amines and amino acids are destroyed and the alkali content of the medium decreases.

According to Manteyfel and others (1949) growth of Bact. formicum on a peptone medium takes place with calcium formiate at pH 7.6 without pH modifications. On the other hand both an alkaline medium (pH 8.6) and an acid one (pH 6.3) are regulated to about pH 7.6. The fact that no alkalization results in a culture at pH 7.6, although formic acid is consumed and a base (calcium) remains, is an indication of the presence of a process that counteracts alkalization. The more strongly alkaline a medium is, the more acetic acid is formed. On the other hand no acetic acid is produced in an acid medium. Its production occurs only when the alkalization has reached a definite degree (pH 7.4). If acetic acid is added to a culture at pH 6.5 it is consumed and the pH value of the medium goes up.

Finally it must still be mentioned that there also are mechanisms in higher plants for regulating the pH value. Sabinin and Minina (1928) observed that when roots of oats, buckwheat, wheat are put in solutions with pH values from 4.5 to 7.7, the pH value of pH 6.0 appeared in one to two hours. The underlying process is complicated; it is a question of a non-equivalent adsorption of cations and anions, an exosmosis of the cations and an elimination

of organic acids. Martinec and Rypacek (1947) and Cetl and others (1952) described a regulation of the pH in tissues of Potamogeton, Vicia faba, etc., in solutions of 0.01N NaOH and HCl with pH values from 3 to 9.

Summary. In cultures on carbohydrate-bearing mediums the production of acid can be so strong that the microorganisms concerned die off. Too strong an acidification may be prevented by means of the formation of neutral products in place of acids or by means of oxidation of the acids to CO_2 and H_2O.

An alkalization of the medium, in cultures on albumin mediums, ensues as a rule due to elimination of ammonia released by the decomposition of amino acids. Some microorganisms are adapted to strongly alkaline conditions; in others special regulating processes prevent the ammonia content from rising too high. The alkali content for example drops with the formation of urea from ammonia. Another process for decreasing the alkali content consists of the production of acids that yield only weakly alkaline ammonium salts with free ammonia.

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CHAPTER 5

GROWTH OF ANAEROBES AND THE REDOX POTENTIAL

I. Theory of Anaerobiosis

Anaerobiosis, life without oxygen, was observed for the first time by Pasteur in the year 1861. He found that living creatures are able to exist when they draw the energy necessary for living not from the oxidation of the substrate with atmospheric oxygen but rather from a transformation of this substrate without the participation of oxygen. He called this way of life fermentation.

Pasteur subdivided microbes into aerobes and anaerobes and differentiated strictly between them: oxygen is a prerequisite for life for aerobes, for anaerobes, on the other hand, it is a poison. Chudyakov (1896) conducted experimental studies on obligate anaerobes, butyric acid bacteria and some viruses. He determined, like Pasteur, that oxygen not only destroys vegetative cells but also spores. However, in addition he came to conclusions that are contrary to Pasteur's opinions. Chudyakov considered the difference between aerobic and anaerobic microorganisms as only quantitative. This point of view is based on the fact that cultures of obligate anaerobes consume oxygen at a low oxygen partial pressure (10 mm). Moreover, anaerobes can be adapted to higher oxygen partial pressures. Thus, for example, butyric acid bacteria that grew at a pO_2 of 5 mm succeeded in adapting to growth at 50 mm in six months. On the other hand, strictly aerobic microorganisms also grow at a low pO_2. Bac. subtilis and Asp. niger still grew, for example, at an oxygen partial pressure of 10 mm. Both anaerobes and aerobes can exist, therefore, at certain oxygen partial pressures.

Beijerinck (1899) supported virtually the same opinion with respect to anaerobiosis. "Respiration patterns" result by cultivating aerobic and anaerobic bacteria in a hanging drop. Aerobes accumulate on the edge of the drop, therefore, closer to the air; anaerobes (butyric acid and saprophytic bacteria) accumulate at some distance from the edge. According to Beijerick's opinion this is proof that anaerobes avoid an excess of oxygen, although they require a slight oxygen partial pressure.

Obligate anaerobes, therefore, are not aerophobic, but rather microaerophilic. Beijerinck doubted that obligate anaerobes can live over an unlimited period of time without any air supply. He assumed that they store a small amount of oxygen required for their growth as a reserve in the cells, so that they can exist for a certain period of time under completely anaerobic conditions.

On the other hand, the opinion is also advocated that life is impossible without oxygen. Windisch, Haehn and Neumann (1953) point out that yeasts, which are facultative anaerobes, do not

grow with a complete lack of atmospheric oxygen. According to their opinion only the energy of the oxidation processes is suitable for the support of life activity.

However, it is not possible to agree with opinions like these. There is a fundamental, qualitative difference between fermentation and respiration. In fermentation an intramolecular displacement of oxygen occurs, due to which one part of the molecule is oxidized at the expense of the other.

At present there is no doubt that this kind of reactions can supply energy for the life of microorganisms. This has been particularly studied in detail in the case of alcoholic fermentation which causes the formation of energy-rich phosphate compounds that are utilized by the microorganisms for the support of their life activity. Atmospheric oxygen does not participate in this. The same is also true of other anaerobic energy processes.

At present this concept is generally accepted. Thus Shaposhnikov (1955), Elsden (1952), Werkman and Wilson (1954), Veselov and others (1954, 1955), emphasize that material for systhesis is supplied by fermentation processes.

Oxidation represents another type of the energy process in which the introduction of a hydrogen acceptor from without is necessary.

Anaerobic and facultative anaerobic microorganisms have a fermentation metabolism in which the substrate is effectively oxidizing and reducing simultaneously.

Aside from this, in anaerobes the hydrogen can be extracted from the substrate to be oxidized and stabilized without participation of a hydrogen acceptor in the form of molecular hydrogen (H_2) as a fermentation product (Gest, 1954. According to the opinion of Shaposhnikov and others the hydrogen is not directly produced from the substrate, but rather by way of formic acid that is accumulated or decomposed to CO_2 and H_2 according to the type of microorganisms concerned.)

Aerobic microorganisms require an additional "oxidator" as a hydrogen acceptor besides the substrate to be oxidized. Atmospheric oxygen assumes this role in them.

Omelyanski (1904, 1953) talks about aerobic or oxidative and anaerobic or fermentative microorganisms that are associated with each other by means of changes. Facultative anaerobes occupy an intermediate position, because they can live according to the air supply of the medium both under aerobic-oxidative conditions and under anaerobic-fermentative conditions.

The question of why oxygen that aerobes tolerate in large amounts is toxic to anaerobes remained unanswered for a long time.

McLeod and Gordon (1923 a, 1923 b, 1925 a, 1925 b) were

the first to treat this problem. Anaerobes are particularly sensitive in the presence of hydrogen peroxide; a 0.0003%-0.0004% H_2O_2 content in the medium already prevents growth. On the other hand aerobes tolerate up to 0.015% H_2O_2. Hydrogen peroxide results in bacteria cultures from the oxidation (dehydrogenation) of the substrate by atmospheric oxygen. According to Wieland oxidation of the substrate (AH_2) occurs as follows:

$$AH_2 + O_2 \longrightarrow A + H_2O_2$$

Aerobic bacteria, which are relatively resistant in the presence of H_2O_2, have a specific ferment (catalase) that reduces hydrogen peroxide to water. Anaerobes, on the other hand, have no catalase available. By cultivating bacteria on blood agar it is possible to establish very small amounts of H_2O_2 that is being formed. Within the growth area of the bacteria the originally chocolate-colored agar takes on a greenish color due to reaction with hydrogen peroxide. Holman (1955) cultivated clostridia on agar mediums with access to air when the surface of the agar was coated over with an extract of beef liver containing catalase.

The hypothesis of McLeod and Gordon, however, is not completely satisfactory. Sherman (1926) referred to the fact that propionic acid bacteria have catalase and, nevertheless, remain anaerobes and are sensitive to oxygen. According to Virtanen and Winter (1928) they produce more catalase, for example, than the facultative anaerobe Esch. coli that forms catalase in an approximately equal amount under aerobic and anaerobic conditions.

Not all obligate anaerobic bacteria form H_2O_2 by contact with air. Bacterioides vulgatus belongs in this group (Vennesland and Hanke, 1940).

Quastel and Stephenson (1926) tried another explanation. Their experiments demonstrated that oxygen does not destroy anaerobes, but rather only stops their life activity. It is possible to pass oxygen for many hours through a washed suspension or a beef bouillon culture of Clostr. sporogenes and then the cells multiply again as the result of a hyperinoculation in a favorable medium, certainly after a period of latency extending up to 66 hours. If compounds with an SH group, acting as a redox system, are added to the medium, the latency period can be shortened.

Other reducing substances, for example glucose, also act in a similar manner. Quastel and Stephenson concluded that anaerobes cannot multiply any longer at a redox potential above a definite limit: that is to say, a low eH is necessary in the medium. They produce in the medium a low redox potential favorable for them, while they release, by means of albumin decomposition, substances with SH groups (like cysteine, glutathione). ([Note:] eH measurements were taken only later in anaerobe cultures by other researchers.)

We are grateful to Kligler and Guggenheim (1937) for experimental data that demonstrate that anaerobes are less sensitive

in the presence of free oxygen rather than in the presence of a high redox potential. Clostr. welchii does not grow under aerobic conditions in the usual beef peptone mediums. However, if a reduction substance, for example ascorbic acid, is added to the medium, growth is also possible under aerobic conditions. The more peptone is contained in the medium, the less ascorbic acid is needed, because the peptone itself acts as a reducing substance (Table 30).

Since the medium comes in contact with air, it contains dissolved oxygen. The amount of dissolved oxygen was determined colorimetrically with an alkaline pyrogallol solution; in beef bouillon with the addition of ascorbic acid it was almost exactly as high as in ordinary beef bouillon. An oxygen content of 95% was tolerated in the presence of vitamin C, while without ascorbic acid, contact with air (oxygen content 100%) was sufficient to inhibit growth. In spite of the presence of oxygen, the redox potential of the medium was lowered. Growth was possible when indigo carmine was reduced to 75% in the medium, corresponding to an eH of -125 mv.

TABLE 30

The influence of various peptone and ascorbic acid concentrations in the medium on the growth of Clostr. welchii (according to Kligler and Guggenheim, 1937)

ascorbic acid in g per l	peptone in %								
	0.0	0.25	0.5	1.0	1.5	2.0	3.0	4.0	5.0
1.0	-	±	+	+	++	++	++	+++	+++
0.2	-	-	±	+	+	++	++	++	+++
0.05	-	-	-	-	-	+	+	++	+++
0.02	-	-	-	-	-	-	-	-	-
0.00	-	-	-	-	-	-	-	-	-

- = no growth + = growth

The objection to a specific action of oxygen (Knaysi and Dutky, 1936) has been refuted by Van Niel (1937).

The importance of the eH as a supporting or inhibiting factor in the growth of anaerobes is acknowledged nowadays (Smith, 1949; Werkman and Wilson, 1954).

It is entirely possible that the role of compounds with an SH group is not only confined to a lowering of the eH. According to Messing's (1934) data, substances with an SH group do not lower the eH substantially in a sterile medium, approximately 60-70 mv. The growth of anaerobes (for example of butyric acid bacteria), on the other hand, is accelerated considerably. Messing assumes that SH compounds activate the reciprocal effect between microbes and the medium, without, however, developing concrete notions in this respect.

It must not be forgotten that substances with an SH group not only lower the eH of the solution but also the eH within the cells. It is conceivable that a slight change of the intracellular conditions affects the life activity of bacteria considerably.

The reason anaerobes do not tolerate a high redox potential of the surrounding medium must depend, according to Engelhardt (1944), on the fact that some vitally important ferments are inactivated at a high eH. Consequently the organism living anaerobically loses its capability for normal nutrition and dies for this reason and not because of poisoning as McLeod assumes.

Coenzyme A, which performs the transfer of acetyl groups, contains an SH group due to whose oxidation processes dependent on coenzyme A are inactivated with the result that in this way the organism loses its ability to live (Schapot, 1954).

The problem of the most favorable redox potential for anaerobes has been studied repeatedly. Aubel and Aubertin (1927) cultivated anaerobes and aerobes in an agar medium on high layer with the addition of various rH_2 indicators: methylene blue that is reduced to 50% at an rH_2 of 14.5; Janus green (green-rose at rH_2 12.0, rose-colorless at rH_2 4.5; neutral red at rH_2 3.0; safranine at rH_2 2.5.

They concluded from the behavior of the facultative anaerobes Esch. coli, Proteus vulgaris, Ps. fluorescens and of the obligate anaerobes Clostr. botulinum, histolyticum, sporogenes, putrificum (designation according to Krassilnikov), perfringens that facultative anaerobic bacteria grow in an rH_2 range of 0-20 and higher and that obligate anaerobes, on the other hand, develop in a narrow range of 0-12.

Yeasts grow in an rH_2 range from 7 to $>$ 20 (Aubel, Aubertin and Genevois, 1929). Growing colonies take on specifically the color of the medium. If the medium is dyed by means of an indicator, the colonies are also dyed; if the medium is colorless due to reduction of the dye, the colonies are also colorless.

Repeated attempts have been made by using indicator dyes to establish the limits of the rH_2 ranges at which growth is possible for anaerobes and aerobes (Aubel, Aubertin and Genevois, 1928; Prevot, 1938). The results vary and have an approximate value at most. For example, Prevot gives rH_2 14-20 for aerobes that grew on the surface of an agar medium, rH_2 7.4-14 for anaerobes of average sensitivity, values under 7.4 for strict anaerobes.

Since the colorimetric method is not satisfactory, an attempt has been made to arrive at more accurate delimitations electrometrically.

Plotz and Geloso (1930) ascertained colorimetrically and electrometrically that Clostr. tetani grows in a beef bouillon culture between rH_2 14 and rH_2 5.5. Growth was not possible either at rH_2 0 (addition of dithionite), or at rH_2 27 (addition of quinone) or at rH_2 15. The upper limit for the germination of spores of Clostr.

tetani lies at about rH_2 17-18 according to Knight and Fildes (1930). ([Note:] Computed by us from the pH and eH values of Knight and Fildes. The different eH values in the medium were obtained by introducing various mixtures of N_2 and O_2.)

According to Vennesland and Hanke (1940) Bacteroides vulgatus grows at rH_2 16.8, but no longer at 18.2 ([Note:] Same method as Knight and Fildes.)

Different eH values can also be adjusted in the medium, according to Hanke and Katz (1943), with the electrolytic method, by means of which oxygen or hydrogen is generated on an electrode inserted in the medium. ([Note:] The second electrode is in another receptacle connected with the culture receptacle by means of an agar bridge. According to the "hook-up" of the direct current source the electrode in the experimental receptacle is either anode or cathode.) The eH can be regulated rather accurately by varying the current intensity. For Bacteroides vulgatus and Clostr. sporogenes rH_2 16.8 (computed by us) resulted again as limit value.

The introduction of oxygen with the simultaneous lowering of the eH by electrolysis proved to be harmless as long as the limit rH_2 value was not exceeded.

Approximately the same limit values resulted in extensive experiments on various types of Clostridium by using the electolytic method at different pH values in the medium (Hanke and Railey, 1945; Table 31).

A greater accuracy can hardly be attained, because it is difficult to maintain the eH value of a growing culture constant over 10 to 30 hours.

An rH_2 of 8 resulted as optimum value for Clostridium types when graduated amounts of sodium thioglycolate and glucose were added to the medium as reducing substances (Reed and Orr, 1943).

Auber, Rosenberg and Gruenberg (1946) found good growth of Clostr. saccharobutyricum (designation according to Krassilnikov) and sporogenes at rH_2 11.2 to 12.4 and exceptionally still at 18.8. In other experiments the cultures were kept under O_2 and N_2. Concerning the O_2 content of the gas mixture growth was detected only with 1.4% O_2 (pH 6.8 eH +116 mv rH 17.4) and below.

The diverse results of electrometric experiments made only on the same types of bacteria demonstrate the difficulties of this kind of experiments. Also the amount of seeding affects the limit potential of growth. The greater the amount of seeding, the higher the limit potential.

We followed the course of rH_2 changes and growth in cultures of anaerobes in order to establish in this way the optimum redox conditions.

TABLE 31

Upper eH limits of growth at different pH values of the medium for three Clostridium types (according to Hanke and Railey, 1945) (rH_2 values computed by us)

Clostr. welchii			Clostr. sporogenes			Clostr. histolyticum		
pH	eH	rH_2	pH	eH	rH_2	pH	eH	rH_2
6.0	106	15.7	6.0	<100	15.4	6.4	85	15.7
6.2	131	16.9	6.2	130	16.9	6.6	90	16.3
6.4	160	18.3	6.4	144	17.8	7.0	60-76	16.0-16.5
6.6	150	18.4	6.6	136-152	17.7-18.2	--**	--	--
6.8	114	17.5	7.0	114	18.9	--	--	--
7.0	90	17.1	--	--	--	--	--	--
7.2	80	17.2	--	--	--	--	--	--
7.4	37-70	15.9-17.2	--	--	--	--	--	--
7.8	0-30	15.6-17.6	--	--	--	--	--	--
Average value 17.0			Average value 17.3			Average value 16.1		

** Determination not made

As is known, a strong drop in the rH_2 appears in the culture in the growth of anaerobes. If an oxidation substance that prevents a lowering of the rH_2 and maintains the redox potential at a definite level is added to the culture, its reaction on cell multiplication can be followed. It is recommended that in experiments of this type, rH_2 indicators be used according to a method employed by Dubos (1929 b). If an indicator is added to a culture in which the rH_2 is dropping, this lowering is stopped at the level of the rH_2 interval at which the added dye is reduced. From the moment at which reduction starts until the time when the dye is almost reduced, it holds the rH_2 at a certain characteristic level. In this way a kind of "plateau" results on the curve of the rH_2 decrease in a graphic representation; after reduction of the entire dye the rH_2 begins to drop again. How long the rH_2 is held at the same level depends on the amount of dye added.

Obligate anaerobic saprophytic bacteria that obtain their energy from connected redox changes of amino acids served as subjects of experiments. (Rabotnova, Toropova and Rabayeva, 1955). Stickland, (1934, 1935) demonstrated that Clostr. sporogenes reduces proline with simultaneous oxidation of alanine during formation of aminovaleric acid. Valine, leucine and α-ketopropionic acid were established as hydrogen donators; in addition to proline glycine can also be a hydrogen acceptor. If usable sugars are present in the medium, they are fermented according to the butyric acid type of fermentation, as was demonstrated by Rodopulo (1946) for Clostr. botulinum and is also probably the case with other sapro-

phytic bacteria.

At the beginning of the experiment the rH_2 value goes down and an intensive cell multiplication starts only after that (Fig. 19). Therefore, first of all the reductive conditions to which growth is tied are produced in the medium.

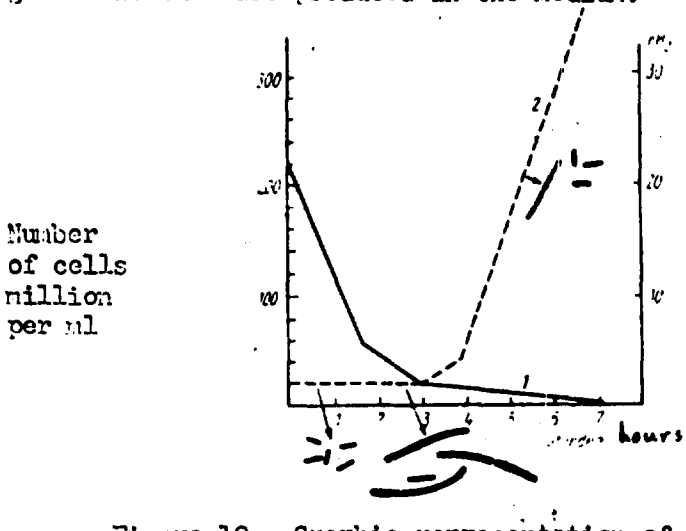


Figure 19. Graphic representation of the rH_2 changes in a culture of Clostr. sporogenes. Number of cells and their morphology.
1 = rH_2;
2 = number of cells.

Various rH_2 indicators were used to buffer the rH_2 value (Table 32); they completely delayed the growth of Clostr. putrificum. Their action was less strong on Clostr. sporogenes; Here neutral red was without effect. Already after 10 hours an intensive multiplication began without the addition of dyes; in the presence of dyes it set in only 24 to 48 hours later.

With a definite germ content the growth of the bacteria could be completely stopped by means of an appropriate concentration of dye (Table 33).

TABLE 32

rH_2 indicators for buffering the rH_2 value

rH_2 indicator	decolorized to 50% at rH_2	molecular weight
neutral red	3.	288
Janus gree	5.5	511
phenosafranine	5.9	322
indigo disulfonate	10.0	498
indigo tetrasulfonate	12.3	611
methylene blue	14.5	305
thionine	16.1	---

TABLE 33

Concentration of rH_2 indicators that stop the growth of anaerobes at a germ content of 2.0×10^7 cells per ml

rH_2 indicator 0.5% solution	Clostr. sporogenes		Clostr. putrificum	
	ml per 10 ml culture solution	concentration in the medium, in mol	ml per 10 ml culture solution	concentration in the medium, in mol
neutral red	1.5	0.0026	0.7	0.0013
Janus green	0.5	0.0005	0.5	0.0005
phenosafranine	1.2	0.0023	0.7	0.0014
indigo disulfonate	2.5	0.0025	2.0	0.0020
indigo tetrasulfonate	2.5	0.0017	2.0	0.0014
methylene blue	1.5	0.0025	1.0	0.0016

The limit concentrations of the various dyes, with the exception of Janus green, were relatively close to each other. The inhibition of growth can be explained by the fact that the dyes keep the rH_2 value at a level that is too high for anaerobes. The dyes themselves are not toxic; in a reduced form in the presence of $Na_2S_2O_4$ they inhibit growth only insignificantly (Table 34). For Clostr. sporogenes the inhibition held good only with phenosafranine also in the presence of sodium dithionite; obviously in this case the dye as such was toxic. Clostr. sporogenes grew only at an $rH_2 < 5$. Neutral red permitted the growth of Clostr. sporogenes, although somewhat retarded. Therefore, multiplication is quite possible at rH_2 3. Clostr. putrificum is still

more strongly anaerobic. It did not multiply in the presence of neutral red. That means that an rH_2 value of 3 is already too high. The action of other reduction substances, ascorbic acid or of hydrogen for example, was analogous to the effect of dithionite.

TABLE 34

The effect of sodium dithionite on the toxicity of the rH_2 indicators

	Clostr. sporogenes			Clostr. putrificum		
rH_2 indicator, 0.5% solution	ml of dye per 10 ml	$Na_2S_2O_4$	No. of cells in mill. per ml after the end of the experiment	ml of dye per 10 ml	$Na_2S_2O_4$	growth
neutral red	1.5	-	146	0.7	-	-
	1.5	0.015	201	0.7	0.015	+
Janus green	0.5		7.8	0.5	-	-
	0.5	0.015	150	0.5	0.015	+
phenosafranine	1.2	-	10	0.7	-	-
	1.2	0.015	10	0.7	0.015	+
indigo disulfonate	2.5	-	-	2.0	-	-
	2.5	0.015	150	2.0	0.015	+
indigo tetrasulfonate	2.5	-	46	2.0	-	-
	2.5	0.015	145	2.0	0.015	+
methylene blue	1.5	-	9.7	1.0	-	-
	1.5	0.015	208	1.0	0.015	+
control	-	-	210	-	-	+

During the time in which a lowering of the rH_2 value from the initial value results until multiplication sets in at a lower rH_2 value, the cells undergo considerable morphological modifications. First they increase in size significantly, especially in length, but they do not divide. As soon as the rH_2 value has reached a certain low level, an intensive cell multiplication takes place. The long filaments that begin to divide immediately at many places are changed into chains of cells that divide. This results in the picture of an ephemeral culture consisting of individual motile cells (Fig. 19).

Immediately after inoculation the rH_2 value was at about 22.

At that value growth started without cell multiplication, that is, this rH_2 value was still not the upper limit. All growth stopped only at rH_2 23.8, as can be seen from experiments with the addition of oxidation substances (Table 35). An addition of dithionite increases the inhibitory action of K_4 $[Fe(CN)_6]$ and $Na_2S_2O_3$ but not of K_3 $[Fe(CN)_6]$.

TABLE 35

Inhibition of growth of anaerobes by adding K_4 $[Fe(CN)_6]$, K_3 $[Fe(CN)_6]$ and $Na_2S_2O_3$

additive		amount of additives in %	rH_2 values at start of experiment	growth
$K_4[Fe(CN)_6]$	Clostr. sporogenes	0.02	25.8	-
	Clostr. putrificum	0.015		-
$K_3[Fe(CN)_6]$	Clostr. sporogenes	0.02	23.8	-
	Clostr. putrificum	0.015		-
$Na_2S_2O_3$	Clostr. sporogenes	0.02	23.8	-
	Clostr. putrificum	0.015		-
Control without additives	Clostr. sporogenes	-	21.6	+
culture in high layer	Clostr. putrificum	-		+
control without additives, aerobic	Clostr. sporogenes	-	24.7	-
	Clostr. putrificum	-		-

The drop in the rH_2 value is not a peculiarity of the culture fluid but rather is bound to the activity of the living cells. Centrifuged young cells, free of culture fluid, again eliminate reducing substances and develop normally when they are put in an appropriate medium.

Stolp (1955) obtained the same results with Clostr. butyricum. According to Stolp the low eH in the culture does not depend

so much on the substances eliminated into the medium as rather on the presence of the living cells themselves. He also found that first an eH drop occurs in the culture and only then does cell fission begin.

Little is known about the nature of the reducing substances eliminated by anaerobes. Quastel and Stephenson (1926) suspect substances with SH groups; Aubel and collaborators (1946) assume that it is hydrogen. Regardless of how the chemical nature of these materials may be constituted, their biological importance is clear: They serve the purpose of providing a favorable medium for the multiplication of bacteria. Anaerobes are widely distributed in nature. They are to be found not only in places where they meet with suitable conditions with a low eH, they themselves provide favorable redox conditions for their growth since they eliminate considerable reduction substances.

The rH_2 limit for spore germination lies somewhat lower than for the growth of vegetative cells. At rH_2 21.8 no germination occurs; at rH_2 20.8 a normal growth of the culture is observed after 24 hours.

All stages of germination can be followed and compared with the rH_2 value of the medium in preparations that are produced hour by hour during the growth of a culture of Clostr. sporogenes from spores. At the start of the experiment the spores, in contrast with the vegetative cells, were not dyed with gentian violet after a one-minute dyeing period without heating. After a two-hour period of incubation they could be dyed without change in size and shape. Then they increased in size and attained the dimensions of giant cells characteristic of the lag phase in the growth of vegetative cells. The rH_2 value dropped simultaneously. The multiplication of the vegetative cells began at rH_2 3-5 (Fig. 20).

The life activity of anaerobes is carried out in a broad rH_2 range from 0 to 22. However, multiplication is possible only at very low rH_2 values from 3 to 5. The redox potential, therefore, is the primary growth limiting factor in anaerobes, and not oxygen or hydrogen peroxide.

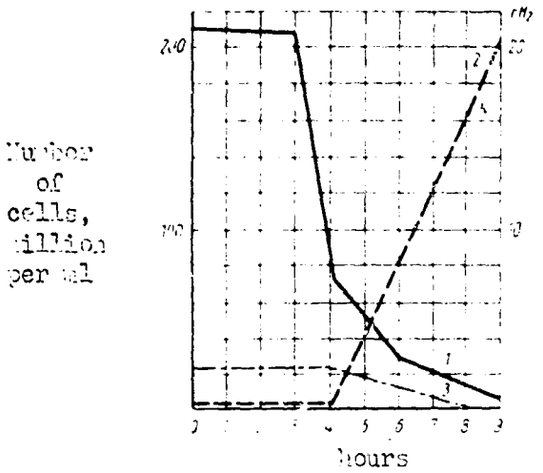


Figure 20. rH_2, spore germination and cell multiplication in Clostr. sporogenes

1 = rH_2; 2 = number of cells; 3 = number of spores

The following conditions, for example, can be created: the eH of the medium is lowered by adding reducing substances or by saturating the medium with hydrogen and the medium is enriched simultaneously by passing air through it or by simple air contact with oxygen. Anaerobes grow under these conditions. They react sooner to the eH of the medium than to the oxygen of the air.

II. The Lag Phase and the Redox Potential in Anaerobic Cultures.

The life cycle of a culture of bacteria is divided into several stages or phases:

The lag phase that starts with seeding in a fresh medium. In this period the introduced cells do not multiply; however, the later multiplication is being prepared in the cells and in the medium.

The logarithmic phase of the most abundant multiplication.

The stationary phase in which death and multiplication of the cells are in balance.

The phase of old age or progressive death of the culture.

The lag phase has been investigated in numerous studies (cf. Porter, 1946). Age and amount of the inoculum, specific type factors and culture conditions affect the length of the lag phase. Up until now there are only vague notions on the origins of the inhibition of multiplication. It is only certain that important changes occur in the cells during this phase and also extend into the medium. The redox conditions also belong to the factors that are important for a normal multiplication. Cultures of anaerobes, therefore, only begin multiplying when the high redox potential of medium, usually present, has been lowered.

This occurs during the lag phase (Rabotnova and Pryanischnikova, 1956).

The duration of the lag phase also depends on the age of the inoculum; the older the inoculum, the longer the lag phase (Figs. 21 and 22).

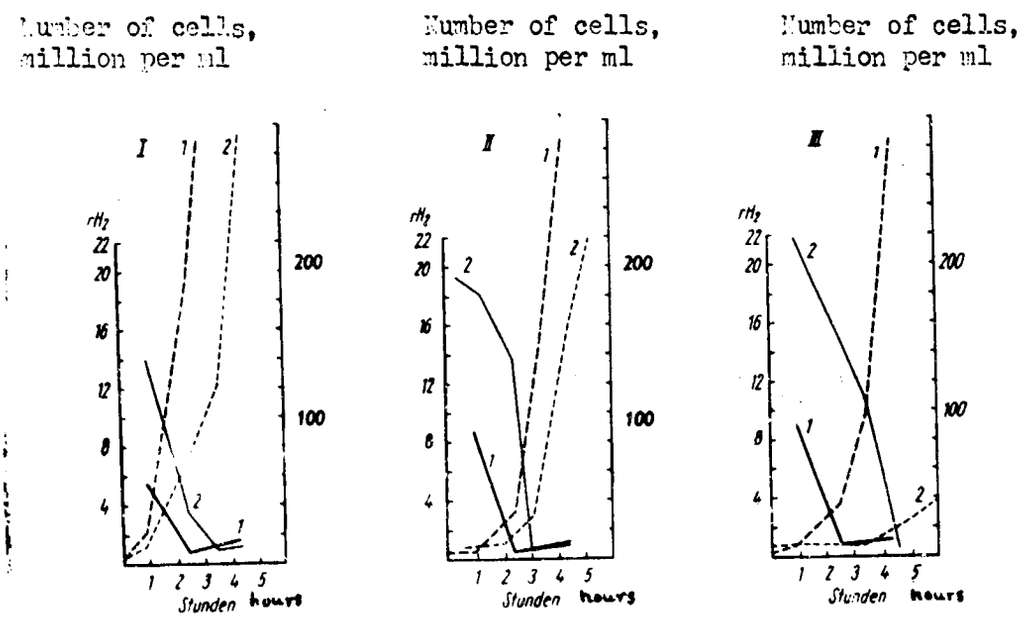


Figure 21, I-III

Effect of dithionite on the duration of the lag phase of Clostr. sporogenes by seeding inoculum of various ages. Inoculum from a six-hour old preculture (I), a 14.5-hour old preculture (II) and a 24-hour old preculture (III). ——— rH_2; ---- germ content; 1 = medium with dithionite; 2 = medium without dithionite.

Therefore, multiplication always began only when the rH_2 value in the cultures of anaerobic saprophytic bacteria had dropped to about 2-3. An addition of dithionite almost completely eliminated the lag phase in a seeding of a six-hour culture; in a seeding of an older culture it was shortened significantly. With a small seeding the lag phase is lengthened considerably (Fig. 23). With the addition of dithionite here also it was extremely or even completely eliminated.

Even though the lag phase was eliminated with a lowering of the rH_2 with dithionite, the stage of the long cell filaments holds good (see page 128), at least for a short time.

By adding an oxidation substance, for example thionine, the rH_2 value was buffered to a high level, at approximately 16. This caused the lag phase to extend over many hours. Ascorbic acid and also partially glucose act like dithionite, that is, they shorten the lag phase with acetone-butanol fermentation bacteria. The lowering of the rH_2 value to almost 0 occurs very quickly in these cultures; however, multiplication begins with a great delay. The butyric acid bacteria of this group are apparently still more definitely anaerobic than Clostr. sporogenes. They require practically a saturation of the medium with reducing substances. Nevertheless, an initially high redox potential may survive and drop.

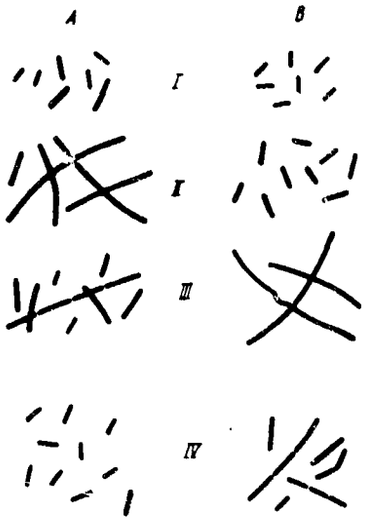


Figure 22. Clostr. sporogenes.

Cell forms from a medium with the addition of dithionite, seeded from a 6-hour old culture (A) and a 24-hour old culture (B). Cell forms of the inoculum (I), of the 10-hour old culture (II), of the 2.5-hour old culture (III) and of the 3.5-hour old culture (IV)

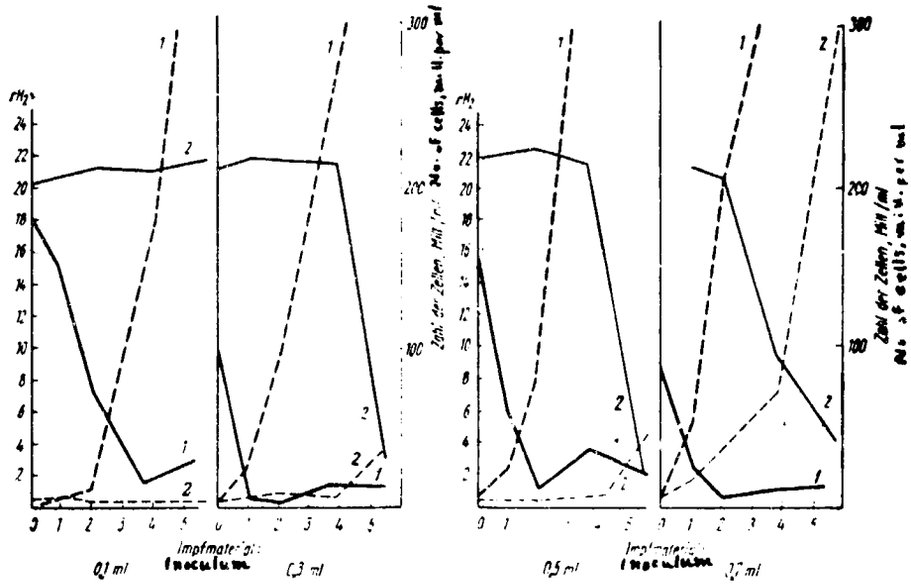


Figure 23. Effect of dithionite on the lag phase of Clostr. sporogenes seeded with different amounts of inoculum.

——— rH_2; ---- germ content;

1 = culture with dithionite;
2 = control without dithionite

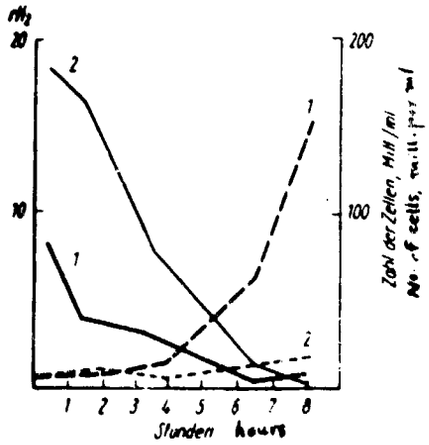


Figure 24. Effect of dithionite on the lag phase with acetone-butanol fermentation bacteria (1% inoculum)

——rH_2;
---- germ content;
1 = culture with dithionite
2 = control without dithionite

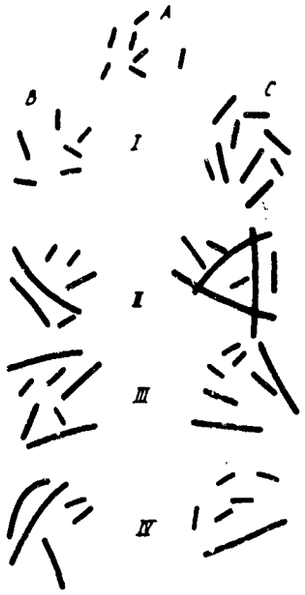


Figure 25. Clostr. acetobutylicum.

Cell form in cultures with and without dithionite. A = inoculum; B = control culture; C = culture with addition of $Na_2S_2O_4$.

I = after 2 hours of culture growth;
II = after 4 hours;
III = after 6.5 hours;
IV = after 9 hours.

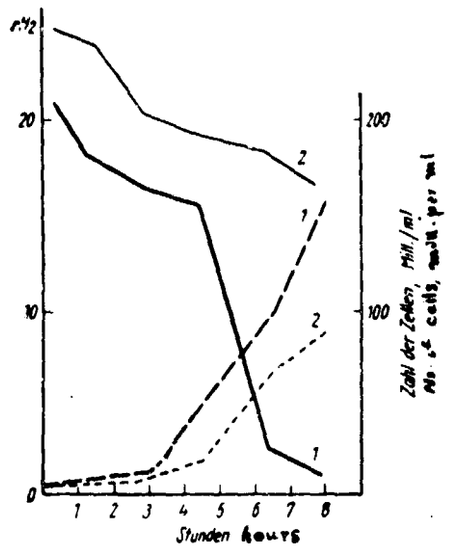


Figure 26. Effect of dithionite on the lag phase with acetone-butanol fermentation bacteria.

—— rH_2; ---- germ content;

1 = culture with dithionite;
2 = control without dithionite

Here also an addition of dithionite accelerated the development of the culture (Fig. 24). During the lag phase the cells increased in size and elongated, less pronounced, however, than with Clostr. sporogenes (Fig. 25).

Observations on redox potential and multiplication of facultative anaerobic acetone-ethanol fermentation bacteria show that multiplication begins already at a relatively little lowered rH_2 value (Fig. 26). An "elongated cell" stage does not occur in the acetone-ethanol bacteria. A certain polymorphism is characteristic of these bacteria. The length of the cells is different at the several ages (Fig. 27). The addition of dithionite lowered the rH_2 value and accelerated multiplication here also as was the case with strict anaerobes.

