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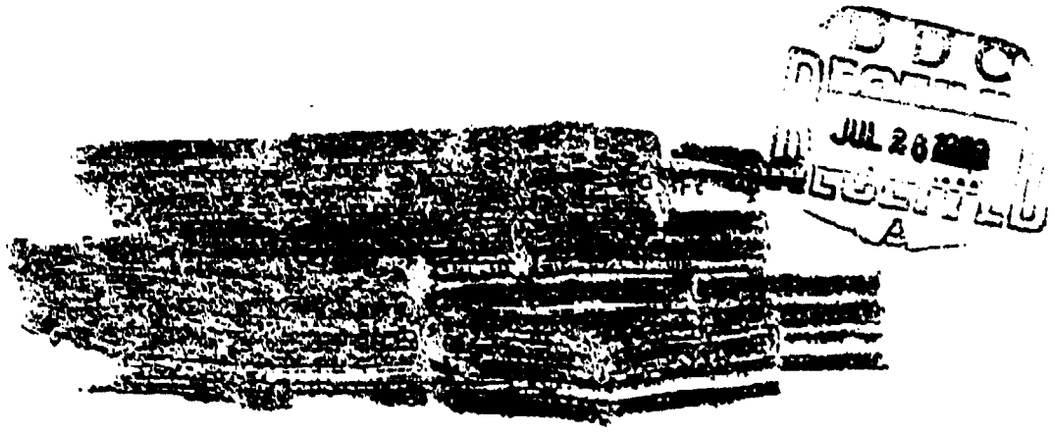

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

APPLICATION OF OXIDATION REDUCTION POTENTIAL TO FERMENTATION

PART I

[Following is a translation of an article by Motoyoshi Kongo, Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University, in the Japanese-language periodical Nihon nogyo kagakkai-shi (Journal of the Agricultural Chemical Society of Japan), Vol 32, 1958, pages A101-105 (Part I) and A113-117 (Part II).]

The development of the theory of oxidation reduction potential, hereafter abbreviated ORP or simply referred to as potential, is mainly due to Clark's work during the years 1920 to 1928. Since then, ORP has been adopted in bacterial research with considerable pro and con arguments, but despite all of this argument, there has developed some direction in this field of research during the years. In fermentation chemistry, despite the fact that oxidation reduction reactions are vitally important to fermentation metabolism, techniques as well as reasoning involving ORP have not been too popular, due to the fact that ORP measurement is difficult and also due to the fact that the analysis of ORP data is equally or even more difficult. As a result, the present paper uses considerable space introducing the methods of using ORP, and at the same time, since much speculation will be related to proven facts in the discussion, the author welcomes any form of criticism from the readers.

1. The Meaning of ORP and Its Use

As it is said that oxidation reduction reactions are present wherever life exists, oxidation reduction systems, hereafter abbreviated redox systems, play a vital role in living organisms. One system can be reduced by receiving an electron (hydrogen in living organisms) from a different strongly oxidizing system. In this transfer of hydrogen from one system to another, energy release is involved. In such reactions, one must have some measure of the reducibility or oxidizability

of a system, and for this purpose ORP is used, the units in use being Eh or rH. This is one way of using ORP.

When platinum is immersed in a reversible redox system, some molecular hydrogen attaches itself to the surface of the metal as atomic hydrogen. This atomic hydrogen goes into solution as an ion, leaving the platinum negatively charged, and as a result a state of equilibrium results among molecular hydrogen pressure, hydrogen ion concentration, equilibrium of the redox system, and the potential of the platinum electrode. If the hydrogen pressure which is in equilibrium with the redox system is high, the abundance of hydrogen means that the system is strongly reducing. At the same time, since there will be more hydrogen ions detaching themselves from the platinum surface, the metal will become more negatively charged. Now the Eh mentioned in the last paragraph is the potential of the platinum measured with respect to a standard hydrogen electrode. One can also define rH as $\log(1/H_2)$, that is the logarithm of the inverse of the equilibrium hydrogen pressure which can be calculated from Eh according to the formulae

$$Eh = (2.3RT/F)\log(H^+/\sqrt{H_2})$$

$$\log(1/H_2) = 2[(Eh/2.3RT/F) + pH]$$

where F : Faraday constant,
R : gas constant,
T : absolute temperature,
H : hydrogen ion concentration, and
H_2: molecular hydrogen pressure in atmospheres.

The method of representation is similar to that for $pH(\log(1/H^+))$. With these definitions, a strongly reducing system with high equilibrium hydrogen pressure has a small rH value, while a strongly oxidising system has a large rH value. rH equals zero corresponds to a hydrogen pressure of one atmosphere, while an oxygen pressure of one atmosphere corresponds to a rH value of about 41. In this manner, various redox systems can be arranged in the order of their relative reducibility or oxidisability. For instance, a succinic acid/fumaric acid system, which is in equilibrium with a hydrogen pressure of $1/10^{14}$ atmospheres, has a rH value of 14. Hydrogen is only transferred from systems with lower rH values to systems with higher rH values. For instance, an ethanol/acetaldehyde system (rH 7.5) can be reduced by a triosephosphate oxidising system (rH 0.8) via DPN (rH 3.4), but it cannot be reduced by a succinic acid system (rH 14). In fermentation metabolism therefore, if at first glance conjugate hydrogen donor and recipient systems apparently coexist, and if the rH value of the hydrogen donor system is higher than the rH value of the recipient system, then obviously a redox reaction, that is fermentation, cannot occur and one must reconsider the type of chemical reaction involved. The redox systems in biological specimens

have rH values between 0 and 25, an exception to the case being adrenalin which has a rH value of 30.7 (pH 0-2), and $E_o' = 380$ mV (pH 7.0). It should be noted that rH expresses intensity and not capacity, that is, it is similar to quantities such as temperature and pH. It should be remembered that the system inside an organism can often have a potential despite the fact that the capacity may be small in many cases.

