1941

The polarographic method in the investigation of several Acetobacter suboxydans fermentation products

Eakin M. Glymph
Iowa State College

NOTE TO USERS

THE POLAROGRAPHIC METHOD IN THE INVESTIGATION OF SEVERAL *ACETOBACTER SUBOXYDANS* FERMENTATION PRODUCTS

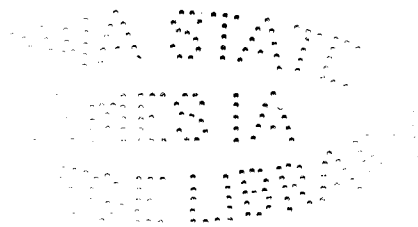
by

Eakin M. Glymph

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject Biophysical Chemistry



Approved:

Signature was redacted for privacy.

In charge of Major work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College

1941

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. E. I. Fulmer for the suggestion of the problem for the investigation and for his helpful advice and criticism during the course of the study and the preparation of this thesis. The assistance of Mr. J. J. Kolfenbach in conducting certain laboratory experiments is very much appreciated.

TABLE OF CONTENTS

INTRODUCTION

The role played by catalytic dehydrogenation in biological oxidation was first pointed out by Heinrich Wieland (1922). In his theory he postulated that respiration proceeds through an activation of hydrogen by specific dehydrogenases and a subsequent transfer of the labilized hydrogen to oxygen as a hydrogen acceptor.

Opposed to this conception of the mechanism for respiration was Warburg (1928) who believed that the activation of oxygen, not hydrogen,was of primary importance. The most generally accepted theories of today embrace both of these mechanisms. It is well known that all oxidations in which oxygen acts as a hydrogen acceptor do not proceed according to the same mechanism and that in many cases the activation of both the substrate and the acceptor is necessary. Even this activation does not always prove satisfactory, and we have entering as a further complicating factor many intermediate hydrogen carriers. According to Oppenheimer (1939), the hydrogen transfer represents the disturbance of an equilibrium whereby the hydrogen of an organic compound, called the donor, is first labilized and then shifted to another compound, the acceptor, in accordance with a thermodynamic potential. If the process involves the final transfer of hydrogen to molecular oxygen as an acceptor, it is called

respiration; on the other hand, if some other substance accepts the labilized hydrogen, it is called fermentation. In either case the general mechanism may be represented by the following simple equation:

$$DH_2 + Acc \longrightarrow D + AccH_2 \qquad (1)$$

The above reaction can proceed spontaneously from left to right only under those conditions of temperature and concentration which will cause it to yield energy. It is from such dissimilation reactions that bacteria obtain energy for growth, reproduction, movement, maintenance of body temperature and the other life processes. Even though the thermodynamic potential may favor the occurrence of a chemical reaction it may be opposed by kinetic hindrances. The reaction can, however, be speeded up greatly by inserting reversible oxidation-reduction systems capable of accepting hydrogen from a donor of lower potential and giving it up again to an acceptor of higher potential.

$$DH_2 + Cat. \rightleftharpoons D + Cat.H_2 + Acc \rightleftharpoons D + Cat. + AccH_2 \qquad (2)$$

By means of this reversible action of the catalyst, the "energy barriers" are destroyed and the reaction proceeds at a more rapid rate.

It might be pointed out that in biological processes a reaction having a thermodynamic potential that is too steep is likely to be inhibited. The interposition of catalysts of intermediate potential causes a more stepwise yielding of

energy which seems more favorable to the living organism. Many of the sugars and their intermediate products of metabolism have very high negative potentials and are not capable of giving their hydrogen directly to molecular oxygen. A whole series of catalysts with graded potentials is required in this case in order that the hydrogen may be transferred from sugar to oxygen. The words of Oppenheimer and Stern (1939) are applicable here, "Considering biological oxidation from the standpoint of energetics we find that the hydrogen in the course of its transfer follows a path prescribed by the thermodynamic potential: starting at a high reduction potential level it sinks to a low level, loosing constantly free energy and performing as much work as possible in accordance with the energy requirements of cell metabolism. The energy is liberated by the chain of coupled reactions which we call oxido-reduction processes. Dehydrogenation, i.e., separation of hydrogen from a donator, in the physiological temperature range is almost always an endothermal process. Only its coupling with hydrogenation yields an over-all reaction with liberation of energy". (page 4)

Treating each of these donor-acceptor reactions separately as an isothermal and reversible process one can determine, from the oxidation-reduction potential of the system, the amount of free energy yielded in each successive transfer of hydrogen. The process may begin with a substance of high re-

ducing power such as a sugar. Oxidation-reduction systems of suitable potentials are provided as catalysts and the hydrogen is first handed over to a system of less negative potential than that of the sugar. The normal potential of zero (at pH 7) is finally reached and the positive range is entered where the respiratory ferment and cytochrome come into play in preparing the hydrogen for its final transfer to oxygen, thereby terminating the chain. In anaerobic metabolism no high potentials are reached as the hydrogen finally is linked to an organic compound which is stable under the existing conditions. The ultimate aim of cell metabolism, however, seems to be the final transfer of organically bound hydrogen to oxygen with the formation of water.