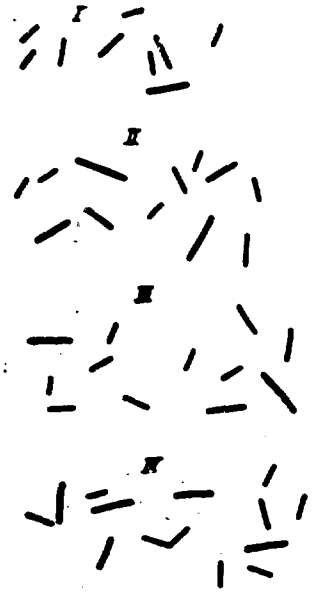


Figure 27. Cell forms of Bac. acetoethylicus

(type of designation not recognized by Bergey or Krassilnikov).
I = inoculum; II = after 2-hours growth of the culture; III = after 4 hours; IV = after 5 hours.

III. Changes in the Redox Potential in the Growth of Anaerobes.

There are numerous observations that refer to the drop of the redox potential in cultures of anaerobes. Pasteur observed already that blue indigo dye is decolorized in cultures of Clostr. cutyricum. Plotz and Geloso (1930) traced the eH and rH_2 values during the growth of Clostr. tetani in a beef peptone medium. The cultures stayed in a vacuum in a special receptacle that was specially suitable for eH measurements and could be sealed hermetically after pumping out the air. (Fig. 28). Contact was made between the calomel electrode and the culture by means of a thin layer of electrolyte that was on the surface of the glass as a film. The rH_2 value had dropped to 10 one hour already after seeding, and as the process went on it dropped to 5.5 (eH -245 mv to 285 mv). During the experiment the pH value went up from 6.8 to 7.5; the higher the pH value, the greater negative rH_2 values were measured.

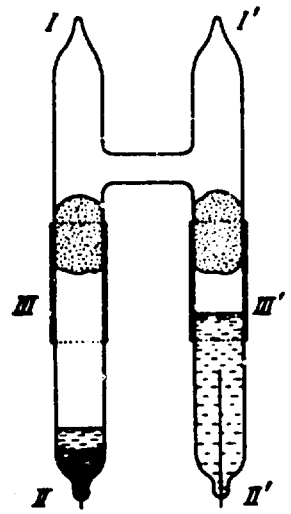


Figure 28. Experimental apparatus for measuring eH during the growth of a bacterial culture in a vacuum (according to Plotz and Geloso, 1930). I and I' = hermetically sealed ends of the tubes through which the air was withdrawn; II = calomel -- half element; II' = bacterial culture; III and III' = insulating material.

The potential built up in the culture is apparently conditioned by a system with an approximately stable rH_2 value, while the eH and the pH value can change. Characterization by means of the rH_2 value, therefore, seems to be justifiable. In a sterile medium the eH fell off slowly when oxygen was withdrawn: up to the 7th day to -80 mv and later to -200 mv.

A similar curve of eH decline resulted also for <u>Clostr. putrificum</u>, <u>Clostr. botulinum</u> and <u>Clostr. sporogenes</u> with an rH_2 drop to 5.5 ± 0.6.

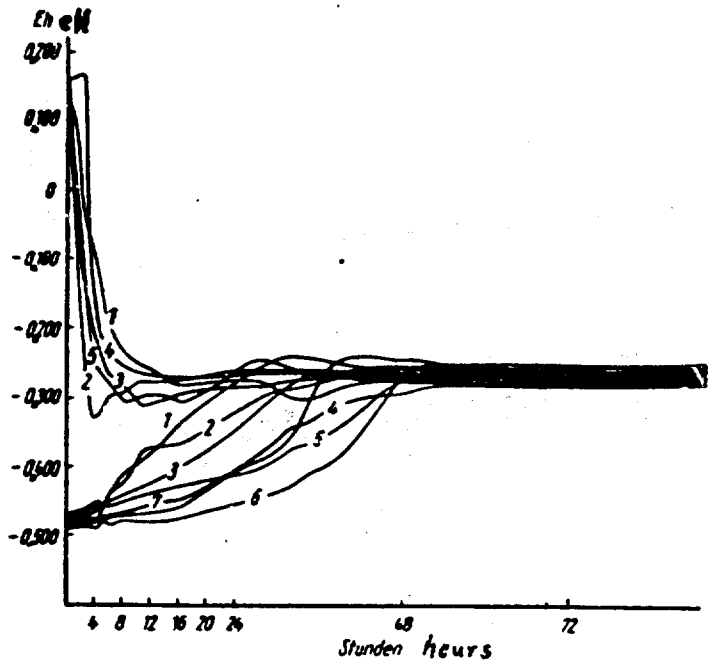


Figure 29. Effect of different eH values in bacterial cultures under anaerobic conditions (according to Plotz and Beloso).

1 = Clostr. bifermentans
2 = Esch. coli
3 - Clostr. histolyticum
4 = Vibr. septique (type of designation not recognized by Bergey or Krassilnikov)
5 = Clostr. putrificum
6 = Clostr. botulinum
7 = Clostr. sporogenes

The limit value to which the potential finally adjusts does not depend on the initial eH of the medium. Experiments on the effect of various eH values at the commencement of growth resulted in higher values being lowered to rH_2 5.5; below this value they are raised to it (Fig. 29). ([Note]: The mediums contained the following additives: 0.2% cysteine (eH -180 mv, rH_2 8.3),

0.03% $Na_2S_2O_4$ (eH -490mv) and titanium citrate (eH -440 mv).

The potential values appeared also in alkaline glucose solutions. A low potential was attained especially rapidly by using platinized asbestos as a catalyzer. Plotz and Geloso suppose that platinum black and the bacteria catalyze the same reaction of sugar dehydrogenation and that this reaction is expressed in a definite rH_2 value.

Gillespie and Rettger (1938 c) obtained other results by studying the eH changes in cultures of Clostr. tetani and Clostr. botulinum in a nitrogen atmosphere. Each of the two types formed a characteristic potential in the medium. With Clostr. tetani the eH dropped to -350 mv (rH_2 3.8); with Clostr. botulinum it dropped to -270 mv to -280 mv (rH_2 6.2). The different eH values were maintained during the entire duration of the experiment. The pH value was almost the same with 7.8 and 7.7 in both cultures.

Different potentials also appeared in cultures of lactic acid bacteria belonging to different types. Lactobac. acidophilus and lactic acid bacteria of the oral cavity produced with cultivation in a nitrogen atmosphere with 0.5%-1% CO_2 a lowering of the eH value in the first case to -100 mv, in the second case to -200 mv. The difference reached 100-110 mv and maintained itself stable. The pH value in the medium was approximately the same in both cultures by means of buffering.

Potential differences also appeared in cultures of Aerobacter polymyxa (type of designation not recognized by Bergey or Krassilnikov) and Bac. macerans in a nitrogen atmosphere. The eH amounted with the first type to -200 mv, with the second to -260 mv to -280 mv. The eH differences between each of four different strains of the same type were less great. It seems possible to use the eH value of cultures as a taxonomic characteristic for type determination.

According to Plotz and Geloso the eH of different cultures, therefore, adjusts itself to a constant value, while Gillespie and Rettger find differences in the individual types. If we consider the graphic representation of the results of Plotz and Geloso (Fig. 29), it is obvious that the final point at which the indications of the electrodes meet actually includes a series of values with differences up to 50 mv. It appears that Plotz and Geloso ignored these differences, while Gillespie and Rettger directed their attention precisely to these slight differences. It is shown, however, by both studies up to what limits the rH_2 value in the cultivation of anaerobic and facultative anaerobic microorganisms is lowered.

Still lower rH_2 values have been observed by other authors. Boyarskaya (1939) determined the eH in cultures of thermophilic anaerobic cellulose oxidizers. The rH_2 value dropped quickly from 29 to 1.5 in medium with 25% feces extract. In other experiments with thermophilic cellulose oxidizers on the same medium with peptone the rH_2 value decreased from 25 to 0.4-2 (Rotmistrov, 1939).

Desulfurizers also follow the general rule. In a medium with sodium lactate as carbon source the rH_2 value dropped from 20 to 7; in a medium with formic acid, from rH_2 21 to 3.5 (Aleschina, 1938). ([Note:] In a medium containing H_2S the possibility of a change in the platinum electrodes due to H_2S must be considered; this could impair the accuracy of the measured values.)

Molland (1944) likewise found in experiments in an argon atmosphere a normal decline of the rH_2 values, greater with obligate anaerobes (Clostridia), less pronounced with facultative anaerobes.

According to Aubel, Rosenberg and Gruenberg (1946) with butyric acid bacteria the lower eH limit in a medium at pH 5 was -274 mv (rH_2 0.9).

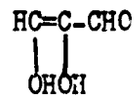
The group of the non-spore forming, strictly anaerobic bacteria of the intestinal tract of animals is different from the saprophytic anaerobes diffused in the soil. Dack and Burrows (1935) found by studying six strains of gram-negative rod-shaped bacteria, that had been isolated from the excrements of monkeys, that an eH of -100 mv appears in cultures of these bacteria in a nitrogen atmosphere at pH 6.4-6.9. That corresponds according to our calculation to an rH_2 value of 10. It is the highest value that we know of in studies on anaerobes.

The Rhodopseudomonas palustris, belonging to the ecological group of purple bacteria, produced with an anaerobic culture in the light in a medium with acetate an rH_2 decrease from 30 to 6, with glucose from 27 to 10 and with thiosulfate from 28 to 18 (Kondratyeva, 1953). The decrease in the rH_2 took place slowly in a period of time of over 100 hours, corresponding to the slow growth of these bacteria. The redox potential has a very low value at 8 in a medium with Na_2S, a strong reduction substance. In this case the rH_2 value in cultures of Rhodopseudomonas palustris did not drop farther but rather rose slowly and after 10 days reached a value of 14. In this case Na_2S is used up by oxidation during photosynthesis.

The question of which factors cause the redox potential to drop in a medium of anaerobes has not been explained up to the present time. It has merely been established that strong reduction substances accumulate in the growing cultures by which rH_2 indicators are decolorized and electrode potentials are lowered.

Quastel and Stephenson (1926) gave thought to compounds with SH groups (cysteine, glutathione, etc.) that are released by proteolysis. Frequently it is a question of hydrogen being released by fermentation. Aubel, Rosenberg and Gruenberg (1946) assume that every anaerobic-living cell is surrounded by an area that is saturated with hydrogen and other reduction substances.

Euler and Hasselquist (1955) suppose that groups of substances of the aldehyde type, having reducing characteristics, are formed by the decomposition of sugar by the microorganisms and also by treatment with alkali "reductone". For this type of substances Euler assumes the following formula:



It is not known if these substances are identical with the ones formed in the metabolism of the microorganisms.

It can be imagined that in the course of a complicated redox process transitory, labile acceptors saturated with hydrogen are formed, which give up their hydrogen easily and have a reduction action. In this connection it must be considered that the oxidized substances, for example CO_2, are stable and can be brought again into the redox process only with an expenditure of energy.

In addition to carbohydrates, as is well known, organic acids and albumin compounds can also be decomposed by anaerobes (Strickland, 1934, 1935).

The biological importance of anaerobiosis lies in the fact that both obligate and facultative anaerobes can utilize, in locations poor in oxygen, organic substance that is not available to aerobes.

Anaerobes certainly do not die in the presence of oxygen within broad limits. Moreover, they do not pass over into the state of anabiosis, but rather at rH_2 values of 20-22 they precipitate reducing substances into their environment until finally a low redox potential, favorable for their growth, is reached. In loose, well aired soil, perforated by capillary cavities, there are in this way microlocations in which anaerobes can grow. Aerobic microorganisms contribute in this respect in that they consume oxygen and on their part also give off reducing substances.

IV. Methods of Cultivating Anaerobes.

1. Importance of the amount of inoculum.

The amount of the inoculum is very important for the cultivation of anaerobes. It is well-known that an abundant seeding is necessary for growing a culture.

The culture fluid in which anerobic microorganisms have grown has a lo eH. The introduction of culture fluid lowers the eH of a fresh medium and prepares it for the cultivation of anerobes. Regulation is absent when too small an amount of inoculum is seeded. The inoculum can be mixed with the medium or added locally without mixing. In the first case the amount of the inoculum must be larger than in the second case. If, for example, it is put on the bottom of the culture receptacle without its being thoroughly mixed with the entire medium, the growth of the bacteria and the lowering of the eH begins from here on out.

The behavior of acetone-butanol fermentation bacteria will

be adduced as an example (Yerusalimski, 1934). In order to start growth in a culture receptacle with 500 ml of culture solution in contact with air, at least 3 ml of a young, active culture are necessary as inoculum. If the inoculation is made with an open ampule containing 0.3 ml of inoculum, growth begins quickly in spite of the small amount of inoculum. The bacteria precipitate hydrogen as a fermentation product which lowers the eH around the ampule and makes it possible for the bacteria to spread out and multiply in the medium.

Solid matter, perhaps strips of filter paper, dried agar, sand, etc. also contribute to locating the inoculum and facilitate the commencement of the culture.

According to experiments by Tschistyakov (1932) acetone-butanol bacteria grow in fluid mediums only after inoculation with about $7 \cdot 10^7$ cells per ml. If additional sand is put into the medium only $1.5 \cdot 10^5$ cells per ml are sufficient.

2. Importance of the Viscosity of the Medium.

The use of a viscous medium creates favorable conditions for the growth of anaerobes. The diffusion of oxygen from the surface into the depth is made difficult in a medium with high viscosity, and a low potential already appears in the medium not far from the surface. Prevot (1938) found that in a beef peptone medium with 1% agar the rH_2 value at the surface is about at 20 and it drops to 7 with increasing distance from the surface.

Rabotnova took electrometric measurements in cultures of acetone-butanol bacteria in a 7% corn mash. The redox potential of a sterile mash held at 9 for a long time at a depth of 4-5 cm, whereas on the surface and in the same period of time it amounted to 20 and more.

Experiments on the eH at various depths of beef peptone mediums with 0.75% agar were performed by Williams (1939). In an alkaline agar (pH about 8) the eH fell from +100 mv at the surface to -150 mv at a depth of 35-40 mm. In an acid medium (pH 5.2) the eH barely changed as the depth increased; it held steady at a position of about +70 mv to +80 mv. Apparently the acid content increases the permeability of a viscous agar for oxygen. The O_2 content of the agar rose under an oxygen pressure of 82 kg per cm^2. The eH amounted to +200 mv at the surface and to ± mv at a depth of 85 mm.

It results from all the observations that the viscosity of the medium is important for its redox state. In general it suffices to add 0.2% agar to the medium for anaerobes to be able to grow with local seeding at the bottom of the culture receptacle.

3. Removal of the Dissolved Oxygen.

A further method for lowering the eH value in the medium is the removal of the dissolved oxygen. A freshly sterilized and quickly cooled medium is used or the oxygen is driven off by boiling.

The oxygen dissolved in the medium may also act indirectly, since it oxidizes components of the substrate. The resulting products in many cases disturb the growth of the anaerobes. Facultative anaerobic bacteria like pneumococci, hemolytic streptococci and Staphyloc. aureus grew in beef bouillon only when at least 1% of the culture fluid was supplied as the amount inoculated (Dubos, 1929 a). Only a fraction of this amount of seeding was necessary with a bouillon freshly sterilized in the autoclave or freshly boiled.

A simple method for excluding contact of the medium with air is cultivation in a Burri tube. ([Note:] Cf. in this respect Janke, A., Arbeitsmethoden der Mikrobiologie [Working Methods in Microbiology]. Dresden and Leipzig: Verlag von Theodor Steinkopff, 1946).

Good results were obtained by cultivating in a vacuum. This procedure was first used by Pasteur. It is possible to evacuate the air from test tubes after inoculation and to seal them hermetically or to put the culture receptacles in a vacuum exsiccator made of glass or metal. ([Note]: When cultivating in vacuum it must not be overlooked that CO_2 is vitally necessary for many bacteria.)

The oxygen may be absorbed by means of an alkaline pyrogallos solution. ([Note]: Pyrogallol is dissolved in soda, not in NaOH or KOH, in order to avoid the simultaneous absorption of CO_2.) This procedure was used for the first time by Nenzki (1879). Today the experiment arrangement described by Buchner (1888) is used mostly.

An apparatus developed by Rabotnova is useful for cultivating under anaerobic conditions with simultaneous eH measurement (Fig. 30).

The enlarged section (2) contains alkaline pyrogallos solution. Part 1 contains the inoculated medium. The S-shaped glass tube (4) is filled with KCl agar and serves as a bridge for the calomel element. The external end is sealed with a firm stopper made of filter paper in order to prevent the agar from drying (5). Electrodes for measuring the eH are fused into the apparatus (3). Part 2 is sealed hermetically with a rubber stopper (6).

In order to guarantee sterility, the individual steps are taken according to the following plan: The S-shaped tube of the apparatus is filled with KCl agar up to the indicated height, after which the external end is closed with a rubber stopper. After the whole apparatus has been wrapped in cotton, it is sterilized. After sterilization and after chilling the agar in the S-tube, the rubber stopper is replaced by a paper stopper that has been dampened in a KCl solution. The inoculated culture medium is put into the inner section of the apparatus; soda solution + pyrogallol is put in the outer, broad section([Note]: 1 ccm of a 20% pyrogallol solution + 1 ml of a saturated Na_2CO_3 solution absorb oxygen from 220 ml of air. It must be observed, however, that the commercial grade "Pyrogallol A for Gas Analysis" absorbs oxygen from the atmosphere of the apparatus only slowly, in the course of several hours.) The pyrogallol is wrapped in paper so that it is not immediately dissolved and absorbs the oxygen already before the receptacle has been sealed hermetically with a rubber stopper. The S-shaped form of the tube proved to be necessary so that the

gas is not forced inwards with the creation of a vacuum within the apparatus after absorption of the oxygen. It is possible, in this type of apparatus, to maintain anaerobic conditions and to perform measurements of the redox potential during the growth of the culture.

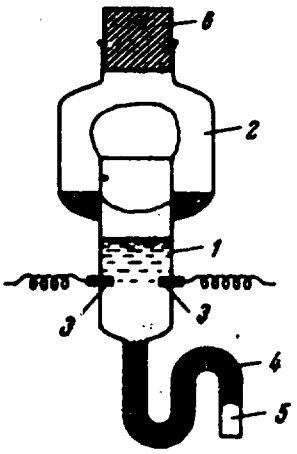


Figure 30. Apparatus for cultivating anaerobes with simultaneous measurement of the eH (according to Zabotnova). Description in text.

Sodium dithionite which absorbs oxygen faster may also be used in place of pyrogallol. 1 ml of a 20% alkaline $Na_2S_2O_4$ solution takes up 10.7 ml of oxygen. 2 ml of a freshly prepared 20% solution of $Na_2S_2O_4$ and 4 ml of 50% KOH are used for a receptacle with a 250 ml capacity. Oxygen is also absorbed by metallic iron (Parker, 1955). If steel-wool is treated with Cu_2SO_4 and a net material, it is oxidized, that is, it absorbs oxygen strongly. By using 10 g of steel-wool a 3-liter exsiccator becomes oxygen-free at 28° C. within 4-5 hours. Since CO_2 is also absorbed at the same time, the atmosphere in the exsiccator must be enriched additionally with carbonic acid. The following combinations of saturated carbonate solutions ensure the CO_2 content, indicated after each one, in the atmosphere of the experiment receptacle.

Na_2CO_3	0.14% CO_2
$Na_2CO_3 + NaHCO_3$	0.60% CO_2
$MgCO_3 + NaHCO_3$	1.40% CO_2
$NaCl + NaHCO_3$	4.50% CO_2
$CaCO_3 + NaHCO_3$	5.00% CO_2
$NaHCO_3$	10.30% CO_2

Another method for absorbing oxygen depends on its catalytic combination by means of hydrogen when platinized asbestos is used (Olkenitzki, 1955).

The following mixture may be used as an oxygen indicator (Parker, 1955): solution A — 3 ml of a 0.5% aqueous solution of methylene blue in 100 ml of water; solution B — 0.5 g of glucose in 100 ml of water at pH 10 (Na_2CO_3). Both solutions are mixed before use. The mixture is dyed as long as the oxygen partial pressure amounts to more than 0.05 atmospheres.

Oxygen may also be replaced by means of an inert gas, for example nitrogen, argon or helium.

The most convenient method of maintaining constant conditions in the atmosphere is to pass inert gases through the medium. In this way the atmospheric oxygen dissolved in the medium escapes very quickly first of all and later also the precipitated gases.

Hydrogen as a reduction substance is not an inert gas and can be used only with this reservation to create anaerobic conditions. Carbonic acid is also not an inert gas: CO_2 is toxic in high concentrations and produces an acidification of the medium.

The gassing method of creating different redox potentials in the medium was used in a series of studies. In this case a gas mixture consisting of purified nitrogen and oxygen is passed through.

4. Reducing Substances.

The often mentioned addition of reduction substances to the medium yields very good results. Pasteur already used this method, in order to maintain growth of anaerobes also with exposure to air. He recommended the addition of sugar to the medium.

Kitasato and Weil (Cf. in this respect the summary in Omelyanski, 1953) suggested the addition of 0.3-0.5% of sodium formiate solution or a 1% pyrocatechin or eikonogen solution. Beijerinck obtained good results with sodium dithionite; Trenkman used 4-10 drops of a 10% Na_2S solution per 10 ml of bouillon; Tarozzi obtained normal growth of the most important pathogenic anaerobes with unimpeded access to air by adding raw, aseptically crushed liver, spleen or kidney tissue to the bouillon.

Reducing substances are always contained in organic complex mediums; they are found also in peptone. The higher the peptone concentration of a medium, the better can strict anaerobes grow with access to air. It is true that peptone alone cannot lower the eH to the required value, even 5% concentrations are insufficient. It is necessary to add still another reduction substance, for example, ascorbic acid (Kligler and Guggenheim, 1937). Glucose is also a useful reduction substance (Wurmser, 1935). An rH_2 of 6-7, that remains constant at pH values of 7-11, results in sugar solutions.

Other sugars also have reducing properties similar to glucose, for example, xylose, arabinose, lactose, galactose, mannose and dioxyacetone. Saccharose is not a reducing sugar and does not lower the eH of a medium.

The reducing properties of various sugars were studied in detail by Aubel, Genevois and Wurmser (1927). They give the following data (at 80° C.):

levulose	at pH 8.2,	eH -260 mv
Glucose	pH 8.2,	eH -400 mv
lactose	pH 8.2,	eH -325 mv
galactose	pH 8.2,	eH -235 mv
levulose	pH 7.5,	eH -180 mv (at 20° C.)

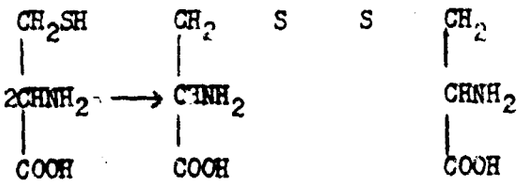
The initial eH value of +250 mv was lowered to ≈100 mv to -200 mv in sterile beef peptone bouillon in the presence of 1% of glucose. It must be observed, however, that in microbiological practice this kind of strong reducing action of sugar cannot be relied on, because except for the consumption of sugar, the formation of acid usually appears and the pH value goes down. The reducing action in an acid medium is considerably less.

The often-mentioned substances with SH groups have strongly reducing properties. They react in the following manner:

$$2R - SH \rightarrow R - S - S - R + 2H$$

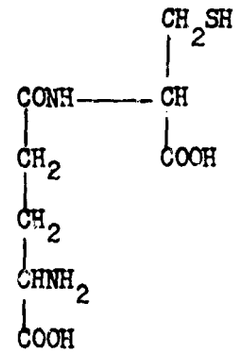
The following belong here:

Cysteine that is transformed into cystine:



Thioglycolic acid:

and glutathione -- the dipeptide of cysteine and glutaminic acid:



The eH value of approximately +4 mv formed by cysteine does not change its magnitude at various pH values. An addition of 0.1% sodium thioglycolate lowers the eH value of the medium from +250 mv to -175 mv to -200 mv; with 0.01% -50mv were reached (Reed and Orr, 1943).

Therefore, all reducing substances only maintain a low eH when the viscosity of the medium has been raised somewhat by the addition of agar. According to observations of Reed and Orr a 0.02% agar concentration is already almost sufficient, 0.05-0.25% act quite well. (The medium remains fluid up to about 0.1% agar.)

Beef peptone bouillon + 1% glucose +0.05% agar + 0.1% sodium thioglycolate is very well suited for cultivating anaerobes in the air (Brewer, 1940).

Substances with SH groups are found in plant and animal tissues. Tarozzi's method, the cultivation of anaerobes in bouillon with the addition of pieces of liver, is based on the action of SH compounds contained in the liver.

When ascorbic acid is used the rH_2 value is about at 14. The oxidized form is unstable, so that the potential depends only on the concentration of the reduced form (Ilienyi and Buesing, 1939· Lemberg, 1956).

Inorganic reduction substances can also be used to lower the rH_2 value. Thus, for example, sulfides and hydrogen sulfide have a strongly reducing action. They are certainly involved as reduction substances only for such microorganisms that tolerate these toxic substances, for example for desulfurizers and sulfur microbes. Small concentrations of H_2S are also tolerated by many saprophytes. Sodium dithionite has strongly reducing properties; however, it is unstable, decomposes with long storage and does not tolerate sterilization in the autoclave. However, since the dry salt invariably eliminates a small amount of the toxic H_2S, it can be considered as sterile and may be added to the culture fluid without prior sterilization. The concentration in the medium should not exceed 0.1%; higher concentrations have a toxic effect. Titanium citrate is a very strong reduction substance. Plotz and Geloso (1930) give the

following method of using it: 3.5 g of titanium chloride are dissolved in 200 ml of M/20 citric acid and neutralized with soda; a few drops of this solution are used in each culture tube.

Another method consists of saturating the medium with hydrogen by means of electrolysis of the culture solution (Hanke and Katz, 1943).

Prevot (1954) suggested a method for the selective enrichment of anaerobes in a microbe mixture. If ten drops of a 0.1% NaN_3 solution are added to the medium, the growth of aerobes is checked as a result of a blocking of the oxidation process, while anaerobes grow very well.

5. Joint Cultivation of Aerobes and Anaerobes.

This method was already proposed by Pasteur and was further developed by Ru, Ponzo, Kedrowski and Scholze (Omelyanski, 1953).

Vinogradski cultivated the anaerobic N_2-fixing Clostr. pasteurianum on a fluid film in a mixture with aerobes.

Clostr. pectinovorum can be cultivated together with Ps. fluorescens (Omelyanski and Kononova, 1926). Clostr. pectinovorum does not grow under normal conditions when seeded in water and straw. However, if it is inoculated simultaneously with Ps. fluorescens, pectic fermentation already begins after 24 hours.

Ps. fluorescens can be replaced by Esch. coli, Bac. mycoides, Bac. mesentericus (designation according to Krassilnikov) and Oidium lactis. Imschenezki (1939) used a similar procedure in growing pure cultures of thermophilic cellulose oxidizers. Esch. coli and cellulose oxidizers were inoculated simultaneously in a medium with feces extract and cellulose and incubated for 24 hours at 37° C. At first only Esch. coli grew, because the redox potential was still too high for the anaerobic cellulose oxidizers. After 24 hours the rH_2 value had dropped from 29 to 17. If the test tubes were then incubated at 60° C., growth of the thermophilic, anaerobic cellulose oxidizers, while the mesophilic Esch. coli dies. The final result was a pure culture of thermophilic cellulose oxidizers.

V. Summary.

The nature of anaerobiosis must be considered as explained at present in its principal characteristics.

Anaerobes are microorganisms that only grow under specific, low redox conditions. The hypothesis seems to be based on the fact that the rH_2 values in cultures of anaerobes during growth of the culture drop down to a value that is characteristic of the individual types, but is determined simultaneously by the culture conditions.

The following factors are important for the production of low redox potentials on which the growth of anaerobes depends: high seeding, addition of solid substances to the medium, increase of viscosity, removal of atmospheric oxygen by means of various methods, addition of reducing substances, joint cultivation with aerobes.

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CHAPTER 6.

GROWTH OF AEROBES AND THE REDOX POTENTIAL

I. The Concept of Aerobiosis.

Microorganisms that grow with access to air are designated aerobic. The concept of aerobiosis with reference to its real importance and its limitations has been discussed very little to date in writings on microbiology.

The following are the principal characteristics:

Aerobes can grow with access to oxygen.

Aerobes need oxygen by itself or they use it as a hydrogen acceptor.

Aerobes, in contrast with anaerobes, are adapted to a higher redox potential that is usually produced by oxygen.

Obligate aerobic bacteria have the tendency to grow in the shape of thin films on the surface of fluid and solid mediums.

Motile microbes accumulate on the surface; however, they can grow in the culture solution. Nonmotile microorganisms, for example obligate aerobic yeasts (yeast-moulds), fungi and Actinomyces grow exclusively on the surface and only sparsely under the surface of the fluid. Only dead cells are deposited on the bottom. Aerobic bacteria that are motile and become nonmotile as they age frequently grow principally with a turbidity in the entire column of fluid. After they have lost their motility they form a thin film on the surface and the medium again becomes clear.

Growth on the surface does not entirely mean, however, that all cells come in contact with air. On the surface of the film, where doubtlessly strongly oxidative conditions prevail, the air has unimpeded access to the cells. But the second cell layer already contains less air, and it is possible that the deeper cell layers live in an environment relatively poor in oxygen, since the oxygen is intercepted by the cells above.

For this reason it is difficult to visualize how the redox conditions under which aerobes grow are constituted, and whether aerobes need absolutely a high redox potential. This question cannot be readily answered, because it is hard to study the action of the potential and of oxygen separately and independently from each other. A higher potential always sets in when oxygen is present. Electrodes and redox indicators show a rise in the rH_2 in the presence of oxygen.

There are observations on the fact that too great a supply of

air is not alltogether favorable for the growth of aerobes.

It was already noticed by Beijerinck (1893) that aerobic bacteria, circumstances permitting, avoid direct contact with air. If a bean is coated over with water in a test tube, after about 24 hours a sharply defined, upwards and downwards, paper-thin "bacteria sheet" appears in the fluid 2-3 cm above the bean. It is formed where the air diffusing down from above and the nutritive material diffusing upwards from the bean produce favorable growth conditions. The bacteria sheet sinks down when oxygen is passed over the fluid; when hydrogen is used it rises.

Bacteria sheets also appear in pure cultures of motile and nonmotile bacteria, for example with Bact. fluorescens non liquifaciens (designation not recognized by Bergey or Krassilnikov), Serr. marcescens, Bact. radicicola (designation not recognized by Bergey or Krassilnikov), Esch. coli and typhus bacteria. In these experiments beef bouillon agar coated over with 0.1% water agar served as source of the nutrient.

Under these conditions aerobes grow predominantly beneath the surface of the substrate in an area in which favorable nutriment conditions prevail.

Egunov (1900) described in detail the growth of bacteria sheets with sulfur microbes. He demonstrated that oxygen is always found and H_2S never over the bacteria sheet, while oxygen is absent under the sheet, but H_2S is present. The level is formed at the contact zone of both gases, of the oxygen diffusing from above and of the H_2S rising from below.

Zycha (1932) demonstrated with tuber bacteria and some other species that the localization of the bacteria sheet depends to a great degree on the composition of the medium. In a mineral salt agar that contains only the slight contamination of the agar as a source of organic carbon, the bacteria distributed in the agar grew only in a thin sheet about 5-10 mm under the surface. When an organic nutrient was added to the medium, for example glucose, peptone or asparagin, the bacteria sheet formed closer to the surface or directly on the surface. In an oxygen atmosphere it formed deep in the agar. Tuber bacteria that are typical aerobes, therefore, avoid direct contact with air on a medium poor in nutrient, while they grow on the surface under otherwise equal conditions in a medium with glucose. Zycha assumed that for the conversion of a larger amount of nutrient more oxygen is needed.

The minimum amount of oxygen necessary for aerobic organisms is very small. According to Chydyakov's (1896) data Bac. subtilis and Asp. niger can still grow with 0.262% of oxygen or a pO_2 of 10^{-5} mm.

Omelyanskiy (1904, 1953) also mentions low limit values (Table 36).

According to Knaysi and Dutky (1934) oxygen deficiency sets in only at a partial pressure of 10 mm in cultures of Bac. megaterium in a rarefied space.

Practical experience with the fermentation of mash by means of yeasts in the alcohol industry (Sabrodski, 1946) shows that yeasts can utilize for their growth the small amounts of oxygen that are adsorbed on the solid pieces of corn-mash or potato-mash.

TABLE 36

Oxygen requirement of some microorganisms (according to Omelyanskiy, 1904, 1953)

	Maximum O_2 content that permits life activity, in atmospheres	Minimum oxygen content, in %
red yeasts	1.68-1.94	0.00016-0.06
Ps. fluorescens	1.94-2.51	0.00016-0.06
Sarcina lutea	2.51-3.18	0.00016-0.06
Pen. glaucum	3.22-3.63	0.06 -0.66

According to the latest studies oxygen deficiency becomes perceptible with aerobes only with a complete withdrawal of oxygen from the surrounding atmosphere to about 0.0001% (Windisch, Haehn and Neumann, 1953).

The determination of smaller amounts of oxygen was possible only by using the polarographic method. According to Longmuir's (1954) data the method worked out by him makes it possible to determine traces of oxygen to 10^{-9} mol. It showed that the intensity of respiration of Aerobacter aerogenes, Microc. candicans, Bac. megaterium, Azotob. indicum, Acetob. suboxidans, Serratia marcescens, Esch. coli and yeasts is reduced to half only with an oxygen concentration of 10^{-6} to 10^{-8} mol.

Aerobes can, therefore, exist at very different oxygen partial pressures, at very low values and at a pO_2 of one atmosphere and more. The optimum values are dependent on the composition of the medium. In many cases a high concentration of oxygen seems not to be optimal. In certain mediums growth is better with a lower oxygen partial pressure in comparison with air.

The question of the optimal redox potential of aerobes has been studied by Aubel, Aubertin and Genevois (1929) in an agar medium with redox indicators. The rH_2 value fluctuated between 14 and 20 near the surface in the growth area of aerobes.

Prevot (1938) assumed that aerobes, like anaerobes have rH_2 optima that are specific to the species and that are occasionally within very narrow limits.

Colorless aerobic sulfur microbes, like Beggiatoa, only grew in enriched cultures after formation of a certain optimal "electrode potential", in experiments conducted by Bahr and Schwartz (1956). The value of this potential may fluctuate for various reasons. Depending on this, the bacterial level shifts in the medium.

Allyn and Baldwin (1930, 1932) and Brown and Baldwin (1933) studied the problem of the redox potential. Tuber bacteria grow in an agar medium in the area in which the eH is optimal. Since peptone, glucose and asparagon act as reduction substances, tuber bacteria grew in mediums that contained these substances nearer the surface than in a mineral salt agar.

Therefore, the composition of the medium is not altogether effective (Zycha, 1932), but rather the redox potential that appears in the medium corresponding to its composition.

If two agar mediums are compared, one of which contains mannitol + KNO_3, the other mannitol + yeast extract, it is seen that in the more strongly reductive yeast extract medium growth results closer to the surface than in the KNO_3 medium (Table 37).

TABLE 37

Localization of the bacteria sheet with tuber bacteria in various agar mediums (the figures give the position of the bacteria sheet in mm under the surface. (According to Allyn and Baldwin, 1930).

substrate	Rhizobium strains from					
	lucerne	clover	peas	beans	soya	lupine
mannitol-nitrate medium	7	6	5	6	13	12
mannitol-yeast extract medium	on the surface				4	2

In a medium with yeast extract and an increasing amount of an oxidation substance ($KMnO_4$) the distance of the bacteria sheet from the surface increases with an increase in the content of oxidation substance, as far as growth generally results (Table 38).

Numerous other experiments pointed in the same direction. Ferrous oxide and cysteine can counteract the action of KNO_3 in a KNO_3 medium. Tuber bacteria do not grow in an agar strongly reduced by cysteine or ferrous oxide. If lumps of agar with an oxidation substance ($KMnO_4$, $KClO_3$, KNO_3, H_2O_2) are laid on the agar layer, growth occurs in the vicinity of the agar lumps. On the other hand if lumps of agar soaked with reduction substances are laid on a too strongly oxidized medium, the bacteria grow around these agar lumps.

TABLE 38

Influence of $KMnO_4$ on the localization of the bacteria sheet with tuber bacteria (according to Allyn and Baldwin, 1930)

$KMnO_4$ concentration in %	0.001	0.002	0.005	0.015	0.020	0.090
Depth in mm at which the bacteria sheets are formed	on the surface	4	6	10 growth of the bacteria sheet only implied	to 10 growth weak	no growth

When solid mediums with a different degree of oxidation are inoculated (mannitol-nitrate mediums with the addition of 0.003%, 0.005%, 0.0075%, 0.010% and 0.050% cysteine) the greatest number of colonies is formed on the medium with 0.003% cysteine, that is, the redox conditions of this medium are optimal for tuber bacteria.

Measurements of the redox potential using polished platinum electrodes gave an eH of +500 mv in the mannitol-KNO_3 medium, +100 mv to ±0 mv in the yeast extract medium. In fluid mannitol-nitrate mediums there was no growth of bacteria; when the eH was lowered by adding thioglycolic acid, growth occurred.

Growth ceased in fluid mannitol-yeast extract mediums when the eH was raised to +500 mv by the addition of hydrogen peroxide. When thioglycolic acid was added as a reduction substance, growth was again possible. If we subsequently compute the rH_2 values, the result is that an rH_2 value of 30-31 is too high for tuber bacteria; at a value of 26-27, however, good growth results.

Rabotnova (1939) attempted to determine the redox conditions within the bacteria sheet of tuber bacteria. The bacteria sheets grew in test tube cultures in a medium with 0.2-0.3% agar. The measurements were taken in situ without any shaking or stirring by using platinum electrodes that were inserted horizontally in the tubes at the height of the expected bacteria sheet.

A mineral salt agar with and without the addition of organic carbon compounds served as medium. ([Note:] 0.25-0.35 g of reducing substances are contained in this medium; these substances result from the partial hydrolysis of the agar during sterilization or are contained in the agar as an infusion.) In numerous separate determinations with Rhizobium strains from vetch and peas rH_2 values between 20 and 29 occurred at the level of the bacteria sheet (Fig. 31).

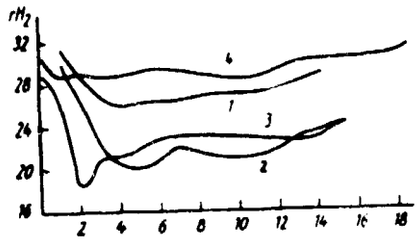


Figure 31. The redox potential in a bacteria level of tuber bactéria from vetch (1,2) and peas (3,4).

1 = Mineral medium with glucose;
2 = with glucose and an extract of Asp. niger containing bios;
3 = mineral medium with glucose;
4 = with traces of glucose.