Actual rH values for individual systems vary slightly from the characteristic standard potentials (rH_o or E_o) of the systems by an amount depending on the ratio of the concentrations of the oxidized form and the reduced form. As can be seen from the following equation, rH_o is defined by putting [reduced form]/[oxidized form] = 1 and the value of rH varies by two units for a single hydrogen atom transfer and by only one unit for a two-hydrogen atom transfer.

$$rH = rH_o - (2/n)\log([\text{reduced form}]/[\text{oxidized form}])$$

where n is the number of hydrogen atoms transferred.

Similarly one has

$$eH = eH_o - (2.3\ RT/nF)\log([\text{reduced form}]/[\text{oxidized form}])$$

In the oxidation reduction reaction between two systems, it is possible for one system to have a higher rH_o value but a lower rH value than the other system when the rH_o values of the two systems are nearly alike, and in such cases, it is possible for hydrogen transfer from the higher rH_o system to the lower rH_o system. Under similar circumstances, it is also possible for two systems to have different rH_o values but the same rH value and to thus be in equilibrium. In using the above formulae, if either the oxidized or the reduced form molecule is dissociated, its effect must be taken into consideration. If either the dissociation constant is very small or the dissociations of the oxidized and reduced form molecules are about the same, the above equations are valid in their given form. On the other hand, however, if upon dissociation the oxidized form has a unit positive or negative charge more than the reduced form, then there will be a unit change in rH (change of two units for n = 1) for a unit change in pH. Since the term representing the effect of dissociation for different values of pH is a constant for a particular system, it can be lumped in the value rH_o or E_o and these new values are indicated by r'H and E_o'. If the degree of dissociation is known, then by measuring the value r'H for a particular pH, the r'H for any pH can easily be derived. Conversely, the dissociation constant can be determined by knowing the ORP for various values of pH. This constitutes another well known use of the ORP. Another application of the ORP is described in section 3.

2. Method of Measurement

There are a number of methods for measuring the ORP of a redox system, such as the method of calculating the standard potential from thermodynamical values, or the method using redox reagents, but most of these methods have various undesirable features, and therefore the electrode method of measurement is normally used. This method consists basically of measuring with a potentiometer the difference in potential of a half cell with a platinum electrode immersed in the object solution and a standard half cell. The standard voltaic cell is one with a mercury electrode with respect to which the potential of a hydrogen electrode is known. With regard to the electrode tank to be immersed in the object solution, if the system reacts rapidly with the electrode but slowly with oxygen, it is not necessary to exclude oxygen, but if the situation is otherwise, then some means must be taken to exclude oxygen from the vicinity of the electrode. For instance, a Thunberg-Borscok vacuum tube is sometimes used, but more commonly a Michaelis tank is employed. The latter is a long-necked bottle with a wide ground mouth with a rubber stopper through which pass the electrode, a KCl agar-agar bridge, titrator tube, glass electrode, air tube, etc. Normally pure nitrogen gas is bubbled through the solution to replace the oxygen, and measurements are made while the nitrogen is being forced through. In the case of flavin and the yellow enzymes which normally exist as oxidation types, they cannot be detected by the normal method of measurement. In such cases, a suitable reducing agent is added, the potential drop is measured with the system evacuated, and ORP must be estimated from the nature of the potential-time curve; or one can aerate with nitrogen, titrate a reducing agent (or first reduce completely and titrate an oxidizing agent), measure the potential corresponding to a certain reduction rate, and then estimate ORP from the potential-reduction rate curve. If such methods are used, one obtains in addition to the standard potential of the system, various characteristics of the ORP. Among redox systems, there are some, such as the base of dehydrogenases, which do not react directly with the electrode, and in such cases, the measurements are made with the addition of a slight amount of redox dye which has a potential near the standard potential. In this case, the dye adopts the ORP of the object system, and the potential at the electrode will give the ORP of the dye.

With beer (1), rice wine (2), grape wine (3)(4), and other fermented solutions (2)(5)(6), the measurement is usually made in a Michaelis tank with nitrogen aeration, and the experimenter adopts the final stable potential following an initial sharp drop in Eh and some fluctuation. With fermented liquor if the activity of the bacteria happens to be weak, then one must consider special measures when taking a sample to make sure that the redox condition of the sample is not changed by coming into contact with air.

The culture potential of bacteria is generally measured by means of immersing a platinum electrode in the culture solution. Many improvements are also being attempted with respect to contact with the potassium chloride agar bridge (7), the aerator (7), simultaneous recording of pH and dissolved oxygen (8), and on other aspects (9)(10) of the method of measurement.