The oxidative dissimilation of alcohols by the acetic acid bacteria offers an interesting case of cell respiration. These bacteria, especially the Acetobacter suboxydans, are noted for their mild oxidative action on alcohols resulting, for the greater part, in a simple dehydrogenation of the alcohol to a ketone or of an aldehyde to an acid. In fact, it was the action of the acetic acid bacteria in converting ethyl alcohol into acetic acid which led Wieland to extend his theory of catalytic dehydrogenation to biochemical oxidations. For example, the Acetobacter suboxydans is able to transform glycerol into dihydroxyacetone with a very high percentage yield. According to the theory of Wieland, this oxidation in-

volves a direct transfer of the activated hydrogen of the substrate to molecular oxygen as an acceptor.

$$\text{glycerol} \xrightarrow[\text{dehydrogenase}]{} + O_2 \rightarrow \text{dihydroxyacetone} + H_2O \qquad (3)$$

The system is possibly much more complex than the above equation would indicate. Shibata (1935) has pointed out that with the acetic acid bacteria the activation of hydrogen alone is sufficient only when an aerobic dehydrogenase and an abundant supply of oxygen are present. When these requirements are not met the cytochrome system may play an important role.

If we consider oxidation-reduction in the light of electron transfer, the activation of the hydrogen consists in the labilization of hydrogen and subsequent liberation of a hydrogen ion, the electron being transferred to the oxidizing agent or acceptor. To represent the transformation of glycerol to dihydroxyacetone according to this mechanism we might write

$$CH_2OHCHOHCH_2OH \rightleftharpoons[\text{dehydrogenase}] CH_2OHCOCH_2OH + 2H^+ + 2e \qquad (4)$$

It is obvious from the above equation that an electrode may be made an acceptor in place of oxygen or some other substance; likewise, by reversing the process the electrode may be made to serve as a donor of electrons.

If a reversible potential for the above reaction could be measured at a dropping mercury electrode, one would have a means of determining the free energy of hydrogenation or dehydrogenation (the free energy for the two processes should be the same and only the sign should vary depending on whether

the electrode was the cathode or anode) by use of the relationship, $\Delta F=-nFE$. From these data one could calculate the over-all energy yield for the conversion of glycerol to dihydroxyacetone when oxygen acts as a hydrogen acceptor by the following coupled reactions:

$$CH_2OHCHOHCH_2OH \text{ —— } CH_2OHCOCH_2OH + H_2 \text{ , } \Delta F = X \qquad (5)$$

$$H_2 + 1/2\ O_2 \text{ ——— } H_2O \text{ , } \Delta F = -56{,}560 \qquad (6)$$

$$CH_2OHCHOHCH_2OH + 1/2\ O_2 \text{ —— } CH_2OHCOCH_2OH + H_2O,$$

$$\Delta F = X + (-56{,}560) \qquad (7)$$

An organic reduction such as that mentioned above may be a truly reversible system yet, due to its electromotive sluggishness, no stable and reproducible potential may be observed for a given ratio of oxidant to reductant. If a reversible enzyme system could be isolated and made to function between the electrode and the solution bearing the reducible substance, the transfer of electrons between the oxidized and reduced forms might be catalyzed, allowing the rapid establishment of equilibrium and resulting in a stable and reproducible potential measurement. The enzyme system functioning in such a manner should be only slightly poised, that is, the oxidation capacity of the enzyme should be of such a magnitude as to allow the establishment of equilibrium without appreciably disturbing the ratio of the oxidized to the reduced forms of the system being studied.

The purpose of the present investigation was to reduce at the dropping mercury cathode a series of ketose compounds produced by the action of Acetobacter suboxydans upon the corresponding polyhydric alcohols. The series studied included dihydroxyacetone, l-erythrulose, l-sorbose, d-tagatose, and a "ketose" formed from i-inositol; the compounds were reduced at various concentrations and pH values. An attempt was made to determine the effect of reduction in the presence of bacterial juices. It was planned to arrange the compounds in the order of their ease of reduction and to determine whether or not this arrangement has any thermodynamic significance.

HISTORICAL

The work of Clark and his collaborators (1928) gave impetus to the determination of oxidation-reduction potentials in organic chemical systems, especially those systems of biological interest. These investigators determined the E_o for a number of reversible dyes by potentiometrically titrating the leuco forms of the dyes with oxidizing agents and the oxidized forms with reducing agents. Among the dyes studied were several sulfonates of indigo, methylene blue, and a number of differently substituted indophenols. The dependence of E_o' on pH for the different cases of organic reductions was clearly emphasized.

Where electromotively active systems are involved, the potentiometric titration method of determining E_o' is highly satisfactory. (An electromotively active system may be described as one which, when an indicator electrode is placed in a solution made up of a given ratio of oxidant to reductant, gives an instantaneous equilibrium potential.) The hemes and hemochromogens, flavins, certain animal and plant pigments, and several hydroquinones fall within this classification. Hematin, an iron-containing porphyrin in which the iron undergoes reversible oxidation-reduction between the ferrous and ferric states, has been studied by Conant et al. (1928). These workers titrated electrometrically the oxidized form of hematin using titanous tartrate as a reducing agent and concluded that the hematin

system behaved reversibly.