The range of fluctuation is wide, because the rH_2 measurement in a bacteria level that often is only a fraction of a millimeter thick presents difficulties. Part of the horizontally arranged electrodes is either above or below the growth zone. However, without doubt the rH_2 value is different at different depths.

In order to establish accurately the rH_2 limits, Rhizob. leguminosarum was cultivated on a mineral medium with 2% glucose, 1% bean meal and 0.8% agar in an atmosphere enriched with oxygen or nitrogen in the exsiccator. The eH of the sterile medium in an oxygen atmosphere was at +360 mv (rH_2 26); in a nitrogen atmosphere, at +270 mv (rH_2 23). Bacteria sheets developed in the inoculated test tubes that were kept together with the sterile controls: At a depth of 2-3 mm in the exsiccator with oxygen; on the surface of the medium in the exsiccator with nitrogen. Obviously the optimal rH_2 must be sought within the above-mentioned limits. The experiments confirm the fact that tuber bacteria do not grow at an indefinite, high rH_2 value, but rather within a certain range whose upper limit lies at rH_2 29. The aerobic Bac. megaterium, studied by Wood, Wood and Baldwin (1935) by using indicator dyes, requires a still lower redox potential.

When the drop in the rH_2 value is obtained in a culture by means of indicators, a distinct plateau is obtained on a curve of the eH changes per unit of time, that is, a section in which the decrease of the rH_2 value ceases. The extent of this plateau is proportional to the amount of indicator added, the height corresponds to the E_0' of the dye (see page 119).

The result was that an eH $\geqslant$ -55 mv is too high (Table 39). Good growth occurred then only when the bacteria could lower the eH without delay to -55 mv, or when the eH was lower from the start. The growth-inhibiting indicator-dyes, had in a reduced form, no effect on the growth of the culture.

TABLE 39

Influence of indicator-dyes on the growth of Bac. megaterium (according to Wood, Wood and Baldwin, 1935)

rH_2 indicators	E_0' (at pH = 7.2)	growth after 24 hours
o-chlorphenol indophenol	+218	-
phenol indophenol	+212	-
o-cresol indophenol	+180	-
1-naphthol-2-sulfonate indophenol	+111	-
thionine	+ 56	-
methylene blue	- 4	-
indigo tetrasulfonate	- 55	+
indigo trisulfonate	- 91	+
indigo disulfonate	-134	+
indigo monosulfonate	-165	+

The addition of a reduction substance (K_2SO_3) to the bouillon works favorably on multiplication. When this substance is added in various concentrations (0.003%-0.03%), mediums with an eH of +450 mv to -50 mv are obtained. The optimal eH for Bac. megaterium was at pH 7.2 within a range of $\pm$0 mv to -50 mv. Growth occurs already on a medium with an optimal eH with small amounts of inoculum in the shortest time. Therefore, Bac. megaterium can no longer grow above a "critical" potential. The unusually low value of 13 results for the redox potential. That means that this typical aerobe not only tolerates a relatively low rH_2, but rather even requires it.

Ilyaletdinov (1954) made similar observations with Bac. cereus and Bac. mesentericus. Methylene blue and dyes with a high E_0' had a toxic action in cultures exposed to the air in a synthetic medium to which sugar and a small amount of yeast autolysate as a vitamin source had been added. while indigo tetrasulfonate and dyes with a low E_0' were innoxious. The toxic action of dyes with a high E_0' appeared only when small amounts of cells (1.5 X 10^4 per ml) were inoculated: with a larger amount of seeding it was absent. Reducing substances are probably introduced with the inoculum so that the oxidizing action of the dyes is neutralized.

Bac. subtilis behaves similarly (Rabotnova). On the other hand with a seeding of 1.0 X 10^5 cells per ml it is sensitive to dyes with an E_0' over -50 mv (rH_2 12.6). Dyes that buffer the redox potential higher than 12.6 prevented growth with small amounts of seeding.

By seeding 3.5 X 10^5 and more cells per ml dyes with a high E_0'

merely delayed the multiplication of cells.

The same was the case on mediums with large amounts of reducing substances (beef bouillon or beef bouillon with sugars) with a small inoculum (1.0×10^5 per ml). The inhibitory effect of the dyes was absent when greater amounts were seeded (Table 40).

Culture occurred in these experiments in a synthetic medium with the addition of glucose, yeast autolysate and indicator dyes at pH 7.1. The culture receptacles were shaken for 48 hours at 30° C. in an agitator.

TABLE 40

The influence of rH_2 indicators on the multiplication of various Bacillus species (germ content in mill. per ml, amount of seeding 1.0×10^5 per ml, duration of experiment 30 hours)

rH_2 indicator	rH_2 of the dye reduced to 50%	beef-peptone bouillon		beef-peptone bouillon with 1% sucrose		
		Bac. megaterium	Bac. cereus	Bac. mycoides, mg per ml*	Bac. megaterium	Bac. cereus
neutral red	3.0	107	432	700	73	900
indigo disulfonate	10.0	105	522	700	56	900
methylene blue	14.5	45	78	200	-	-
thionine	16.1	40	224	200	73	500
control	-	93	430	800	69	1000

* Bac. mycoides has a filamentous growth; consequently the number of cells cannot be ascertained by direct count as with the other cultures, but must be computed from the weight of the mass of bacteria in mg per ml.

All the above-mentioned species of bacteria are typical aerobes and grow without difficulty on the surface of solid mediums. It is to be assumed that atmospheric oxygen does not penetrate the interior of the cells, while the rH_2 indicators are diffused and produce an oxidation in the interior of the cell that prevents growth.

The preference for more or less reducing conditions and the resistance power of aerobes with respect to a low rH_2 in the medium have been verified by further observations.

Dubos (1929) found an acceleration of growth in the presence of reduction substances in facultative aerobic staphylococci and streptococci.

Although yeasts are not included among obligate aerobic organisms, they multiply greatly in fluid mediums only with access to air.

Collingsworth and Reid (1935) demonstrated that an addition of 0.02% thioglycolic acid or 0.1% $Na_2S_2O_3$ as reduction substances to a synthetic medium with a small amount of seeding makes a faster growth possible than without addition of these substances.

Nitrifying bacteria can also exist in locations with a very low rH_2 value. Lyubimov (1937) studied the distribution of nitrifying bacteria in the mud of water-bodies and determined at the same time the redox potential of the mud with the electrometric method. The result was that nitrifying bacteria are found in the mud to a water-depth of 30 m. On the whole they are found down to 5 cm beneath the surface of the mud. Here the rH_2 was at 15-16; on the surface of the mud it amounted to 20. 0.15 mg per liter of oxygen was dissolved in the water above the mud. In colonies grown on silicic acid gel and with reference to the nitrification process, nitrifiers from the surface and from a depth of 2 meters showed no appreciable differences. They were also active in deep mud layers. The result of isolation was that it was a question of a Nitrosomonas strain.

Kingma Boltjes (1935) found that in cultures of Nitrosomonas and Nitrobacter with an air deficiency the eH dropped in the first case to -40 mv and in the second case to -160 mv with an initial eH of the medium at +300 mv. However, the nitrifiers were not killed off because of that. After remaining for a rather long time under anaerobic conditions the cells began to multiply again when they had access to air.

All the studies indicate that aerobic microorganisms both tolerate reductive conditions and grow with access to air. Growth under reductive conditions does not at all mean that aerobic bacteria are able to do without oxygen. Obviously some aerobes need at the same time oxygen and also reducing substances that lower the redox potential in the medium. The rH_2 limit value lies considerably higher for aerobes than for anaerobes that multiply only in a range of rH_2 0-5, although they remain capable of living at a higher rH_2.

The reason aerobes avoid strongly oxidative conditions is probably based on the fact that, just like anaerobes, they have important ferment systems that are active only in a reduced state.

Brown and Snell (1954) also support this point of view and refer to coenzyme A whose SH group is active only in a reduced state. Additions of reduction substances (Na_2S) increase the activity of coenzyme A.

The problem of the requirements of aerobes for oxygen in conjunction with the importance of the redox potential is more complicated than it seemed to be originally.

Knaysi and Dutky (1933, 1934) tried to determine the limits of the need for oxygen in aerobic bacteria and their requirements for the redox potential in comparison with each other. Growth continued at an eH of -160 mv when sodium sulfite was added as a reduction substance to a bouillon culture of Bac. megaterium. The medium was free of oxygen under these conditions. Without a doubt the reduction substance causes,

in addition to a lowering of the eH value, a bonding of oxygen. Bac. megaterium could not grow under these conditions. For a second experiment an oxidation substance (Fe^{III} ammonium citrate) was chosen that supports in a vacuum without access to oxygen an eH just to -40 mv, a value that is high enough for Bac. megaterium. However, the bacteria did not grow in a vacuum on this medium. A favorable eH alone, without oxygen, therefore, still did not ensure growth, that is, aerobes as such need oxygen.

If aerobes do not utilize oxygen for the creation of a high redox potential except as such, this means that not every hydrogen acceptor whatsoever is usable, but precisely oxygen. Thus, for example, Fe^{III} ammonium citrate cannot replace oxygen as hydrogen acceptor for Bac. megaterium. The question arises whether any other oxidation substance can replace oxygen. Methylene blue is frequently used as hydrogen acceptor in experiments on oxidative ferments.

Wieland and Bertho (1928), for example, oxidized alcohol to acetic acid with washed acetic acid bacteria cells that were no longer growing. With quinone as hydrogen acceptor oxidation occurred twelve times faster than with atmospheric oxygen; with methylene blue it took place more slowly. On the other hand, according to Stokes (1952), methylene blue cannot replace oxygen in the oxidation of acetates with a suspension of Esch. coli.

The problem also remains open whether other hydrogen acceptors can replace oxygen for all the processes of life activity, including multiplication.

There are also optimal ranges for oxygen just as there are for nutrients. The same is true of the eH of the medium. It is higher than the eH for strict anaerobes; its upper and lower limits, however, have been determined only in individual cases.

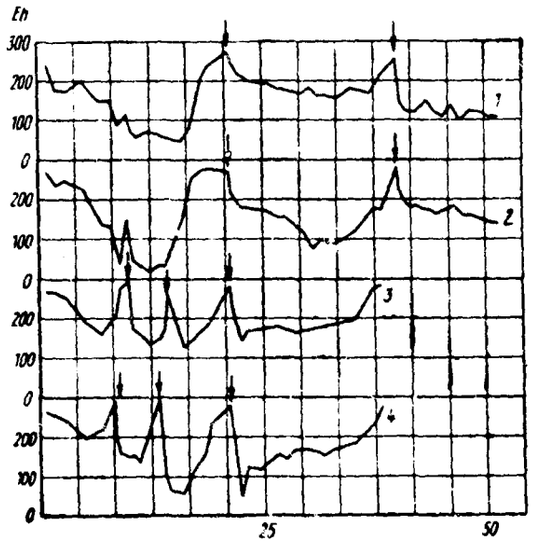
II. Changes in the Redox Conditions in Cultures of Aerobic Microorganisms.

In general, changes in the redox potential in cultures of different species of aerobic microorganisms agree in their principal characteristics: The potential drops off more or less rapidly, remains constant for some time and then rises again. The course of the drop in potential, duration of the minimum and progress of the rise in the eH are different, however, in the individual species.

Obligate aerobes, like nitrifying bacteria, according to Kingma Boltjes (1935), lower the eH of the medium during the logarithmic growth phase. With Nitrosomonas the potential dropped from eH 200 mv to 80 mv; with Nitrobacter, from 250 mv to 100 mv. After exhaustion of the oxidizable substrate (NH_4 and NO_2) the eH went back up to the original height. It went down again when the multiplication rate increased after the addition of oxidizable substances (Fig. 32).

The drop in the eH may be absent with the slow growth of nitrifiers. Zobell (1935) cultivated nitrifying bacteria at a temperature of 20° C. At pH 8, in this case, the eH fluctuated between 280 mv and

350 mv. According to our calculation this corresponds to an rH_2 value of 24-28. A lowering of the eH was not observed, which perhaps can be explained by the fact that at the low temperature of 20° C. (Kingma Boltjes cultivated nitrifiers at 30° C.) the solubility of oxygen is greater; however, growth and elimination of reducing substances take place slowly so that the reducing substances are oxidized again in the same measure as they are former.



days

Figure 32. Course of the eH in cultures of nitrifiers (according to Kingma Boltjes, 1935).

The arrows indicate the moment of the repeated addition of ammonium with Nitrosomonas (1 and 2) and of nitrite with Nitrobacter (3 and 4).

Acetic acid bacteria are also obligate aerobes. According to Lyubimov (1950) the rH_2 in cultures of Acetob. xylinoides (designation not recognized by Bergey or Krassilnikov), vini acetati (idem) and curvum (idem) in a yeast-water ethanol medium remained at the same level until the typical bacterial film had formed on the culture solution. Then the potential dropped. With species that form a thick film (for example with Acetob. xylinoides), the rH_2 reached the low value of 6-8 that remained constant for a rather long time (Fig. 33).

With Acetob. vini acetati that forms a thin film that sinks easily the redox potential attained values of 7-9, however, then it rose again rapidly. With Acetob. curvum the redox potential dropped to about 10 (Fig. 34).

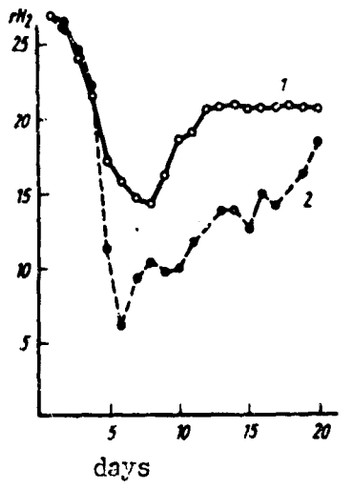


Figure 33. Course of the eH in a culture of Acetob. xylinoides (average values from 5 experiments) (according to Lyubimov, 1950)

1 = upper electrode in the bacteria film
2 = lower electrode under the bacteria film

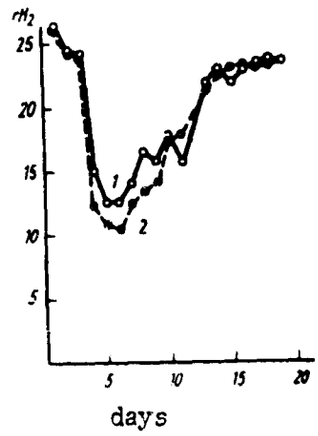


Figure 34. Course of the eH in a culture of Acetob. curvum (average values from 4 experiments) (according to Lyubimov, 1950)

1 = upper electrode

2 = lower electrode (both beneath the bacteria film

When Acetob. xylinoides was cultivated in a medium that was only 7-8 mm thick, the slime film soon almost completely filled the medium. Measurements with one of the electrodes inserted in the film gave a decrease in the rH_2 value to 13.7 within 24 hours followed by a new rise to about 20.

Phycomycetes displayed, as aerobic organisms, typical growth only when there was contact with air on the surface of the substrate.

Observations by Labrousse and Sarejanni (1929) on cultures of fungi on agar mediums showed that strongly reductive conditions develop in the agar so that methylene blue, indigo disulfonate, Nile blue, Janus green and neutral red are reduced. The rH_2 values in the medium went down to 5 (Janus green) and further down to 3 (neutral red).

The decrease in the rH_2 value in Phycomycetes can also be established with the electrometric method (Kanel, 1935). In a culture of Rhizopus nigricans in a synthetic medium with 10% glucose the redox potential dropped to rH_2 under the fungi layer, remained at this level for 19-20 days and then went up again. The rH_2 reached a value of 5-6 in the same medium with another Rhizopus strain.

According to experiments by Rabotnova aerobic Actionomycetes follow the same general rule. Actinomyces globisporus grew without access to air in the already-described experiment receptacle for cultivating anaerobes (cf. pp 138, 182). The drop in the rH_2 appeared under the film surface after 48 hours on a glucose-peptone medium. The redox potential dropped from 31 to 21 after the third day and to 16.3 on the sixth day. It remained at 15-17 until the seventeenth day and then rose again more or less rapidly. Occasionally the rH_2 value went down slowly, but always to the above-mentioned value.

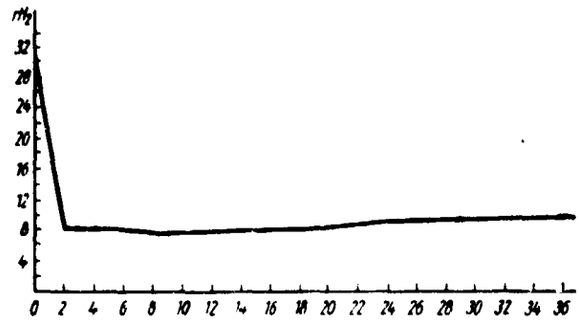
The redox potential also decreased very much under the bacteria sheet of tuber bacteria (Rabotnova, 1936). It reached the value of 8 on a medium with glucose and remained at this level for 30-40 days (Fig. 35).

The rH_2 value fell to 12-14 on the same substrate with the addition of yeast extract. The oxygen of the air was obviously used up in the bacteria sheet and did not reach under it.

On a mineral medium with nitrates and mannitol the redox potential under the growth area dropped very slightly, reaching about 23-28 (Fig. 36).

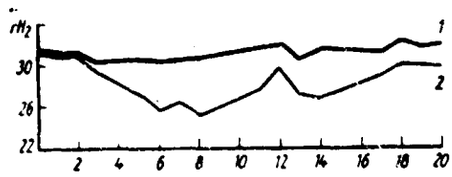
Therefore, depending on the composition of the medium, the redox potential can decrease in varying large amounts under a bacteria sheet. It can reach very low values (rH_2 7-8) on some mediums.

While the decrease in the rH_2 value in cultures of strict aerobic bacteria is especially surprising, it is quite understandable in facultative aerobic bacteria, since facultative aerobes can change their metabolism to anaerobiosis at a low redox potential.



days

Figure 35. The redox potential in a culture of Rhizob. leguminosarum on an agar medium.



days

Figure 36. The redox potential in a culture of Rhizob. japonicum on a mannitol-nitrate agar.

1 = electrode values at a depth of 3 mm.

2 = at a depth of 9-10 mm.

Esch. coli, a typical representative of the facultative aerobes, lowered the eH almost to the potential of a hydrogen electrode. Esch. coli can grow not only under aerobic conditions, but also with complete exclusion of air, for example in a hydrogen atmosphere (Clifton, Cleary and Beard, 1934; Clifton and Cleary, 1934).

As long as the bacteria were multiplying, the potential maintained its low value. When multiplication ceased, the eH went up. In continuous culture experiments performed by the above-mentioned authors the number of cells capable of living a long time (up to [illegible] days) did not decrease; during this time the eH also remained low.

A sizable decrease to -200 mv occurred in cultures of lactic acid bacteria by growing in milk (Frazier and Whittier, 1931).

Shaposhnikov and Sacharov (1929) observed decoloration of methylene blue and reduction of S and NO_3 in growing cultures of Lactobac. delbrueckii. This also indicates a decrease in the rH_2.

According to experiments by Hewitt (1930 a-d, 1950) in some pathogenic bacteria there is a relationship between the course of the eH curve and the ability of the bacteria to form hydrogen peroxide. The eH dropped here also, then either rose again rapidly, for example with hemolytic streptococci, or remained for a long time at a low level with other species, for example Corynebact. diphtheriae.

In culture of hemolytic streptococci the eH fell during the logarithmic growth phase to +160 mv and then rose again, especially when air was admitted to the culture, to values that were higher than the initial ones (Fig. 37). H_2O_2 could be established in the culture.

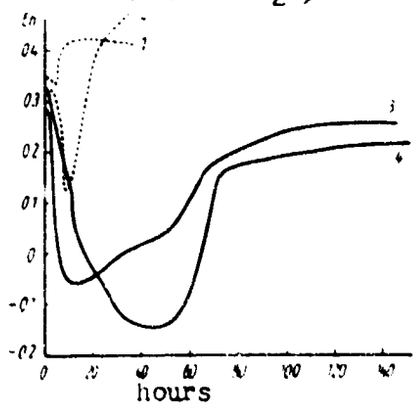


Figure 37. The eH in bacterial cultures supplied with air (according to Hewitt, 1950).

1 = Pneumococci; 2 = Streptococci; 3 = Staphylococci; 4 = Corynebact. diphtheriae.

Pneumococci, which are catalase-negative, produced hydrogen peroxide and attained a higher eH, 420-510 mv, in a culture supplied with air than the catalase-positive streptococci. When a catalase preparation was added to a Pneumococci culture, the eH decreased more than 100 mv.

Since the amount of the eH decrease in cultures of Streptococci is specific of the species, according to observations by Barnes (1956), an attempt was made to utilize eH values for characterizing individual species. Differences in reduction ability can be determined in a simple way by using indicators. The eH in the culture does not always follow the form of a curve with a descending and a rising section. According to Hewitt (1931, 1950) the addition of lysing agents led to a sizable drop in the redox potential due to lysis. The introduction of lysozyme to Micrococ. lysodeicticus (designation not recognized by Bergey or Krassilnikov) acted in this manner. With the dissolution of the protoplast reducing substances obviously go into solution and affect the potential. After death of the culture the potential rose again (Fig. 38).

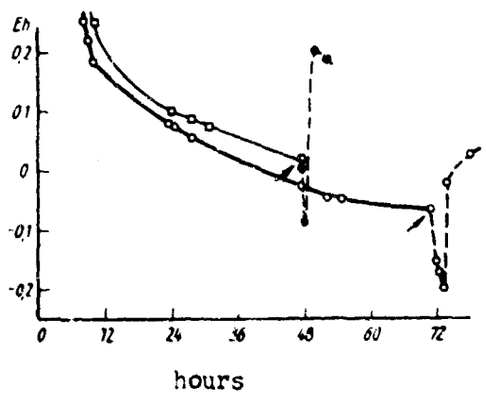


Figure 38. The eH in a culture of Micrococ. lysodeikticus (according to Hewitt, 1950).
The addition of lysozyme is indicated by an arrow.

In a culture of Bact. dysenteriae (designation according to Krassilnikov) containing bacteriophages growth was absent and the potential did not change. When the phages were added to an already growing culture, the eH decreased strongly above all, and rose again with the death of the culture.

Cultures of Azotob. chroococcum showed, according to Rybalkina (1937), a twice-repeated lowering and rising of the

eH value, so that a curve with two maxima was produced. According to microscopic observations the eH fluctuations seemed to be related to the growth stages of the culture. The second lowering of the eH value was always associated with the release of the cell content. The more cells died, the more pronounced was the second decrease of the potential (Fig. 39).

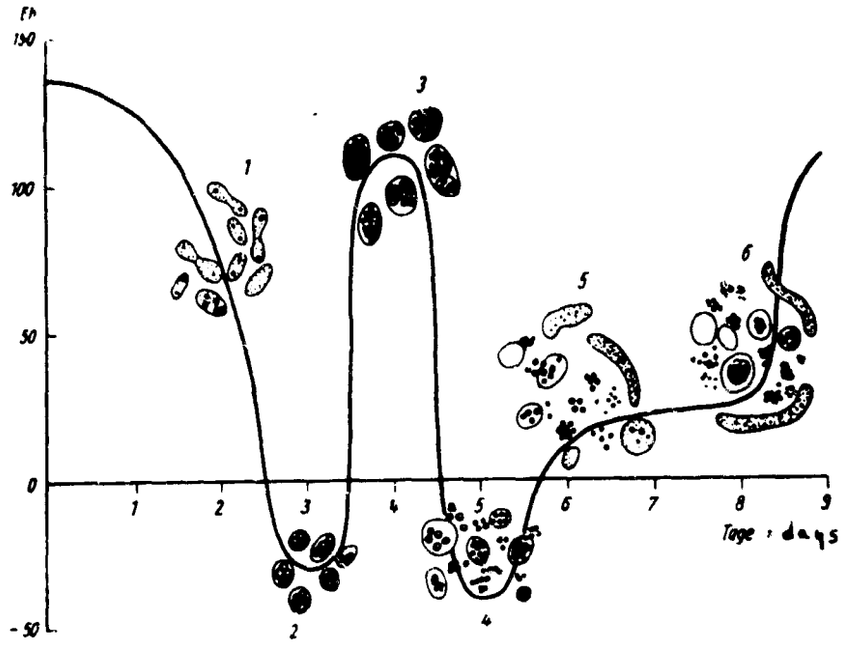


Figure 39. Course of the eH in a culture of Azotob. chroococcum (according to Rybalkina, 1937).

1 = young cells;
2 = granular cells;
3 = + ripe, granular cells;
4, 5, 6 = autolysis stages

With Azotob. agile (designation according to Krassilnikov) the cells survived for a long culture time. The second drop and the second rise of the potential were missing here. Zabotnova (1941) obtained the same results with both species, although the potential differences were not so pronounced (Fig. 40).

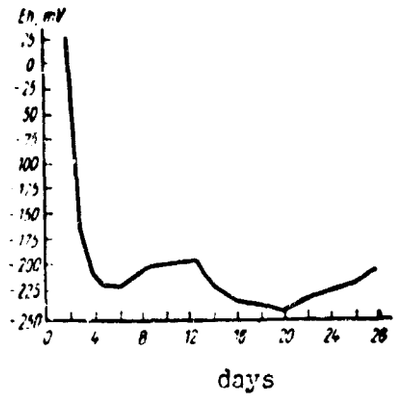


Figure 40. The eH under the area of growth of Azotob. chroococcum on an agar medium (according to Zhotnova, 1944).

Potential shifts also appear in the blood and the living tissues of plants and animals. Reiss (1943) and Vles (1943) determined for example the redox potential of blood in vivo and in vitro. The rH_2 values of blood in the veins of a dog were at about 20-21; in the cubital veins of a human, at 22.2. With a supply of air in vitro the eH goes up to 300 mv. Under anaerobic conditions it sank to +20 mv and lower.

Pavlov and Issakova-Keo (1929) determined the eH in hen's eggs. As the embryo grew the eH dropped from +240 mv to +100 mv; when growth stopped, it went up. In the opinion of the authors, therefore, it must be assumed that the embryo gives off reducing substances in its development.

The eH drops in a germinating seed as a result of accumulation of SH compounds and ascorbic acid (Virtanen and Tautanen, 1952).

There are various notions on the causes of the decrease in the eH in cultures of aerobes.

Coulter and Isaacs (1929) assume that all the oxygen dissolved in the culture fluid is consumed in cultures of typhus bacteria in the logarithmic phase. As confirmation it is determined that the eH went down to 85-90 mv (pH 7.6) in a sterile bouillon evacuated with nitrogen. With the death of the cells the potential went down still farther to -145 mv as a result of the elimination of reducing substances given off by the autolyzing cells (Fig. 41). When the culture was continuously and intensively supplied with air, the drop in the potential stopped, although the cells were multiplying.

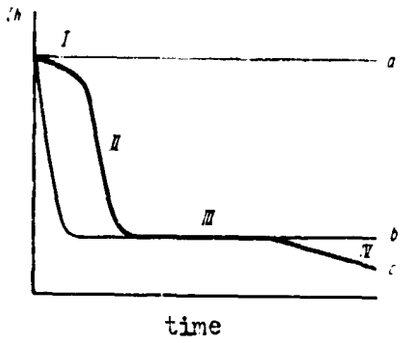


Figure 41. The eH in a culture of typhus bacteria (according to Coulter and Isaaks, 1929).

a = in a culture supplied with air;
b = in sterile, air-exhausted bouillon;
c = in a culture without air supply;

I = lag phase; II = logarithmic growth phase;
III = stationary growth phase; IV = period of death of cells and enrichment of the medium with reducing substances from the dying cells.

The decrease in the eH was greater on a medium with glucose than on the same medium without glucose. This is explained by the release of a greater amount of reducing substances due to decay of a greater amount of cells. The cessation of the decrease in the potential in the culture supplied with air depends, according to Coulter and Isaaks, on the fact that the formation of a low potential is conditioned by the consumption of oxygen in respiration. The potential is lowered by reducing substances only in later growth stages of the culture.

Tsiotsiouvo (1939) is of the opinion that the consumption of oxygen due to respiration is not the only cause of the decrease in the eH value. It must be added to this that the cells of aerobes, just as cells of anaerobes, eliminate reducing substances during metabolism. Comparative measurements of the rH_2 in cultures of tuber bacteria and in sterile agar mediums in an air and a nitrogen atmosphere demonstrated that the rH_2 values in the bacteria cultures decrease more than can be explained by the consumption of the oxygen dissolved in the medium (Fig. 42).

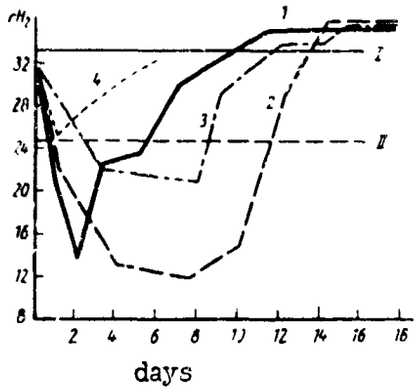


Figure 42. The redox potential in growth of tuber bacteria on a medium according to Zycha.

I = rH_2 of a sterile medium under aerobic conditions;
II = under anaerobic conditions;

1 = Rhizob. leguminosarum;
2 = Rhizob. meliloti;
3 = Rhizob. japonicum;
4 = Rhizob. trifolii (very weak growth and no formation of bacteria sheet).

If it is considered that the rH_2 values do not decrease in the same degree in differently composed mediums, it must be assumed that the reducing substances eliminated by the bacteria either act in different ways or are given off in different amounts.

The discharge of reducing substances can also be determined in other ways. If a porcelain bougie is inserted in a beef bouillon culture of Esch. coli, a fluid free of microorganisms diffuses in it. The presence of reducing substances is ascertained in this culture fluid by means of an electrode. The redox potential went down to 5 for example, while it amounts to 20-25 mv in a sterile beef bouillon in a nitrogen atmosphere.

The reducing substances given off by a growing culture can perhaps lead to the formation of such low potentials that in spite of an air supply to the culture the eH goes down just as in mediums not supplied with air. Hewitt (1931) described a case in which the decrease in the eH was even greater than in the parallel culture not supplied with air.

In beef bouillon supplied with air Microc. lysodeikticus absorbed oxygen so intensively and gave off reducing substances

so abundantly that the eH value, compared with a culture not supplied with air, dropped more than 100 mv (Fig. 43). It is worth noting that the electrode is more sensitive in the presence of the reducing substances eliminated by the bacteria than in the presence of atmospheric oxygen.

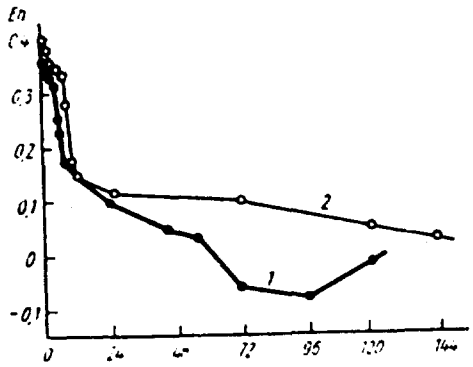


Figure 43. The eH of an aerobic culture of Micrococ. lysodeikticus supplied with air (1) and of one not supplied with air (2) (according to Hewitt, 1931).

The reducing substances discharged by Staphyloc. aureus can be "titrated out" with potassium ferricyanide in the culture (Clifton, 1933). However, the culture filtrate contained no reduction substance. Obviously the reduction substance is localized on the surface of the cells and is oxidized when it reaches the milieu. Clifton concludes from his experiments that the reducing substances are formed only in the presence of an oxidizable substrate, because in washed suspensions without a hydrogen donator no decrease in the eH occurred.

The height of the potential in the culture has a dynamic character. It depends on the proportion of the speed of discharge of reducing substances by the organisms and their oxidation. After the drop in the eH in cultures of aerobes it begins to rise again when multiplication stops and the substrate is exhausted; no more oxygen is consumed, and the elimination of reducing substances ceases.

III. Growth of Aerobes, Depending on the Redox Potential of the Culture Medium.

Certain contradictions result from the study of the redox potential in cultures of aerobes: On the one hand aerobes need atmospheric oxygen; on the other hand they avoid an excess of

oxygen and give off reducing substances of an unknown nature into the substrate.

The observations discussed up until now still do not make it possible to answer the question of at what rH_2 interval existence and multiplication of aerobes is possible. It should be assumed that this interval is very large, because the life activity of aerobic microorganisms takes place with the reciprocal action of two opposing forces: the force of oxygen as an oxidation substance and the force of the reducing substances given off by the cells.

In order to have an idea of the rH_2 value occurring with multiplication, the rH_2 measurement should be taken only when all cells are under approximately the same conditions. This is the case in a culture of facultative aerobic bacteria that grow uniformly in the entire medium without forming a film. Obligate aerobes can be studied only in cultures that are stirred continuously in order to prevent the formation of a film. In order to be able to compare the redox potential of the medium with the growth of the culture, the determination of the increase in the number of cells must take place simultaneously with the rH_2 measurements. In addition various redox conditions must be present in the medium in order to comprehend the influence of the redox factor on multiplication.

Rabotnova studied rH_2 changes and the course of multiplication of Azotob. chroococcum by cultivating it in a thin layer (Fig. 44). At the beginning of the experiment the rH_2 value in the medium was at about 29. During the course of growth of the culture it dropped in 114 hours to 16. Down to rH_2 20 the cells multiplied briskly, then autolysis set in. Greater morphological changes in the cells, for example the formation of giant cells occurring in anaerobes, were absent. The cells increased only little in size, at the most to double in size. A reticular structure was visible in the protoplasm by dyeing with gentian violet. After that autolysis appeared and the cells decayed.

Ascorbic acid was used for lowering the rH_2 value. The initial rH_2 of the medium was lowered from 29 to 20 by adding 0.05%, 0.1% and 0.2%. During growth of the culture it went down further to 16, in some experiments even to 11 (Fig. 44). Moreover, multiplication occurred very energetically down to rH_2 11.

The maximum cell crop was greater in a strongly reduced medium than in a medium without addition of a reduction substance. In one experiment the maximum number of cells reached 3.5×10^9 per ml; however, without ascorbic acid, only 3.0×10^3 per ml.

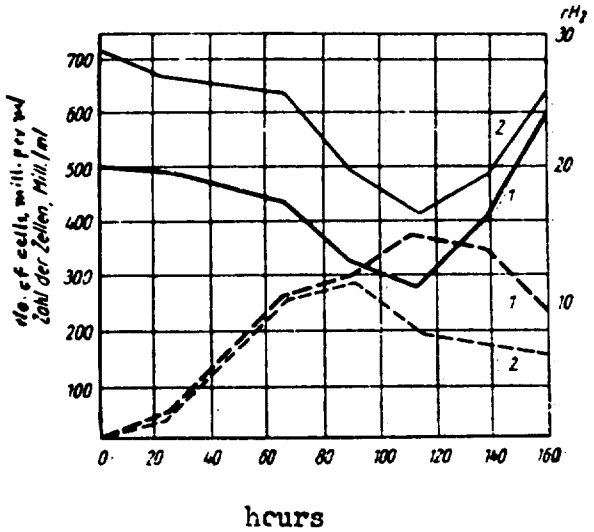


Figure 44. rH_2 changes and cell multiplication in a culture of Azotob. chroococcum. ——— = rH_2 ---- = number of cells; 1 = with 0.15 ascorbic acid;
2 = control without reduction substance.

Azotobacter, therefore, grows in the unusually broad rH_2 range of 11-29. Growth occurs somewhat more intensively at an initial rH_2 value lowered to 20.

Azotobacter also grew in a medium that contained dissolved hydrogen. Up to 6.0 × 10^7 cells per ml grew with a seeding of less than 1.0 × 10^6 cells per ml. Growth was indeed better with an oxygen supply. Under these conditions Azotobacter is obviously less sensitive to the rH_2 value in the medium.

In order to raise the redox conditions in the medium, the cultures were shaken in an agitator at a rate of 100 shakes per minute.

The thorough mixing of the medium can have a very great effect on the number of cells produced, when the cells give off toxic metabolic products. With constant agitation the cells do not succeed in building-up a zone that is enriched with these substances. The cells can grow better consequently. Veleminsky and Butschovitz (1929) demonstrated this in experiments with yeasts. A constant stirring without an air supply, performed in a specially constructed apparatus, increased the number of cells to the same degree as occurred with an air supply simultaneous with agitation.

However, these results can hardly be applied to Azotobacter. Azotobacter oxidizes organic substances to CO_2 and H_2O and forms no toxic metabolic products. It is to be assumed rather that the better oxygen supply obtained by stirring the culture has a favorable effect.

In order to explain how much Azotobacter depends on a high redox potential, the redox potential was lowered by adding reduction substances (ascorbic acid and dithionite). The addition was made at the start of the experiment and half-way through the experiment, in order to maintain a low redox potential even with a heavy supply of air. Lowering the rH_2 value to 20 by adding ascorbic acid did not have, however, any appreciable effect on multiplication of the cells (Table 41).

TABLE 41

Effect of the redox potential on the growth of Azotobacter in a shake culture.

time in hours from beginning of seeding	without additive		0.033% $Na_2S_2O_4$		0.1% ascorbic acid	
	rH_2	No. of cells in mill. per ml.	rH_2	No. of cells in mill.per ml.	rH_2	No. of cells in mill.per ml.
2	29.9	No growth	26.4		20.5	No growth
23	28.9		26.5	18	20.4	
56	28.6	74	16.3	112	19.3	34
72	27.9	300	24.3	523	20.4	421
101	29.6	1204	24.9	753	20.2	1049
119	29.7	746	23.9	1060	25.0	838

The slow commencement of growth can be attributed to the low experiment temperature of 20° C. Growth occurred in an approximately equal strength in a range of rH_2 20-30.

Not too much significance must be ascribed in these experiments to the differences in the maximum number of cells. Multiplication occurred very rapidly and autolysis set in just as fast, so that the amount of the maximum crop depended mostly on the moment of taking the specimen. The maximum values of 1204, 1060 and 1049 million cells per milliliter must be considered equivalent.

The cell yield with a good supply of oxygen was twice as large as in cultures at rest in which obviously not enough dissolved oxygen was available.

Azotobacter, therefore, is relatively less sensitive to the redox conditions of the mediums, however, it reacts strongly to dissolved oxygen.

The stimulating effect of ascorbic acid, very pronounced in stationary cultures, was missing from the shake culture.

It could be assumed that for Azotobacter, cultivated on nitrogen-

free mediums, the results were affected not only by the dissolved oxygen but also by dissolved atmospheric nitrogen. However, in a medium with combined nitrogen [$(NH_4)_2SO_4$] that is more easily assimilated than atmospheric nitrogen the same laws prevailed.

A comparison between the growth of Azotobacter in fluid and solid mediums was interesting. Azotobacter was cultivated in Petri dishes with 20 ml of agar. The growing cells were washed off, counted and transferred to 1 ml of agar (Table 42).

TABLE 42

Growth of Azotob. chroococcum in a fluid medium and on agar after 48 hours at 30° C.