3. Growth and ORP

The study of ORP in the field of bacteriology originally consisted of measuring the potential of the bacterial culture environment, that is, one was interested in the effect of ORP as an environmental factor on bacterial growth, and also in the variation in the ORP of the culture resulting from metabolism.

A. Limiting Potential for Bacterial Growth

It is quite natural that the study of the limiting potential for bacterial growth had for its first subject anaerobic bacteria since as early as 1929 it was generally concluded anaerobic microorganisms required for their growth a strongly reducing environmental condition, and ORP was employed to indicate the reducing character of the culture or environment. Now the ORP of a culture is determined by the oxygen pressure and the redox system in the culture. The experiment is generally performed with aeration with a mixture of nitrogen and oxygen, or also with the addition of a particular redox system or systems. The results obtained have been that with eight cultures of spore-forming bacteria, growth would be inhibited by rH larger than 12 (Eh -60 mV, pH 7.0); for Clostridium tetani the maximum ORP for growth being rH 15 (Eh +36 mV, pH 7.0) or rH 17 (Eh +110 mV, pH 7.2); for Cl. sporogenes, rH 14; for the Buthanobacterium clostridium, +300 mV (pH 6.8) with nitrogen-oxygen aeration and +335 mV (pH 6.8) with the addition of potassium ferricyanide; for Cl. Welchii, +125 mV (pH 7.2); for Bacerodes vulgatus, +150 mV (pH 6.6) (9); for Cl. saccharobutyricum, +116 mV (pH 6.8) (11); and for Cl. perfringens, +230 mV (12). These results show that there must be some low ORP before anaerobic bacteria will grow. On the other hand, aerobic bacteria can grow even if the ORP is high, and it has been pointed out (13) that Asp. niger and other bacteria often grow better the higher the ORP. Exceptions to this case of course are aerobic bacteria such as Diplococcus pneumoniae, some hemolytic Diplococci, Rhizobium, Bacillus megathrerium, etc., which require a lowering of the ORP for growth to start. That is, in these cases if the ORP is maintained higher than a certain level after inoculation of the culture, by means of oxidizing the culture or by means of addition of a redox system or systems, then so long as the bacteria is incapable of reducing the system and lowering the ORP, growth will be inhibited despite the abundance of oxygen. It is interesting to know that a purely physical quantity such as ORP is closely related to the phenomenon of

biological growth, but we are still left with the problem of truly understanding this relation. As a start we can probably stipulate that the redox system generates some electromotive force which can be observed as a potential at the electrode, and that the ORP measured is an indicator of the oxidation-reduction state in the culture. As one aspect of the problem, the metabolism of a bacterial system depends on the enzymes associated with the system, and it is known that while aerobic bacteria can work on redox systems with high standard potentials, anaerobic bacteria can work only on redox systems with low potentials. Also aerobic bacteria at the same time must first work on low level oxidized forms. In order to work on systems with low potentials, the potential of the environment must be brought down to below some level. For instance, the reaction which synthesizes alanine from pyruvic acid and ammonia in the presence of glucose operates only when rH is less than 20, and it is assumed that at higher potentials, the glucose is oxidized by oxygen and there is no hydrogen available for the synthesis of alanine. Also under high ORP conditions, the SH radical cannot remain in its reduced form and therefore the various activated enzymes cannot function (14). Examples such as this reveal the significance of the limiting potential. It should also be mentioned that the potential of the environment poses a necessary but not necessarily a sufficient condition for the functioning of the various biochemical reactions involved in the growth process.

B. Culture Potential

With both aerobic and anaerobic bacteria, a reduction process occurs in the culture before the start of any growth. That is, the ORP drops and it reaches a minimum either at the beginning or half way through the period of maximum growth, after which the potential may either rise or remain near the minimum level. With bacteria such as Diplococcus pneumoniae, hemolytic Diplococci, or Lactobacillus casei (15) which produce and accumulate hydroxides, the potential progressively rises. Generally speaking, the minimum potential of aerobic bacteria is higher than that of anaerobic bacteria. In Table 1 are given the potentials observed with the cultures of various fermentation microorganisms. It is seen that the ORP is an aid to classification in the case of Lactobacilli and Aerobacter. In the case of Cl. butyricum, it has been reported (10) that a particular strain which sporates faster than others can be differentiated by the fact that its ORP has a slow decline and a rapid rise. Little is known of what electromotively active redox system is responsible for the culture potential observable at the electrode. In any event, it can be assumed that the electromotive redox system reflects the combined effect of the redox systems involved in the metabolism of a particular bacterial strain and the concentrations of these systems. In other words, the culture potential is a function of some group of redox systems and their interaction. For instance, there is a distinct difference in potential

between the Acetobacter action which oxidizes acetic acid and that which oxidizes ethanol, and similarly there is a difference in potential between the respiration of yeast and the alcohol fermentation with yeast.

Table 1. Culture Potential of Fermentation Microorganisms

NOT TRANSLATED

Legend to Table 1, page 7:

1. Top yeast
2. Yeast
3. Beer yeast, baker's yeast
4. Lactobacilli
5. Aeration with air
6. Air
7. Aeration
8. Oxidation of acetic acid,
   Ethanol - acetaldehyde - acetic acid
9. Anaerobic
10. Aerobic
11. Suspension
12. Culture containing biotin
13. None
14. Glucose
15. Mannitol
16. Arabinose
17. Calcium gluconate
18. Cane sugar (N_2 aeration)
19. Glucose (suspension)
20. *R means redox reagent; E is electrical measurement; and E + R is electrical measurement with redox reagent added.