By the application of spectrophotometric methods to the study of the oxidation of hemoglobin to methemoglobin, Conant and Scott (1928) were able to show that the system hemoglobin/methemoglobin was reversible. Although they had difficulty in getting consistent and reproducible results, the E_0 value found for the system agreed favorably with the value obtained by the electrometric titration method. Schmidt (1938) has determined the potential of the above system using a reversible dye instead of the potentiometric technique. He allowed exactly known ratios of the oxidized to the reduced form of the dye to come to equilibrium with the hemoglobin/methemoglobin system. By having the dye in great excess he was able to poise the potential at different levels. The hemoglobin was determined by the Van Slyke method and the E_0 of the system calculated from the following equation:

$$E'_0 \frac{MetHb}{Hb} = E_{dye} - 0.06 \log \frac{MetHb}{Hb} \qquad (8)$$

Cytochrome c is another metalloporphyrin which has been found to be electromotively active. Coolidge (1937) observed that his preparation gave very unsatisfactory potentials at an inert electrode; however, he was able to determine an average value for E_0. Statz, Sidwell, and Hagness (1938) described a spectrophotometric method for measuring the oxidation-reduction potential of pure cytochrome c. The E_0 value obtained was found to agree closely with that obtained by Wurmser and Wurmser

(1938) who used the potentiometric method

The organic chemical systems whose oxidation-reduction potentials may be accurately determined by direct potentiometric measurements are limited. There are, however, a relatively large number of electromotively sluggish but truly reversible systems whose potentials may be determined by more indirect methods. Most of these indirect methods require the use of dyes.

Oxidation-reduction indicator dyes have been used in biological oxidation studies in a number of interesting ways. Needham and Needham (1925) attempted to measure the average oxidation-reduction potential within an intact living cell using an indicator dye. In one experiment these investigators, with the aid of micromanipulators and a micro-injection pipette, were able to inject the oxidized form of a buffered indophenol dye into an amoeba. It had been found by preliminary experiments that this dye was not completely reduced within the cell interior. When equilibrium between the dye and the cell interior was established the color of the amoeba was compared with micro test tubes (of about the same thickness as the amoeba) containing various ratios of the oxidized to the reduced form of the dye. The potential of the dye ratio in the tube most closely approximating in color that of the cell was taken as the equilibrium potential within the cell. There are a few rather serious objections to this method: first, the dye may show a toxic effect, and second, the amount of dye necessary to cause visibility within the cell might

be great enough to cause an appreciable shift of the natural state of equilibrium.

A reversible dye may also be used as a "potential mediator". No stable electrode potential is observed when an indifferent electrode is immersed in an electromotively sluggish system made up of a given ratio of oxidant to reductant. If, however, the electromotively sluggish system is allowed to come to equilibrium with a small amount of an electromotively active dye, a stable potential will be registered. In such a case the amount of dye used should be too small to affect to any detectable degree the ratio of oxidant to reductant in the system under investigation. The potential measured at the electrode will be that of the dye, but since the dye is in equilibrium with the sluggish system it will also be the potential of this system. Many investigators of biological systems have employed this technique.

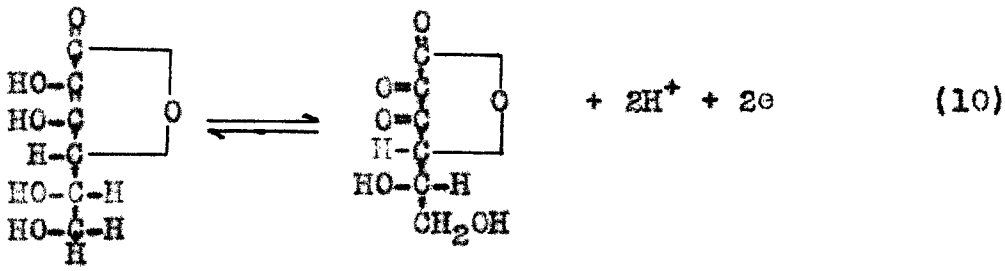
Reductone/oxyreductone is an example of the type of system described above. The equilibrium between the oxidant and the reductant may be represented by the following equation:

$$H-\overset{OH}{\overset{|}{C}}=\overset{OH}{\overset{|}{C}}-\overset{H}{\overset{|}{C}}=O \rightleftharpoons H-\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}-H + 2H^{+} + 2c \qquad (9)$$

Wurmser, Mayer and Crépy (1936) have determined the $\underline{E'_o}$ of the the above system employing the potentiometric technique in conjunction with an electromotively active dye. The study of the system involved many difficulties as the compounds are unstable in certain ranges of pH. The measurements were carried out

using Thunberg-Borsook tubes. The dyes found applicable were alizarin blue and the mono- and disulfonates of indigo. The above workers have shown that the $\underline{E'_o}$ shifts with pH as is predicted by the equation for the system.