Experiment No.	No. of cells in a fluid medium (million per ml)		No. of cells on agar (million per ml)
	stationary culture	shake culture	
1	200	-	-
2	69	1070	1500
3	76	670	1000
4	220	900	6600

Similar observations were made on the changes in redox conditions and the increase in the number of cells with Bac. subtilis. Bac. subtilis usually forms a surface film in a bouillon culture. There are strains, however, with which the film appears only in old cultures; during the first days of development they grow submerged in the medium. Moreover with careful supervision of the morphological characteristics and the physiological behavior these strains did not differ in any way from the Bac. subtilis described in Bergey's and Krassilnikov's manuals.

It was possible, by using these strains, to study the rH_2 in the medium and to determine at what rH_2 value multiplication of the cells occurs. The rH_2 went down from 23 to 17 (in some experiments to 14) by cultivating on beef-peptone bouillon in a thin layer.

During this time the number of cells went up to 2.0×10^8 per ml; then autolysis set in (Fig. 45).

It is worthy of note that even in a thin layer, with direct action of air, a very low redox potential appears occasionally.

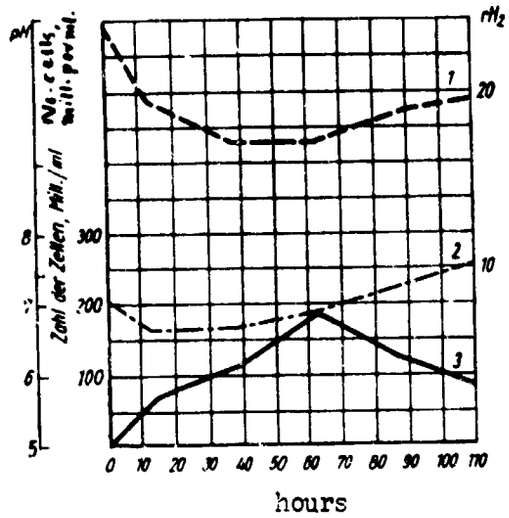


Figure 45. Growth of Bac. subtilis on beef-peptone bouillon in a thin layer.

1 = rH_2; 2 = pH; 3 = number of cells.

An even greater lowering of the rH_2 value, down to 12 and 5, can be established by means of colorimetric determination of the redox conditions (Table 43). Janus green changed color from dark green to raspberry red; indigo tetrasulfonate was decolorized. This indicates a drop in the rH_2 value to under 12. The aerobe, Bac. subtilis, can multiply, therefore, in a medium whose rH_2 value is very low, although only for a short time and even in the logarithmic growth period.

TABLE 43

Reduction of rH_2 indicators during the growth of Bac. subtilis

rH_2 indicator	ml of 0.5% color solution per 30 ml of the medium	color of the indicator after			
		0 hrs.	24 hrs.	36 hrs.	47 hrs.
neutral red	0.05		red		yellow (alkaline)
Janus green	0.05	dark green		raspberry red	

phenosafranine	0.06			pink
indigo tetrasul-fonate	0.05	light blue	clear-ing	reduction
methylene blue	0.05	light blue	reduction	
thionine	0.045	violet	reduction	

This showed up still more clearly by cultivating Bac. subtilis in beef-peptone bouillon and 1% sucrose. Lowering of the rH_2 and increase in the number of cells still took place here very rapidly (Fig. 46).

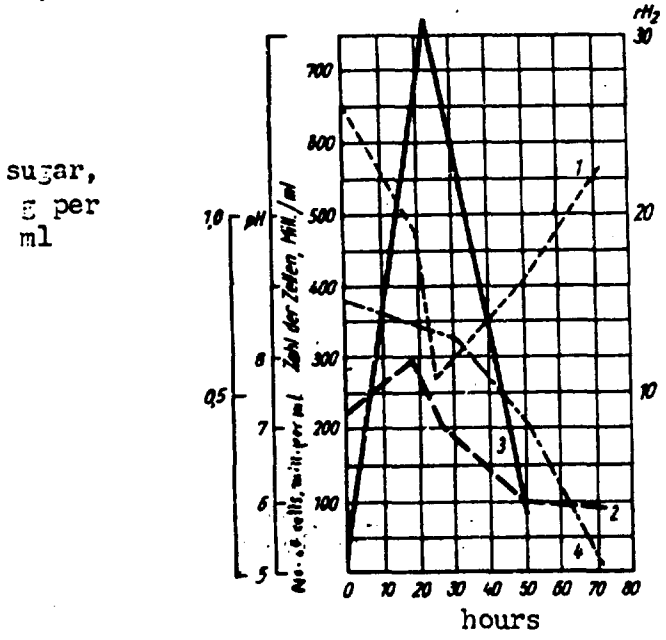


Figure 46. Growth of Bac. subtilis in beef-peptone bouillon with 1% sucrose.

1 = rH_2; 2 = pH; 3 = number of cells; 4 = sucrose content.

Changes set in already in the first hours after commencement of the experiment (Fig. 47). The beginning of the logarithmic phase coincided with the sharpest decline of the rH_2 value. The cell content was changing already during the lag phase: The cells of the inoculum developed into filamentous cells; giant forms that survived up to 24 hours appeared in a still greater amount than in cultures of anaerobes. The behavior of Bac. subtilis seems to indicate that the cells are not as sensitive to the redox condition

of the medium as obligate anaerobes. With Clostr. sporogenes multiplication first appeared at a definite, very low rH_2 value; at high values the cells did not divide but rather only grew. Bac. subtilis on the other hand grows and divides when the redox potential drops from 25-28 to 10-12.

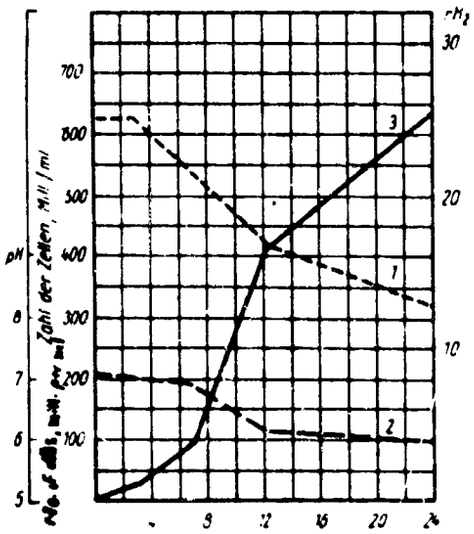


Figure 47. First growth stages of Bac. subtilis in beef-peptone bouillon + 1% sucrose. Development of the culture:

1 = rH_2; 2 = pH; 3 = number of cells.

A decrease in the amount of air supply to the subtilis cultures decreased multiplication significantly (culture in high layer, Fig. 48).

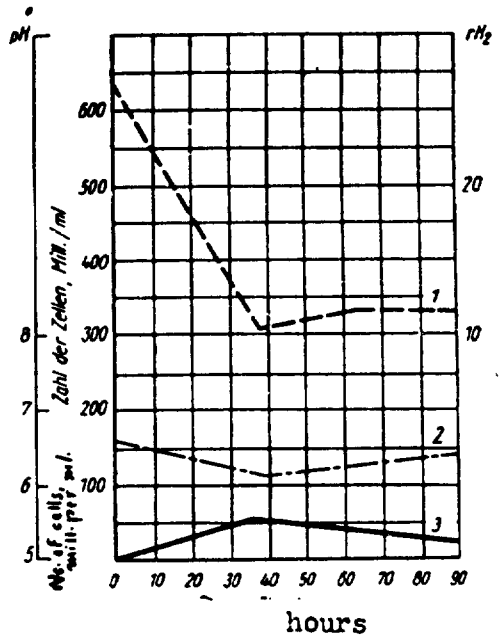


Figure 48. Growth of Bac. subtilis in beef-peptone bouillon in high layer. 1 = rH_2; 2 = pH; 3 = number of cells.

There are only few studies on the redox conditions in cultures of Actinomycetes. According to Konova (1954) Actinomycetes grow in submerged culture at rH_2 values between 20 and 28. Kramli, Kovacs and Matkovics (1954) established a lowering of the eH in surface cultures of Streptom. griseus.

Zabotnova, Duyanova and Asova studied the effect of rH_2 changes under the surface film of Act. globisporus by growing the culture in a fluid medium. In order to attain a rise in the redox potential under the surface film, 5% H_2O_2, 0.01 mol $KMnO_4$ or 0.12 mol $K_3[Fe(CN)_6]$ were added at intervals drop by drop. The additions took place after the surface film had formed and the rH_2 decrease had begun. However, the redox potential could not be held at a definite level with this method. Six to seven hours after addition of the oxidation substance the eH went down to about 70-90 mv; erratic changes appeared, therefore. While the redox potential held at about 15 in control cultures, it fluctuated between 15 and 26-28 with the addition of the oxidation substance. The different rH_2 values had no effect on the growth of the culture (Table 44).

TABLE 44

Weight of the yield in the growth of Actinomycetes in the presence of oxidation substances.

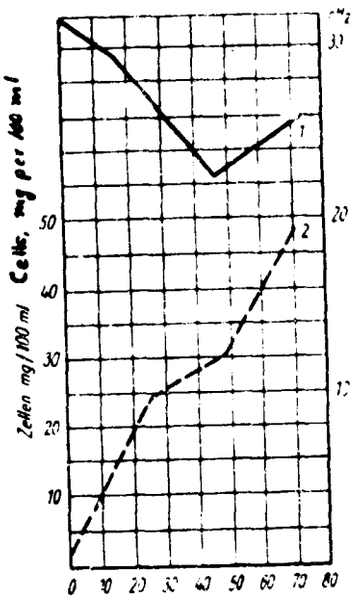
Oxidation substance	Experiment I	Experiment II
	Wet weight of the mycelium in g	
H_2O_2	0.1260	0.2239
$K_3[Fe(CN)_6]$	0.1622	0.2142
$KMnO_4$	0.1022	0.1926
Control	0.1434	0.1737
Without additive		0.1170

With a submerged culture the redox potential in the medium dropped from 32 to 22 during the growth of the culture in spite of an air supply. An increase in the mycelium size occurred at rH_2 30-32 and also at rH_2 22-25 (Fig. 49). In some cases the redox potential with an air supply dropped to 15-16 in experiments with other strains.

In order to establish the upper limit of the rH_2 value that still allowed growth, culture took place with a supply of oxygen. It showed that in this case no decrease in the rH_2 value appears as it does with a supply of air. In some experiments the redox potential went up to 30, in others it remained constant at 25-28 (Fig. 50). The growth was also normal under these conditions.

An rH_2 value of 29-30 obviously still cannot be assumed as upper limit for the growth, on the other hand a lowering of the rH_2 value does not seem to be at all necessary for normal growth. Act. globisporus consequently must be included among the microorganisms that are considerably insensitive to the rH_2 of the medium.

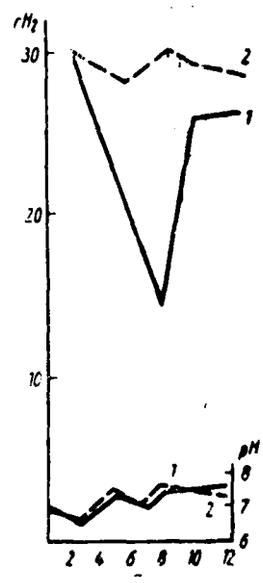
The course of the rH_2 changes and of the multiplication were also studied with facultative aerobic bacteria. Growth with the denitrifying Ps. aeruginosa in a bouillon culture was vastly independent of the eH changes that appeared (Fig. 51).



hours

Figure 49. Growth and redox potential of Act. globisporus in submerged culture.

1 = rH_2;
2 = weight of yield



days

Figure 50. Redox potential and hydrogen ion concentration in cultures of Act. globisporus with a supply of air and oxygen.

1 = air; 2 = oxygen. The pH values given below show that the growth of the culture is normal in both cases.

In a culture of acetone-ethanol bacteria in a 30% potato mash with the addition of $CaCO_3$ the redox potential dropped rapidly from the initial value of 25 with a simultaneous greater multiplication of the cells that also continued at rH_2 6 (Fig. 52). Multiplication was also possible in the wide range of rH_2 25 to 4-5.

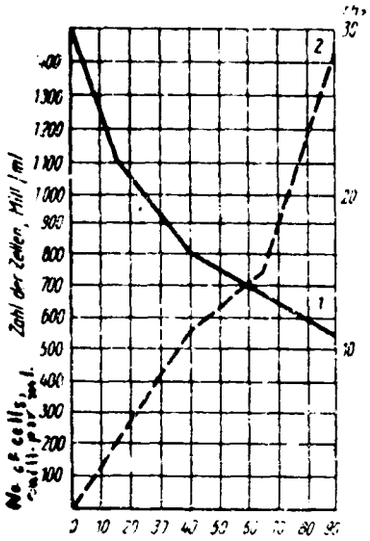


Figure 51. Redox potential and multiplication with Ps. aeruginosa in meat-peptone bouillon.

1 = rH_2;

2 = number of cells.

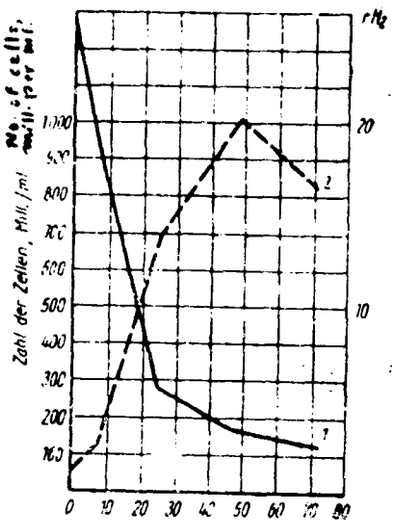


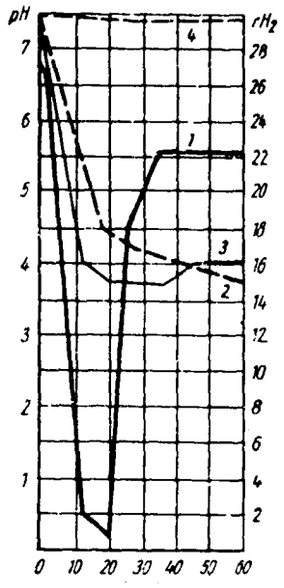
Figure 52. Redox potential and multiplication of cells of Bact. aceto-ethylicus (designation not recognized in Bergey or Krassilnikov).

1 = rH_2;

2 = number of cells.

Esc[illegible] coli is another typical facultative aerobe that grows in fluid [illegible]ums in turbidity. In mediums containing peptone coli behaved like saprophytic bacteria and decomposed peptone. In mediums with sugars a fermentation occurred with the formation of CO_2, H_2, volatile and non-volatile acids. In both mediums the growth was good; rH_2 measurements during growth of the cultures showed, however, that in both cases the redox conditions developed completely differently (Fig. 53).

The redox potential in a medium with sugar dropped very quickly from 27 to 1 during the logarithmic growth phase. Then the culture died as a result of much acid formation (pH drop to 4), and the redox potential went up again. In a medium with peptone the rH_2 values went down from 30 to 15. The pH value held steady at 7.5. The culture remained viable for a long time, and the low rH_2 value remained unchanged. Therefore, growth was possible in the range of rH_2 30-1.



hours

Figure 53. Redox potential and hydrogen ion concentration in a culture of Esch. coli.

1 = rH_2 on beef-peptone bouillon + sugar;

2 = pH; 3 = rH_2 in beef-peptone bouillon;

4 = pH.

All the results of experiments indicate that aerobic, especially facultative aerobic microorganisms, are able to multiply within a broad rH_2 range.

IV. A Procedure for Differentiating Obligate Aerobic Microorganisms from Facultative Aerobes.

Although the terms "aerobe" and "facultative aerobe" are often used, sufficient data are frequently lacking on whether a species should be classified in one or the other group.

A procedure for establishing the obligate or facultative aerobic behavior consists of cultivating with the exclusion of oxygen. We use for this purpose an anaerobe receptacle in a simplified form (Fig. 54) and we determine the multiplication by means of turbidity measurement or by determining the number

of cells per ml. By cultivating in mediums with carbohydrates the fermentation power can be established; in beef-peptone mediums, the ability for anaerobic decomposition (Table 45). Strains of the same species can display a different behavior with respect to facultative anaerobic growth. Of the strains belonging to various species that we examined in our experiments Bac. megaterium behaved like an obligate aerobe; Bac. cereus and mycoides, like a facultative aerobe. We found both obligate aerobes and facultative aerobes among strains of Bac. subtilus. Azotob. chroococcum, Actinom. globisporus and Acetob. schattenbachii (designation not recognized by Bergey or Krassilnikov) behaved like obligate aerobes in similar experiments with appropriate mediums.

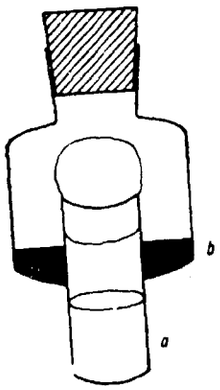


Figure 54. Culture receptacle for cultivating microorganisms with air excluded. The inoculated culture medium is put in the narrow section (a), and a pyrogallol solution in the wide section (b).

TABLE 45

Multiplication of various species of aerobic bacteria under anaerobic conditions with an inoculum of 1.3 X 10^6 cells per ml.

	in beef-peptone bouillon		in beef peptone bouillon +1% glucose		in beef peptone bouillon + 1% sucrose	
	duration of experiment, in hours	no. of cells in mill. per ml.	duration of experiment, in hours	no. of cells in mill. per ml.	duration of experiment, in hours	no. of cells in mill. per ml.
Bac. subtilis strain 9769			49	277		
Bac. subtilis	72	4	96	40	96	8
Bac. megaterium	30	7			30	3
Bac. cereus	30	100	30	111	30	235
Bac. mycoides	30	400*			30	235*

* Weight of yield in mg per l.

V. Methods of Cultivating Aerobes.

It is necessary to ensure an abundant supply of air for cultivating aerobic microorganisms. This is not always simple, because atmospheric oxygen is only moderately soluble in water. The most effective procedure for providing the aerobes with air is to cultivate them on the surface of solid mediums. Porous, solid mediums like bran, sawdust, etc. make possible a much better supply of air than for example agar mediums.

Aerobic microorganisms grow in stationary fluid mediums in the form of a surface film, so that the upper cell layers are well provided with oxygen and the ones underneath less and less so as depth increases. Physiological studies on aerobic microorganisms are made difficult due to the differences in the supply of oxygen within a culture. The effect of various factors can be ascertained only when all the cells are under the same conditions. In recent years the submerged culture method, first developed by Kluyver and Perquin (1933) for fungi, has had increasing attention paid to it.

The simplest way to provide air under laboratory conditions consists of introducing air by means of a glass tube. The air, sterilized through a cotton-wad filter, is pumped through the culture fluid

with a compressor or is sucked through by means of a water-jet pump. The use of fine-pored filters is recommended in this case.

Lyubimov (1954) suggested the use of a cylindrical culture receptacle with a double bottom in which the false bottom consists of a porous porcelain plate.

Some mediums foam, especially with a fine air supply. In such cases the air bubbles are increased in size or substances are added to check the formation of foam, for example oleic acid, soy oil, castor oil and other oils, tributyl acetate or higher alcohols.

In technical firms that are concerned with the mass-cultivation of aerobic microorganisms, aerating is used to a large extent in submerged cultivation, in the production of press-yeast for example.

Aeration is also improved by means of shake culture. Agitators are used in which the culture flasks are secured to a rotating or forward band backward moving stand. The number of revolutions or oscillations amounts to 100-300 per minute. The procedure is approximately equivalent to an aerated submerged culture. With rotating agitators only small amounts of liquid are put in the flasks, about 40 ml in a 250-ml flask. The culture solution is spread by the rotation in a thin, elastic layer on the walls; it is broken up in the backward and forward motion due to the uninterrupted shaking.

Another method of stirring consists of using a rapidly rotating propeller stirring device. The medium draws in air continuously due to uninterrupted whirling motion; it is well mixed and aerated. However, in using the propeller stirring device the rotating axle always passes through the neck of the receptacle, and the maintenance of sterile conditions is, therefore, made difficult and requires special equipment. With a magnetic stirring device there is a magnetic core in the culture receptacle, that is sealed in a glass capsule and is sterilized together with the medium. The impulse is produced from the outside by means of an electromagnet mounted under the culture receptacle and its motion is transmitted to the magnetic core. One disadvantage of the magnetic stirring device consists of the fact that it heats up gradually and warms the culture receptacle to 30°-35° C.

Fremel, Vyatkin, Stankov and Smirnova (1954) conducted extensive research on methods of submerged cultivation of aerobes. In cultivating Asp. niger for obtaining fungus amylase, cultivation in a drum rotating in a horizontal position was the equivalent of an uneconomical aeration in a tank with 100 m^3 of air per hour per 1 m^3 of medium.

In view of the moderate solubility of oxygen in water, the intensity of stirring is of great importance in the cultivation of aerobic microorganisms. Smith and Johnson (1954) assume that aeration always becomes the limiting factor, if maximal cell yields are desired. They determined the amounts of the oxygen dissolved in the medium by various aerating methods by means of sulfite oxidation (Table 46). It was shown from the example of Serratia marcescens in a medium with 4% glucose + 2% citrate that with an air supply intensity of 0.5 mm of O_2 the maximal cell yield reaches 9 mg per ml, whereas with 9 mm of O_2, it amounts to 23 mg per ml, which corresponds to 1.7×10^{11} cells per ml. The

other laboratory methods of providing air are, therefore, insufficient for a strong aerobe like Serratia marcescens. An adequate aeration is ensured only by using agitators and flasks with special equipment for breaking-up the fluid.

The principle of watering porous surfaces was used to produce a good contact of the culture fluid with the air. This procedure using beechwood chips has been known for a long time in the production of vinegar. Rabotnova (1944) described a laboratory model of a vinegar cask. A similar apparatus was developed by Pasynski and Neymark (1952) for other fermentations.

The measurement of the redox potential or the determination of the oxygen by means of the polarographic method (Zeidler and Taubeneck, 1956) may be used to verify the saturation of the medium with oxygen.

Tödt's (1958) method for the electrochemical determination of the dissolved oxygen depends on the fact that dissolved oxygen acts on the potential of an element consisting of a precious metal and lead and zinc (cf. also Windisch, Garsuch and Heumann, 1958).

TABLE 46

The action of various methods of aeration on Serratia marcescens (according to Smith and Johnson, 1954)

receptacle	volume of liquid	aerating method	Intensity of air supply. Air volume: 1 volume of solution in 1 minute	action of aeration m mol O_2 per liter in 1 minute
test tube (18 mm X 150 mm)	10 ml	unstirred medium	-	0.03
Erlenmeyer flask (500 ml)	20 ml	unstirred medium	-	0.32
idem	20 ml	agitator 250 RPM	-	1.1
idem	10 ml	idem	-	2.0
idem	50 ml	idem	-	0.60
idem	100 ml	unstirred medium	-	0.10
idem	100 ml	agitator	-	0.27
idem*	20 ml	agitator	-	2-9.5

flask (18 liters)	15 l	supplied through tubes 8 mm in diam.	1.0	0.60
idem	15 l	fine aeration area of 120 cm^2	1.0	0.60
fermentation vat (30 liters)	15 l	stirring device 500 RPM fine aeration	1.0	2.0
fermentation vat (100 gallons)	250 l	stirring device 250 RPM fine aeration	1.0	1.0
fermentation vat (3.5 liters) *	1.5 l	stirring device 1900 RPM fine aeration	3.3	10.0

* Special flask with equipment for breaking-up the fluid by shaking.

Hanke and Katz (1943) proposed a method, hardly used until now, for maintaining anaerobic and aerobic conditions in the medium:

The inserted a platinum electrode in the culture medium and connected a direct current supply to one of the two poles. A second electrode was inserted in a receptacle with KCl. The two receptacles were connected over a KCl-agar bridge. With a direct current supply gases that are formed by electrolysis of the water are precipitated at the electrodes: hydrogen at the cathode, oxygen at the anode. Since both electrodes are in different receptacles, the culture fluid in them is saturated either with hydrogen or with oxygen depending on the direction of the current.

Along the lines of the same principle, Rabotnova described an experiment set-up for producing different redox potentials in the medium.

A 12 volt direct current is supplied to a potentiometer and from there it can be drawn with various voltages. The current of the electrode in the experiment receptacle is fed to an ammeter. The second terminal is connected to the second electrode that is inserted in a saturated KCl solution. The receptacles with KCl and with the culture are connected with each other by a U-tube filled with KCl-agar. The area of the electrodes amounts to about 1 cm^2. The culture receptacle electrode is in a vertical position, so that settling cells are deposited as little as possible on it. The electrode intended for eH measurement, inserted in the culture fluid, indicates an eH that is dependent on the degree of saturation of the medium with oxygen. The current intensity can be changed in the range of +20 mA. to -20 mA., and it depends on the resistance in the circuit. The culture medium and the agar bridge produce rather strong resistances. The diameter of the

U-tube that contains the agar bridge is very important. Changes in the diameter in the range of 2-7 mm already affect the current intensity considerably.

With this arrangement eH values from -900 mv to +900 mv can be attained without difficulty in a sterile medium. The eH that is present in mediums in which microorganisms are growing affects their life activity. Microorganisms with a strong reduction power prevent the occurrence of higher eH values when the culture is multiplying actively and is giving off reducing substances. Their reduction power decreases with increasing age of the culture. If the need for maintaining the eH at a definite level arises, the current intensity is used as a regulating factor.

The potential at the electrode used for obtaining oxygen or hydrogen reaches values of 1.5 to 2.0 volts and more. With a potential like this not only can an electrolysis of the water be expected, but also the electrolysis of some salts. In fact, an alkalization appears when the medium is trated with hydrogen, and an acidification at the electrode that is emitting oxygen. High voltages on the electrode must be avoided as much as possible. When the medium is saturated with oxygen, chalk may be added and the electrode can be inserted in the chalk sediment at the bottom of the receptacle. In this way acidification is avoided. Alkalization of the medium with hydrogen is usually less to be feared, since smaller voltages are used for decreasing the eH in the medium than for raising it. This is understandable since the life activity of the bacteria is also oriented toward a decrease in the potential, therefore, usually it must be only slightly intensified.

It was to be explained by the use of this method whether microorganisms react to the enrichment of the medium with oxygen due to electrolysis as they do to aeration, and whether the passage of current has any detrimental or disturbing effect.

Yeast was put in two beakers with 25 ml of beer wort. The beer wort was covered with a layer, 3-4cm thick, of sterile vaseline oil, in order to reduce the supply of air from the surface. One beaker received the electrode that served to enrich the medium with oxygen, the other beaker served as control. Occasionally we also used a flask with a thin layer of beer wort (aerobic conditions) as a second control. Both beakers and the flask also contained electrodes for eH measurement. After a few hours the eH value of the medium in the experiment receptacle either rises or goes down. In the control beaker without access to air the eH went down (the rapidity depends on the amount of inoculum); under aerobic conditions it held at 120 mv.

In an experiment in which 2X 10^6 cells per milliliter were inoculated in beer wort (Sacch. cerevisiae, strain Ja) the potential could be held without difficulty at the same level. With a higher cell seeding (5.2 X 10^6 cells) of another strain (Sacch. cerevisiae XIII) it had to be regulated much more. The increase in the rH_2 value due to electrolysis led to an increase in the cell yield in comparison with the anaerobic experiment. In the aerobic control 58.6 million cells per ml grew; in the anaerobic culture, 33.8 million per ml; and with electrolytic enrichment of the medium with oxygen, 46.4 million per ml (Fig. 55).

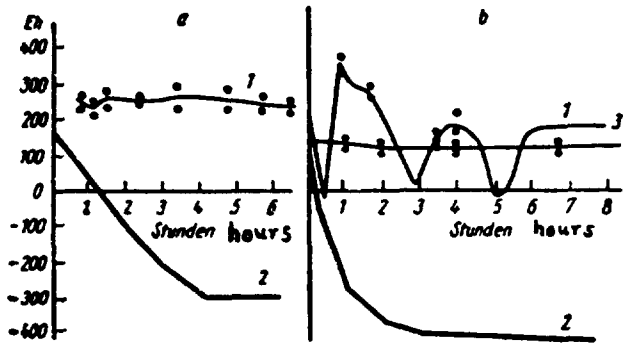


Figure 55. Behavior of the eH in a culture of Sacch. cerevisiae.

a. Inoculum of 2 X 10^6 cells per ml.
b. 5.2 X 10^6 cells per ml.

1 = without air supply, but with enrichment of the medium with oxygen due to electrolysis of the medium;
2 = under anaerobic conditions;
3 = under aerobic conditions with a thin layer.

Enrichment with hydrogen reduced multiplication; however, it could not completely stop it (Table 47).

TABLE 47

The yield in yeast cells at various rH_2 values produced electrolytically in the medium.

organism	experiment conditions	duration of experiment in hours	pH and rH_2 changes during the experiment: pH	rH_2	no. of cells, mill. per ml.: beginning of exp.	end of exper.	relative increase
Sacch. cerevisiae, strain Ja	+ (anaerobic control)	3	5 5.3 → 4.7	27 → 10 6.2	36.5	132.8 89.6	3.7 2.5
	+ (anaerobic control)	5	5.4 → 4.1 5.4 → 4.6	22 → 30 7 → 3.7	16.8	140.8 100	8.4 5.92
	- (anaerobic control)	4	5.3 → 5.4 5.3 → 4.4	2 → -4 7.2 → 3.6	58.8	152.8 190	2.6 3.6
	+ (aerobic control) (anaerobic control)	6.5	5.3 → 5.0 5.3 → 4.6 5.3 → 5.0	27 → 16 21 → 12 6.4 → 4.6	17.8	128.8 1953.6 75.2	7.2 8.6 4.2
Torula utilis	+ (aerobic control) (anaerobic control)	6	5.3 → 5.0 5.3 → 4.6 5.3 → 5.1	30.5 → 18.5 19.9 → 15.7 7.7 → 4.8	14.4	53.6 75.6 26.9	3.8 5.2 1.9
	- (aerobic control)	5.5	5.3 → 5.6 5.3 → 4.7	-2 → 1 11.6 → 4	10.4	30.8 44.0	3 4.2

Sacch. cere-visiae, strain VII	+ (aerobic control)	6.5	5.3	5.3	20	30	5.2	46.4	8.7
			5.3	4.9	23			58.6	11.3
	(anaerobic control)		5.3	5.7	4.5			33.8	6.2

+ = anode in the culture receptacle (O_2 supply);

- = cathode in the culture receptacle (H_2 supply).

Yeasts, therefore, reacted to an increase in the eH by means of electrolysis in the same way as to atmospheric oxygen.

Yeasts taken from the experiment receptacles showed a normal appearance under the microscope. All the cells formed spore groups, while this was the case with only about half the cells under anaerobic conditions. Dead cells, that were identified by staining with diluted methylene blue solution, were present in a much smaller number than in the anaerobic control.

The obligate aerobic chlorophyll-free alga Prototheca reacted to the oxygen supplied by electrolysis in a medium with glycerin (Table 48):

TABLE 48

Cell yield of Prototheca after 24 hours at various rH_2 values produced electrolytically.

experiment conditions	pH	rH_2	no. of cells (mill. per ml) at beginning of experiment	at end of experiment	remark
+	3	22-30	0.67	1.64	under anaerobic conditions 1.04
+					
anaerobic	5	27	1.49	2.92	
control	7.5	10		2.12	
+					
anaerobic	6.5	32	0.71	2.12	
control	7.5	13		0.96	

+ = anode in the culture receptacle (O_2 supply)

The redox potential of a medium in which microorganisms grow can, therefore, be raised electrolytically, in which case aerobes react to the eH increase with increased multiplication. It is important to trace the hydrogen ion concentration and to prevent any excessive acidification. Conditions for the growth of anaerobic bacteria can be created by enriching the medium with hydrogen with the same method. Hanke and Katz (1943) used this procedure for cultivating anaerobic microorganisms. Bact. vulgatus and Clostr. sporogenes grew in mediums supplied with air when a low eH was maintained by electrolysis (cathode in the culture receptacle).

Since by enriching the medium electrolytically with hydrogen or oxygen an electrolysis of parts of the medium appear and may produce uncontrolled and undesired changes, this method must be used with caution, in which case tensions above 1.5-2 volts on the electrodes should be avoided.

Sadoff (1955, Sadoff and others, 1956) further improved the method and drew attention to its superiority over older methods of supplying air. Pure oxygen and hydrogen obtained electrolytically can also be used for introducing in the culture receptacle.

Two nickel electrodes are inserted in a U-tube with a diameter of 3 cm. The size of the cathode is 5 X 2 cm; the size of the anode, 10 X 2 cm. The u-tube is filled with a strong wash. A 12-volt direct current is supplied by a rectifier. The oxygen and hydrogen given off can be immediately introduced in the culture receptacles.

VI. Summary.

Aerobes can grow within a broad pO_2 range from O_2 pressures of more than one atmosphere down to minimal values that are measured in millimeters or fractions of millimeters. Dissolved oxygen is still sufficient in a concentration of 10^{-8} mol. However, there is a definite, narrow range of the optimum oxygen concentration that is under the oxygen partial pressure of air. This range shifts depending on the composition of the medium. Redox potential measurements showed that aerobes also favor reductive conditions. Various species of bacteria probably differ from each other considerably in this respect.

Aerobes require oxygen on the one hand and a low redox potential on the other. By this means the characteristic growth of aerobes in the form of a fine surface film and within the medium also becomes clear. With this kind of growth most of the cells develop under a somewhat lower oxygen partial pressure in comparison with air and at the same time come in contact with the medium in which the redox potential is lowered due to the life activity.

Numerous observations on the rH_2 changes in cultures of various aerobes indicate that strongly reductive conditions are produced in the medium. This is to be attributed to the absorption of the dissolved oxygen and to the elimination of unknown metabolic products with reductive characteristics.

The rH_2 limits in which the aerobes multiply can be excluded from the course of the redox potential and of the multiplication of the cells in cultures of aerobes. The result was that all species studied change the redox potential in the medium strongly and are able to grow within a broad rH_2 range. The rH_2 of the medium obviously has no critical significance. The question of what relationships exist between intracellular redox conditions and the redox potential of the medium remains to be clarified. It may be assumed that the external conditions have only a slight effect when they do not shift the intracellular redox conditions. Obviously the cells have a buffering mechanism available that protects the protoplasts from detrimental rH_2 changes. Only when the buffering capability is no longer sufficient do oxidation substances infiltrating into the cell raise the intracellular rH_2 and prevent further growth.

Facultative aerobic organisms are still less sensitive to the redox potential in the medium than obligate aerobes.

In cultivating aerobic microorganisms it is necessary to provide a good supply of air to the culture. This can be accomplished by means of agitating and stirring the medium constantly, by means of passing air through it or by means of electrolytic saturation of the medium with oxygen. In practice it is scarcely possible to arrive at an optimum supply of air, because the moderate solubility of oxygen in aqueous mediums excludes the formation of too strongly oxidizing conditions.

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CHAPTER 7

REDOX POTENTIAL AND METABOLISM (BIBLIOGRAPHICAL SURVEY)

I. Influence of the Nature of the Metabolism on the Redox Potential.

The first observations on the redox potential in cultures of microorganisms already showed that there frequently is a connection between the nature of the eH curve during growth of the culture and certain metabolic processes.

Plotz and Gelosc (1930, cf. Chap. 5) found that in cultures of various anaerobic bacteria one and the same final potential is produced. They suspected that this potential is characteristic of a definite type of sugar decomposition.

We find detailed data on metabolic reactions and eH in the medium in studies by Kluyver and his collaborators. Elema, Kluyver and von Dalfsen (1934) cultivated Micrococ. denitrificans on a medium simply composed of nitrates, lcohol and mineral salts. Under anaerobic conditions an easily reproducible potential formed on the electrodes. It remains at the same level as long as nitrites are present in the medium. If the nitrite content in the medium is exhausted due to further reduction, the potential goes down. From this, the authors concluded that the cells give off substances in the medium that represent a reversible redox system and form the redox potential on the electrode.

Nitrites as such are not able to produce the potential that occurs in the medium with their presence. Its formation is obviously connected with a process in the course of which they are produced.

If cyanide is added to the medium, the course of the denitrification process is altered: The hypothetical stage of hydrogenation of the hyponitrite is omitted, which affects the size of the redox potential.

Therefore, it was first established by Kluyver and his associates that the size of the potential in the medium has a relationship to the metabolic reactions of the microorganisms.

Kluyver and Hoogerheide (1934, 1936) studied yeasts. As is well-known, it is sufficient to raise the O_2 partial pressure in order to attain a metabolic change in yeasts: They pass over from fermentation to respiration (Pasteur effect). ([Note:] Kostytschev (1919) found that yeasts also do not completely produce fermentation with aeration, but rather they breathe out 1/3 of the required sugar and ferment 2/3. The continuation of fermentation is probably exclusively the result of a zymase excess and is without any particular advantage to the yeasts, because the respiration energy is

sufficient. The same behavior was observed in the alcoholic fermentation of species of Mucor. According to Meyerhof (1925), Torula and other wild yeasts cause a strong fermentation under anaerobic conditions, while they pass over completely to respiration with an air supply as the most economical way to utilize the sugar. The fermentation function is strongly developed and the respiration power diminishes due to cultivation methods and selection. Aeration only partially prevents fermentation. Thus only one molecule of sugar is breathed out by brewer's yeast in the presence of oxygen, while 3 to 4 are fermented.) A study on whether and how the change-over acts on the potential was made in a Warburg apparatus in which the eH, in addition to the gas exchange, was determined with the aid of built-in gold electrodes.

None of the yeasts with a different fermentation process that were studied was able to breathe in a nitrogen atmosphere; they were forced to change over to fermentation. In this case the eH in the suspension of all the yeasts amounted to 80-100 mv. In air a fermentation resulted only in the amount that was characteristic of each species of yeast. With Sacch. cerevisiae respiration is the condition. With air, fermentation equals 0.18. The eH in yeast suspensions with 3% glucose under aerobic conditions amounts approximately to +160 mv. With Sacch. marxianus respiration is the condition: fermentation with an air supply equals 5. The eH amounts approximately to +240 mv. The corresponding values for Torula candida are 7.5 and +280 mv and with Cryptococcus dermatidis, 11.5 and +300 mv. In a lactate solution, that is in the presence of a non-fermentable substrate, only Sacch. cerevisiae can breathe, in which case the eH amounts to +320 mv. The same is true with other non-fermentable substrates (alcohol, acetate).

Atmospheric oxygen also acts on the electrode, but the effect of the specific metabolic reaction is stronger. ([Note:] Polished gold electrodes are excessively insensitive to dissolved gases like O_2 and H_2.) The effect of oxygen on the electrode depends on the density of the suspension being studied; the denser the suspension, the faster the eH drops. In all the above-performed experiments a standard suspension of 400 mg of fresh yeast per 100 ml of phosphate buffer (pH 5.4) was used.