C. Aerobic and Anaerobic Bacteria

The difference between aerobic and anaerobic bacteria can be more exactly defined by the limiting potential at which the bacteria can commence growth. For instance, aerobic bacteria can grow with the culture ORP at the normal level (higher than +200 mV, normally +300 mV, pH 7.0), while anaerobic bacteria will not grow unless the ORP is below a certain level. Normally the starting level must be about E_h = -200 mV. As another example, Escherichia coli has standard potentials for both a high and a low level redox system, and as a result, growth can occur at any ORP level, and the culture potential can drop to nearly rH = 0. Since anaerobic bacteria will grow only while the ORP is low, a favorable culture environment can be created by lowering the ORP by the addition of substances such as liver extract, Na-thioglycollate (21), $Na_2S_2O_4$ (22), and ascorbic acid (10)(23). Or on the other hand, one can mix aerobic and anaerobic bacteria, in which case aerobic growth will first dominate, but with the lowering of the culture ORP, the growth of anaerobic bacteria can also occur as the ORP drops below a certain level. Examples of such combinations are Clostridium butyricum and karakusakin /Translation unknown but probably a cellulase-producing bacteria./ (24), Bacteroides vulgatus and Alcaligenes fecalis (9), and Lactobacillus and Aerobacter cloacoae (25). It is reported that 0.1-0.2% of agar is added to an anaerobic culture medium (26) sometimes which apparently helps

keep the ORP low (27). As just described, bacteria, through their metabolism processes, act as reducing agents, and as a result, when a culture medium is inoculated with this bacteria there will be stress between the ORP of the medium and the reducing action of the bacteria. It has been known for a long time that bacterial growth improves with the number of bacteria in the culture medium and this can be easily explained by the fact that the reducing action of many bacteria can combine to rapidly lower the ORP in the vicinity of the cells, and through redox reactions this condition spreads itself throughout the medium. With few bacteria, growth may or may not occur depending on whether the metabolism of the few bacteria is adequate or not with respect to lowering the ORP of the medium. From such a viewpoint, the addition of agar should prevent the dispersion of both the bacteria and its reducing action, and thus improve conditions. With a sufficient concentration of bacteria, it should be possible to inoculate a medium with a higher than limiting potential, and still have growth. For instance, in the transplanting of Cl. butyricum or related buthanol-producing bacteria, the seed concentration is 2% or more so that growth is possible even in an aerobic condition. In contrast to this, a single spore will not grow readily unless the ORP of the medium is reduced, and it was found that the addition of a piece of liver was quite effective (26).

It should be an interesting problem to see what, if anything, would happen to aerobic or anaerobic bacteria if the culture condition was changed from aerobic to anaerobic or conversely. Naturally the effect would depend on the type of bacteria as well as the degree of aeration. In the case of Bacteroides vulgatus for example, the ORP of the medium rises considerably as the oxygen-to-nitrogen ratio in aeration is increased, but no harm is experienced so long as the growth is vigorous. When the ORP is further increased to -250 mV (pH 5.6-6.4), however, both growth and acid formation stop, and the culture loses its activity. With Cl. butyricum, aeration causes a rapid rise in the ORP and autodigestion sets in, but when the aeration is discontinued after about five hours, the ORP drops again and normal logarithmic growth without any phase lag is observed (10). With Cl. acetobutylicum (29), Cl. saccharobutyricum (30), or Cl. sporogenes (30), aeration unless it is excessive hinders growth but not fermentation. With the first of the above species, the flavin in the fermenting solution is always in its reduced form irrespective of whether there is or is not aeration, and the ORP is low. It is seen that there is considerable difference between the concept of aerobic in the sense of aerating a culture and the concept of aerobic or anaerobic from the viewpoint of the ORP. This difference is even more pronounced than in the previously described case of an anaerobic medium under aerobic conditions. With the anaerobic E. coli, aeration will raise the ORP to a level higher than 500 mV, but growth is improved rather than inhibited because of the change from fermentation to respiration under such conditions (10). In contrast, the

aerobic Bac. megaterium can be started at +30 mV (pH 7.0) and after about six hours the potential will have dropped by 270 mV and growth will start. If at this time nitrogen is introduced, the ORP level drops again slightly and growth stops (10).

(Continued to Part II)

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APPLICATION OF OXIDATION REDUCTION POTENTIAL TO FERMENTATION

PART II

4. The Standard Potential of Various Redox Systems

Considerable data are now available with respect to the standard potential of various redox systems not to mention the systems within living organisms. Examples of biological redox systems are shown in Table 2. The standard potentials of FAD and DPN differ depending on whether the compounds are free or bound to some protein. In the case of FAD, the potential can also differ with the protein combination, for instance there is a difference of 400 mV between old yellow enzyme and xanthine oxidase. It should also be noted that the ORP is linearly related to the free energy change associated with redox reactions, that is $-\Delta F = nFE$, and therefore a correspondence between this free energy and the standard potential of a redox system can be established. One can therefore estimate the energy release accompanying the transfer of hydrogen from one system to another by taking the difference in values between the two systems. For instance in alcohol fermentation, roughly 9,000 calories of free energy is released per molecule of alcohol, and if this is respirated, about 47,000 calories is produced.