Ascorbic acid and its first oxidation product form a system very closely related to the one just discussed. The equilibrium is represented by the following equation:



(10)

Ball (1937) reported that much of the earlier work done on the determination of the oxidation-reduction potential of the above system is unreliable. The values given, in many cases, have been based upon the concentration of the reductant alone, but such values are thermodynamically meaningless. Some other investigators have failed to give consideration to the instability of dehydroascorbic acid within certain ranges of pH. Ball has shown conclusively, however, that ascorbic acid is the reductant of a reversible system which is sluggish in electromotive activity. He titrated ascorbic acid with quinone and with potassium ferricyanide using certain dyes as "potential mediators". The $\underline{E_d}$ values were determined over the pH range 1.0 to 8.6 and the $\underline{E_o}$ value was found to be +0.390 volt as 30°C. The po-

tentials determined in the alkaline range were unreliable because of the instability of the initial oxidant. The value of $\underline{n}$ in the equation below was found to be two over the pH range studied

$$E = E_o - \frac{RT}{nF} \ln \frac{(Red)}{(Ox)} \qquad (11)$$

There is still another type of system which normally appears entirely irreversible, but which is reversible in the presence of the proper enzyme and an electromotively active dye. The succinate-enzyme-fumarate system has been reported to be an example of this type by Wishart (1923), Quastel and Whetham (1924), Thunberg (1925), and Borsook and Schott (1931). The enzyme preparation used by the latter investigators was a dehydrogenase extracted from beef heart and from beef diaphragm. The oxidation-reduction potential was determined potentiometrically using methylene blue in most cases as the "mediator dye". The succinate/fumarate ratios used were 9:1, 5:5, and 1:9, and the measurements were carried out over a pH range of 6.10 to 7.47. Equilibrium readings were taken after about one hour. The $\underline{E_o}$ value reported by Borsook and Schott for the reaction

$$\text{Succinate}^{=} \rightleftharpoons \text{Fumarate}^{=} + 2H^{+} + 2e \qquad (12)$$

was -0.437 volt at 25°C. Parks and Huffman (1932) have shown this value to be in excellent agreement with that calculated from thermal data.

Barron and Hastings(1934) have determined the oxidation-

reduction potential of the system lactate-enzyme-pyruvate potentiometrically over the pH range 5.73 to 7.79. This system was found to be reversible in the presence of the enzyme alpha-hydroxyoxidase when pyocyanine and cresyl violet were added as "mediators". The normal potential was found to be +0.248 volt at 35°C.

Since this thesis deals with the reduction of ketone groups to alcohols, it should be of especial interest to note that Wurmser and Wurmser (1936) have shown the isopropyl alcohol/acetone system to behave reversibly in the presence of the proper dehydrogenase enzyme. The alcohol dehydrogenase used was precipitated by saturated ammonium sulfate from Lebedew's yeast juice. By the electrometric measurement of the equilibrium potentials established between isopropyl alcohol and acetone at different ratios in the presence of the enzyme and an electromotively active dye, these investigators were able to show the value of E_o at 35°C. to be +0.176 volt. Several hours were required, however, in order for equilibrium to be established. Lehmann (1934) has demonstrated the reversibility of the system ethyl alcohol-dehydrogenase-acetaldehyde using a similar alcohol dehydrogenase prepared from Lebedew's dry yeast.

It would appear from the foregoing discussion that many other biological systems now considered irreversible might prove to be reversible in the presence of the proper enzymes acting as catalysts.

Although a large number of oxidation-reduction reactions of organic compounds have been shown to be reversible, by far the greater number fall into the irreversible classification. Much effort has been directed toward an electrochemical formulation of these irreversible processes, but the results in some cases are of doubtful value. Conant (1926) was the first to make extensive studies in this direction. He investigated the reduction of a few 1,4-diketones, certain azo dyes, and several nitro compounds. It was suggested by Conant that all oxidation-reduction processes might be reversible under the proper conditions and that in the final analysis the distinction between reversible and irreversible reactions is probably only one of rates.

In order to study the relation between the speed of an irreversible reduction and the potential of the reducing agent employed, Conant used a very interesting technique. An equimolecular mixture of the reducing agent and its oxidized form was placed in an electrolytic cell and the potential of the system was measured at an inert electrode in the usual way. The compound under examination was then introduced, and the shift with time of the potential of the reversible system was observed. The rate of change of the potential was then a function of the speed of the irreversible reduction. If no change occurred, it was concluded that there had been no reaction. By definition, then, the "apparent reduction potential" (A.R.P.) of a compound

was considered to be the potential of a "critical reagent" which would cause a twenty to thirty per cent reduction in thirty minutes.

It should be pointed out that the above reductions take place in two steps one of which is reversible, the other irreversible; therefore the over-all reaction must, of necessity, be irreversible. As emphasized by Conant, it is only when some irreversible process controls the amount of material undergoing a subsequent irreversible transformation that the speed of the reaction will be governed by the free energy or potential of the reagent.