Kluyver supposes that a certain potential characterizes simultaneously a certain metabolic type. He assumes that the formation of a potential on the electrode, inserted in the microbe cultures, depends on unknown redox systems that are given off by the cells. The relationship between the reduced and the oxidized form of these systems depends on the metabolism. Yeast cells apparently give off a series of different redox systems. However, only those that are partially reduced can act on the electrode. Completely oxidized or reduced systems cannot cause the formation of a reproducible potential on the electrode. If there are no partially reduced systems, the addition of an indicator in their place can determine the build-up of the potential. While Kluyver found an eH of +60 mv in the fermentation of yeasts, Fromageot and Desnuelle (1935) obtained a value of -40 mv. By adding Nile blue, which is already effective in a 0.0001% concentration, Kluyver also obtained an eH value of -40 mv (rH_2 9.5). ([Note:] Fromageot and Post, 1937, performed electrometric eH measure-

ments in suspensions of Sacch. cerevisiae. They started with values that other writers had obtained by determining the redox potential with alcoholic fermentation. By this means they could not find, however, a stable rH_2 with different amounts of inoculum and pH values at the start. The lowest rH_2 of 4.2 was observed in the presence of indigo trisulfonate; the highest rH_2 of 12.6, by adding methylene blue).

According to the anticipated eH values, Kluyver added mixtures of indicators to the mediums: for low eH values, a universal indicator with Nile blue, brilliant alizarin blue, Janus green, phenosafranine and neutral red in concentrations of 0.001%; for higher values, a mixture of gallocyanin, thionine, brilliant cresyl blue and methylene blue.

The indicators were verified in the example of lactic acid fermentation. Lactic acid bacteria form different and frequently poorly formed potentials in the rH_2 range of 5-12, due to lactic acid fermentation. By adding the universal indicator the rH_2 was precisely in the range of 5.0 to 5.8 in all cultures.

Cosic (1936) attempted to apply Kluiver's method to acetic acid fermentation, but achieved no unequivocal results. Janke (1937) concerned himself with the same problem, and rightly indicated that the metabolism of bacteria must not be judged alone according to the gas exchange. He studied the eH in a suspension of Acetob. ascendens (designation not recognized by Bergey or Krassilnikov). Ethanol or acetaldehyde were used as a substrate, which was enriched by oxidation of ethanol as intermediary product according to air supply conditions in greater or lesser amounts. The eH was about 100 mv higher with oxidation of acetaldehyde than with ethanol.

The denser the suspension, the more acetaldehyde was formed. Consequently, its oxidation is slowed, the alcohol is oxidized and the eH goes down. In less dense suspensions acetaldehyde is not stored up, but is oxidized, and a higher potential results, as is characteristic for the oxidation of acetaldehyde.

When acetaldehyde is added to the medium, the eH does not change. This can be considered an indication that acetaldehyde does not act directly on the electrode; the oxidation process of the acetaldehyde proceeding from the bacteria is much more effective. Janke's data coincide with Kluyver's concepts on a biocatalyser that is given off into the medium by various metabolic reactions and causes the formation of the potential.

Moreover, according to Wurmser (1926) there is a connection between metabolism and eH in the medium. He studied the energy relationships between redox potential and the course of the metabolism and computed the energy released by oxidation of glucose under various conditions of air supply, that is at various rH_2 values. The computation was based on the usual atmospheric conditions, that is pH_2O= 0.019 and pCO_2 = 0.0003. The oxygen partial pressure was changed from the natural atmospheric value ($pO_2 = 10^{-0.67}$) to a hydrogen atmosphere ($pO_2 = 10^{-35.2}$). In the presence of air the energy output

in the oxidation of glucose amounts to 681 Cal. (Fig. 1). When a reaction that is associated with oxidation due to oxygen occurs with a lowered rH_2, invariably found in living organisms, the energy output becomes smaller than with free access to atmospheric oxygen. With a decrease in the rH_2 value the reaction is replaced by another one that cannot exist under normal aerobic conditions in addition to the first, oxidative reaction.

Based on these considerations Wurmser computed the redox potential of plant cells. He started with the fact that the cells can form alanine from the products of glucose decomposition, but on the other hand they also completely oxidize the glucose:

$$1/6C_6H_{12}O_6 + 2CH_3COCOOH + 2\ NH_3 = 2CH_3CHNH_2COOH + CO_2 + H_2O.$$

Obviously alanine is formed when the rH_2 has gone down so far that the oxidation of 1/6 molecule of glucose yields no more energy than its oxidation at the expense of α-ketopropionic acid. Alanine is formed from it, for which purpose 58 cal. are necessary; consequently, at the proper rH_2 the oxidation of 1/6 molecule of glucose must have yielded 58 cal. and 348 cal. from a whole molecule. According to Wurmser (see Fig. 1) an rH_2 value of 22 corresponds to this energy output. In the cells in which alanine is formed, the rH_2, therefore, should not exceed the value of 22. Intracellular rH_2 measurements gave in fact approximately this value.

Promageot and Desnuelle (1935) determined the eH colorimetrically in yeast suspensions. Under anaerobic conditions in a buffer solution of pH 6.4 the eH fluctuated between -145 mv to -185 mv and the rH_2 between 6.6 and 8.0.

If the concentration of alanine and α-ketopropionic acid in the culture is taken into account, then this redox potential suffices completely from the thermodynamic point of view for the reduction of α-ketopropionic acid to alanine in the presence of NH_3.

Without doubt there is a connection between the metabolic reactions in growing microorganisms and the rH_2 value in the culture medium. The size of the redox potential depends on the nature of the metabolic reactions.

On the other hand, metabolism is also dependent on the redox potential. According to Wurmser, certain reactions are possible only under specific redox conditions. The redox potential is not only an effect but also a determining factor in various metabolic processes.

II. Redox Potential and Ferments.

It is a fact that the formation and function of various ferments depend on the redox conditions. Slovsov (1916) found that more lipases and fewer protinases are formed in anaerobic cultures of staphylococci than in aerobic cultures. According to Salitor and Pyatova (1937) the production of catalase is reduced by cultivation in a high layer. In cultures of Bac. macerans more than double the

amount of amylase was formed with aeration (Daniels and Stahly, 1946).

The activity of proteolytic enzymes is inhibited, according to experiments with papain, by oxidation substances; it is increased by reduction substances (Bersin and Logemann, 1933). The same relationship has also been found in other protinases (Greenberg and Winnich, 1940).

Reiss (1935, 1936, 1938, 1939) studied the dependency of fermentation activity on the eH value. Papain contains an SH group (Pa-SHO that is inactivated by oxidation substances (Pa-S-S-Pa) and is reactivated by reduction substances. Experiments with various oxidation and reduction substances showed that proteolysis measured by means of formol titration is absent at rH_2 23.4 (eH +400 mv, pH 4.8), rH_2 23.6 (eH +365 mv, pH 5.5), rH_2 23.3 (eH +310 mv, pH 6.3) and rH_2 22.8 (eH +268 mv, pH 6.8). It took place unimpeded below these rH_2 values.

Gelatin proteolysate, obtained by means of papain at $rH_2 < 23$, shows characteristics of a synthesizing activity at a high rH_2 value. If bichromate, potassium persulfate, sodium perborate or H_2O_2 is added to the proteolysate at 37° C., a decrease in the formol nitrogen (Fig. 56) and a condensation of amino acids (polypeptide) takes place. This process occurs at an eH of +410 mv to +450 mv and is not changed until an eH of +570 mv. It is stopped only at eH $>$ 570 mv. Up to 15% of free amino acids can be condensed. The eH values of +450 mv to +570 mv correspond to an rH_2 of 28.5 to 33.3.

The autolysis of spleen tissue was studied in a similar manner. The optimum eH was about +200 mv at pH 3.8 and about +100 mv at pH 4.9 (fig. 57). Above and below those two eH values autolysis was weaker, especially at the higher eH. At an eH of +600 mv no more autolysis appeared.

Barley protinases followed the same general rule. Weak proteolysis was detectable at eH +328 mv and pH 6, eH +226 mv and pH 7 and eH +176 mv and pH 8 (corresponding to rH_2 23.22 and 22, as computed by us).

In silkworms the optimum of the protinases, in comparison with gelatin, was at pH 8.0-8.5 (Reiss and Achard, 1943; Achard and Reiss, 1943). Gelatin hydrolysis was studied in a range of eH -550 mv to +330 mv. The optimum eH was between -250 mv and -300 mv (rH_2 7). At $rH_2 > 26$ albumin systhesis could be detected. rH_2 values from 16 to 25 were established in living caterpillars, pupae and butterflies. The redox potential was, therefore, in quite another range than is assumed for the optimum occurrence of synthesis and hydrolysis.

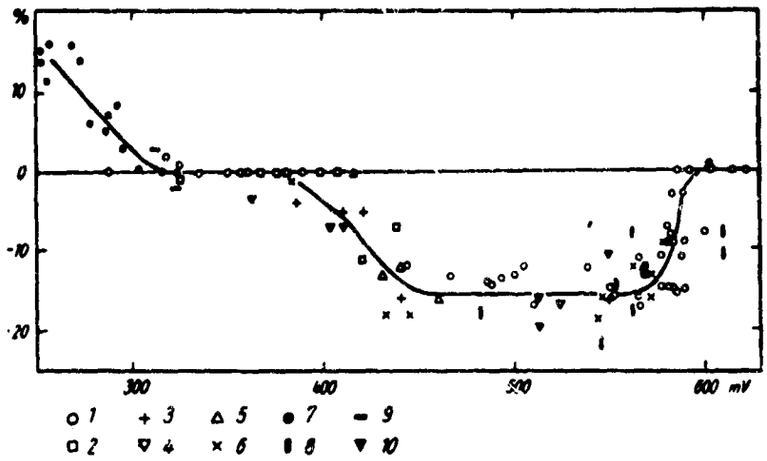

Figure 56. The dependency of the amount of detectable nitrogen in formol titration on the eH in gelatin proteolysis by means of papain (pH 4.9) (according to Reiss).

1 = H_2O_2; 2 - ferricyanide;

3 = iodate; 4 = perborate;

5 = bichromate; 6 = persulfate + bichromate;

7 = no additive; 8 = H_2O_2 without glycerin;

9 = no additive, no glycerin; 10 = bichromate without glycerin.

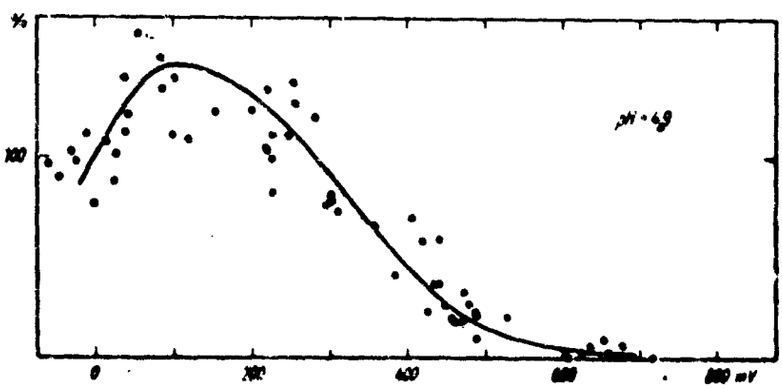

Figure 57. Autolysis of rat spleen at pH 4.9 depending on eH (according to Reiss).

In experiments with highly purified urease (Sizer and Tytell, 1941) the optimum reaction, measured by the formation of CO_2 at pH 6.8, was between eH +100 mv and +200 mv. The same result was obtained with a 20% to 70% increased activity by adding oxidation and reduction substances to the medium ($KMnO_4$, 0.0001-0.000,006 mol; H_2S, 0.05-0.0025 mol; and a mixture of $Na_2S_2O_4$ and $K_4[Fe(CN)_6]$, 0.005 mol). By using various substances they obtained a scale of eH values between -257 mv and +539 mv (Table 49). In these experiments also the eH proved to be just as important for the occurrence of enzymatic reactions as the pH. Surely here also there is an action on the sulfhydryl group.

TABLE 49

eH values by adding oxidation and reduction substances to the medium (according to Sizer and Tytell, 1941).

substance	concentration in mol	eH in mv
$K_3[Fe(CN)_6]$	0.005	539
$K_3[Fe(CN)_6] + K_4[Fe(CN)_6]$	0.0025	443
$K_4[Fe(CN)_6]$	0.005	335
$Na_2S_2O_3$	0.005	224
KCNS	0.005	205
KCN	0.005	194
thiourea	0.005	168
thioglycolic acid	0.005	61
cysteine	0.005	5
H_2S	0.025	47
H_2S	0.125	8
H_2S	0.25	-20
Na_2S	0.005	-24
Na_2S	0.01	-82
$Na_2S_2O_4$	0.00125	-89
$Na_2S_2O_4$	0.0025	-160
$Na_2S_2O_4$	0.005	-206
$Na_2S_2O_4$	0.01	-257

When unpurified urease was used the eH had a considerably weaker effect on the activity. Apparently the ferment was protected from the action of unfavorable eH values by the by-products.

Hellerman, Perkin and Clark (1933) pointed out earlier that crystalline urease is inactivated by oxidation substances (for example, iodine, or by aeration in the presence of copper as a catalyzer). A subsequent addition of sulfides or thioglycolic acid reactivated the ferment. Yeast invertin did not modify its activity at eH values from -270 mv to +600 mv. It is inactivated due to strong oxidation substances only at eH values over +600 mv (Sizer, 1942). Phosphatases from beef lungs and beef liver, which are effective both in an alkaline and in an acid environment, also did not modify their activity at eH values from -500 mv to +350 mv. They were inactivated only at eH +650 mv. The process was reversible here also with the addition of reduction substances. Apparently the inactivation is connected with an oxidation of amino acids of the ferment, probably of the tyrosine. According to experiments by Fiegenbaum (1942) fungus sucrase is inactivated by H_2O_2 and $Na_2S_2O_4$. Maltase and yeast sucrase are inhibited or completely inactivated by H_2O_2 but are stimulated by $Na_2S_2O_4$.

According to Lipmann (1933) the inhibition of glycolysis by saturating with oxygen or by adding oxidation substances is based on a reversible oxidative inactivation of the ferments concerned. In the presence of air dichlorophenol-indophenol forms an eH of +100 mv which stops the glycolysis. According to Lebedev, fermentation takes place at pH 6.6 and eH +60 mv to +80 mv in a yeast maceration broth. If the eH is adjusted to a higher level of +160 mv by means of naphtholsulfonate-indophenol with an air supply, the fermentation ceases. If thionine is added to the maceration broth, a lower potential is formed, at which fermentation again takes place.

Boyland (1930) studied the activity of a zymase preparation with the addition of various rH_2 indicators (Table 50).

TABLE 50

eH and CO_2 discharge (pH 6) by action of zymase on glucose (according to Boyland).

Added rH_2 indicator	eH during the reaction	CO_2 in mm^3, discharged within 20 min.
indigodisulfonate	-30	0.16
indigotrisulfonate	-20	0.10
indigotetrasulfonate	-10	0.52
methylene blue	10	0.47
thionine	30	0.40
naphtholindo-2,6-dibrom-phenol-2-sulfonate	140	0.18
naphtholindophenol-2-sulfonate	130	0.10

leukoindigodisulfonate	-100	0.08
leukomethylene blue	0	0.42
leukonaphtholindophenol-2-sulfonate	120	0.12

The optimum eH for zymase action was within the range of -10 mv to +30 mv. This optimum occurred with rH_2 indicators both in an oxidized and in a reduced form.

In Lipmann's (1934) experiments the optimum was approximately in the same range.

The research conducted by Engelhardt and Sakov (1943) and Engelhardt (1944) contributed substantially to an understanding of the Pasteur effect. They studied individual partial reactions out of the chain of reactions that lead to the formation of alcohol and CO_2 from sugar.

Hexosediphosphate as starting substance is not oxidized but only fermented. Therefore, a Pasteur effect is not possible here. Only two reactions take place up to the formation of hexosediphosphate: The isomerization of glucose monosulfate to the fructose derivitive and its secondary phosphorylation. In Engelhardt's experiments the isomerization was insensitive to oxidation and reduction substances, but not the esterification reaction. An inhibition of the corresponding ferment, of the phosphorylase, was observed in an eH range of +50 mv to +200 mv, independently of the oxidation substances used (rH_2 indicators, iodine, ferricyanide, quinone, oxidized ascorbic acid, cytochrome + cytochrome oxidase). The inhibition of the phosphorylase was reversible. Consequently the formation of the diphosphoric ester during the course of the reaction is the part in which the Pasteur effect is effective.

Ferments that catalyze vital metabolic processes are also inactivated at a definite rH_2 value in anaerobes. Anaerobes probably cannot exist above a certain eH value for this reason. Engelhardt assumes that obligate anaerobes cause the formation of essential intermediary products of metabolism in the presence of oxygen. They have lost the capability of oxidative metabolism; the capability of inhibition of anaerobic decomposition is preserved, however. The inhibiting mechanism that lies in the Pasteur effect cannot, just as with yeasts, be replaced by oxidative decomposition.

According to Aubel and Perdigon (1940) cell suspensions of Clostr. butylicum form only ethanol and acetic acid from carbohydrates under aerobic conditions; under anaerobic conditions butyric acid also appears. The enzymes on which the condensation of C_2 to C_4 compounds depends are inactivated or destroyed by aeration.

A suspension of washed cells of Clostr. butylicum in a glucose solution, according to Aubel and Houget (1939), takes up oxygen that probably is combined with intermediary metabolic products and prevents the syntheses required for growth.

In this way Engelhardt's opinion is confirmed: Anaerobes die off when a certain rH_2 value is exceeded, because vitally necessary ferments are disturbed in their function. As recent studies show, the Pasteur effect operates also in other fermentation organisms.

Baskett and Hinshalwood (1951, a and b) found that Bact. aerogenes is able to growth both as an aerobe and as an anaerobe with numerous carbon compounds (glucose, arabinose, citrate, α-ketopropionic acid, glycerin and inositol). Acids are formed under anaerobic conditions. If the bacteria are removed from aerobic conditions and put under anaerobic conditions, life activity stops at first, but then starts up again. Obviously existence under anaerobic conditions requires the development of a new ferment mechanism. Multiplication occurs more slowly with an anaerobic manner of living. If the bacteria are brought from anaerobic to aerobic conditions, no disturbance results: oxygen automatically suspends the fermentative zymosis system and starts the oxidative system that already was previously present in the cells functioning again.

By changing the redox conditions it is possible to interfere experimentally with the course of metabolic processes.

III. Changes in Metabolism by Means of Control of the Redox Conditions.

Already long before the notion of redox conditions was current in biology the fact was known that metabolic processes in microorganisms could be altered by means of varying the air supply. Although there was no determination of the redox potential in older studies, changing the air supply is really one of the simplest methods of affecting the redox conditions.

Pasteur already pointed out the regulating role of aerobiosis conditions. He found, in his studies on the production of beer (Pasteur Vallery-Radot, 1928), that yeasts are not different from the higher plants and that their fermentation capability only comes to light when they are compelled to live under special circumstances.

Fermentation occurs in high layer with an oxygen deficiency. On the other hand aeration of the medium is necessary for the yeasts to multiply. Under these conditions the yeasts utilize sugar more economically for developing their own body (Pasteur effect).

Observations on the metabolism of moulds under aerobic and anaerobic conditions are found in studies by Kostytschev and his collaborators (Kostytschev and Afanasyeva, 1917; Kostytschev, 1921-23; Kostytschev and Afanasyeva, 1921-23).

Moulds, which are aerobes, tolerate anaerobiosis for a short time. In this case they go over from oxidative metabolism to fermentation. Kostytschev cultivated mould layers under aerobic conditions, then immersed them in a culture solution and replaced the air in the flask with hydrogen. Asp. niger and Pen. glaucum under these conditions fermented sugar to equimolecular amounts of alcohol

and CO_2. The moulds lived only a short time under anaerobic conditions. The fermentation lasted 24-48 hours, although not very intensively. After three days the mycelium died off and succumbed to autolysis. The amount of alcohol produced amounted to 250-525 mg in a 5% sugar solution. Alcohol was also produced from a series of other nutrients; of course, a prerequisite was the addition of chalk to the medium. Although the fungi tolerate an acid reaction well under aerobic conditions, they ferment the substrate under anaerobic conditions only when neutralized with chalk.

Kostytschev found the formation of alcohol under anaerobic conditions with glycerin, mannitol, tartaric acid, lactic acid and quinic acid. In some cases reducing substances could be detected in the medium. From this Kostytschev concluded that the above-mentioned substances are converted into sugar before fermentation.

Alcoholic fermentation was not a normal process for the fungi that were examined. The fermentable substances were consumed in greater amounts than corresponded to the amount of the two fermentation products. Therefore, still other products not taken into account by the authors must have been produced.

Intensified aeration of fluid mediums causes certain alterations in the biochemical activity of yeasts. Kostytschev and Faermann (1928) found that yeasts ferment substances not affected by usual cultivation, when air is introduced into the culture. They observed the formation of CO_2 and alcohol frommannitol and glycerin, although in small amounts. With a 15 g amount of yeast in 16 ml of water only 131 mg of CO_2 and 126 mg of alcohol were produced. Probably a reserve substance (glycogen) was formed from annitol and glycerin and was then fermented.

In order to explain the mechanism of fermentation, Neuberg and his collaborators (1928) changed the normal course of the reaction in various ways, among others by creating aerobic and anaerobic conditions.

Neuberg conducted his research on a biochemical basis. He did not work with growing cultures but rather used dense cell suspensions in which multiplication was prevented due to a nutrient deficiency. Here also products were formed that did not appear under normal conditions.

Simon (1930) attempted to adapt acetic acid bacteria to anaerobic conditions (Table 51) and came to the conclusion that acetic acid bacteria have the zymase complex. In sugar-free controls neither alcohol nor CO_2 was produced.

TABLE 51

Production of alcohol and CO_2 in suspensions of acetic acid bacteria (according to Simon, 1930.)

	medium	Cell amount in g (net weight)	alcohol g per 100 ml	CO_2 in ml per increment
Acetob. suboxydans in nitrogen atmosphere	1.8 g glucose + 150 ml water	2.5	0.1150	11.5
	40.9 g glucose + 230 ml water	5.4	0.1978	7.0
Acetob. suboxydans in air	1.8 g glucose + 150 ml water	2.65	0.1242	7.5

Rabotnova was unable to establish a production of alcohol, on the other hand, when she cultivated bacterial layers of Acetob. xylinum under anaerobic conditions on a high-quality culture medium (grape-juice with 1% glucose).

Haehn and Engel (1929) attempted to establish a lactic acid fermentation with Acetob. xylinum in accordance with the concepts of Neuberg and Simon (1928) and Simon (1930). Although they themselves allege that under anaerobic conditions neither growth nor lactic acid formation can be established, they did attach positive importance to the small amounts of lactic acid that result after the transfer of the completed layers into anaerobic conditions. However, it must rather have been a question of a postmortal autolysis than of a fermentation.

Anhagen and Neuberg (1933) and Anhagen and Anhagen (1934) changed the alcoholic fermentation of yeast into a lactic acid fermentation by adding glutathione. However, the conditions do not correspond in any way to the conditions of the normal life activity. A magnesium hexosediphosphate was used as substrate, and the yeasts used were plasmolyzed with toluene. Here also the amount of lactic acid was very small in comparison with the amount of yeast consumed. Ten to 100 milligrams of lactic acid corresponded to one gram of press-yeast. Although in some cases pure yeast cultures were used, in most of the experiments the authors used surface-fermented and below-fermented brewer's yeasts.

With Esch. coli a more intense formation of lactic acid was tied in with the addition of glutathione as a reduction substance (Cattaneo and Neuberg, 1934). Nevertheless, the experiments of Neuberg and his collaborators showed that microorganisms can change the operation of their ferment array depending on the external conditions.

Although Neuberg does not go into the redox potential, it is the active factor in his experiments, since the creation of anaerobic conditions in the experiments with acetic acid bacteria and the addition of glutathione in his research on yeasts and Esch. coli cause a lowering of the redox potential. Under the given experiment conditions it is not possible, however, to conclude with certainty that the bacteria studied change the nature of their metabolism. By using large amounts of cells, as Neuberg used, it is possible that the substances found had been formed from the cell substance itself. Moreover, the possibility exists that processes take place in dense suspensions without nutrient that can be considered as physiological artifacts.

Neuberg was able to establish a change in fermentation with multiplying, normal cells; namely, in the experiments on alcoholic fermentation in an alkaline environment and in the course of alcoholic fermentation in the presence of bisulfite.

In a neutral to weakly alkaline reaction, acetic acid appears among the products, a substance that is not characteristic of alcoholic fermentation. Simultaneously more glycerin is also formed as a by-product.

Neuberg's method of raising the glycerin output in alcoholic fermentation by adding bisulfite has attained industrial importance. We are not dealing here with damaged cells that change their metabolic processes under unnatural conditions, but rather with normally growing cultures. However, the fermentation takes another course due to the action of the bisulfite. Up to 20%-30% of the fermentable sugar is converted into glycerin. During the first world war great amounts of glycerin were produced in Germany in this way in accordance with a patent of Konnstein and Luedecke.

The directed influence of metabolic processes is a current problem in modern microbiology. The redox conditions, which are measurable quantitatively as redox potential, are of great importance in this connection. Under the direction of Uspenski various researchers concerned themselves with experiments on the metabolism of microorganisms and the changes caused by rH_2 shifts. Kusnetzov (1932) worked with Asp. niger whose metabolism had already been frequently studied. Neither citric acid nor oxalic acid is formed in the medium at $rH_2 > 17$. The sugar was probably completely oxidized to CO_2 and H_2O. When the fungus layer was put in a nitrogen atmosphere with an rH_2 value of 12.8-14, the formation of ethanol occurred in the cultures. When the growing mycelium was transferred to a hydrogen atmosphere, the potential in the cultures dropped to rH_2 2 and the formation of citric acid stopped. ([Note:] Oudlet, 1936, studied the rH_2 changes in cultures of Asp. niger strains that produced different yields of citric acid. He found citric acid formation with active strains, although in all cultures an rH_2 value of 9-12 was measured under the fungus layer. Oudlet concluded from this that the formation of citric acid is not connected with the redox potential of the medium. However, he did not take into account the fact that the mycelium is subject to far stronger oxidative conditions on the surface th[illegible]arged in the medium and that precisely this part of the my[illegible]as citric acid. Inactive strains do not

form any acid, which cannot, however, be associated with an unfavorable rH_2 value.)

Kanel (1935) conducted similar research on Rhizopus nigricans. The fungus formed only lactic acid and alcohol at rH_2 0-8, but no fumaric acid. A mixture of lactic acid and fumaric acid was produced at $rH_2 > 8$. Another Rhizopus strain formed lactic acid under anaerobic and aerobic conditions ($rH_2 = 30$ with aeration.)

Krasina (1936) studied the decomposition of formic acid by bacteria. Formic acid fermenting bacteria were first described by Omelyanskiy (1953). He cultivated them under aerobic conditions and found a decomposition of formic acid to carbonic acid and hydrogen:

$$Ca(HCOO)_2 + H_2O \rightarrow CaCO_3 + CO_2 + 2H_2$$

Stephenson and Stickland (1933) isolated bacteria that decomposed formic acid to carbonic acid and methane under anaerobic conditions in a nitrogen or hydrogen atmosphere:

$$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$$

Krasina supposed that both other courses of the processes are determined by the size of the redox potential. A strain of bacteria isolated from slime was subject of research. The redox potential in the medium dropped gradually from rH_2 22 to rH_2 17-19 by cultivating in a medium with 0.1% peptone and 2% $Ca(HCOO)_2$ under aerobic conditions. $CaCO_3$ was deposited simultaneously on the sides of the receptacle. Gas was not formed. The decomposition of the formic acid occurred with carbonic acid formation:

$$Ca(HCOO)_2 + O_2 \rightarrow Ca(HCO_3)_2$$

If the rH_2 value drops to 14, gas formation also appears; that is, the decomposition of formic acid is changing.

Another series of experiments was started under stronger anaerobic conditions, in accordance with Omelyanskiy's studies. The rH_2 value went down to 11-12. A gas analysis resulted in 76.8% hydrogen and 21.6% CO_2. Under these conditions the formic acid was decomposed in accordance with the formula worked out by Omelyanskiy.

If the experiments were performed according to the method of Stephenson and Stickland in a hydrogen atmosphere, the rH_2 value fell to 6 and lower. In this case methane also appeared as a fermentation product, among others. Its percentage is small with 2.4%-2.7% alongside 8.2%-8.5% CO_2; however, it is evidence of change-over of the bacterial metabolism. The decomposition of formic acid occurs, therefore, in different ways in the same culture at a different rH_2 value.

Korotschkhina (1936) studied the denitrification process in Ps. fluorescens under various redox conditions. She introduced different rH_2 values in the medium by supplying air, nitrogen and

hydrogen and by adding various carbon sources. The following redox potentials were measured in a medium with calcium citrate: with a supply of air, rH_2 31.8-35.2; with nitrogen, rH_2 20-21 and with a supply of hydrogen, rH_2O. The rH_2 dropped to 13-14 in a medium with alcohol. The denitrification process took place, however, approximately uniformly in every experiment.

Consequently, denitrifiers seem to be organisms whose metabolic nature is not altered by rH_2 changes in the external medium. In this connection they recall the behavior of Rhizopus strain x (according to Kanel) that also did not react to rH_2 changes.

However, a statement of the number of cells at various rH_2 values is missing from Korotschkhina's study. The only result was probably the extent to which the bacteria are insensitive to the redox conditions of the medium.

Krebs (1937) described metabolic processes dependent on the air supply in gonococci and staphylococci. α-ketopropionic acid served as substrate. The experiments were performed with washed suspensions. Although the redox potential was not determined quantitatively, the results are worth mentioning.

$$CH_3COCOOH + 1/2O_2 \rightarrow CH_3COOH + CO_2$$

Under anaerobic conditions, in an N_2 and CO_2 atmosphere, on the other hand, the following dismutation process occurred:

$$\begin{array}{ccc} CH_3COCOOH & H_2 & CH_3CHOHCOOH \\ + & \| & \longrightarrow \\ CH_3COCOOH & O & CH_3COOH + CO_2 \end{array}$$

The influence of the eH on butylene glycol fermentation was studied further. The fermentation of sugar with the formation of neutral C_4 compounds, acetylmethyl-carbinol and butylene glycol is characteristic of Aerob. polymyxa (designation not recognized by Bergey or Krassilnikov), Aerob. aerogenes, Aerob. cloacae, Bac. subtilis and others. These substances can result from condensation of acetaldehyde.

Stahley and Werkman (1942) studied the influence of acetaldehyde in Aerob. polymyxa (designation not recognized by Bergey or Krassilnikov) on the output of 2,3-butylene glycol (BG), a more strongly reduced substance ($CH_3CHOHCHOHCH_3$), and acetylmethyl-carbinol (AMC), a more strongly oxidized substance ($CH_3CHOHCOCH_3$). With a supply of air more AMC is formed from glucose; on the other hand, with anaerobic conditions, more BG. eH measurements during the development of the culture yielded the result that even with aeration the eH drops to -300 mv. When a large part of the glucose has been fermented, the eH rises again quickly. The eH change shows up also in the formation of the fermentation products. In the first period, at a low eH, BG is formed principally and is oxidised later to AMC. The authors concluded that BG and AMC represent a redox

system that reacts reversibly depending on the conditions.

In experiments with Aerob. indologenes (designation according to Krassilnikov) Brewer and Werkman (1940) studied the fermentation of citric acid at pH 6.5 in air and in nitrogen, with a macrorespirometer (Table 52). BG was formed under anaerobic conditions, but no AMC. Concerning organic acids, acetic and succinic acid, that were oxidized with aerobiosis to CO_2 and H_2O, were detected.

Paretsky and Werkman (1947) found the same dependency of AMC and BG formation on the redox conditions in Aerob. aerogenes. They were able to increase the yield of AMC at the expense of BG. Methylene blue seems to buffer the eH in the medium to the level at which the formation of AMC prevails.

Orlova (1950) cultivated Aerob. aerogenes in high and low layers of a medium containing sugar, so that more or less aerobic conditions were given. There was no eH measurement, but the redox conditions were different without doubt. BG was accumulated in the beginning under aerobic conditions. After 40 hours the amount of BG diminished and AMC appeared. BG is oxidized to AMC. Under anaerobic conditions no AMC is formed, but the percentage of BG keeps on increasing (Table 53).

TABLE 52

Fermentation of citric acid by Aerob. indologenes under aerobic conditions. Fermentation products in m mol per 100 m mol of fermented citrate (according to Brewer and Werkman, 1940)

atmosphere	N_2	air
time in days	6.3	10.6
fermented citrate	50	50
H_2	41.6	1.7
CO_2	169.3	363.5
formic acid	0	0.2
acetic acid	158.7	73.1
acetylmethyl-carbinol	0	traces
2,3-butylene glucol	2.0	0.9
ethanol	0.7	0.7
succinic acid	14.1	0
lactic acid	1.1	1.0
absorbed O_2	0	218
fermented C, %*	92.61	86.7
Index O/V	1.01	0.98

* % of citrate-carbon captured in the fermentation products.

TABLE 53

Fermentation products of Aerob. aerogenes under anaerobic and aerobic conditions (according to Orlova, 1950)

experiment conditions	duration of experiment in hrs.	fermented sugar in m mol per l.	fermentation products in m mol per l					no. of bacteria in mill. per ml.
			acetylmethylcarbinol (AMC)	2,3-butylene glycol (BG)	volatile acid	lactic acid	ethanol	
aerobic	133	99.25	12.3	24.3	8.3	43.5*	43.8	237
anaerobic	133	85.76	0	42.37*	16.03	23.7	29.8	122

*after 91 hours.

Neish, Blackwood and Ledingham (1945 a and b) studied a strain of Bac. subtilis that also formed neutral C_4 products. The culture took place with a supply of air and of nitrogen (Table 54). The eH was not measured. Corresponding results were also obtained with Bac. glycolacticum, which is closely connected with Bac. subtilis (Taha, 1955).

TABLE 54

Fermentation products with Bac. subtilis after 9 days under aerobic and anaerobic conditions, in m mol per 100 ml of fermented glucose (according to Neish and others, 1945)

fermentation products	in nitrogen	in oxygen
2,3-butylene glucol	57.38	33.35
acetylmethyl-carbinol	1.66	33.63
glycerin	39.91	3.66
ethanol	12.88	7.38
lactic acid	19.96	1.77
formic acid	5.56	1.03
acetic acid	0	4.99
butyric acid	0.35	1.24
CO_2	122.00	207.58
carbon, combined	11.03	34.00
H_2	0	0
C in %	9[illegible].0	93.0
fermented glucose	100.0	98.9
redox index	1.04	2.24

Bac. subtilis behaves like Aerob. aerogenes (cf. Orlova, 1950). Under aerobic conditions more AMC is formed, whereas with anaerobicsis 2,3-butylene glycol predominates. The output is greater

under aerobic conditions. As for acids, there was no agreement between Bac. subtilis and Aerob. aerogenes.

According to Maksimova (1954) the formation of diacetyl ($CH_3COCOCH_3$), another neutral C_4 product, depends on the redox conditions. This substance is produced by aroma-producing bacteria, for example Streptoc. diacetilactis (designation according to Krassilnikov). These bacteria are used in the manufacture of butter. Diacetyl occurs in cultures that do not lower the eH much during growth. In cultures of Streptoc. diacetilactis the rH_2 never drops below 7, while rH_2 values of 4-5 appear with the ordinary lactic acid bacteria. While with lactic acid bacteria generally the more strongly reduced neutral products BG and AMC appear, with Streptoc. diacetilactis moreover the more strongly oxidized diacetyl is formed. A rise in the rH_2 in the culture due to aeration causes increased diacetyl formation.

In recent years research was conducted in Hungary on the redox potential in cultures of microorganisms that have an industrial application. In this connection a relationship between eH changes in the culture and productivity resulted. Thus, for example, it was established that the increased yield of antibiotics with Streptom. griseus is connected with a specific eH in the culture (Kramli, 1954). Kramli and Szabo (1956) made the same observation with regard to the formation of riboflavin in cultures of Eremothecium Ashbyi and Kramli and Lantos (1956) concerning the formation of ergosterol by yeasts.

According to Kramli the curve of the rH_2 changes in microbian cultures is a characteristic of their physiological state. In the production of antibiotics the presence of a bacteriophage or of a foreign infection is evident from the nature of the rH_2 curve (Kramli, Kovacs, Matcovics, Natonek, Pulay and Turay, 1954). According to Mohelska-Myshikova (1955) the eH is also a characteristic in cultures of acetone-butanol bacteria that reacts in the culture under abnormal conditions. The observation of rH_2 changes during the growth of a culture under production conditions therefore, also has practical significance.

The eH in Staphylococci cultures can be used to differentiate strains, according to Kramli, that are resistant or sensitive to antibiotics. When antibiotics are added to a sensitive strain, the eH in the culture drops less sharply than without antibiotics. In the culture of a resistant Staphylococci strain the eH is the same with and without antibiotics (Kramli, Stur and Turay, 1955).

IV. Summary.

Changing the redox conditions is a means of affecting the metabolic process in microorganisms. To date this field of the physiology of microbes has still not been studied methodically.

Consequently it is necessary to assemble new facts and new observations on the problem of the significance of changes in redox conditions for the various types of life activity.

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CHAPTER 8

DEPENDENCY OF METABOLISM ON THE REDOX CONDITIONS OF THE CULTURE MEDIUM (EXPERIMENTAL RESEARCH)

We studied the effect of redox conditions on the metabolism of microorganisms that have practical importance. The ability resulting from a change in the air supply conditions and from the addition of oxidation and reduction substances to affect metabolic processes was analyzed. The influence of the nutrient conditions, of the composition of the medium, of the pH value and of other factors was disregarded.

Microorganisms that are the concern of soil biology were subjects of research, because their life activity affects the fertility of the soil, and also microorganisms that are important for the fermentation industry.

Without doubt that redox conditions have a great effect on the activity of the microflora of the soil. The air supply of soils may be very different, their structure and moisture content may change, which affects the redox state. The life activity of the microorganisms also has a great effect. When decomposable organic substances reach the soil, the intensified activity of aerobic saprophytes may cause the consumption of the oxygen in the vicinity of these substances and the accumulation of reduced substances. Soil microorganisms therefore, live under constantly changing redox conditions and anaerobes under anaerobic conditions. However, it is necessary to know what happens to anaerobes under aerobic conditions and vice versa with aerobes in the absence of an air supply or at a low eH. This type of research is able to expand our knowledge of the role of the various microbes in the substance cycle in soil formation.

In connection with fermentation organisms we investigated problems current in the field of technical fermentation. Thus, for example, it is important to control the output in the production of solvents, alcohols and other neutral substances. Until now only one method of regulating the glycerin yield in alcoholic fermentation has been worked out. The production of considerable amounts of glycerin was achieved by adding bisulfite. We surmise that further success can be attained in this field. Thus the addition of a reduction substance in certain bacterial fermentations increases the output of reduced products. On the other hand, under certain conditions a rise in the redox potential that can be achieved by an intensified aeration causes oxidized products to be stored.