5. Biochemical Function of Microorganisms and ORP

Considering the arrangement of redox systems in Table 2, one can assume that there will be some suitable ORP range for the proper functioning of a given set of redox reactions for a selected set of redox systems. In fact, it has been shown that such ORP ranges exist for example for aerobic reactions such as involving glucose, yeast extract, and redox dye, or for various other enzyme reactions. Other examples are the -350 mV potential required for gas generation with E. coli (36), the rH 22-24 required for nitrogen fixation with Azotobacter agile (16), the rH 7-8 required for ethanol oxidation with Acetobacter, and the rH 20-22 needed for the oxidation of acetic acid (17).

6. Analysis of the Mechanism of Fermentation

We would like to consider the standard potentials of redox systems tabulated in Table 2 not only as the results of some analysis but as the basis for obtaining a better over-all understanding of apparently orderly biological functions. For instance in a bacterial culture, one notes that the time variation of the potential of the culture must reflect the dynamical changes occurring in the culture, and therefore if one can construct step-by-step the redox processes presumably responsible for the changes in culture potential, there is the possibility that the mechanism of the entire redox metabolism system can somehow be clarified. Although the exact nature of the culture potential is not

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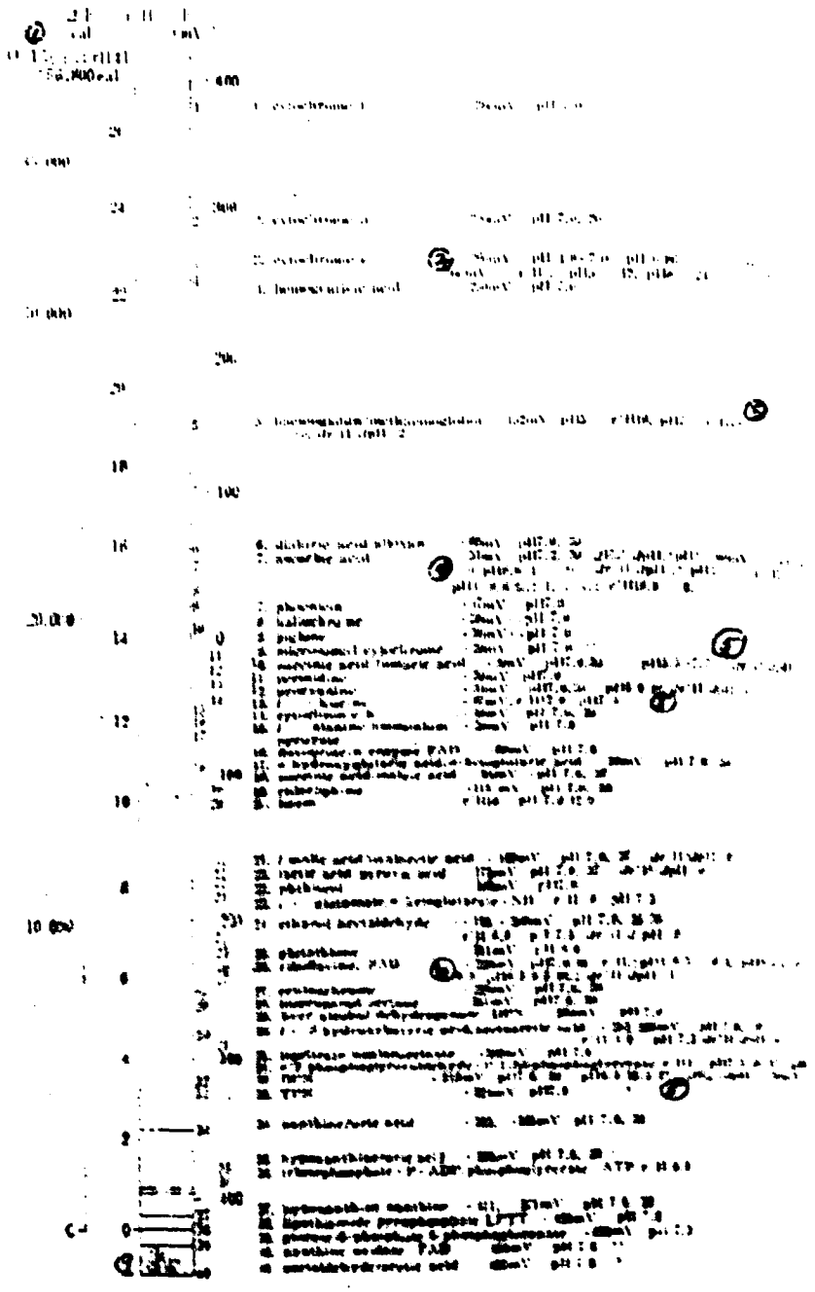

Legend to Table 2, page 13:

1. rH = 41 for O_2 1 atm pressure
2. (+260 mV) (pH 4.0-7.0); for pH 6.86, dE_o'/dpH = -60 mV (31); r'H = 19(pH 5), 24(pH 8), thereafter $dr'H/dpH$ = 0
3. r'H = 16(pH 5), 19(pH 7), and in between $dr'H/dpH$ = 2
4. (+51 mV) (pH 7.2, 30° C), dE_o'/dpH varies from 60 mV to 30 mV at pH 4 and remains constant to pH 8.6; $dr'H/dpH$ = 0 with r'H = 13 up to pH 4, $dr'H/dpH$ = 1 in range pH 4-8.6, but 0 again for higher pH with r'H = 8.8
5. range
6. (-220 mV) (pH 7.0, 30°), r'H = 6.4(pH 1-6.5), 9.3(pH 9.5-13.0); $dr'H/dpH$ = 1(pH 6.5-9.5)
7. H_2 excess potential

yet clear, it at least can be assumed that this potential is due to some electromotive redox system connected with the microorganism, and that this particular redox system is capable of moving in and out of cells such that this system reflects the ORP inside of a cell. Now the potential inside a cell depends on the various redox systems existing in the cell. For instance assume that there are two redox systems in the cell, one with a high standard potential and the other with a low potential, and also assume that oxidation reduction occurs through the electromotive redox system. Then if the reduction by the low potential system and the oxidation by the high potential happen to equal each other, the ratio of the oxidised form to the reduced form in the electromotive redox system will remain constant, and the measured ORP will remain at some level within the potential range for this system. On the other hand, if either the high or the low potential redox system in the cell tends to dominate over the other system, then there should be a corresponding rise or decrease in the ORP. Here the concentration of the electromotive system should be low for better sensitivity, since the shift in potential would be small for high concentration. The initial drop in culture potential normally experienced stays within the limits of the potential range for the electromotive system, the level differing with the relative intensities of the high and low systems in the cell. Even if the high and low potential systems undergo oxidation reduction reactions through a non-electromotive redox system but say that an electromotive redox system existed separately, then so long as the potential ranges are not dissimilar, the state of the former will be reflected in that of the latter almost instantaneously, and the apparent result will be the same. Typical examples of the above situation are seen in alcohol fermentation and in homolactic acid fermentation. When not just two but also other redox systems work on the electromotive system, it is assumed that the measured potential reflects a compounded effect. Therefore in systems which are not as complicated as when respiration is occurring or which are on the other hand not as simple as in alcohol fermentation, one can often deduce from the potential curve what redox systems tend to be more dominant in their effect

at what times. This is one way of using ORP. An application of this method to buthanol fermentation was already reported in this journal (5), and although details will not be repeated here, let us suffice it to say that aeration with nitrogen during the ORP measurement was necessary since otherwise the generation of hydrogen gas would have dropped the culture potential down to and held it down at -420 mV (pH 7.0). Also with alcohol fermentation, the process becomes complicated when rice malt is used on a starch substrate instead of on sugar. A report on this subject may be found in last year's issue of this journal (2). In a rapid yeast wort making process [Process peculiar to Japanese rice wine making. Reader should consult reference on Japanese brewing practice.], the potential rises and stays at the level rH 19-21 up to the stage in the process where saccarification and lactic acid formation are most active, but it then declines with the addition of yeast and as fermentation becomes active. It should be noted here that since yeast has a redox system with a high standard potential and also since there is a high potential redox system contained in the rice mold amylase, the culture potential does not immediately drop. About a day after the batch has neared the end of its fermentation and has been cooled, the potential reaches a minimum of rH 11, but it slowly rises again during its period of storage. Similarly with the mash, after the third and final addition of ingredients, saccharification is fairly active for two or three days during which time the potential rises to about rH 13, but with fermentation taking over, the potential drops and reaches a minimum of rH 7 after the froth has subsided. However, with the fermentation nearing maturity, the potential slowly rises again, it rises sharply with the addition of alcohol, and later it also rises upon contact with the air at the top of the tank. Slow oxidation reduction reactions among the various systems tend to lower the potential, but filtration and removal of bottom sediment which allows air contact causes the potential to rise again. As just described, the progress of this parallel double fermentation process is clearly reflected in the variation of the ORP. Data are also available showing the difference in potential variation between successful and unsuccessful yeast wort cultures, but the details will not be given here for lack of space. On the subject of buthanol fermentation, there is also a study which attempts to relate the number of transplants of a culture to an increase in the rate of fermentation, by comparing the ORP values of such cultures, and the reader is referred to the original article (6) for details.

7. Study of the Movement of Microorganism Colonies

When there is a mixed culture consisting of various different types of bacteria or molds, it seems that the culture potential might be useful in determining which bacteria or mold might be dominant at any particular time. A good example of such a mixed culture is that consisting of rice mold and yeast in the brewing of Japanese rice wine. Here so far as the rice mold is concerned, the amylase produced by the

mold is the active component. There is an experimental report giving culture potentials measured during the making of yeast wort by the sanpai [Translator's note: Abbreviation of yamaoroshi haishi or a simplified yeast culture-making process where the yamaoroshi or grinding step is omitted.] process (2). According to this report, the growth of Lactobacilli and nitrate-reducing bacteria causes the potential to drop. The action of these bacteria weakens as saccharification progresses and the potential rises to a maximum, but it drops again as fermentation takes over.