Michaelis (1935) and later Michaelis and Schubert (1938) have investigated the reversible two-step oxidation-reduction of organic compounds. The intermediates formed during reduction when only one electron has been added are called semiquinones. These semiquinones are free radicals and as a result of their instability they readily undergo dimerization or dismutation. When dismutation occurs, one oxidized and one reduced molecule are formed from two molecules of the semiquinone. Michaelis (1935) has postulated that most organic reductions might conceivably proceed according to this two-step mechanism. He has summarized this conception of oxidation-reduction in the following statement:

> Were it not for the existence of intermediate radicals, we might say that oxidations in organic chemistry are

> of necessity always bivalent. Whenever this bivalent oxidation can be brought about by two successive univalent oxidations, then the kinetics of such a reaction will be greatly enhanced. The thermodynamic possibility of the univalent oxidation, and the existence of the radical as an intermediate step of the complete bivalent oxidation, will depend on the dismutation constant. If this constant is very large, it is equivalent to saying: The formation of the radical involves a very high step in energy. Only if, due to resonance, the formation of the radical requires relatively little energy, will the bivalent oxidation run smoothly. The reason why the oxidation of organic compounds is frequently very sluggish, even when an oxidant of thermodynamically sufficient oxidative power is applied, is that the oxidation has probably, as a rule, to go through two univalent steps; and to go through the intermediate step means, in general, climbing over a large energy hill, except in those cases described above in which the semiquinone formation constant is relatively large. It is the task of all catalysts and enzymes concerned with oxidation-reduction processes to ease the climb over this energy hill, or to convert the substance to be oxidized into some form, or into some compound, in which the intermediate radical will have a stronger resonance and so a greater stability than it has in its original form. (page 104).

The foregoing presentation is only intended to be a brief review of the principles and methods which have been used in oxidation-reduction studies in organic and biological chemistries. For a more complete discussion reference is given to the monographs by Michaelis (1930) and Wurmser (1930). This work is more concerned with the relatively newer polarographic method developed by Heyrovský (1923) for studying oxidation-reduction processes.

Although the discovery and earlier development of the polarographic method was due largely to the work of Heyrovský

(1936) and his school at the Charles University in Prague, Czechoslovakia, much credit is also due Hohn (1937) in Germany, Semerano in Italy and Shikata in Japan. The fact that more than five hundred publications have appeared in the literature during the short period since its discovery is indicative of the importance of the method. A detailed discussion of the fundamental principles involved in the polarographic method has been given in a review by Kolthoff and Lingane (1939). The application of the procedure in organic chemistry has been reviewed by Müller (1939[a]).

The method is based upon the interpretation of current-voltage curves obtained by electrolyzing dilute solutions of electroreducible or electroöxidizable substances between two electrodes, one of which is a large,quiet,non-polarized pool of mercury, the other a small capillary tube from which mercury drops slowly fall. Each separate mercury drop is then an electrode which is exactly reproduced by its successor. From these current-voltage curves the substance under investigation may be determined both qualitatively and quantitatively at the same time if the proper experimental conditions exist. With the original apparatus Heyrovský was able to vary the electromotive force applied to the cell from zero to the full voltage of the battery by varying the resistance of a rheostat connected in parallel with the battery. Using such equipment, current-voltage curves were obtained by gradually applying an electromotive

force and noting, by means of a galvanometer connected in series with the cell, the amount of current passing through the cell at each setting of the resistance. The applied voltage was measured with a potentiometer.

The fact that the above method of obtaining current-voltage curves with the dropping mercury electrode is rather laborious and time consuming, led Heyrovský and Shikata (1925) to design an automatic recording device which gave continuous curves. Light reflected from a moving-coil galvanometer mirror was made to fall through a slit onto photosensitive paper wound on a revolving drum protected by means of a light-tight housing. The revolution of the drum was synchronized with the changes in applied voltage. As the galvanometer was deflected by current flowing through the cell a curve was automotically traced on the photosensitive paper. The instrument was called a "polarograph" and the curves so obtained, "polarograms". Many improvements in the design of the original apparatus have been made. Polarographs of later design may now be obtained from most of the American scientific instrument companies, but they are not essential for much of the research in this field.

The analysis of a typical current-voltage curve will make clearer the principles involved in the polarographic method. The lower curve in Figure 4 was obtained by reducing 5 x 10^{-4}M cadmium chloride in 0.1 M potassium chloride at the dropping mercury electrode. If the dropping electrode is the cathode,

reduction occurs and the "wave" obtained is a "cathodic wave"; on the other hand, if the dropping electrode is the anode, oxidation takes place and an "anodic wave" is observed. In the reduction mentioned above, it can be seen that only a very small current, the residual current, flowed through the cell until a voltage of about 0.575 was reached. The nature of this residual current has been thoroughly investigated by Ilkovič and Semerano (1932), Ilkovič (1936[a], 1936[b]) Maas (1938) and others. The residual current has been found to be due to a "condenser current" and also to a very small "faradayic current".

Referring again to Figure 4, it is observed that at a voltage of 0.575 continuous electrolysis began. This involved the discharge of cadmium ions at the dropping mercury cathode to form a very dilute amalgam and the dissolution of mercury at the large quiet anode resulting in the formation of calomel. The current did not increase indefinitely but reached a limiting value of four microamperes at a voltage of about 0.625.