I. Denitrification and Redox Conditions of the Medium.

Denitrifying bacteria have a negative part in the metabolic balance of soil: They give off NO_3-nitrogen essential for the fertility of the soil as N_2 in the air. Denitrifying microorganisms

are facultative aerobes, that is they live under aerobic and anaerobic conditions. Under aerobic conditions they oxidize the substrate by means of atmospheric oxygen. In culture solutions without nitrate they grow with a film-formation on the surface. In nitrate mediums they grow submerged within the entire volume of fluid even without access to air. The question arises in this connection as to the extent to which denitrification depends on the presence of oxygen.

Fedorov (1949) assumes that by loosening the soil in soil cultivation, denitrification ceases as a result of the intensified aeration. However, this must be doubted.

According to Lloyd and Cranston (1930) denitrification begins only under anaerobic conditions. Nevertheless, a very slight formation of gaseous nitrogen was also observed when the medium consisted of only one layer, that is with an abundant supply of air.

Rusakova and Butkevitch (1941) observed a decline in denitrification due to the action of oxygen in experiments on sea-water denitrifiers. If Ps. denitrificans is cultivated anaerobically and with an N_2-O_2 mixture, it is found, according to experiments that Sacks and Barker (1949) performed with washed cell suspensions, that the activity of the denitrifying enzyme from cells that are cultivated in mediums saturated with oxygen amounts to only 29% of the activity of cells cultivated anaerobically. The reduction of NO_3 to NO_2 proved to be less sensitive. According to the studies made by Sacks and Barker, oxygen acts in a two-fold manner on denitrification: It impedes the formation of nitrate and nitrite reducing ferment systems and decreases the speed of reduction when such systems are present. Therefore, denitrification is generally considered as an anaerobic process whose course is retarded by aeration.

However, contrary opinions have also been advocated. Thus Korotschkina (1936) established in growing cultures of Ps. denitrificans that when air is introduced (rH_2 35) denitrification does not cease. A delayed change of the nitrates into nitrites was merely observed in comparison with experiments in a hydrogen atmosphere. Unfortunately, Korotschkina did not study the multiplication of the cells. Therefore, it is not clear whether the weaker denitrification under aerobic conditions was connected with an intensified multiplication of the cells.

Korsakova (1941) cultivated Achromob. siccum (designation not recognized by Bergey or Krassilnikov) and Ps. aeruginosa under aerobic and anerobic conditions. It turned out that denitrification at the beginning of growth (in the first 24 hours) with equal intensity under aerobic and anaerobic conditions. However, under anaerobic conditions denitrification took place until the nitrates had been consumed, while under aerobic conditions it stopped already before that. Obviously the organic substance was exhausted more quickly under aerobic conditions, because the oxygen in the air was also available for its oxidation. If the amount of organic compounds in the medium is increased five to ten times, denitrification continues longer also under aerobic conditions. Therefore, anaerobic conditions are not a prerequisite for denitrification. Respiration and denitrification can take place simultaneously. Skerman, Lack and Millis (1951) also assume, in

accordance with experiments on a Pseudomonas strain, that oxidation processes can occur side by side with the oxygen of the air and the oxygen of the nitrates, but that the free oxygen is favored. Kluyver (1954) concludes, however, from these experiments that small traces of oxygen already interrupt denitrification.

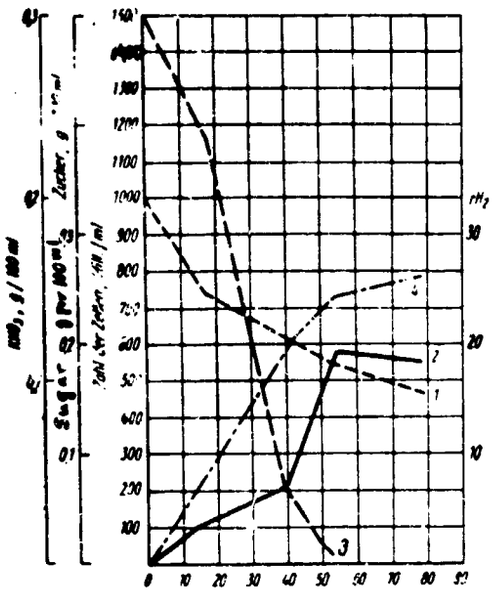
Species of the genus Pseudomonas and Achromobacter, belong to denitrifiers. Results divergent from one another can possibly be explained by the fact that there are differences in the behavior of various species and strains of denitrifiers. Thus according to Pinghui Liu (1952) only 26 of 45 Pseudomonas aeruginosa strains, that agreed with each other with regard to pigment formation, were capable of denitrification. In Meiklejohns's (1949) opinion denitrification is controlled by the redox potential of the medium. Obligate aerobic organisms do not reduce nitrates, although it is possible that denitrification to N_2 may take place at a sufficiently high redox potential. Denitrification to nitrogen occurs with facultative aerobes. Anaerobes denitrify down to NH_3 at a low eH. Therefore, a gradation depending on the eH is assumed. However, other microorganisms appear in each type of denitrification. It should be interesting to trace the denitrification. It should be interesting to trace the denitrification of one and the same strain at different rH_2 values.

Rabotnova and Bobkova studied Ps. aeruginosa (unpublished). This species is especially suitable for this kind of experiments. They are able to utilize numerous different substances as carbon sources (carbohydrates, acids, alcohols, aromatic compounds, hydrocarbons -- Rabotnova, Ulubekova and Magnitskya, 1950, demonstrated that Ps. aeruginosa can utilize bitumen, petroleum and rubber --, peptone and others), which indicates a high adaptability of the ferment systems. With a culture in beef bouillon + 0.3% KNO_3 and in a mineral medium with 0.3% carbohydrates + 0.3% KNO_3 multiplication was possible within a very wide rH_2 range (from rH_2 2 by saturating the medium with hydrogen to rH_2 25-30 by aerating the medium or by saturating it electrolytically with oxygen). Denitrification occurred simultaneously under aerobic and anaerobic conditions. Therefore, with a supply of air the cells also use the atmospheric oxygen in addition to the nitrates for oxidizing the substrate.

Growth of the bacteria under strictly anaerobic conditions on an alkaline pyrogallol solution is restricted due to the denitrification process, that is to say when the nitrates are consumed, multiplication stops (Fig. 58).

The bacteria formed a film at the surface contiguous to the medium under a vaseline oil layer five to six cm thick that indeed made the access of air difficult but did not prevent it completely. Denitrification was detectable at the start of growth of the culture, while the cells multiplied after 60 hours due to the atmospheric oxygen that diffused through the vaseline (Fig. 59).

With an unimpeded supply of air denitrification occurred very rapidly in 500-ml flasks with 250 ml of medium; but they multiplied also after the nitrates had been used up and the organic substance was utilized further (Fig. 60).



hours

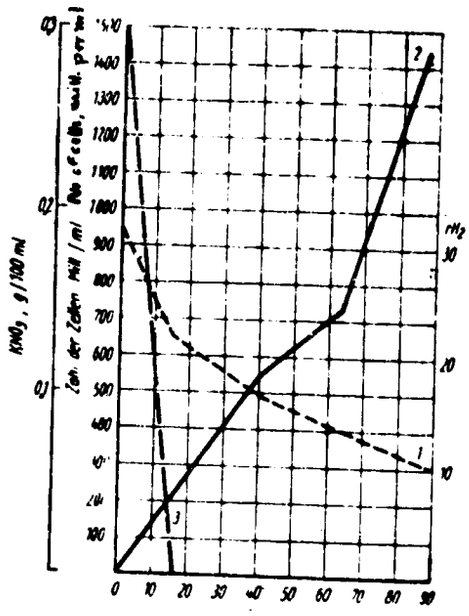
Figure 58. Growth of Ps. aeruginosa under anaerobic conditions in a mineral medium with glucose.

1 = rH_2

2 = number of cells in mill. per ml;

3 = amount of KNO_3 in g per 100 ml;

4 = sugar consumed in g per 100 ml.



hours

Figure 59. Growth of Ps. aeruginosa with difficult access to air under a vaseline oil layer in beef peptone bouillon.

1 = rH_2;

2 = number of cells in mill. per ml;

3 = amount of KNO_3 in g per 100 ml.

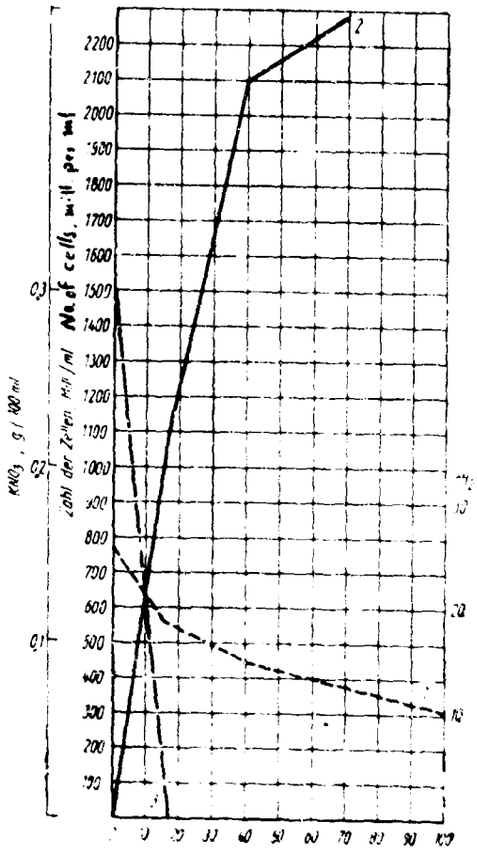


Figure 4. Growth of Ps. aeruginosa with free access to air in beef peptone bouillon.

1 = NH_3;

2 = number of cells in million per ml;

3 = amount of $NaNO_3$ in g per 100 ml.

Denitrification and oxidation of the substrate by means of atmospheric oxygen take place simultaneously with an air supply. The organic substance in the medium is used up rapidly, and the culture becomes stationary (Fig. 61).

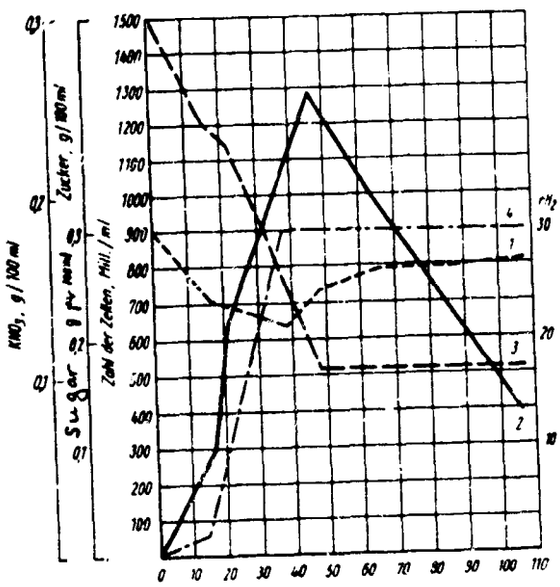


Figure 61. Growth of Ps. aeruginosa in a synthetic medium with glucose and with an air supply.

1 = rH_2;
2 = number of cells in mill. per ml;
3 = KNO_3 content in g per 100 ml;
4 = sugar consumed in g per 100 ml.

It is worth noting that saturating the medium with hydrogen and the corresponding lowering of the rH_2 value to 1-2 affects neither the multiplication of the cells nor denitrification (Fig. 62). A periodic addition of H_2O_2 and $KMnO_4$ to stabilize the rH_2 at 30 has no effect. Growth stops only at rH_2 40, attained by electrolytic saturation with oxygen.

Qualitative changes in metabolism by modifying the redox conditions and the degree of aeration could not be determined. Moreover, a high redox potential was characteristic of denitrification, except when the process occurred without a supply of air. The oxidative nature of denitrification is supported by means of the verification of a cytochrome system in denitrification that occurs with aerobic organisms, while it is usually absent with anaerobes (Hamen and Vernon, 1955).

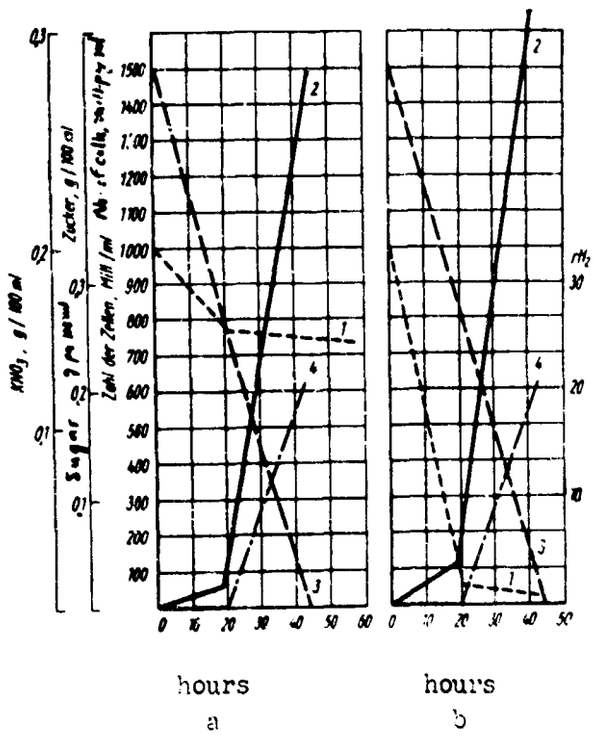


Figure 62. Growth of Ps. aeruginosa in a synthetic medium with glucose with free access to air (a) and with hydrogen saturation (b).

1 = rH_2;

2 = number of cells in million per ml;

3 = KNO_3 content in g per 100 ml;

4 = sugar consumption in g per 100 ml.

It is immaterial for denitrifiers whether atmospheric oxygen or oxygen from the nitrates serves as hydrogen acceptor.

These observations give rise to the widely held opinion that loosening the soil and increased aeration prevent nitrogen losses due to denitrification. It must be assumed that denitrification occurs under more or less aerobic conditions, when an organic substance is present. However, due to the activity of nitrifying and N_2 fixing microorganisms the nitrogen loss by denitrification is compensated under aerobic conditions.

II. Redox Potential and Assimilation of Molecular Nitrogen.

The importance of N_2 fixing microbes for soil fertility is generally well-known. It is mainly a question, in so far as the soil is concerned as a location, of Azotobacter species, Clostr. pasteurianum, some Cyanophyceae that also occur in the soil, the tuber bacteria of legumes and the Actinomycetes in the Rhizothamnidia of alders, of eleagnaces and some other plants.

Among Russian microbiologists, Vinogradskiy, Omelyanskiy, Kostytschev, Butkevich and Timiryasev have primarily participated in research on N_2 fixation and N_2 fixing microorganisms. The biochemical process of N_2 fixation has not yet been explained to date, in spite of a scarcely observable number of studies. With respect to the difficulties in this research the circumstances can only be compared with those found in photosynthesis. According to recent results (Vinogradski, 1952; Fedorov, 1952; Newton, Wilson and Burris, 1953) nitrogen assimilation is a reduction process that is connected with the oxidation of organic compounds in an as yet unknown way.

We conducted research on whether there is a relationship between the redox potential of the medium and nitrogen assimilation. In this connection some clues for the possible process of N_2 fixation have come up (Rabotnova, 1941).

1. Correlations between N_2 Fixation and Redox Potential of the Medium.

According to Burk (1934) nitrogen fixation can be kept in check if the pN_2 partial pressure drops below 0.5 atmospheres. In this case the speed of the N_2 fixation decreases proportionally to the pN_2 decrease. Thus the speed of nitrogen fixation at a pN_2 of 0.215 ± 0.002 atmospheres over the bacteria suspension is about twice as slow as with the normal nitrogen content of the atmosphere. If pN_2 drops so far that the N_2 fixation is slowed, a higher eH is formed than with a sufficient nitrogen pressure. Figure 63 shows an apparatus in which the eH can be measured by cultivating Azotob. chroococcum in a gas mixture at a partial vacuum, when pO_2 corresponds to the atmospheric pressure, but pN_2 amounts to 0.04 atmospheres instead of the normal value of 0.8 atmospheres. The same eH is present in a sterile medium in the air and at a decreased pN_2, and the total amount of nitrogen in the below-described apparatus is sufficient for the nitrogen requirement of Azotobacter. Consequently a decrease in N_2 fixation causes a considerable increase in the eH and the redox potential in the medium (Fig. 64).

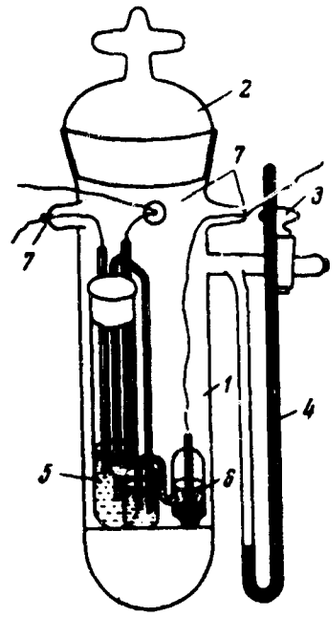
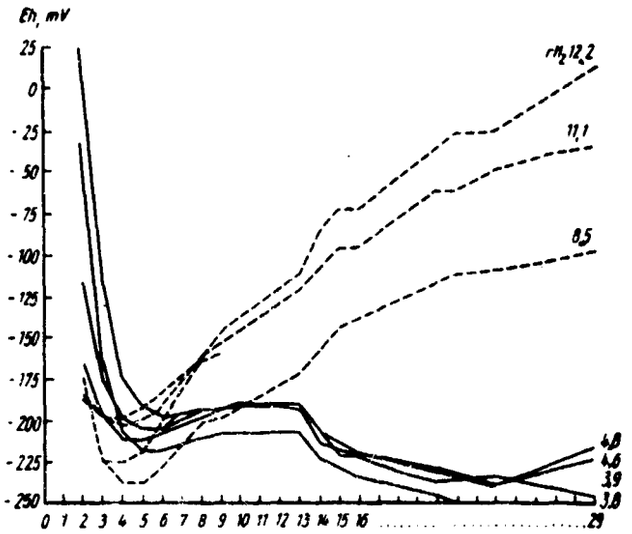


Figure 63. Apparatus for cultivating bacteria under anaerobic conditions and in various gas mixtures. (1) cylinder that is closed by a lid (2); air is pumped out through cock (3) and the gas introduced. This operation may be traced by means of a mercurial manometer (4). In the cylinder there is a test tube as culture receptacle (5) provided with electrodes, calomel electrode (6), connection to the potentiometer (7).



days

Figure 64. The eH in a culture of Azotob. chroococcum in a nitrogen-free medium with glucose:
..... = in air;
-------- = at pN_2 0.04 atmospheres.

The rN_2 values at the end of the experiment are given in the graphic representation. [The last figure on the abscissa must mean 29, not 29.]

The question of whether tuber bacteria can also fix nitrogen without symbiosis with legumes was studied from many aspects (Zabotnova, 1940) but is still unanswered at present. By cultivating tuber bacteria in various mediums an increase in the combined nitrogen was able to be established clearly only with a most precise procedure and by not using heavy N_2 (Zabotnova, 1946).

Vinogradski (1926, 1952) determined a very slight nitrogen increase within the margin of error, but constant, by using Kjeldahl's micromethod by cultivating tuber bacteria on mediums containing plant extract. Vinogradski assumes that pure cultures can also fix nitrogen, that this capability is, however, very slight, since the conditions for N_2 fixation are unfavorable.

Fedorov (1952) determined that pure cultures of tuber bacteria, therefore, fix nitrogen, when not more than two to three milligrams of combined nitrogen are contained in 100 ml of culture medium. This amount is sufficient to start growth; later, after exhaustion of the combined nitrogen, the bacteria can still fix 2-3 mg of nitrogen from the air per gram of organic substance used.

Rabotnova's (1941) experiments showed that during cultivation a higher eH appears also in tuber bacteria -- as in cultures of Azotobacter, although not very pronounced -- at an initially lowered redox potential. A difference in the eH was present at various pN_2 by cultivating on nitrogen-rich or nitrogen-poor mediums (Fig. 65).

In cultures of Azetob. schuetzenbachii (designation not recognized by Bergey or Krasilnikov), which does not fix nitrogen, no eH differences appeared at normal and decreased pN_2. Therefore, cultures of tuber bacteria react to N_2, although they cannot fix it for certain in a detectable manner. Nitrogen is not an inert gas for them. The experiments are an indirect proof that tuber bacteria also contain ferments in a pure culture that are apparently related to N_2 fixation.

2. Experiments on the Mechanism of Nitrogen Fixation by means of Azotobacter.

There are two points of view on the course of nitrogen fixation by Azotobacter. Kostytschev and Vinogradski conceived of nitrogen fixation as a two-phase process: First, reduction of N_2 to ammonia, then consumption of the ammonia in the formation of organic nitrogen compounds.

Newton, Wilson and Burris (1953) arrived at the same opinion in experiments with heavy nitrogen.

On the other hand the point of view is advocated (Fedorov, 1952) that the nitrogen is combined immediately in organic compounds.

If nitrogen fixation occurs according to Fedorov's concept, the amount of fixed nitrogen had to be in a simple relationship to the amount of cell substance. The amount of nitrogen that can be fixed by a cell had to be extremely constant. If on the other hand N_2 fixation as NH_3 and albumin formation are processes not immediately connected with each other, this kind of relationship is not to be expected.

It seems very likely that both processes are adjusted to each other, but that the relationship of both to each other can be disturbed. The conditions in the cell, for example, could be more favorable for the formation of NH_3 or of another intermediary product than for growth and the synthesis of the amino acids. We attempted to compute from data in writings on the subject the amount of nitrogen that can be fixed by a cell.

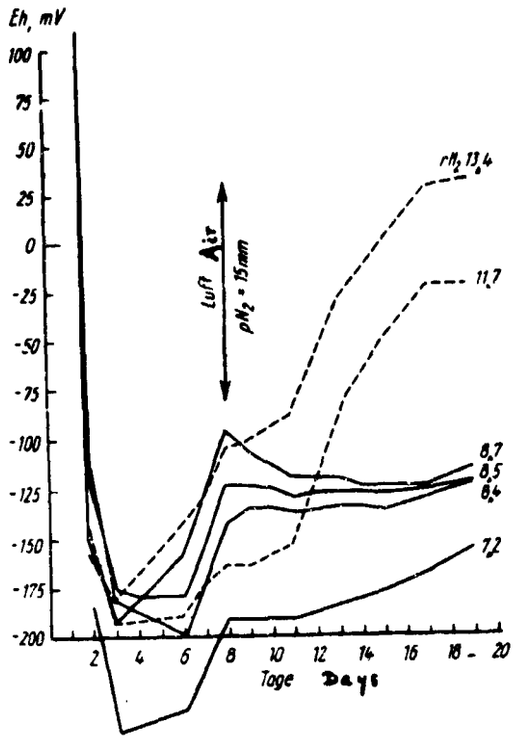


Figure 65. Course of the eH in a culture of Rhizob. leguminosarum in a nitrogen-free medium.

——— = eH with a culture in air;
- - - - - = culture first in air (8 days), then at pN_2 15 mm.

The figures on the curves indicated the rN_2 values after the end of the experiment.

It follows from Plinkov's (1948) experiments on the effect of the pH values on the growth and the nitrogen fixation of Azotobacter that at pH 6-8 approximately the same amount of nitrogen per unit of weight of the cell-mass is combined, at pH 6.1[illegible] per mg of cells, 0.049 mg of N_2 and at pH 7.15, 0.044 mg of N_2

Fedorov (1948) ascertained the number of cells and the amount of fixed nitrogen in a growing culture of Azotobacter during 16 days. Independently of the age of the culture, always about the same amount of nitrogen was assimilated by one cell (Table 55).

TABLE 55

Amount of nitrogen in mg $N_2 \times 10^{-10}$, fixed by an Azotobacter cell during growth of the culture (according to Fedorov, 1948)

days	2	4	6	8	10	12	14	16
amount of fixed N_2 per cell in mg $\times 10^{-10}$	2.8	3.9	3.6	3.7	3.8	3.6	2.8	3.4

Although N_2 fixation and growth are different processes, they are, nevertheless, in harmony with each other. When factors change, disturbances may appear that affect the metabolism. Redox conditions must be thought of first of all.

Rabotnova, Kondratyeva, Nette and Arones (1949) studied the process of nitrogen fixation per cell at various rH_2 values. They used gas mixtures with a different O_2 content, besides oxidation and reduction substances, with an inoculum of 30-50 million cells per ml of medium and an experiment time of up to 12 hours. The result was that rH_2 values in the range of 20-27 or eH values between 174 mv and 405 mv affect the life activity of Azotobacter considerably. The rH_2 range of 22-25 was optimum for nitrogen fixation. Multiplication and nitrogen fixation were checked at higher rH_2 values.

Nitrogen fixation was inhibited more than multiplication at lower rH_2 values. The amount of fixed nitrogen, computed on one cell and one gram of consumed sugar, was different under the various conditions (Fig. 66).

It is difficult to imagine that the composition of the bacteria bodies is changed; it is well-known that the nitrogen content is fairly constant in the cells. Obviously here it was a matter of the formation of an intermediary product of nitrogen fixation that was stored up in the medium. Since the nitrogen was determined in the culture in the aggregate, that is to say, in the cells and in the medium, this question could not be answered by means of the above experiments.

3. Enrichment of the Substrate with Nitrogen Compounds through the Growth of Azotobacter.

Kostytschev, Ryskaltschuk and Schvezova (1926) found ammonia in cultures of Azotob. agilis with sugar or mannitol as carbon sources which led to the assumption that ammonia could be an intermediary product of nitrogen fixation. However, since the purity of the culture had not been verified, the presence of ammonia-forming bacteria in addition to Azotobacter could not be excluded with certainty.

The experiments of Kostytschev and Shelumova (1931) were repeated later with Azotob. vinelandii in which case special care was devoted to culture purity. Twenty-five to twenty-seven milligrams of nitrogen were assimilated from two grams of mannitol. Ammonia was again found in cultures on solid and liquid mediums. According to Kostytschev, ammonia is formed at the commencement of growth of the culture as the first product of nitrogen fixation in an amount of one to two milligrams of nitrogen per 150 milliliters of medium. After the culture has stopped growing, ammonia is also released by autolytic deamidization.

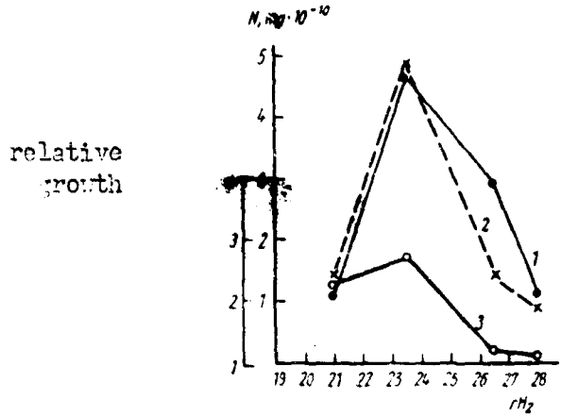


Figure 66. N_2 fixation and cell multiplication at various rH_2 values.

1 = nitrogen fixation with no increase in amount of cells;
2 = nitrogen fixation with cell multiplication;
3 = relative cell multiplication.

Winogradski (1952) also assumed that nitrogen fixation results from a reduction of the nitrogen. He supposes that first hydrazine and later ammonia are formed. Ammonia can be utilized by Azotobacter in the same way as by other microbes. Under specific conditions a disturbance of the fixation process, that is, the formation of ammonia and the consumption of ammonia in anabolism, is possible when growth is inhibited by alkali salts of organic acids. In this case ammonia is enriched in the medium.

Vinogradski detected ammonia in cultures of Azotob. chroococcum, Azotob. agilis and Azotob. vinelandii that grew on silicic acid gel with the addition of alkali salts of organic acids. In addition he found ammonia secretions from killed cells. Here also ammonia has a double origin: on the one hand it is the product of nitrogen fixation and on the other hand, the product of an autolytic deamidization. The amount of ammonia discharged amounts to 5 mg per Petri dish with living cultures and fractions of a milligram with dead cells.

However, there is a contradiction between Kostytschev's and Vinogradski's studies. Kostytschev observed ammonia secretions in cultures on mediums containing sugar, while Vinogradski points out that no ammonia is given off here, because it is completely consumed by the strongly growing culture. Ammonia secretions, according to his experiments, occur only in the presence of alkali salts of organic acids when multiplication does not keep up with nitrogen fixation.

Butkevich and Kolesnikova (1941) detected ammonia in a shake culture of Azotob. chroococcum in a medium to which glucose had been added until exhaustion of the energy-supplying substance. The quantities determined here are unusually large with up to 15 mg of ammonia per 100 ml of culture medium. According to the authors' opinion the formation of ammonia cannot be due to autolysis.

According to Fedorov (1948, 1952) the nitrogen is first combined in an enzyme that can not be separated from the living cell. HN ___ NH groups are formed that are taken over by keto acids. Amino acids are formed from them. Amino acids are also given off in the medium when sufficient keto acids or other unsaturated compounds are present. According to this opinion ammonia cannot be considered as an intermediary product in N_2 fixation. Fedorov cultivated Azotob. agilis in mediums with the addition of sugar and sodium salts of α-propionic acid, aconitic acid and fumaric acid as well as quinic acid and citric acid that are converted by decomposition into compounds with a double bond. After 2-4 weeks up to 60%-70% fixed nitrogen (with reference to the total amount of nitrogen in cells + culture solution) was detected in the culture solution used, not in the form of NH_3 nitrogen, however. Considering the long time the experiment lasted it could, however, have been a question of nitrogen compounds that are released only by autolysis.

There are also contrary results. Minenkov (1928) cultivated Azotob. chroococcum with aeration in a culture solution with the addition of mannitol and on sand that was soaked with the same solution. An ammonia secretion was not detectable. Roberg (1935) found a nitrogen enrichment in the amount of 0.5-1.5 mg of nitrogen per 100 ml, in the culture solution of Azotobacter cultures that was used. A later considerable increase was attributed to autolysis. Rabotnova and her collaborators also studied the problem of a discharge of nitrogen compounds into the medium partly with freshly isolated strains of Azotob. chroococcum, partly with the combined strains Azotob. chroococcum 54 and agilis 22D. In order to avoid autolysis, young, two to three-day old cultures were studied, and older cultures were

used only in a few cases. Cultivation took place with an uninterrupted supply of air, so that all cells were exposed to approximately the same conditions. Nitrogen determinations were made with reference to the whole culture and the cell-free filtrate. NH_3 -free air was used for aeration. The pH value of the medium during growth of the culture remained practically unchanged with carbohydrates and calcium salts of organic acids. An alkalization of the medium up to pH 9 and higher, depending on the anion consumption, occurred with sodium salts of organic acids. This agrees with Vinogradski's (1952) finding on silicic acid gel plates.

When sucrose, mannitol or calcium lactate were used as carbon sources, Azotobacter multiplied especially well. The nitrogen fixed from the air was used up in forming amino acids and did not appear in the medium. Growth was less good with sodium salts of acetic, butyric and lactic acid, although nitrogen was fixed intensively. A considerable part of the combined nitrogen could be detected in the filtrate of the culture. If the number of cells and the amount of the nitrogen fixed in the culture (filtrate + cells) are compared, it is evident that the less nitrogen falls to the lot of the individual cell, the better the growth of the culture. Inversely the nitrogen content of the individual cells is higher with a poorer growth (Table 56).

TABLE 56

Dependency of the amount of nitrogen fixed by a cell on the intensity of multiplication (Azotob. Beijerinckii)

no. of cells in million per ml.	fixed nitrogen per cell in 10^{-10} mg	carbon source
to 400	1-2	sucrose
220 - 280	1-1.4	calcium lactate
180	2.5-3.5	mannitol
100	3-4	sodium lactate
60 - 70	4-5	sodium butyrate
60 - 70	6-8	sodium acetate

In the case of weaker growth of the culture, part of the fixed nitrogen is given off into the medium in spite of the higher nitrogen content of the cells. Two milligrams and more nitrogen could be detected per 100 ml of filtrate. If the amount of nitrogen per cell is computed; that is subtracting from the total amount of nitrogen in the culture the nitrogen in the filtrate, it turns out that there is a normally constant amount of nitrogen of 1-3 X 10^{-10} mg (average value) per cell.

It must be especially pointed out in this respect that combined nitrogen could be detected already in the culture filtrate after about 24-hours growth of the culture, when an autolysis of the cells could not yet be present.

Our results confirm the theory of Kostytschev and Vinogradski according to which nitrogen fixation takes place in two phases. First synthetis of a stable intermediary product occurs, which is consumed in the formation of amino acids and proteins. When the intermediary product is not consumed sufficiently fast, as is the case in mediums with alkali salts of organic acids as carbon sources, nitrogen compounds are given off into the medium. The stronger the growth, the less the medium is enriched with nitrogen. In contrast with the results arrived at by Vinogradski, the primary fixation product is not only ammonia. The filtrate did not produce, with Nessler's reagent, the characteristic yellow coloration, but rather a greenish to pale yellow precipitate. The chromatographic test for amino acids with ninhydrin, performed during the second growth stage of the culture, was also negative. However, by aerating the culture a small amount of ammonia escaped. We assume that Azotob. chroococcum and Azotob. agilis also give off nitrogen compounds into the environment, independently of autolysis, in their natural location under certain conditions.

III. Influence of the Redox Conditions on the Autotrophic and Heterotrophic Way of Life of Chlorella.

The importance of soil algae in the cycle of soil substances has had little attention paid to it up until now. Therefore, we considered it opportune to study the way of life of the widely diffused soil alga, Chlorella.

Chlorella can live as an organism containing chlorophyll, but it is able also to live carbon-heterotrophically with various organic carbon compounds (Artari, 1903, 1906). According to Genevois (1927) Chlorella also has a fermentative metabolism available to it. Myers (1947) studied its heterotrophic manner of subsistence. It is a question of an "oxidative assimilation" of the organic compounds that takes place in accordance with the following equation:

$$C_6H_{12}O_6 + O_2 = 5(CH_2O) + CO_2 + H_2O.$$

Obviously Chlorella utilizes sugar extremely economically: 5/6 of the sugar is assimilated, 1/6 is decomposed to CO_2.

An organism with so many different varieties of capability is particularly suitable for studying the dependency of metabolism on external conditions. It can be assumed that diversity of metabolic processes, characteristic of Chlorella, developed as an adaptation to changing living conditions. In this connection Chlorella for example is in contrast with lactic acid bacteria, that have little adaptability and are homofermentative and pass over to a state of rest when conditions are unfavorable for fermentation. It is to be expected that the capability of oxidative assimilation of organic compounds depends on the air supply. Chlorella oxidizes the substrate under aerobic conditions, while under anaerobic conditions it possibly changes over to fermentation.

Rabotnova and Konova (1950) studied the conditions under which photosyntnesis occurs in Chlorella vulgaris. Growth in a mineral medium

with light was weaker than in beer-wort. The best growth was achieved in considerably diluted beer-wort from 1° beer.

Chlorella vulgaris did not grow under anaerobic conditions, although its life activity was not suspended and sugar decomposition and acid formation continued (Table 57).

TABLE 57

Growth of Chlorella vulgaris in a synthetic medium under anaerobic conditions (number of cells per ml: at the beginning, 2 million; at the end of the experiment, about 700,000). Duration of the experiment: 20 days, dark culture.

pH		amount of sugar (g per 100 ml)		acid content of 40 ml of fermentation substrate (ml 0.1 n NaOH)	
starting value	final value	starting value	final value	total	volatile acids
6.9	5.8	0.9	0.5	8.4	6.3

The behavior was the same in light and darkness.

The effect of the redox potential on the nature of the metabolism was traced in mixtures of N_2, O_2 and CO_2, that is with various conditions of aerobiosis. The result was that Chlorella lives heterotrophically under microaerophilic conditions; 92% to 100% of the increase in quantity was made from the consumption of sugar. In a nitrogen + air atmosphere carbon autotrophy and heterotrophy were developed in an equal amount. Under strongly aerobic conditions, that is in an oxygen atmosphere, only 15% of the amount of all the cells was formed from the assimilation of sugar, whereas the greater part resulted from photosynthesis (Table 58).

TABLE 58

Relationship between auto- and heterotrophic subsistence in Chlorella vulgaris under various rH_2 conditions

Experiment No.	gas mixture	rH_2 in light	rH_2 in darkness	increase in quantity due to sugar consumption, in %
1	N_2+CO_2	21.2	19.3	92.0
2		22.3	21.9	100.0
1	N_2+air	22.5	20.7	59.0
	$+CO_2$	24.6	23.0	39.8
1	O_2+CO_2	23.9	25.3	15.6

Therefore, autotrophic and heterotrophic ways of life are highly dependent on the redox conditions. When the supply of air is improved, Chlorella changes over from the utilization of organic carbon compounds to photosynthesis. The result for conditions in the soil was that soil algae like Chlorella vulgaris can exist not only on the surface of the soil but also in deeper strata; that is to say, without light and with an oxygen deficiency. In the first case they are concerned with the formation of an organic substance; in the second case, with its consumption.

IV. Acetone-Butanol Fermentation, Depending on the Redox Conditions.

Acetone-butanol fermentation has great practical importance. Under certain conditions a control of the fermentation process might make it possible to increase the output of valuable fermentation products.

1. Prevention of the Formation of Neutral Products.

It has already been mentioned that the process of acetone-butanol fermentation can be affected by changing the reaction of the medium. If the acids that appear in fermentation are neutralized with chalk, the phase of the formation of neutral products (second phase) is eliminated, and acetic acid and butyric acid are accumulated in the medium. The process is also affected by the nitrogen compounds in the medium (Yerusalimski, 1934, 1935). Fermentation occurs normally in mediums containing albumin. In mediums with simple nitrogen compounds, for example peptone and amino acids, the formation of neutral products is inhibited, and acids appear.

According to Rabotnova neither neutralization of the medium nor supply of certain nitrogen compounds is alone decisive. The addition of the various substances in certain concentrations is the main cause of the elimination of the second phase, only higher concentrations inhibit the multiplication of the cells and the fermentation of the carbohydrate. This phase is particularly easy to affect by adding various salts. In this case a strong acidification occurs (down to pH 3.9). Fermentation ceases due to the unfavorable acid content, so that many unfermented carbohydrates are left over. The type of fermentation shift in this case is always the same and does not depend on the substance that it has produced. The fact is worth noting that when the concentration of the added substances is increased, fermentation suddenly changes; that is, without a gradual transition from normal fermentation to fermentation without the formation of neutral products.

From the start of fermentation on, different salts were added to the fermentation substrate. The course of the fermentation was sufficiently characterized by the amount of acetone formed and of the unfermented substances, by the pH and the acid content. Accordingly it is possible to decide whether the fermentation has occurred normally or without the second phase (Table 59).