8. The Transformation of Fermentation by the Addition of Redox Dyes

Redox dyes are used not only for dying specimens and as an ORP reagent, but they are also used quite often in the study of various dehydrogenases and in the study of so-called methylene blue respiration (increase in oxygen consumption). Examples are the increase in oxygen consumption due to flavin in Lactobacilli metabolism; a similar type of increase in oxygen consumption due to a redox dye in a system of baker's yeast; or in the case of yeast extract solution, the inhibition of fermentation due to a redox dye with a relatively high standard potential; or the promotion of aerobic decomposition of sugar by a redox dye with a fixed standard potential; or with Azotobacter, the inhibition of respiration by a redox dye (37). There have also been attempts to use redox dyes for the classification of bacteria such as for example the attempt to isolate different species of Rhizobium by the reducing action on dyes with different standard potentials (38). It has also been shown that lipid formation by yeast is aided by the addition of quinone derivatives (39). Mentioning other phenomena which are somewhat related to fermentation, one has the fact that the absorption and release of H^+ and K^+ in yeast is affected by a dye (40), and also the fact that acid formation in a culture inoculated with E. coli is inhibited by the addition of a dye with a high standard potential (41). Also in a culture of Acetobacter xylanogenum, the oxidation of sorbitol to sorbose is promoted in the deep as well as the shallow parts of the culture medium with the addition of methylene blue of suitable strength (42). It has also been shown that bacteria belonging to the genera Clostridium or Eubacterium, which are known to produce acetic acid and another acid or propionic acid and lactic acid, can be developed into a stable mutant which produces nothing but acetic acid and lactic acid by means of transplanting the bacteria 20 to 40 times in a culture medium containing a redox dye (43). There is also a paper reporting that the addition of flavin or phenosafranin to the extract of Lactobacillus delbrueckii will produce dismutation of pyruvic acid, but it does not say that this method has been used effectively in the actual fermentation process. From the standpoint of the standard potentials of redox systems, if we consider fermentation to be the oxidation reduction reactions involving more than simply two redox systems, then taking two systems which have a potential difference and injecting a redox system,

which standard potential lies between the potentials of the two systems, the oxidation reduction reaction between the two systems should be facilitated. For instance in buthanol fermentation, the main system responsible for producing buthanol has a standard potential at about rH_0 = 4.8 while the acetone producing system is associated with the system at rH_0 = 0. Now let us assume that the introduction of a redox system, for instance a dye, with rH lying somewhere between rH_0 = 0.8 (hydrogen donor) and 4.8 would increase the yield of lactic acid and buthanol. Actually this is found to be the case as depicted in Table 3, where it can be seen that the acetone forming process has changed over into the buthanol forming process.

Table 3. Change in Solvent Ratio in Buthanol Fermentation Due to Redox Dye Addition

	Dye			
Solvent (% yield to sugar consumed)	None	Neutral Red (20) (44) (E_0' = -325 mV) 6.0 mM	Benzyl Viologen (E_0' = -359 mV) 0.4 mM	Methyl Viologen (E_0' = -446 mV) 1.2 mM
Acetone	9.0	3.8	1.8	1.8
Buthanol	19.4	29.0	29.0	28.7
Ethanol	3.8	1.5	3.4	4.2
Total solvent	32.2	34.3	34.2	34.7

Remarks: Washed live bacteria used. Glucose substrate concentration 3-4%, fermentation temperature 37° C, sugar consumption always more than 98%.

As to how this transformation was brought about, there is no decisive explanation at present, but one line of reasoning could be as follows: Say that the flavin which was added to the extract of Lactobacillus delbrueckii is responsible for the hydrogen transfer between the FAD of the pyruvic acid dehydrogenase and the FAD of the lactic acid dehydrogenase (45), and that the hydrogen transfer between the hydrogen donor system and the buthanol forming system was aided by the dye added to the system in a manner resembling that in which hydrogen is consecutively transferred even down to the oxygen level by the action of FAD and flavin added to DPN oxidase, the latter obtained by filtering a culture of Cl. kluyveri (46); or from another point of view, it could also be reasoned that potential of the environment was strongly attracted toward the standard potential of the buthanol forming system.

9. The Practical Application of ORP

Here are given some examples of the use of the ORP in the fermentation industry.

For instance in buthanol fermentation, when corn mash is acid-hydrolized in a copper vessel, the existence of copper ion sustains a high ORP level which is unfavorable to fermentation. It is therefore necessary to add powdered iron, which has a low standard potential, in order to reduce the ORP and to allow normal fermentation to occur (47). In this fermentation, two minima are experienced in the culture potential, and it has been pointed out (19) and also proven (6) that the culture at a time of low potential forms a good starter.