This limiting current is caused by an almost complete state of concentration polarization. When the discharge of reducible material begins, the concentration in the immediate vicinity of the electrode is diminished. This establishes a concentration gradient between the body of the solution and the depleted area around the electrode giving rise to diffusion into the depleted area. As the applied voltage is increased

more material is reduced in a given instant and the concentration gradient is made steeper causing a more rapid diffusion. Finally, at the limiting current, all the material reaching the electrode in a given instant is being reduced. In other words, at this point the concentration in the depleted area becomes so small that the difference in concentration between this area and the body of the solution approaches a constant average value equal simply to the concentration in the body of the solution. Since the concentration gradient becomes constant at this point, the rate of diffusion also becomes a constant. The amount of electroreducible material reaching the electrode in a given instant will determine the amount of current that will flow through the cell. In the case of an electrially-charged reducible substance, it must be remembered that there are two forces acting to bring the substance to the electrode surface, a diffusion force and an electrical migration force. Normally then, the limiting current is the sum of a diffusion current and a migration current. If an "indifferent electrolyte" is added in large excess, the current will be carried almost entirely by the added electrolyte and the limiting current then becomes strictly a diffusion current. An "indifferent electrolyte" is one which reduces at a more negative potential than the substance being investigated. In the presence of a relatively large amount of indifferent electrolyte, all other factors being constant, the diffusion current is directly proportional to the concentration

of reducible substance in the body of the solution. This is the principle upon which quantitative polarography is based.

Ilković (1934) has derived an equation for the diffusion current.

$$I_d = 0.627\ nFD^{1/2} Cm^{2/3} t^{1/6} \qquad (13)$$

In this equation, I_d represents the diffusion current in amperes, n is the number of faradays of electricity required for the reduction of one mole of the substance in question, F is Faraday's constant, D is the diffusion coefficient of the reducible substance in centimeters squared per second, C is its concentration in moles per ml., m is the weight of mercury in grams flowing from the capillary per second, and t is the time in seconds required for the formation of each drop.

Lingane and Kolthoff (1939) have shown conclusively that the above equation holds in the case of inorganic reduction. Maas (1938) found that the diffusion current was not directly proportional to the concentration of reducible material if the drop-time was shorter than four second. Lingane and Kolthoff (1939) reported the optimum drop-time for quantitative polarography as being between three and six seconds. The latter authors have shown the product $m^{2/3}t^{1/6}$ of equation 13 to be almost a constant over the potential range from zero to -1.0 volts when the drops were formed in 0.1M potassium chloride, provided the pressure on the dropping mercury was maintained constant; at higher potentials it decreased slightly.

The phenomenon of concentration polarization is not characteristic of the dropping mercury electrode alone; however, this electrode does offer a number of unique advantages. The average current becomes steady immediately at each new setting of applied voltage, and is independent of the time of electrolysis. The high hydrogen overvoltage on mercury makes possible the reduction of substances up to a potential difference of -2.0 volts against the standard hydrogen half-cell provided the proper indifferent electrolyte is chosen. Unlike any other electrode, the dropping mercury electrode has a continually exposed fresh surface of mercury during the life period of each drop. In addition, the life period of an electrode is very short thus eliminating troublesome aging effects so that the results obtained are perfectly reproducible. Kolthoff and Lingane (1939) reduced several inorganic ions using a platinum micro electrode in place of the dropping mercury electrode.

In the case of many current-voltage curves, the current passes through a very distinct maximum after which it falls to the "limiting current value". The cause of these maxima is still one of the unsolved problems of polarography. Heyrovský (1934) attributed the maxima to an adsorption of the electroreducible substance on the growing mercury drop. Other authors have attributed them to an electrostatic stirring effect at the surface of the mercury drops. Notwithstanding the fact that the exact cause of maxima is unknown, they may be suppressed or

eliminated by adding certain substances more highly adsorbed than the electroreducible substance in question.

The polarographic potential which characterizes an electroreducible or electroöxidizable substance has, in the past, been defined in a number of ways. Müller (1939[a]) lists these different methods, the most important being the potential at a 45° tangent to the curve. All of these older methods were found unsatisfactory, however, since the potential was found to vary with the concentration of the substance under investigation and with the drop-time. Later Heyrovský and Ilkovič (1935) introduced the "half-wave potential" which was found to be independent of such factors as drop-time and concentration. The half-wave potential is the point of inflection on the current-voltage curve half way between the residual and the limiting current. These authors showed that the polarographic half-wave potential bore a direct relationship to the potentiometrically established $\underline{E_o}$ in inorganic reductions.

In order to make clear the above relationship Müller's (1939[b]) presentation of the original derivation of Heyrovský and Ilkovič is followed in the discussion given below.

The current at any point on the current-voltage curve may be given by equation 14 when the current is governed only by the rate of diffusion.

$$I = K(C - C_o) \qquad (14)$$

In the above equation, $\underline{I}$ is the current, $\underline{C}$ is the concentration in the body of the solution, $\underline{C_o}$ is the concentration at the interface, and $\underline{K}$ is the diffusion current constant. When the limiting current (the maximal diffusion current) is reached, $\underline{C_o}$, the concentration at the electrode/solution interface, becomes negligibly small compared to $\underline{C}$ and,

$$I_d = KC \qquad (15)$$

The concentration of the reaction product, $\underline{C_a}$, at the interface is also proportional to the current $\underline{I}$, and Heyrovský and Ilkovič give the relation,

$$C_a = kI \qquad (16)$$

where $\underline{k}$ is again a diffusion constant.

If the same laws which govern reversible reactions at other electrodes are in operation at the dropping mercury electrode, the potential at any point on the curve should be given by the equation

$$E = E_o - \frac{RT}{nF} \ln \frac{C_a}{C_o} \qquad (17)$$

Concentrations are used in equation 17 for simplicity.