TABLE 59

Influence of salts on the course of acetone-butanol fermentation

substance added	concentration at which normal fermentation results		concentration at which the second phase is eliminated	
	mol	%	mol	%
KNO_3	0.03	0.3	0.04	0.4
$KMnO_4$	0.0095	0.15	0.01114	0.17
$Na_2S_2O_3$	0.01	0.25	0.012	0.30
$CuSO_4$	0.000044	0.0075	0.000059	0.010
KCl	0.1	0.75	0.13	1.0
K_2SO_4	0.1	1.5	0.14	2.0

The basis for the interruption of fermentation in the first phase is the inability of the bacteria to form neutral products. The result of this is an excessive acidification. If fermentation takes place by adding an excess of chalk in the presence of salt concentrations that cause the elimination of the second phase, then the fermentation process does not stop, but rather the carbohydrates present are fermented and acetic and butyric acid are produced. Therefore, fermentation occurs in the same way as with chalk without the addition of salt. Small amounts of chalk make the additions of salt ineffectual. When only a little chalk is acced a total of 0.1%) fermentation occurs with the normal yield of neutral products.

In industrial fermentation, also, small additions of chalk seem to have a favorable effect, particularly when little suitable substrates are used, like molasses or hydrolysates. Apparently in acetone-butanol works in the USA this method is used with the fermentation of molasses (Langlyke, Smythe and Perlman, 1952).

A strong effect on the fermentation of acetone-butanol causes considerable changes in the fermentation process. It is possible, however, by means of a weaker action to cause finer changes in the fermentation process. This can be achieved by adding non-toxic substances or small amounts of specifically acting substances.

2. Changes in the Proportion of the Fermentation Products to Each Other.

According to Wilson, Peterson and Fred (1930) the proportion of the fermentation products can be modified by changing the nitrogen source. The addition of ammonium salts to mediums with organic nitrogen compounds causes a diminution in the acetone output. The alcohol yield is increased somewhat by adding butyric and propionic acid (Simon and Weizmann, 1937). More acetone is obtained by adding acetic

acid; with other strains, more acetone and butanol (Bernhauer and Kuerschner, 1935).

When acetic acid was used with radioactive carbon it turned out that acetic acid changes into butanol (Wood, Brown and Werkman, 1945). Its conversions in acetone-butanol fermentation are complicated, however, and have not yet been completely explained.

The kind of substance to be fermented also has an influence on the final output of fermentation products (Johnson, Peterson and Fred, 1931). When a strongly reduced compound, like for example mannitol ($C_6H_{14}O_6$), is fermented, relatively more butanol and hydrogen are formed, and when a more weakly reduced substance, like glucose ($C_6H_{12}O_6$), is fermented, less butanol and hydrogen and more acetone are formed. The fermentation of the more strongly oxidized calcium gluconate produces a still greater yield of acetone.

3. Dependency of the Redox Potential on the Course of Acetone-Butanol Fermentation.

In order to be able to study the relationships between the redox conditions of the medium and the nature of the fermentation, the first point to explain is in what way the redox potential of the medium depends on the course of the acetone-butanol fermentation. For this purpose we traced the rH_2 changes in normal fermentation and in fermentation without the formation of neutral products.

Werkman and his collaborators (Reynolds, Coil and Werkman, 1934) determined that the potential in normal fermentation with a large output of neutral products is lower than in fermentation with a smaller output of neutral products. It follows from the work of Johnson, Peterson and Fred (1931) that eH values dropped rapidly and quite considerably in the induction period of the fermentation.

Rabotnova (1944) conducted methodical research on the redox potential in normal acetone-butanol fermentation and in fermentation with the addition of chalk or in a peptone medium, that is, by checking the formation of neutral products. With normal fermentation in a 7.5%-8% corn-mash the redox potential of the medium decreased rapidly. Negative rH_2 values down to -4 frequently appeared during the first 10-15 hours. Then the redox potential went up again to 0 and maintained this value during the further progress of the fermentation. An rH_2 value of 0 also appeared in the fermentation of potato mash, but without negative values occurring first and usually with a rise of the redox potential toward the end of the fermentation.

The relationship between gas formation and the rise in the rH_2 value at the end of the fermentation was peculiar. In the normal course of fermentation, that is when the discharge of gas occurred very rapidly and was over after a short time, the redox potential always went up at the end of the fermentation (Fig. 67).

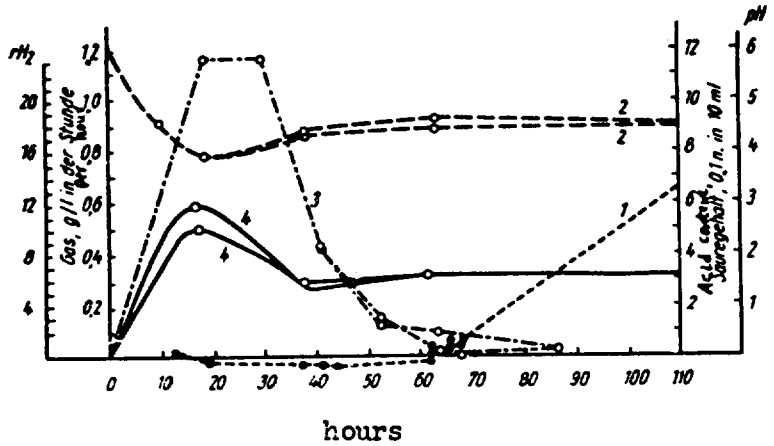
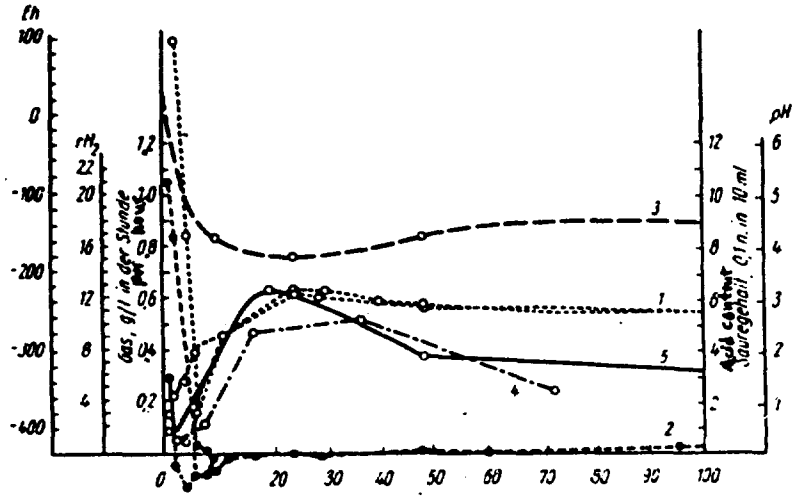


Figure 67. Normal acetone-butanol fermentation in 7% corn-mash (rH_2 measurements start 12 hours after start of experiment).

1 = rH_2;
2 = pH;
3 = gas formation in g per 1 per hour;
4 = acid content, 0.1 n in 10 cm^3

As already mentioned, chalk suppresses the second phase of fermentation, that is, the formation of neutral products. The rH_2 curve is no different in this case from the curve in the normal course of fermentation; it depends on the kind of gas discharge. Fermentation also stops in the first phase in peptone mediums. The redox potential went down when the gas discharge had reached its peak and then went up again. The course of the fermentation was complete and rapid in mediums with added peptone and chalk. The elimination of gas was hardly different than in normal fermentation, but no neutral products were formed, and the rH_2 changes were the same as in normal fermentation. When the discharge of gas stopped after a short time, the rH_2 went up at the end of the fermentation. The redox potential remained for more than 100 hours at 0 when the discharge of gas lasted a long time.

When the elimination of gas occurred only slowly and continued for a long time, the rH_2 value remained constant for a long time (up to 100 hours) (Fig. 68).



hours

Figure 68. Acetone-butanol fermentation in 7% corn mash with gas formation occurring slowly

1 = eH;
2 = rH_2;
3 = pH;
4 = gas, grams per liter per hour;
5 = acid content, 0.1 n in 10 ml.

The experiments showed that the course of the rH_2 changes in the medium depends first of all on the intensity of the gas discharge. As soon as the gas (half is hydrogen) is given off intensively, the redox potential of the medium drops to 0 and characterizes the hydrogen atmosphere thus, independently of how the fermentation occurs and whether or not neutral products are formed.

The metabolic processes could not be sufficiently characterized in detail by means of the redox potential in acetone-butanol fermentation. It merely indicates the presence of a larger or smaller amount of hydrogen in the medium. That does not mean, naturally, for one thing, that there are no other redox systems in the medium that are tied in with metabolism in the cell. However, they do not act on the electrode, because the platinum electrode is considerably more sensitive than the hydrogen one. Since there has been no success to date in isolating enzymes of the acetone-butanol fermentation from the living cell

(Simon and Weizmann, 1937) or to detect them in the medium, it must be assumed that no reducing substances except H_2 that act on the electrode potential are in the medium.

The potential was measured in a fermentation substrate after the end of the fermentation. The rH_2 value was very low at the end of the gas discharge, because dissolved hydrogen was still present. After standing for 20 days the hydrogen had escaped from the fermentation substrate, oxygen went into solution and the rH_2 went up to 20-25 (Table 60).

TABLE 60

Anaerobic final values of the redox potential after the end of the various types of fermentation (duration of observation: 3-8 days at 40° C.)

fermentation	age of the fermentation substrate in days	addition of chloroform*	rH_2	pH
normal, occurring	6	+	9.60	4.60
in an 8% corn-	6	-	9.80	4.20
mash (formation	7	+	8.00	4.70
of neutral sub-	7	-	8.00	4.70
stances)	3	+	9.45	4.48
	3	-	9.52	4.40
		average	9.00	
in a corn-mash with	14	+	7.9	5.55
the addition of	14	-	9.7	5.6
chalk (no forma-	7	+	8.9	5.51
tion of neutral	7	-	9.3	5.61
substances)	20	+	8.60	5.73
	20	-	8.52	5.72
		average	8.7	
in a peptone me-	9	+	9.6	4.05
dium (no forma-	9	+	9.66	4.03
tion of neutral	9	+	10.8	4.03
substances)	16	+	7.95	4.01
	16	-	8.42	4.01
	6	+	7.28	4.01
	20	+	8.0	4.17
	20	-	8.24	4.62
		average	8.7	

* For interrupting the fermentation, see Text, page 246.

After the dissolved gases had been removed from the fermentation substrate in a vacuum at room temperature, it was put in a nitrogen atmosphere, in order to prevent the action of the atmospheric oxygen on the electrode. The redox potential slowly took on a stable value that

was independent of the initial value. The anaerobic potential remained at an rH_2 value of 8-9 with normal fermentation and with fermentation without the formation of neutral products. In individual experiments there were discrepancies of up to 2 rH_2 units that obviously were due to uncontrolled conditions of the experiment setup.

Considerable differences in the redox potential in normal fermentation and in fermentation with elimination of the second phase could not be detected. In all cases the rH_2 was between 7.5 and 9.5. It was also determined during fermentation, after the fermentation had been interrupted by the addition of chloroform and the hydrogen present in the medium had been removed. In this case also a potential appeared whose value did not differ from the rH_2 value after the conclusion of fermentation. The rH_2 values in normal fermentation and in fermentation with exclusion of the second phase likewise did not differ considerably from each other.

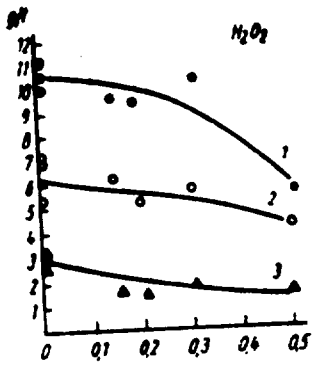
Since the method used did not allow an accurate determination of the potential and discrepancies of ± 2 rH_2 units and more appeared in various experiments, it is possible that there are finer differences between the two fermentation processes.

It was ascertained, by measuring the rH_2 in a sterile medium under anaerobic conditions, that the rH_2 values in an 8% corn-mash dropped rapidly at the beginning and more slowly later, until after 8-10 days they remained at a value that corresponds approximately to the rH_2 value of glucose solutions under anaerobic conditions. The redox potential was more stable in a peptone medium. It remained at a value of 8-9.

IV. Dependency of the Proportion of the Fermentation Products to Each Other on Oxidation and Reduction Substances. ([Note:] R. Sumarukova participated in the as yet unpublished studies).

The proportion of the fermentation products to each other can be altered by adding oxidation or reduction substances to the fermentation substrate at the start of a decline in the formation of acid, that is at the beginning of the second phase (Fig. 69). In order to avoid damaging the cells, we repeatedly added the oxidation and reduction substances to the medium specifically in small, single doses.

Dithionite in a concentration of 50-100 mg per 150 ml of medium increased the butanol output to a maximum of about 25%. The output of ethanol was increased only slightly. The amount of acetone remained unchanged. The effect of dithionite was especially evident when the output of neutral products remained relatively small. The main reason for a not completely satisfactory fermentation process in these experiments was that the temperature in the incubator was somewhat over 37° C. As has already been mentioned, this caused a decrease in the amount of butanol and generally in the total amount of neutral products. If many neutral products were formed, the action of the dithionite was weaker. Dithionite, therefore, made it possible to compensate for a loss of fermentation products due to too high a temperature.



ml of a 10% solution per 150 ml

Figure 69. The effect of an oxidation substance (H_2O_2) on the output of neutral products in acetone-butanol fermentation. Abscissa: amount of oxidation substance (H_2O_2) added.

1 = butanol;
2 = acetone;
3 = ethanol.

Glycerin in amounts of 0.5-1 g per 150 ml of medium also increased butanol yield by about 10%-20%. The acetone and ethanol output remained unchanged. Sodium formiate in only small concentrations reaised the output of butanol about 14%-15%; with higher concentrations its action was toxic. Hydrogen peroxide checked the entire fermentation considerably, especially the formation of butanol. Electrolytic saturation of the medium with hydrogen acted in the same way.

The proportion of fermentation products in acetone-butanol fermentation, however, can be changed only with an accurately determined dosage of the active substance, especially of a reduction substance. The addition of reduction substances after termination of the first growth period causes an increase in the output of a more strongly reduced fermentation product, butanol. The addition of oxidation substances inhibits fermentation without causing the output of a more strongly oxidized product, acetone, to increase.

V. Acetone-Ethanol Fermentation and the Multiplication of Bac. acetoethylicus (designation not recognized by Bergey or Krassilnikov), Depending on the Redox Potential of the Culture Medium.

Acetone-ethanol fermentation has achieved no practical significance to date; nevertheless it deserves attention in view of the increasing requirement for acetone by industry.

Acetone-ethanol bacteria are facultative aerobes. They produce fermentation in high layer of a culture medium and form colonies on the surface of agar mediums.

Until recently there were no data on whether oxygen is an inert gas for them or is involved in their metabolism. Sorokin (1952) ascertained that with Bac. acetoethylicus a respiration linked to the cytochrome system takes place and that oxygen is incorporated in the metabolism of the carbohydrates. It resulted from experiments with inhibitors that carbohydrates are able to ferment or to breathe.

Sodium fluoride checks growth under aerobic conditions by blocking the enolase. KCN as a heavy metal poison inhibits the cytochrome system. If KCN is added together with sodium fluoride, growth is also interrupted under aerobic conditions, because both fermentation and respiration are eliminated.

Therefore, acetone-ethanol bacteria belong to the facultative type of aerobes that are able to change the nature of metabolism according to the conditions: under aerobic conditions they cause a glycolytic decomposition and oxidation processes; under anaerobic conditions, only the glycolytic decomposition.

As has already been mentioned, the ratio between the fermentation products can be modified easily by means of pH changes. A change in the strength or the glucose content of the medium also affects fermentation. Thus the ratio between the fermentation products acetone-alcohol can be decreased from 1:4 to 1:2.3 by increasing the concentration strength from 0.7% to 2.8% (Yarmola, 1948). According to Koslova (1940) the addition of acetic acid to a carbohydrate medium causes the acetone output to increase. The principal fermentation products in the decomposition of potato-mash with glycerin are ethanol and gases; only a little acetone is formed (Yarmola, 1949).

When oxygen is involved in metabolism, according to Sacharov (1930) the air supply conditions have a great effect on the course of acetone-ethanol fermentation. Unfortunately the redox conditions in the medium were not determined quantitatively. Sacharov cultivated acetone-ethanol bacteria in a potato medium with an air supply in a thin layer with methylene blue as oxidation substance.

The consumption of sugar and the growth of the bacteria were not affected appreciably by aeration, but the formation of acetone dropped about 30%-35%. It was concluded, therefore, that the formation of acetone is an anaerobic process that is disturbed by oxygen. However, it was not taken into account that acetone may have been lost with aeration.

Rabotnova and Mamontova studied how fermentation is affected by rH_2 changes in the medium (unpublished). Since H_2 occurs abundantly

(cf. Chap. 6) in a high quality medium like potato-mash with the addition of chalk, it was chiefly necessary to study how the medium's redox potential can be raised. A culture medium that had a layer 1 cm thick in a Vinogradski flask with a 25 cm diameter showed no difference, when the rH_2 was measured by means of platinized platinum electrodes, in comparison with a high-layer medium in a test tube. In both cases the electrodes produced with fermentation a completely similar drop in the redox potential. The rH_2 dropped from the initial value (rH_2 23-25) to 14-17 eight hours after inoculation, and after 24 hours reached a value of 2-3 that remained constant for as long as gas discharge lasted.

It could be assumed that the electrodes do not indicate the correct results, because they become saturated with hydrogen due to the affinity of platinum for this gas and do not react to the presence of oxygen or of oxidizing compounds. Therefore, we took colorimetric measurements and compared the behavior of the rH_2 indicators in fermentation in high and low layer. The result was an indeed small but unmistakable difference: in the test tube culture Janus green was completely reduced after 10 hours, corresponding to an rH_2 of 5; neutral red was reduced after 24 hours, which means that the rH_2 value had dropped to under 3. Two zones could be observed in the low layer of the medium. In the upper layer, which was in direct contact with the air, the redox potential went down under 12 in the first 10 hours, while at the bottom it fell to 5. After 24 hours the difference balanced out somewhat. In the upper zone the rH_2 value amounted to approximately 5 and in the lower one to less than 3.

Also by cultivating acetone-ethanol bacteria in a layer approximately 1 cm high, the redox potential in the medium can be raised only slightly by means of hydrogen saturation. Only when oxygen is passed through the medium in the test tube can the rH_2 values -- mainly in the upper half of the medium -- be held only partially between 12 and 5.

Under less aerobic conditions the multiplication of the bacteria was not worse in the presence of air but rather better than in high layer. ([Note:] The number of cells was determined by direct count in the preparations.) The maximum values of bacteria count were about 8% higher with raised rH_2 values. On the other hand the consumption of sugar when air was supplied was about 10% less than under less aerobic conditions (Table 61).

TABLE 61

Cell multiplication and sugar consumption with varying amounts of aeration of the cultures (culture in thin layer, with a supply of air or by introducing oxygen. Control without aeration in a test tube 4 cm in diameter with a layer thickness of 20 to 25 cm.)

experiment number	number of cells in mill. per ml		increase in yield under aerobic conditions, %	sugar consumption, g per l		decrease in sugar consumption under aerobic conditions, %
	with aeration	without aeration		with aeration	without aeration	
1	1040	950	8.8	37.2	39.0	4.5
2	1000	903	10.7	--	--	--
3	1099	998	10.0	33.0	38.9	15.1
4	1140	1113	3.0	30.5	35.5	14.0
5	1076	992	8.5	37.1	38.3	3.0
6	1091	1011	7.0	41.9	48.0	16.7
7	1074	996	12.0	--	--	--
8	1076	1036	4.0	40.9	45.2	9.5
Average			8.0	Average		10.4

Aeration and increasing the redox potential also caused a change in the proportion of the fermentation products to each other. Under aerobic conditions as much acetone was formed as under strongly anaerobic conditions, to some extent somewhat less. If the volatility of acetone is taken into consideration, there can indeed be no doubt that more acetone escapes when oxygen is supplied or from the thin layer of the medium at 40° C. than from the test tube. Acetone, therefore, is indeed not formed less under aerobic conditions than under anaerobic ones. Perhaps the formation of acetone is even greater. This opinion is confirmed by Fateeva (1952). On the other hand under aerobic conditions the amount of alcohol was always smaller than under anaerobic conditions.

The total amount of volatile acids was the same under more or less aerobic conditions; only the proportion between the individual acids was changed somewhat. The quantity of acetic acid was larger at first during growth of the culture, but later it decreased rapidly. This corresponds to the two phases of acetone-ethanol fermentation, in Shaposhnikov's (1940) opinion. During the intensive multiplication a more strongly oxidized product is stored up in the medium. When multiplication occurs more slowly, the hydrogen of the carbohydrates that was previously used in synthesis of the albumins is utilized for fermentation products. At the same time the amount of a more strongly reduced product than acetic acid, namely acetone, increases. The maximal amount of acetic acid was always greater under aerobic conditions than under more anaerobic ones.

Formic acid in acetone-ethanol fermentation is produced in approximately the same amount as acetic acid, in which case its total amount increases from the beginning to the end of the fermentation. The amount of acid formed under aerobic conditions was always less than under anaerobic conditions (Table 62).

TABLE 62

Production of acetic acid and formic acid in acetone-ethanol fermentation, depending on the air supply (ml 0.1 n per 100 ml of fermentation substrate).

experiment no.	with aeration		without aeration	
	maximal amt. of acetic acid	amt. of formic acid at the end of the experiment	maximal amt. of acetic acid	amt. of formic acid at the end of the experiment
3	17.0	15.0	15.2	15.7
6	19.7	16.2	14.8	18.2
7*	17.0	13.8	9.0	20.0
8	19.1	14.9	15.2	17.9

* Strictly anaerobic conditions in an anaerobe culture receptacle.

It was to be expected that the ratio of ethanol to acetic acid

would be changed in the decomposition of the sugar molecule, depending on the redox conditions. More acetic acid is formed under strong oxidative conditions; if the conditions are more strongly reductive, more alcohol is produced. If the amount of alcohol and of acid is expressed in m mol, the quantity of alcohol is always much larger than the amount of acetic acid. This is especially evident at the end of the fermentation when the amount of alcohol increases, whereas the acetic acid that is converted to acetone decreases more and more. The ratio alcohol: acetic acid, however, is smaller under strongly aerobic conditions than under anaerobic ones, especially during and at the end of fermentation, when acetic acid is consumed. On the other hand, in the first hours of the fermentation process, when acetone is not yet being stored up, the formation of acetic acid is particularly encouraged under aerobic conditions (Table 63).

TABLE 63

The ratio of alcohol to acetic acid (in m mol) during acetone-ethanol fermentation under various experiment conditions

experiment conditions	duration of experiment in hours			
	24	48	72	96
Vinogradski flask	5.04	--	25.9	--
test tube	5.8	--	41.7	--
supplied with O_2	5.7	14.6	--	35.1
test tube (control)	8.8	24.0	--	41.6
hydrolytic saturation with O_2	3.4	13.4	25.5	26.8
test tube (control)	4.6	25.0	40.4	44.4
test tube	4.4	16.3	29.3	41.8
strictly anaerobic	8.7	35.6	43.5	108.7

In spite of the relatively slight possibilities of an effect of the redox condition it can be determined that at a somewhat higher rH_2. therefore. with aeration, the multiplication of acetone-ethanol bacteria is helped. The consumption of carbohydrates diminishes. The formation of acetic acid becomes greater in the first fermentation phase and the total amount of formic acid, less. Concerning the formation of neutral products, since evaporation under various conditions of air supply could not be determined quantitatively, there are merely well-founded opinions that under aerobic conditions more acetone is formed and under less aerobic conditions, more alcohol.

VI. Multiplication and Acid Formation with Homofermentative Lactic Acid Bacteria. Depending on the Redox Conditions.

The thermophil Lactobac. delbrueckii is a homofermentative

lactic acid bacterium that is used industrially for obtaining lactic acid. The only fermentation product is lactic acid. The growth of Lactobac. delbrueckii under various redox conditions has been studied only insufficiently to date.

According to Genevois and Nicolayev (1934) lactic acid fermentation with streptococci and Lactobac. bulgaricus is connected with an rH_2 value of about 5 (Janus green and phenosafranine were reduced). In the presence of air lactic acid was formed only slowly or not at all.

Kluyver and Hoogerheide (1934, cf. Chap. 7) also assume that the various lactic acid bacteria lower the redox potential to one and the same level of 5-5.8.

Also according to Kasanskaya's (1951) studies Lactobac. delbrueckii (strain WDSch.) tends toward anaerobiosis. In a tall cylinder the output of cells amounted after 24 hours to 124 million; in a thin layer, to 65 million. The same was also determined with acid formation. In the cylinder 15.0 M-equiv. of triose were detected; in the thin layer, on the other hand, only 5.25 M-equiv.

According to Bertho and Glueck (1932) washed suspensions of Lactobac. delbrueckii form H_2O_2 in the presence of air. There in McLeod's opinion they are anaerobes.

On the other hand, Davis (1933, a, b) demonstrated that some representatives of lactic acid bacteria, among others Lactobac. casei, do not form any hydrogen peroxide and that, therefore, oxygen is an inert gas for them. Fermentation takes place uniformly in oxygen and nitrogen atmospheres.

In experiments by Chaix and Flamens (1953) on Bac. coagulans entirely the same amounts of cells were produced after 20 hours under aerobic and anaerobic conditions. Growth occurred more rapidly under aerobic conditions. Cytochrome could be determined in the cells in cultures under aerobic conditions, which was not the case under anaerobic conditions. Bac. coagulans, therefore, behaves, according to the culture conditions, like a typical aerobe or anaerobe. The relationship with oxygen, therefore, apparently is different in the various species and strains of homofermentative lactic acid bacteria.

The reaction of Lactobac. delbrueckii to oxygen and to the redox potential not only has theoretical importance but also practical significance. If oxygen is an inert gas for these bacteria, the fermenting fluid can be thoroughly mixed in the receptacles by means of compressed air. If the bacteria are sensitive to oxygen, this procedure is not usable. Therefore, it seems to us necessary to study the dependency of growth and fermentation on the degree of oxygen supply, in which case rH_2 is used as a quantitative measurement.

1. The Air Supply Intensity of the Medium and its Effect on Multiplication and Acid Formation with Lactobac. delbrueckii.

We made our studies (jointly with Shkundova and Gretschushkina, unpublished) on the industrially used strains XI, XII and WDSch, iso-

lated by Kleymenova (1940), the first two of which form acid strongly, while the third one is less active. Other strains were also used in this research. Beer-wort (3° beer) was used as a medium with 1% crushed malt without the addition of chalk. Cultivation took place in a Vinogradski flask or in high layer in test tubes. Uniform distribution of the crushed malt in test tubes was achieved by means of horizontal stratification. Experiment temperatures were 50° C. and in some cases 37° C.

Previous observations on strains XI and XII had shown that the number of cells usually reaches its maximum after 30 to 50 hours, after which autolysis begins. The main amount of acid is also formed in the first 48 hours, then the acid content increases only slightly. Accordingly we determined the acid content and number of cells after 48 hours, and we only partially continued the observations (Table 64).

TABLE 64

Effect of the air supply on multiplication and acid formation by various strains of Lactobac. delbrueckii. Temperature: 48°-52° C. The acid content was determined by titration and calculated for lactic acid.

strain	experiment condition	% of lactic acid after hours			number of cells after 48 hrs. in mill. per ml.
		48	72	190	
XI	flask	1.07	--	1.13	302
	test tube	0.665	--	0.70	96
XII	flask	0.75	--	--	290
	test tube	0.58	--	--	254
WDT3	flask	0.52	0.54	--	97
	test tube	0.42	0.42	--	59
Savodskoy	flask	0.48	--	0.51	176
	test tube	0.35	--	0.37	80
WDSch.	flask	0.31	--	--	90
	test tube	0.37	--	--	63
9595	flask	0.21	0.24	--	23
	test tube	0.42	0.42	--	58

The individual strains differed considerably with respect to acid formation and multiplication. Multiplication and acid formation took place more intensively with more active acid formation (strain XI, XII. WDT3 and Savodskoy) under aerobic conditions. Weaker acid producers

(strain WDSch. and 9595) on the other hand formed more acid under anaerobic conditions. Lactobac. delbrueckii, therefore, reacted only weakly to oxygen, which is particularly amazing, because it generally grows better aerobically than under anaerobic conditions.

2. Multiplication, Acid Formation and Redox Potential in the Growth of Lactobac. delbrueckii under Strictly Aerobic and Anaerobic Conditions.

The experiments under strictly anaerobic conditions were performed in the apparatus, already described (cf. p 230), that permits eH measurements during the growth of the culture. The eH dropped in the first 24 hours from 200 mv to zero and subsequently remained unchanged at this level. The pH value at the end of the experiment amounted to 3.2-3.4. From this it followed that the rH_2 value in the culture was between 7 and 9. All the strains are able to form acid and to multiply under strictly anaerobic conditions (Table 65).

TABLE 65

Acid content and cell output with Lactob. delbrueckii under anaerobic conditions at rH_2 7-9.

Strain	after 48 hours		after 100 hours	
	lactic acid, %	no. of cells, mill. per ml.	lactic acid, %	no. of cells, mill. per ml.
XI	--	236 452	1.0	198
XII	0.94 0.98	383 330	1.0	174
Sadovodskoi	--	--	0.76	200
WDSch	.	--	0.42	185

When Lactobac. delbrueckii grew under aerobic conditions in a Vinogradski flask in a 1 cm thick beer-wort layer (3° beer), the rH_2 in the medium did not drop or dropped only slightly and remained at a value of about 28 during the entire duration of the experiment. Nevertheless, multiplication and acid formation did not differ appreciably from the values that had been observed in experiments under anaerobic conditions (Table 66).

TABLE 66

Acid content and number of cells of Lactobac. delbrueckii Strain XII under aerobic conditions (thin layer) after 48 hours at rH_2 28.

experiment No.	acid content, %	No. of cells, mill. per ml
1	0.71	150**
2	0.45	--
3*	0.79 0.81	562*** 532
4*	0.75	474

* Experiment at 37° C.
** Initial number of cells: 5.3 mill. per ml.
*** Initial number of cells: 8.4 mill. per ml.

For purposes of comparison, parallel experiments were performed with strain XI under aerobic and under anaerobic conditions ([Note]: In order to compensate for evaporation losses, the experiment receptacles were filled with distilled water at the end of the experiments to the initial volume.) In this case culture growth and acid formation were better under aerobic conditions than under anaerobic ones (Table 67.)

TABLE 47

Acid formation and number of cells under aerobic and anaerobic conditions with Lactobac. delbrueckii, strain XI (beer-wort from 6° beer).

Experiment conditions	acid content in % after 48 hrs.	acid content in % after 220 hrs.	No. of cells after 48 hrs in mill. per ml
aerobic	1.41	1.92	252
anaerobic	1.05	1.17	242
strictly anaerobic	1.02	1.17	186

3. Growth of Lactobac. delbrueckii by saturating the medium with Hydrogen and Oxygen.

In the rH_2 range of 7-28 an appreciable effect on multiplication and acid formation could not be detected. Because of this the question was raised of how Lactobac. delbrueckii reacts when the rH_2 is lowered experimentally under 7 or raised over 28 by saturating the medium with hydrogen or oxygen by means of electrolysis of the water (Table 68). ([Note]: The apparatus for cultivating under anaerobic conditions that has already been described (cf. Fig. 63) was used in this type of experiment. The pulled-off lower end of the receptacle is filled with KCl agar and inserted in a glass with a saturated KCl solution. In another glass there is a platinum electrode in KCl.

Both vessels are connected by a U-tube. One of the electrodes is connected with the negative pole of the current supply and the other electrode, that is in the KCl, with the positive pole. The medium in the receptacle is then saturated with hydrogen. The rH_2 was measured two to three times during the experiment. After it had come to the level given in Table 68, it no longer changed appreciably.

TABLE 68

Acid formation and multiplication of Lactobac. delbrueckii at an rH_2 of 1 and less after 30 hours at 42° C.

Strain	Experiment Conditions	rH_2	pH	% of lactic acid (according to titratable acid content	No. of cells in mill. per ml
XI	Control	6.2	3.1	0.98	236
	saturation with H_2	0.6	3.43	0.89	390
	Control	9.0	3.02	1.03	336
	saturation with H_2	0.4	3.10	1.05	296
XII	Control	15.3	3.28	0.5	451
	saturation with H_2	1.0	4.11	0.48	416

However, here also no appreciable effect on growth and acid formation could be detected.

The electrolytic saturation of the medium with oxygen always results in a certain acidification of the culture solution; therefore, beer-wort with the addition of chalk was utilized for these studies. An rH_2 of approximately 30 results with electrolytic saturation with oxygen. It is evident from the first two experiments (Table 69) that the bacteria lowered the redox potential somewhat, in which case multiplication and acid formation occurred. Growth, therefore, was considerably worse than in the control experiment with H_2. In the next two experiments we prevented the rH_2 from dropping below 30 by means of a stronger current. In this case no growth occurred in general.

TABLE 69

Acid formation and multiplication of Lactobac. delbrueckii by saturating the medium with oxygen.

Experiment No	Experiment conditions	rH_2 after 24 hrs.	rH_2 after 48 hrs.	Final pH value	Acid content (according to Frideman), % of lactic acid	No. of cells in mill. per ml
1	Control	15.2	17.8	4.4	1.02	280
	saturation with O_2*	30.6	20.4	4.7	0.72	198
2	Control*	13.6	22.2	5.1	1.72	124
	saturation with O_2	30.6	22.1	4.5	0.33	20
3	Saturation with O_2**	30		5.0	0.0	no multiplication
4	Saturation with O_2***	25	32	5.0	0.0	no multiplication

* Duration of experiment: 48 hours.
** Duration of experiment: 30 hours.
*** Duration of experiment: 55 hours.

In one of the experiments the receptacle was filled with 40 ml of culture solution and shaken at about 100 RPM. The receptacles in the control experiments contained nitrogen instead of air (Table 70).

It is evident from Table 70 that in an rH_2 range at 25 and between 27-29 multiplication and acid formation still occur intensively. Lactobac. delbrueckii first stops growing when the medium is strongly saturated with oxygen and at $rH_2 > 30$.

TABLE 70

Acid formation and multiplication of Lactobac. delbrueckii in shake culture

Experiment Conditions	rH_2		pH		Acid content % of lactic acid	fermented maltose mg per 100 ml	No. of cells mill. per ml.
	Initial value	After 50 hrs.	Initial value	After 50 hrs.			
In air	27.4	25.8	5.44	3.45	0.434	520	316
	29.5	25.3		3.54	0.425	471	358
In nitrogen	21.0	16.3	5.44	3.36	0.451	552	351

4. Growth of Lactobac. delbrueckii with Various Air Supply in the Presence of Chalk.

Up to now observations were made on the acid formation in beer-wort without chalk. We repeated them in beer-wort with chalk. Strains XI and 9595 were used for these studies. Experiment temperatures were 37° C. and 48° C. Aerobic conditions were created in Vinogradski flasks with a beer-wort layer 1-1.5 cm. thick. In some of the flasks a layer of vaseline 6 cm. thick was applied on the surface of the medium. Since vaseline-oil, however, does not shut off the medium absolutely from atmospheric oxygen, the experiments must be evaluated as such with impeded air supply. Fermentation took place well, both in the presence of chalk and without the addition of chalk, with an air supply and under the vaseline layer, however, somewhat better with a supply of air (Table 71).

The cultures developed approximately uniformly at 37° C. and 48° C. Fermentation was just as slight when the acids formed were neutralized with chalk depending on the air supply as without the addition of chalk.

TABLE 71

Acid formation by strains XI and 9595 of Lactobac. delbrueckii by cultivation in beer-wort with chalk at 37° C. and 48° C. (every other parallel set of experiments).

Culture	Experiment conditions	% lactic acid after		Experiment temperature in °C.
		48 hrs.	96 hrs.	
9595	Aerobic	1.49	2.47	
		1.67	2.43	
				37

	Anaerobic	0.86 0.68	2.19 2.19	
	Aerobic	1.18 1.18	2.30 3.83	48
	Anaerobic	0.99 1.08	1.13 1.13	
XI	Aerobic	1.65 1.62	6.57 5.87	37
	Anaerobic	2.29 1.79	5.58 5.58	
	Aerobic	2.71 2.71	4.65 5.69	48
	Anaerobic	3.71 2.77	7.07 4.93	

5. Analysis of Acids Formed by Lactobac. delbrueckii at Various rH_2 Values.

Some lactic acid bacteria considered as homofermentative are able to produce fermentation of the heterofermentative type, depending on the pH value. In addition to lactic acid, the only fermentation product in an acid medium, volatile acids were still formed in an alkaline medium (Gunsalus and Niven), 1942; see Chap. 4).

We studied whether a similar change in the type of fermentation also occurs in Lactobac. delbrueckii, depending on the redox potential.

In the following experiments the total titratable acid content and the lactic acid content were determined simultaneously in accordance with Frideman's method. In addition, the amount of fermented sugar was related to the amount of lactic acid formed in order to clarify whether the sugar consumption was the same under aerobic and anaerobic conditions for lactic acid formation (Tables 72 and 73).

TABLE 72

Lactobac. delbrueckii (strain XII): lactic acid formation and total acid formation in the medium under aerobic and anaerobic conditions

Experiment No. *	Experiment Conditions	Acid content after 48 hrs.			Acid content after 120 hrs. in Exper. 1; after 185 hrs. in Exp. 2.			No. of cells mill. per ml.	
		after titration, %	according to Frideman, %	% lactic acid of the total content	After titration, %	According to Frideman, %	% lactic acid of the total acid content	48 hrs.	120 hrs.
1	Aerobic	0.73	--	--	0.86	0.75	87	245	120
	anaerob.	0.53	--	--	0.58	0.54	93	108	93
	strict anaer.	0.30	--	--	0.49	0.42	86	120	54
2	Aerobic	1.39	1.44	103	1.56	1.62	104	--	--
	anaerob.	0.83	0.90	108	0.90	0.81	90	--	--
	strict anaer.	0.77	0.81	105	0.83	0.94	113	--	--

* Experiment 1 in beer-wort, 3° beer; experiment 2 in beer-wort, 6° beer.