The ORP of grape wine during the course of fermentation and of the final product has been measured and studied often (48). A typical example of the change in ORP in this case is as follows: As a grape starts to ripen, rH = 22.7-25.0, but it declines to 18.0-20.0 as the fruit ripens (49). When the grape is crushed, the contact with air results in a slight increase in the ORP to 22. At the stage of roughly maximum saccharification, the potential is about 14, and this drops to a minimum of about 7 at the point of maximum fermentation activity. At the end of fermentation, the value rises again to 9, and it jumps to 15 as the wine comes into contact with air during transportation. When blended with brandy, pasteurized, and cooled, the value is about 18 to 20, but this gradually declines during storage and aging (50). If rH should rise during storage, this often means a loss of color or a brownish discoloration and the development of murkiness from suspended iron rust. It is said that rH ranges between 16.0 and 21.5 for a quality product, but that a spoiled product will smell of the urine of vermine, taste strange, and the value of rH will often exceed 25 (4). Therefore in order to keep the ORP down, ascorbic acid to the amount of 20 to 100 mg/l is often added after the main fermentation, and it is reported that this improves both the bouquet and the clearness of the wine (51). There is also a patented process for adding pyroacemic acid-lactic acid, a low standard potential redox system, for accelerating the aging (52). There is also a report on aeration with hydrogen gas, which is said to decrease rH by 5 units down to about 16, and that the bouquet was greatly improved upon tasting after about 25 days (53). As just described, it can be seen that the ORP can be used as a guide for the proper management and control of the fermentation process as well as for improving the quality of the end product or products (54).

In making whisky, it has been reported that the ORP decreases during storage and at the beginning of the aging process.

With beer, the value of rH drops to a low of around 7 to 10 during the stage of most active fermentation (55). Following this period,

if care is not exercised, rH can rise to about 16 to 17 and cloudiness will occur. At this rH level, yeast, particularly wild strains grow well (56), protein oxidation will also occur and contribute to the cloudiness. It is also said that the so-called "sunlight odor" will be enhanced if rH is higher than 13. Another report states that the change in taste due to pasteurization is due to the oxygen concentration preceding the pasteurization, and that this deterioration in taste can be avoided by the addition of a reducing agent prior to the pasteurization process. In any event, it is important to avoid contact with air as much as possible and to prevent any rise of the ORP. At the same time, one must consider not only the value of rH but also buffering of the potential, that is a beer with a large buffering capacity is desired. The reducing power of the beer, including buffering capacity, is measured by the Indicator Time Test (abbreviated ITT; measurement of the rate of reduction with a high standard potential dye added to the substrate). With a carefully produced product, ITT is about 100 seconds, but with poor control and exposure to air, ITT can sometimes exceed 1,000 seconds. Dark beer is fairly abundant in potential-buffering matter, and upon investigating oxidation reactions in the dark beer which apparently do not alter the level rH = 10, it was found that an activated sugar with a standard potential of around 9 or 10, which sugar is produced during the processes of making wheat germ and also malt liquor, combined with oxygen to become the oxygen source for the yeast (57). It is also stated that an excess of this activated sugar can result in the precipitation of cysteine, glutathione, and redactone.

As it has been previously reported in this journal, attempts are being made to utilize ORP in the management of fermentation and the end product in the brewing of Japanese sake or rice wine (2). Generally the ORP of synthesized wine is high with rH about 18.5-21.0, while the rH of true wine is somewhat less. With new rice wine, rH is about 11.0-13.5 before pasteurization, with the value rising to about 15.5 to 17.0 after six to nine months after pasteurization. In synthetic wine making, makers are allowed to introduce up to 5% in terms of rice of yeast wort, mash, and rice wine, but quite often the bouquet changes for the worse after this addition. This can be prevented by ORP control, that is, since the mixing of a large amount of high ORP synthetic wine with a small amount of low ORP additives would normally result in a sudden change in the ORP of the combined system and a simultaneous deterioration of bouquet, one can consider methods to avoid this sudden change in the ORP such as for instance blending the two after the synthetic wine has fully matured.

In alcohol fermentation, it is said that a high ORP reduces the fermentation activity of the yeast and the alcohol yield (58). Even in the refining of alcohol with potassium permanganate, a study has been made on the change in ORP and the suitable conditions for this process (2).

Although this is not true fermentation, a study has been done on the ORP function in the manufacture of black tea, that is, the functions of various components during the oxidation process have been investigated (59).

In another area of research, work is being undertaken to study the effect of antibodies by analysing the ORP curve of cultures of disease-causing microorganisms. The curves are assumed to be characteristics for specific antibodies, and it is reported that more direct knowledge can be obtained in this manner than through cellular measurements (60). It has also been reported that methylene blue enhances the antibiotic action of penicillin, and that the addition of any dye with a higher or lower standard potential inhibits this action, so that now a relationship has been established between ORP and the effectiveness of penicillin (61).

With respect to germicides, the effect of low ORP on germicidal effect has been argued (62), and it has been pointed out that the germicidal effect of chlorine or chloridated compounds parallels the potential effect (63).

10. Conclusion

Through this rather brief review of the use of ORP in research on fermentation, one can see that we have just started in this field of investigation. Since oxidation reduction is the principle reaction in fermentation, the ORP approach to investigating the energy metabolism in fermentation reactions and the mechanism of such reactions would seem to be promising. Within the framework of ORP, it is also possible to collate the information on individual redox systems, and to obtain a synthetic picture of the entire structure. Naturally it would be quite questionable to nonchalantly apply our simple knowledge to the complex systems in a living cell, but at the same time, our efforts must be in this direction. With respect to application of the ORP approach, there are without doubt many areas of research which have not yet been touched, and the author hopes that the reader will show interest in and undertake research in this field.

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