Substituting the values of $\underline{C_o}$ and $\underline{C_a}$ found from equations 14 and 16 respectively into equation 17

$$E = E_o - \frac{RT}{nF} \ln \frac{kKI}{KC-I} \qquad (18)$$

or

$$E = E_o - \frac{RT}{nF} \ln \frac{I}{I_d - I} + K' \qquad (19)$$

At the half-wave potential, $I = I_d - I$ so the logarithmic term drops out.

$$E_{1/2} = E_o + K' \qquad (20)$$

It may be seen from equation 19 that the polarographic half-wave potential is equal to the normal electrode potential plus a constant. Müller (1939[b]) stated that this constant may be positive, negative or zero in metal ion reductions depending, probably, upon the nature of the amalgam. The validity of equation 19 was first verified experimentally by Tomes (1937) who showed that a graph of E against ln $I/I_d - I$ gave a straight line the slope of which was equal to RT/nF for the reduction of various metal ions.

Müller and Baumberger (1937[a], 1937[b]) have demonstrated that in well-buffered solutions the polarographic half-wave potential is equal to the potentiometrically established E'_o of simple reversible organic oxidation-reduction systems. These authors examined quinhydrone polarographically. One-half of the quinhydrone curve was observed to be "cathodic" and one-half "anodic". This was to be expected since quinhydrone dissociates in solution into equivalent amounts of quinone and hydroquinone. At the midpoint in the curve where the curve

changes from "anodic" to "cathodic" no current flows; this is the half-wave potential and corresponds to the E_0' for quinhydrone as determined by the usual potentiometric methods.

Müller and Baumberger further showed that the "anodic curve" obtained when hydroquinone alone was present in solution was identical in appearance with the "cathodic curve" obtained when only quinone was present. The half-wave potentials in both cases were the same and also the same as that obtained with quinhydrone. These authors state that, "Whenever the half-wave potential is a constant, whether the curve is anodic or cathodic, the system is perfectly reversible in a thermodynamic sense".

It should be pointed out here that the midpoint on a current-voltage curve is not the true half-wave potential. In order to obtain the latter value, the small IR drop in the solution must be subtracted from the applied voltage. This is shown in the following equation:

$$E = V - IR \qquad (21)$$

where E is the true potential at the electrode surface, V the applied voltage, I the current, and R the resistance of the solution between the electrodes. This correction is usually very small. As most organic molecules are not charged, the indifferent electrolyte is not added to eliminate the migration current but to lower the resistance in the cell.

Müller (1940[a]) has investigated the polarographic oxidation

and reduction of quinhydrone in buffered and unbuffered solutions. The importance of buffering with primary acid buffers in organic oxidations and reductions at the dropping mercury electrode was clearly exhibited. Lewis (1938) defines a primary acid as one which dissociates without requiring activation energies; in other words, the ionization process is rapid, a property which is essential for buffering action at a dropping mercury electrode. Müller (1940[a]) has also made clear the fact that the pH value in the immediate vicinity of the electrode may be widely different from that in the body of the solution in the case of unbuffered or improperly buffered solutions.

Most organic reductions which have been studied polarographically fall into the class designated as irreversible; however, smooth S-shaped curves are often obtained as well as regular shifts in half-wave potentials with pH. As pointed out by Müller and Baumberger (1939), this suggests the selection of a reversible process from a reduction which, on the whole, is irreversible. These authors speculated that the reversible step represented at the electrode might involve the direct addition of an electron to form a free radical which undergoes subsequent dimerization or dismutation. Michaelis' (1935) investigation of this type of reduction has already been presented. Müller (1940[b]) has shown the dropping mercury electrode to be applicable to the detection of inter-

mediate radicals in the case of reversible reductions. On the other hand, the reversible step mentioned above might involve the reduction of hydrogen ions to hydrogen atoms followed by addition of the hydrogen to the organic compound.

The final criterion of reversibility is that the reduced form of the compound be oxidized at the same potential as that at which the oxidized form is reduced. The sluggishly reversible systems mentioned earlier which have been studied polarographically do not fulfill this requirement. Kodiček and Wenig (1938) have shown that ascorbic acid can be oxidized polarographically but not reduced.

Müller (1939[a]) has found that Conant's A.R.P, corresponds more closely to the deposition potential on a polarographic curve than to the half-wave potential in the cases which have been studied. He suggested calling the half-wave potentials in cases where one reversible step is evident "polarographic apparent reduction potentials", P.A.R.P. Müller and Baumberger (1939) have demonstrated the P.A.R.P. of the pyruvate ion to be -1.0 volt at pH 7. The "apparent oxidation potential" of the lactate ion is +1.0 volt according to Barmore (1929) who used Conant's method to determine the value given. These two potentials may be brought together to form a reversible system by means of enzymes. This fact had been previously demonstrated using a platinum indicator electrode. Müller suggested that the dropping mercury electrode might also be used as an indicator electrode in these enzyme equi-

librated systems. The polarographic method of studying biological oxidation would allow investigations of systems with potentials in the range of hydrogen overvoltage on mercury, that is, potentials as negative as -2.0 volts referred to the standard hydrogen half-cell.