TABLE 73

Lactic acid formation and fermentation of sugar by means of Lactobac. delbrueckii (strain XII) at different rH_2 values.*

Experiment conditions	Time in hrs.	pH	rH_2	Titratable acid content (lactic acid) mg	Lactic acid: According to Frideman, mg	Lactic acid: % of the total acid content	Fermented sugar: mg	Fermented sugar: per 1 g lactic acid	No. of cells mill. per ml
Aerobic	0	5.7	25.7	--	--	--	--	--	--
	4	5.62	25.0	--	--	--	--	--	70.8
	21	3.5	21.1	621	617	99.4	--	--	790
	43	3.42	20.5	891	940	105	--	--	839
	67	3.4	20.5	891	940	105	--	--	964
Anaerobic	0	5.7	26.5	--	--	--	--	--	--
	4	5.53	25.8	--	--	--	--	--	37
	21	3.45	15.4	396	382	96.5	--	--	254
	43	3.36	16.0	594	541	91.5	--	--	331
	57	3.33	17.7	675	652	98.2	--	--	354
Aerobic	0	5.58	26.8	--	--	--	--	--	--
	17	3.45	20.8	394	418	106	725	1.73	537
	44	3.33	24.0	639	556	87	921	1.65	652
	68	3.28	23.5	648	647	100	921	1.41	883
Anaerobic	0	5.53	27.9	--	--	--	--	--	--
	17	3.44	13.3	315	297	94.5	458	1.54	260
	44	3.19	16.0	522	491	89.0	611	1.24	538
	68	3.28	17.7	585	560	96.0	842	1.50	505

O_2 supply	0	5.36	23.0	--	--	--	--	--	--
	10	3.93	24.0	183	155	84.5	--	--	294
	42	3.54	24.7	468	463	99.0	670	1.44	890
	66	3.54	2.16	468	463	99.0	670	1.44	498
H_2 supply	0	5.44	25.1	--	--	--	--	--	--
	10	3.98	1.1	150	127	110	177	0.95	276
	42	3.45	7.6	576	564	97.5	618	1.09	681
	66	3.55	16.5	576	564	97.5	618	1.09	568

* Cultivation took place in beer-wort from 6° beer with crushed malt without the addition of chalk. Aerobic conditions were produced by using Vinogradski flasks with a layer 2.5 cm. thick. The anaerobic conditions are virtually the same with impeded access to air. Beer-wort was filled in test tubes to a height of 25 cm. and supplied with oxygen and hydrogen.

Lactobac. delbrueckii only forms lactic acid independently of the air supply conditions and of the rH_2 value. Theoretically one gram of lactic acid per gram of fermented sugar is formed by homofermentative lactic acid fermentation. In our experiments under aerobic conditions a little more than 1.5 grams of sugar were necessary, however, to form one gram of lactic acid and under anaerobic conditions, a bit less than 1.5 grams of sugar. Since more cells are formed under aerobic conditions, it is probably that the greater consumption of sugar is related to this. Moreover, it has not been studied whether neutral fermentation products are formed under aerobic conditions or an oxidation of the sugar occurs.

It does not follow from the behavior on solid substrates that contact with atmospheric oxygen does not injure the cells. After seeding a diluted bacteria suspension (strain XII) in a wort agar in high layer, uniform colonies grew in the entire agar in the form of white dots in the vicinity of which the chalk was dissolved. In high layer agar without the addition of chalk very small colonies, visible only microscopically, grew; they were uniformly distributed in the culture tubes and consisted of filamentous cells. There were no colonies on the surface, however.

The sparse growth on the surface of solid substrates did not seem to be conditioned by atmospheric oxygen, but rather by the fact that the acid being formed was diffused only beneath, but with colonies in agar, in all directions.

The experiments with Lactobac. delbrueckii have demonstrated that these bacteria grow independently of the air supply and of the redox potential of the medium in an rH_2 range of 0-30 and that they ferment sugar to lactic acid. The individual strains, however, showed certain differences. Active strains multiplied better under aerobic conditions than under anaerobic ones; they also formed more acid and consumed more sugar for the formation of one gram of acid under aerobic conditions. The less active strains multiplied better under anaerobic conditions and in this case likewise formed more acid. No changes in the fermentation process were caused by the air supply and the redox potential.

VII. Multiplication and Fermentation with Heterofermentative Lactic Acid Bacteria, Depending on the Redox Conditions.

It has already been shown with the example of lactic acid streptococci that the fermentation of glucose takes place better under anaerobic conditions, in which case more lactic acid and alcohol are formed than under aerobic conditions (Nefelova, 1952). The redox potential, however, was not determined quantitatively by means of rH_2 measurement.

Rabotnova (unpublished article prepared jointly with Gretschuschkina) performed experiments on Betabact. pentoaceticum (designation not recognized by Bergey or Krassilnikov), a representative of the heterofermentative lactic acid bacteria, similar to the experiments performed with Lactobac. delbrueckii. The sugar was completely decomposed by fermentation in wort with the addition of 2% chalk. More fermentation products were stored up, because the pH value held at a

favorable level for the bacteria of approximately 5.5 (Table 74). Toward the end of the experiment the number of cells dropped off as a result of autolysis. Chemical changes usually stopped after 48 hours, and the composition of the medium hardly changed at all any more. An extensive neutralization of the acids being formed was achieved by using chalk and the best aeration of the culture solution occurred in shake culture (rH_2 about 30). In order to create anaerobic conditions, the flasks were filled with nitrogen. However, since a slight exchange of gas with air occurred during the supply of gas, only an incomplete exclusion of air was guaranteed.

Fermentation occurred in the broad range of rH_2 17.7-32. Between rH_2 29-32 multiplication and sugar consumption took place to a lesser degree than at lower rH_2 values.

The proportion of fermentation products to each other changed with the redox potential: under more anaerobic conditions the lactic acid portion was greater; under strong aerobic conditions, lesser.

Similar experiments were also performed in a medium without chalk. In this case we were able to achieve anaerobic conditions by using a layer 20 cm. thick, since the medium did not have to be shaken continuously as was necessary in experiments with chalk. The rH_2 amounted to 11-12 in the experiments without the addition of chalk, while in an experiment set up simultaneously by using a thin layer 1.5 cm. thick the rH_2 lay at 24. An rH_2 of 16-17 was achieved in a shake culture in a nitrogen atmosphere, and rH_2 values of 29-31 by means of shaking the medium in air.

The pH value in the medium without the addition of chalk dropped on an average from 5.8-6.0 to 3.5. Fermentation usually stopped after 48 hours (Table 75).

TABLE 74

Heterofermentative lactic acid fermentation of Betabact. pentoaceticum in wort with the addition of chalk at under various conditions of aeration (all experiments were performed in shake cultures)

Experiment conditions	In nitrogen				In air	
rH_2	17.7-20	20-25	25	29-30	29.5-30.5	28-32
Duration of experiment in hours	48	52	69	52	48	69
No. of cells in mill. per ml.	1,869	--	595	--	389	364
Fermented sugar in m mol	24.6	31.2	13.2	8.3	3.6	3.66
Lactic acid in m mol	20.2	20.6	4.69	2.8	0.9	1.2
Alcohol in m mol	4.0	4.3	1.8	1.65	1.25	1.75
Acetic acid in m mol	1.3	2.2	1.4	1.3	1.1	1.5
pH	5.7-5.9	5.4	5.2-5.7	5.5	5.5-5.7	5.0-5.8

TABLE 75

Heterofermentative lactic acid fermentarion of Betabact. pentoaceticum in wort without chalk under various conditions of aeration.

rH_2	11-12	16-17	24	29-31
Experiment Conditions	High layer medium in test tube	Nitrogen atmosphere in shake culture	Culture medium in thin layer in flask	Air in shake culture
Duration of experiment in hrs.	80	72	80	72
No. of cells in mill, per ml	398 401	1.167	471 462	478
Fermented sugar in m mol	15.0 17.5	16.2	12.8 15.9	7.0
Lactic acid in m mol	6.3 6.6	6.8	4.1 3.35	2.3
Alcohol in m mol.	3.25 4.25	5.7	1.76 2.0	2.56
Acetic acid in m mol.	0.8 1.0	1.3	1.2 1.7	1.4
CO_2	2	2.13	--	--
pH	3.38 3.45	3.5	3.52 3.47	4.27

The general rules were about the same in fermentation without chalk as in fermentation with the addition of chalk. Fermentation occurred both at rH_2 11-12 and at rH_2 29-31. Multiplication and sugar consumption were less under aerobic conditions than at an rH_2 of 16-17. Furthermore, it is worth mentioning that multiplication was also less at the lowest rH_2 values of 11-12 than at an rH_2 of 16-17. Under anaerobic conditions lactic acid was the predominant product, while under aerobic conditions its portion became smaller in comparison with the other fermentation products. Alcohol assumes the second place quantity-wise; under aerobic conditions the output became smaller. Acetic acid was not formed in as large a quantity as lactic acid and alcohol.

The general rules that Nefelova (1952) determined with lactic acid streptococci also apply, therefore, to Betabact. pentoaceticum.

Heterofermentative bacteria of the Betabact. pentoaceticum type grow in a wide rH_2 range from 11-12 to 30-32. Under strong aerobic conditions growth and fermentation were prevented, however. The proportion of the fermentation products to each other changed at the different rH_2 values. Under anaerobic conditions lactic acid was the predominant product of fermentation. Under aerobic conditions, however, lactic acid was produced in about the same amount as acetic acid and alcohol. The proportion of alcohol to acetic acid changed also according to the redox conditions. While acetic acid was always formed in the same amount, the quantity of alcohol was larger under anaerobic conditions.

VIII. Influence of the Redox Potential on the Formation of Antagonistic Substances with Streptomyces globisporus.

Many Actinomyces have achieved importance as producers of valuable antibiotics. However, little is known on the formation of antagonistic substances, depending on the redox potential.

Streptom. globisporus an antibiotic that is effective against Bac. mycoides. It is known that Streptom. globisporus is able to grow in a wide rH_2 range. We determined the activity of the antibiotic substance formed in cultures at various rH_2 values from the size of the sterile zones after transferring the agar plates from the Actinomyces culture to a bed of Bac. mycoides.

For the culture we (joint study with Kuligina, unpublished) used an agar medium with 20% potato extract, 1% glucose and 0.5% peptone, the oxidation and reduction substance, and rH_2 indicators were added (Table 76).

TABLE 76

The amount of antibiotic formed from Streptom. globisporus at various rH_2 values of the culture medium.

Experiment No.	Medium	rH_2	Age of culture in days	Diameter of the sterile zone in cultures of Bac. mycoides in mm
1	with $Na_2S_2O_4$	under 8	3 8	7 - 9 8 -11.5
	with $KMnO_4$	over 21	3 8	9 -10 10 -11.5
	control	about 14	3 8	9 -10 9 -11

2	with $Na_2S_2O_4$	7-8	4 9	29-35 47-48
	with $KMnO_4$	over 23.7	4 9	28-35 45-47
	Control	13-14	4 9	36 47
3	with $Na_2S_2O_4$	7	6	28-33
	control	13-14	6	32

The experiments on solid mediums demonstrated that rH_2 changes within the range of 7-23.7 and above do not affect growth and the formation of the antibiotic.

Then some experiments were performed by submerged cultivation in a fluid medium with aeration and saturated simultaneously electrolytically with oxygen and hydrogen (Tables 77 and 78).

The result was that saturating the medium with hydrogen has no effect on the growth of the Actinomyces and does not prevent the formation of the antibiotic. An rH_2 value of 35 was reached by saturating electrolytically with oxygen. The antibiotic was still present in the medium after 63 hours.

TABLE 77

Growth of Streptom. globisporus and amount of antibiotic at a changed redox potential(submerged cultivation) H_2 = electrolytic saturation with hydrogen.

Experiment No.	Age of culture, hr.	pH		eH		rH_2		Sugar consumption g per 100 ml		Mycelium weight, mg per 10 ml		Diameter of the zones, in mm.	
		control	H_2	Control	H_2	Control	H_2	Control	H_2	Control	H_2	Control	H_2
1	22	--	--	--	--	--	under 8	0.17	0.16	12	13	--	--
	29	--	--	--	--	--		0.17	0.17	16	16	14-19	14-18
2	0	7.3	7.3	349	216	26.6	22	--	--	--	--	--	--
	19	7.4	7.6	264	-165	23.8	9.6	0.17	0.18	--	--	--	--
	27	7.5	7.9	221	-258	26.6	7.0	0.20	0.19	11	11.3	--	--
	44	7.7	8.1	160	-320	21.1	5.2	0.23	0.23	15.3	16	19-17	15-17

TABLE 78

Effect of oxygen on the growth of Streptom. globisporus (submerged cultivation) (electrolytic saturation with O_2).

Age of culture in hrs.	pH	eH	rH_2	sugar consumption, g per 100 ml	weight of mycelium, mg per 10 ml	diameter of the zones in mm
0	7.3	390	28	--	--	--
15	6.8	332	25.1	--	--	--
20	6.8	291	23.4	0.087	1	--
39	6.6	230	21.1	0.150	2	--
63	7.9	500	35.5	0.214	4.8	18

IX. Summary.

The principle of active intervention in the course of fermentations is still quite little used in technical microbiology.

Neuberg' experiment with glycerin fermentation in yeasts has already demonstrated the possibilities of the application of this principle. Our experiments with acetone-butanol fermentation have adduced a further verification of this. The addition of dithionite to the medium succeeded in increasing the butanol output; this is a method whose technical applicability must still be examined.

The problem arises from our experiments on the significance of the redox potential, which was brought up under facultative aerobes: Those microbes that are able to live both with an air supply and anaerobically are called facultative aerobes or anaerobes.

Metabolism in facultative anaerobes or aerobes can occur in two ways: Under anaerobic conditions the organism performs a fermentation metabolism, while with an air supply it changes over to oxidative metabolism. When oxygen is an inert gas to microorganisms they grow independently of it, whether oxygen is present or not. The behavior with respect to oxygen must, therefore, be studied in each facultative aerobe, which has scarcely been done until now. The so-called "Pasteur-effect", that is, the capability of passing over from anaerobic to aerobic metabolism in the presence of air, in which case the sugar consumption decreases, has been determined only with yeasts.

It has been found for homofermentative lactic acid bacteria of the Lactobac. delbrueckii type that they behave rather indifferently with respect to oxygen. Under both aerobic and anaerobic conditions they multiply and carry on the fermentation process with gradual differences in the individual species. All the strains studied by us can be classified in the species of facultative aerobes for which oxygen is essentially an inert gas.

Heterofermentative lactic acid bacteria, like Betabact. pentoaceticum, also grow well under aerobic and anaerobic conditions, in which case the proportion of the fermentation products to each other shift somewhat.

Oxygen is also important for acetone-ethanol bacteria and denitrifiers. This is particularly evident with the latter. Under anaerobic conditions they live by denitrification; that is, by means of oxidation of the substrate with the oxygen of the nitrates. Under aerobic conditions denitrification does not take place, and they carry on in this case oxidation processes, possibly respiration processes, that are tied in with oxygen. We observed no case in our experiments that was completely switched over from one type of metabolism to the other by changing the air supply conditions. The ability to utilize combined oxygen also holds with an air supply.

Yeasts with strongly pronounced fermentative characteristics behave like this. Under anaerobic conditions they only ferment carbohydrates, while under aerobic conditions they also breathe. As

Kluyver has demonstrated there are yeasts that are not fermentation organisms and live mainly aerobically, but also perform a fermentation within certain limits, when they live with air excluded. Many moulds also behave like this.

Among microorganisms we find all shades from obligate anaerobes to obligate aerobes. The following groups can be distinguished (Table 79):

TABLE 79

Survey of redox conditions among which different metabolic processes take place.

Processes	Agent	rH_2 range in which the process is possible	rH_2 change in the medium observable in growth of the culture	Author
Anaerobic:				
acetone-butanol fermentation	Clostr. acetobutylicum	0-2	20-0	Rabotnova
anaerobic saprophyte	Clostr. sporogenes Clostr. putrificum***	0-5	22-0	Rabotnova
hydrogen fermentation of formic acid	Bact. formicum***	7*-14	22-6	Krasina
bacterial photosynthesis	Chromatium	not over 15	--	Nefelova
methane fermentation of formic acid	Bact. formicum***	0-7	22-0	Krasina
facultative anaerobic:				
acetone-ethanol fermentation	Bac. aceto-ethylicus	0-20	25-3	Rabotnova
homofermentative lactic acid fermentation	Lactobac. delbrueckii	0-30	28-7	Rabotnova

heterofermentative lactic acid fermentation	Betabact. pentoaceticum	11*-32	28-5	Rabotnova
lactic acid fermentation of fungi	Rhizopus	0-32	30-5	Kanel
alcoholic fermentation	yeasts	10-15	25-10	Kluyver
alcoholic fermentation	Asp. niger	12 and less		Kusnetsov
alcoholic fermentations	Asp. niger	--	25-9	Guldet
aroma forming	Streptoc. diacetilactis	not under 7	22-7	Maksimova
denitrification	Ps. aeruginosa	0-30	30-10	Rabotnova Korotschkina
bacterial photosynthesis	Rhodops. palustris (Athiorhodaceae)	1-26	30-8	Kondratyeva
aerobic:				
albumen decomposition	Bac. subtilis	12*-25	25-10	Rabotnova
nitrogen formation	Azotobacter	22-30	30-10	Rabotnova
aerobic photosynthesis	Chlorella	24 and more	--	Rabotnova
antibiotics production	Streptom. globisporus	5**-35	31-11	Rabotnova
citric acid fermentation	Asp. niger	12**-17	--	Kusnetsov
nitrification	Nitrosomonas	20-30	--	Zobell, Lyubimov

	Nitrosomonas	--	24-13	Kingma Boltjes
	Nitrobacter	--	24-9	Kingma Boltjes
acetic acid fermentation	Bact. xylonoides***	--	27-6	Lyubimov
oxidation of formic acid	Bact. formicum ***	17-22	--	Krasina

* It is possible that the process takes place at a lower rH_2 value.
** In the presence of only small amounts of oxygen.
*** Designation according to Krassilnikov.

a. Obligate anaerobes that live exclusively by anaerobic processes, that is at the expense of redox reactions without participation of oxygen as hydrogen acceptor. Oxygen is toxic for them under usual culture conditions. Saprophytic bacteria and acetone-butanol bacteria, for example, belong here. They multiply only at very low rH_2 values of 0-5, although they are viable at an rH_2 up to 20.

b. Organisms that perform anaerobic fermentative processes exclusively, but also grow with a supply of oxygen that is an inert gas for them, for example lactic acid bacteria of the Lactobac. delbrueckii type. They are viable in the rH_2 range of 0-30, independently of the redox potential, and perform an intensive fermentation.

c. The most extensive group is composed of organisms that perform both oxidative and fermentation processes and pass over more or less easily from one type of processes to another, according as atmospheric oxygen is present or not. Some of them have become predominatly adapted to an aerobic, oxidative way of life (yeasts, some spore-forming aerobes), and anaerobic conditions are less favorable for them. Others have adapted themselves, judging from existing evidence, equally well to fermentative and oxidative processes (Esch. coli) and, circumstances permitting, perform them simultaneously. Furthermore, there are organisms that tend more to anaerobic, fermentative processes, but with an air supply are capable of oxidative processes, liko, for example, the acetone-ethanol bacteria. All these microorganisms live in a wide rH_2 range of 0-30.

d. Organisms that live primarily with oxidation processes and even if they do not multiply are able to live in any case under anaerobic conditions and maintain their metabolism to a limited extent. There are fungi that are able to live in an rH_2 range of about 5-30. Chlorella vulgaris, for example, also belongs to this group; it prefers aerobic conditions, but is capable of living without access to air, although under these conditions it does not multiply.

e. In addition, mention must be made of the obligate aerobes that require oxygen as a hydrogen acceptor and as a necessary component of the medium. Without an air supply they have at most a very reduced life activity. They survive anaerobic conditions when they

are in a state of rest or with a very limited metabolism. The following belong to this group: Azotobacter, Bac. subtilis and some spore-forming aerobic saprophytic bacteria, nitrifiers, iron microbes and Acetobacter. Their preference for oxidative conditions with an rH_2 of 20-30 is characteristic of these microorganisms, although they also survive an rH_2 drop to 10-20.

f. An exceptional position is occupied by those microorganisms that exhibit an oxidative metabolism; however, as hydrogen acceptor they do not use free oxygen but rather combined oxygen or both. Denitrifiers are able to utilize O_2 and NO_3 simultaneously as acceptor. They are viable in the wide rH_2 range of 0-30. Desulfurizers use the oxygen of sulfates under anaerobic conditions. As obligate anaerobes they prefer a low redox potential. Methane bacteria require carbonic acid as hydrogen acceptor in an anaerobic way of life.

Included herein are also bacteria with photosynthesis pigments that oxidize the substrate under anaerobic conditions with the aid of the hydroxyl ion formed by photolysis of water. The oxidative nature of the metabolism of these bacteria finds expression in the fact that they contain cytochrome and that some of them adapt themselves easily in darkness to oxidative metabolic processes with participation of atmospheric oxygen. Consequently these microorganisms utilize two sources of energy -- light and the oxidation of organic compounds.

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Chapter 9

SUMMARIZING OBSERVATIONS ON THE RELATIONS BETWEEN MICROORGANISMS AND SUBSTRATE (pH AND rH_2)

Two factors are always to be considered in studying the interrelationship between microorganisms and their nutritive substrate: The adaptation of the microorganisms to the medium and the change of the medium by the growing microorganisms.

I. Active Acidity.

The adaptation to the medium is shown, for example, by the fact that many species are able to grow in an acid medium, for example Thiobac. thiooxidans; others, for example sulfur microbes of the Beggiatoa type, in an alkaline medium, while most microorganisms prefer the neutral range. The pH range in which growth is possible may be narrow or wide in the individual species, in which case the conditions in their natural habitat are codetermining.

In so far as the question of their adaptation to acid or alkaline pH ranges is concerned, it might be conceivable that acid-preferring microorganisms have a relatively acid protoplasm; alkali-preferring ones, on the other hand, have a relatively alkaline protoplasm. Moreover, the possibility exists that the adaptability to acid or alkali mediums results from the ability of the cell not to let H and OH ions penetrate the interior of the cell; that is, to preserve the neutral reaction of the protoplasm in acid and alkaline mediums.

Experimental data, for example measurements of the intracellular pH value in organisms that prefer an acid or an alkaline environment, do not exist to date. The second assumption, however, has a greater degree of probability, because the total activity of the protoplasm and of the metabolism in its entirety do not differ in principle from each other in acid-preferring and alkali-preferring microorganisms. Both the internal (intra-cellular) conditions and the external conditions found in the medium are significant for adaptation to the medium.

1. Intracellular Conditions.

The intra-cellular environment is a physical and chemical system complexly organized from cytoplasm, cell nucleus, chondriosomes, vacuoles, etc. Therefore, it is hardly possible to talk about the pH value of the cell content as a whole. Earlier studies were too crude in their method to be able to examine these conditions in detail. Therefore, they provide us with only approximate values; nevertheless, leads can be [illegible] from them.

In older plant cells (parenchymal cells) the cell sap forms the major part of the cell content; the protoplasm is limited to a thin film wall. The cell sap is only weakly buffered; the reaction can be neutral or acid, even down to a pH range of 1-2. A change in the CO_2 content can indeed modify the reaction. As far as the pH value of the cytoplasm could be ascertained, it was at pH 6-7.

The living cells of the animal body consist almost only of protoplasm. A neutral reaction resulted from pH measurements by means of microinjections. The protoplasm of the large egg cells of marine echinoderms that attain a diameter of 150μ was unusually strongly buffered, as was brought out by injecting acids and alkalis. If the buffering finally breaks down and the intracellular pH value is changed, the cells die off.

Some studies, certainly little reliable, of bacteria cells difficult to study due to their small size indicate a neutral reaction of the cell content. Here also the buffering is strong: In staphylococci with an intra-cellular pH value of 7.5 it hardly changes, even though the cells have adapted their growth to an acid environment (pH 3.5).

Generally it must be considered that the protoplast is viable only in a definite, relatively narrow intracellular pH range that is stabilized by buffer systems. Moreover, it is obvicusly a question of the buffer characteristics of the albumin substances and of the carbonates.

If acids are formed in the metabolism, they often appear in the large vacuoles in plant cells or they are precipitated as calcium salts.

Acids being formed in the cells of the animal body can be given off to the exterior or be oxidized subsequently. In bacteria whose metabolism frequently results in the formation of acid, the acids are discharged into the surrounding medium.

2. Hydrogen Ion Concentration of the Culture Medium.

In the medium the originally present pH value is changed and regulated by the metabolism of the microorganisms. The following general rules pertain to growth in mediums containing carbohydrates:

In the simplest case, with homofermentative lactic acid bacteria, there is no possibility for regulating the acid content. Lactic acid is formed until the cells finally die off due to the increasing acidity of the medium.

Lactic acid bacteria on the other hand, have adapted themselves to higher acid contents. The pH value can drop to about 3 without the cells dying off. When the alcohol supply is exhausted, acetic acid is oxidized to CO_2 and H_2O; the acidity of the medium decreases again.

Finally there are bacteria that regulate the hydrogen ion concentration of the medium automatically, since they form neutral products instead of acids. When the pH value drops in the formation of acids from carbohydrates by acetone-butanol fermentation bacteria, an enzymatic conversion of acids being formed to neutral products starts. The same is the case with the butylene-glycol fermentation of Aerob. aerogenes in which 6.3 also is the critical pH value. The accumulation of formic acid by Esch. coli and acetone-ethanol bacteria occurs only in alkaline and neutral mediums; in acid mediums it is decomposed to CO_2 and H_2. According to Ashby there is also a pH control in Azotobacter cultures whose mechanism has not yet been completely explained.

In practice the output of neutral products in industrial fermentations can be increased if after the critical pH value is reached acids are added continuously to the medium. This is the procedure for example in the acetone-butanol industry. The end-product for additional acids is draff that is left over after the distillation of neutral products. They should not be added already at the start of fermentation but rather only when the cells have multiplied and the mechanism for the formation of neutral products has taken up its activity. In principle this procedure can be followed in all fermentations when it is desired to increase the yield of neutral products.

Active pH regulation also appears in metabolic mechanisms that allow alkaline by-products to be formed. Thus sporogenic aerobes, for example Bac. subtilis, Bac. mesentericus and Bac. megaterium transfer ammonia to the less alkaline urea (cf. Chap. 4).

In many cases acids are formed from peptone and neutralize the ammonia. Manteyfel, Bogdanova and Chetverikova (1949) made similar observations on Bact. formicum (designation not recognized by Bergey or Krassilnikov) (cf. Chap. 4).

According to Sierakovski (1924) in the decomposition of peptone by Esch. coli the CO_2 being formed by an alkaline reaction fixes the ammonia. In mediums with carbohydrates there is no regulation in Coli bacteria, so that the cells die off due to the formation of acid.

Gale and Epps (1942) ascertained that Esch. coli on albumin mediums has a special fermentative mechanism for the automatic regulation of the pH. They found that depending on the pH value at which the Esch. coli is cultivated a special set of enzymes is formed. In an acid reaction carboxylases are produced that break down the acids and thus lower the acid content. The amines being formed also contribute to the neutralization of the medium. On the other hand deaminases are produced by cultivation in alkaline mediums and the ammonium salts of the corresponding fatty acids result from deaminization of the amino acids.

Of course there are microorganisms that have no mechanisms for actively regulating the pH, for example Proteus vulgaris and Bact. alcaligenes that tolerate high alkalinity in albumin mediums.

II. Redox Conditions.

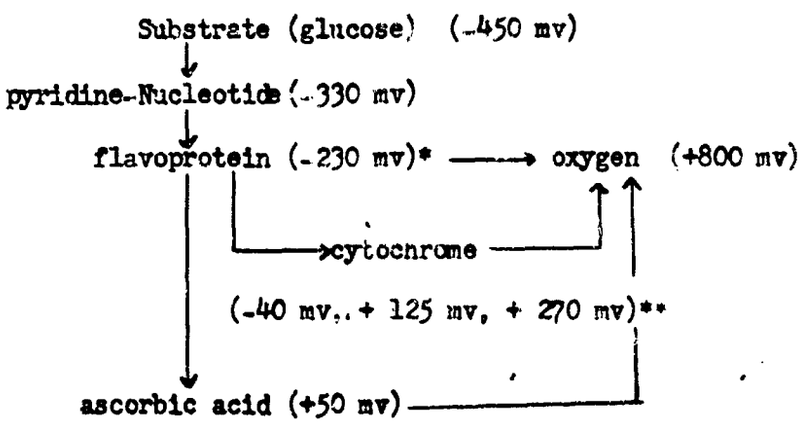
1. Intracellular Redox Potential.

The redox conditions within the cell can be studied by means of vital staining or microinjection. Until the present time measurements have been undertaken primarily on the egg cell of echinoderms.

Under aerobic conditions the rH_2 values in animal or plant cells are at approximately 20; under anaerobic conditions they drop to 6-7 and lower, without causing the cells to die off (cf. Chap. 3).

That can be explained by the fact that atmospheric oxygen is replaced in its role as hydrogen acceptor by other acceptors belonging to the cell; in this way an oxygen deficiency can be compensated at least temporarily. We can talk about a "buffering" of the vitally necessary oxidation processes.

There are various substances that transfer the hydrogen or the electrons in the biogenetic oxidation of the substrate to the oxygen. The following outline expresses it for example in a very simple form:



* According to Teorell (1951) E_0' of flavin = -185 mv.

** According to Payl (1951).

The chain of the transferrers may differ according to the type to which they belong. Pyridine nucleotide and flavoprotein are characteristic of aerobes and anaerobes. Moreover, cytochromes with different E_0' occur in most microorganisms. Instead of cytochromes, polyphenoloxidases or ascorbic acid can transfer hydrogen from flavoproteins to oxygen. A direct transfer of flavoprotein to oxygen is also possible. The substrate, which gives off hydrogen or electrons, has a low potential: for glucose, for example it is at -450 mv. Then hydrogen is passed on to transferrers with increasingly higher potentials until finally it reaches the oxygen that shows a very high redox potential with +800 mv.

It may be assumed that the hydrogen transferrers also have a regulating, kind of buffering action, since they "store" electrons or hydrogen. However, there are scarcely any data on the amount of

transfer substances in the cell that could be significant in this connection.

Pyridine nucleotide is present in amounts of 0.5 mg in one gram of fresh yeast. Flavins are synthesized by many microorganisms and occasionally are given off abundantly into the medium (acetone-butanol bacteria, Eremothecium Ashbyi and others). Iron prophyrins (hemoglobins) related to the cytochromes are able to store up oxygen. According to Postgate (1956) desulfurizers contain cytochromes up to 3% of the net weight. Here, according to Postgate, the cytochromes could perform a protective function with respect to the oxygen dissolved in the medium.

According to Moss (1956) the cytochrome A_2 content in Aerob. aerogenes in cultures with various oxygen concentrations from 10^{-3} to 10^{-6} mol is larger the less oxygen is present.

Lenhoff, Nicholas and Kaplan (1956) also have found in Ps. fluorescens a dependency of the cytochrome content on the aeration conditions. That indicates also that, depending on the external conditions, the cell is able to regulate the amount of transfer substances that, as has already been said, participate in buffering the oxygen.

2. Redox Potential of the Culture Medium.

Anaerobes grow at an extremely low redox potential; aerobes require higher rH_2 values. Anaerobes are able to produce actively a low redox potential in the medium, favorable for them. If we transfer anaerobes into a fresh medium, the redox potential, before multiplication starts, drops from 20-22 to very low values (1-5).

The adaptive nature of the ability to give off reducing substances seems to result also from the following comparison: saprophytes and pathogenic anaerobes, widely diffused in nature, in the soil, in mud, etc., as a rule lower the eH of the medium. On the other hand anaerobic representatives of the specific microflora of the intestinal tract of animals, that are only to be found there and have become adapted to the anaerobic conditions of their habitat, change the eH only slightly when cultivated in vitro. Apparently this ability has not been developed in them because there was no need for it.

It must still be added that these microorganisms can be cultivated in the laboratory only with difficulty and quickly die. The reason for this probably lies in the oxidizing action of oxygen that they cannot compensate.

Aerobes live in a wide rH_2 range. Many of them multiply with an abundant air supply at rH_2 values up to 30 (Actionomyces, Azotobacter). They lower the rH_2 value simultaneously to 7-8. That is due to the consumption of the dissolved oxygen and to the discharge of reduction substances into the medium.

If we compare the curves of cell multiplication in aerobes and of the drop in the rH_2 value with each other, a decrease in multiplication is never established when the rH_2 goes down. Aerobes are

apparently not very sensitive to the rH_2 values in the medium.

It must be realized, however, that values over 20-22 were never found for the intracellular rH_2. Only cells with chlorophyll, that give off oxygen during photosynthesis, do not have a high redox potential, while the rH_2 in the surrounding medium, saturated with O_2, may reach values of 30-35 and more. The production of reducing substances in the cell constitutes a protection against an excessive oxidative action of the oxygen. Experiments with redox indicators point in the same direction (cf. Chap. 6).

Therefore, aerobes also prefer a relatively low redox potential. That is explained by the fact that many vitally important enzymes contain SH groups or other radicals that can be oxidized. They must live at a low rH_2 so that these enzymes may not be inactivated. Although they require oxygen, an excess of oxygen in the cell is detrimental to aerobes. They protect themselves from the oxygen by means of a reductive barrier.

On the other hand, an addition of foreign reduction substances stimulates multiplication considerably. We have observed this in Bac. subtilis, in Azotobacter and in lactic acid bacteria. There are indications that yeasts can be stimulated to multiply by means of reduction substances. Virtanen and Rautanen (1952) demonstrated that pea seeds without storage cotyledons can be grown when ascorbic acid or other reduction substances are added.

Moreover, we are of the opinion that the biological reason for the formation of "redox substances" also lies in the fact that they represent reservoirs or buffer systems that receive the hydrogen of oxidizable substances when there is an oxygen deficiency. The oxidation process, the energy source of life, must not be interrupted. The hydrogen acceptor (oxygen) nevertheless may frequently be absent. This may be demonstrated, for example, by the fact that in spite of the slight solubility of oxygen in water, aerobes live in it and are able even to multiply, which is due to the fact that they mobilize hydrogen acceptors. We have established these reducing reserve substances electrometrically in live, multiplying cultures of microbes. At present the cultures are taking in oxygen voraciously and it is constantly deficient.

These substances act not only in the cell, but are also given off into the external medium where they accumulate in considerable amounts. They take over the hydrogen quite rapidly, but they bring about its combination with the oxygen only slowly. Just as yeasts can live for some time on the glycogen supply stored up in the cell, there are also aerobes that live for a long time on the hydrogen reserve acceptors. These act as a buffer between the cell and the oxygen (cf. also Chap. 9, II, 1). Without doubt the transfer systems of the oxidative processes, like cytochromes, belong to them, and probably other presently unknown substances.

Baumberger's (1939) experiments are a confirmation of this opinion. This author observed simultaneously the state of the cytochromes in the cell (by means of the absorption spectrum) and the pH in the

medium as he was studying yeasts under aerobic and anaerobic conditions. In this case there was complete agreement between the condition of the cytochromes and the eH in the medium: The cytochromes are reduced and the eH lowered with an oxygen deficiency; with access to oxygen the cytochromes are oxidized and the eH goes up. However, it is well worth noting that with a deficiency of oxygen the eH in the medium went down lower than could be the case due only to the cytochromes. Therefore, the low eH outside is not only conditioned by the cytochromes but also by other substances from the chain of hydrogen transferrers with a low E_0'. The fact that these systems are established outside the cell is important. This means that the surrender of hydrogen to oxygen does not occur within the cell but rather outside or on their surface.

It is indeed possible that not only cytochromes, flavin or pyridine nucleotide are given off into the medium (Legge, 1954, assumes, of course, that cytochrome oxidase appears extra-cellularly). These substances are very complex; all the constructive capabilities of the cell must be mobilized for their synthesis. It is conceivable that simpler hydrogen acceptors are given off into the medium as a buffer, that take the hydrogen over from the pyridine nucleotide or from the flavins. Since suspensions of aerobe cells are able to lower the rH_2 quite sharply under aerobic conditions (to rH_2 = 5 to 10), it cannot, therefore, be doubted that the buffer substances show low E_0' values.

To date there are no precise data for solving this problem. The study of the substances given off by microbe cells into the medium is a very interesting problem of microbiology that at present has not been solved in any way.

Therefore, microorganisms are able to modify the pH and rH_2 of the surrounding medium, depending on their requirements. They have this ability to a considerably greater degree than other organisms. Higher forms of life have available numerous devices for maintaining stable conditions within the body. Evolution has taken the direction of an increasing separation of the inner from the outer medium. This is evident, for example, with regard to the temperature relationships with the environment.

The evolution of microorganisms took a different course. Because of their small dimensions stable internal conditions cannot be guaranteed with the requisite certainty. Regulation of the living conditions, therefore, occurs not within but outside the cell. Many functions that take place within the body in higher organisms occur in the environment in microorganisms. Thus also some stages of metabolism, for example some phases of the oxidation process, take place in the medium or on the surface of the cell.

In their natural habitat we frequently run across a sudden change from aerobic to anaerobic conditions. After a heavy rain, for example, anaerobic conditions appear immediately in the soil instead of aerobic ones. Organic substances in the soil (animal corpses, dead plants) come to produce reducing substances in the environment as a result of the energy consumption of oxygen and of the discharge of reducing substances by live growing heterotrophic aerobes. In fact, experiments have shown that many aerobes and anaerobes can tolerate con-

siderable variations in the redox conditions. This does not exclude that in other cases, for example in many sulfur bacteria that are adjusted to special habitats, pH and rH_2 limits are laid down quite narrowly (Kaplan, 1956; Baas-Becking and Wood, 1955; Bahr and Schwartz, 1956).

A change in the rH_2 conditions within the possible limits for their growth can cause changes with regard to their metabolic processes in many microorganisms (cf. Chaps. 7 and 8).

This can be seen particularly clearly in various fermentation processes. With a change in the redox conditions a switch from respiration to fermentation and vice versa can be observed, above all. Oxidative conditions, therefore, a high redox potential of the medium, in yeasts, fungi and bacterial microorganisms bring the enzymes primarily into action to catalyze the utilization of carbohydrates, in which case body substances and CO_2 are formed in the end effect. In addition the fermentation enzymes are more or less eliminated. When no oxidative processes can occur, the fermentation enzymes come into operation to guarantee multiplication at the expense of the anaerobic fermentation. With anaerobic fermentation various acids and neutral products are formed that are specific for each species of microorganism.

The quantitative and qualitative composition of the fermentation products can be changed considerably by cultivating the microorganisms under various redox conditions. This is seen especially clearly in fermentations in which neutral C_4 compounds are produced. Fermentations like this were observed in Aerob. aerogenes and in sporogenic aerobes. With a greater degree of oxidation more strongly oxidized acetylmethyl-carbinol is stored up; with a higher degree of reduction, 2,3-butylene glycol.

Similar shifts can be observed with acetone-butanol fermentation and acetone-ethanol fermentation: strongly reduced products are stored up in greater amounts under strongly reduction conditions.

A clear shift in the correlation of the products can be observed in heterofermentative fermentation. Under strongly reductive conditions, more lactic acid is formed; under strongly oxidative conditions, relatively more acetic acid. The proportion of acetic acid and alcohol is also changed: under strongly reductive conditions relatively more alcohol is formed.

Knowledge about the pH and rH_2 values as factors that cause natural changes in the metabolic processes, therefore, hold out the possibility of controlling these processes.

However, it is not always simple to produce changes in metabolism experimentally. Buffer properties can counteract the pH and rH_2 change.

Therefore, the study of factors like temperature and radiation in which such a direct countereffect on the part of the organism as with pH and rH_2 is scarcely to be expected would be all the more important. These two factors have not been drawn on up until now as a means for the controlled change of the metabolic processes.

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