Heyrovský and Smoleŕ (1932) were the first to attempt the reduction of sugars at the dropping mercury electrode. These investigators reported that aldoses were not reducible but that the ketose sugars, fructose and sorbose, were both reducible at -1.80 volts in neutral or slightly alkaline solutions. The reduction potential given by the above authors is referred to the normal calomel half-cell as a reference electrode, but they do not state whether the value is a half-wave potential or the potential at a 45° tangent to the curve. The aldoses studied were glucose, galactose, mannose, rhamnose, l-arbinose, and lyxose. The disaccharides sucrose, maltose, and lactose were also found not to reduce. The rate of inversion of sucrose in acid was followed polarographically by analysis of the invert sugar from time to time. The height of the polarographic curve in the analysis of a honey was found to be proportional to the amount of fructose present.

Heyrovský and Smoleŕ (1932) attempted to determine the number of electrons involved in the reduction of levulose by comparing the height of the curve obtained with that obtained

for the reduction of a solution of barium chloride of equal molarity. The values were determined at various temperatures but are of doubtful value as the differences in diffusion coefficients were not taken into consideration.

Cantor and Peniston (1940) found that, contrary to earlier reports, aldoses were reducible at the dropping mercury cathode. These authors used larger concentrations of sugars than had formerly been employed and the reduction observed was attributed to the presence of an "aldehydo" form in the equilibrated solutions. The sugars studied were d-glucose, d-mannose, d-galactose, l-allose, d-xylose, l-arabinose, d-lyxose and d-ribose. The amount of "aldehydo" form was estimated under various conditions of pH and concentration.

Winkel and Proske (1936) have determined the effect on the P.A.R.P. of substituting hydroxyl groups in the alpha and beta positions to a ketonic carbonyl group.

Adkins and Cox (1938), Cox and Adkins (1939) and later Baker and Adkins (1940) have investigated the relative oxidation-reduction reactivities of a series of ketones using the polarograph as an analytical tool to determine the composition of the equilibrium mixtures in systems of the type,

$$R_2CO + R'_2CHOH \rightleftharpoons R_2CHOH + R'_2CO \qquad (23)$$

These investigators discovered that systems of the above type reached equilibrium in the presence of aluminum t-butoxide as a catalyst. By equilibrating each of the several ketones

with an alcohol chosen as a standard, an order showing the relative oxidizing strength of each of the ketones was arranged. Adkins and Baker (1940) have determined the oxidation potentials in volts of several ketones by comparing the oxidizing power of these carbonyl compounds with quinones in a strictly reversible process.

The polarographic method has been used by Kerr (1940) to study dextrose, maltose, beta-amylose and cornstarch. Evidence is submitted that the carbohydrates may possibly exist in the free "aldehydo" form and the theory is proposed that they may in turn ionize as acids.

Shikata and Shoji (1927) reported that reducing substances in commercial fermentation products, such as "sake", "shoyu", wine and beer could be detected by means of the polarograph. Some of the reducing substances found were cinnamaldehyde, furfuraldehyde, and acetaldehyde.

EXPERIMENTAL

Materials

Dihydroxyacetone

The dihydroxyacetone used in the investigation was prepared by the action of *Acetobacter suboxydans* on glycerol. The method of carrying out the fermentation and recovering the product, dihydroxyacetone, was based on that given by Underkofler and Fulmer (1937). The medium contained per 100 ml.: 6.0 grams glycerol, 0.50 grams yeast extract, and 0.30 grams primary potassium phosphate. After a seven-day period of incubation at 28°C., the product was recovered.

The crude dihydroxyacetone was dissolved in boiling absolute alcohol. A little Norite was added to remove the traces of color and the solution was filtered with suction. Most of the alcohol was distilled off and the residual small volume of solution was cooled in a well stoppered flask to prevent the access of moisture as traces of water prevent crystallization. The cold solution was shaken and when crystallization was complete (seeding is often necessary) the dihydroxyacetone was filtered off rapidly, using suction. The crystals were washed with cold absolute alcohol. The recrystallization was repeated several times; the final product was dried in a vacuum desiccator over calcium chloride.

l-Erythrulose

The l-erythrulose used was formed by the action of Acetobacter suboxydans upon meso-erythritol according to the procedure described by Whistler and Underkofler (1938). The medium contained 3.0 grams of meso-erythritol and 0.5 grams of yeast extract per 100 ml. of solution. As erythrulose is a sirup and difficult to recover in pure form, the experiments with this compound were carried out using the diluted fermentation liquor.

d-Tagatose

The d-tagatose was furnished by Dr. L. A. Underkofler of the Chemistry Department of this college. It was prepared by a method described by Reichstein and Bosshard (1934) involving epimerization of d-galactose with anhydrous pyridine.

l-Sorbose

The l-sorbose was produced by the action of Acetobacter suboxydans upon sorbitol. The fermentation procedure was according to that described by Fulmer, Dunning, Guymon and Underkofler (1936). The crude product obtained was recrystallized a number of times from alcohol-water solutions and finally the pure sorbose was dried in a vacuum desiccator over calcium chloride for several days.

"Ketose" from i-inositol

The "ketose" compound was furnished by Dr. W. H. Pitcher of this laboratory and was formed by the action of Acetobacter suboxydans on i-inositol. The compound was a pure product recovered from its phenylhydrazone derivative.

Indifferent electrolytes

All of the materials used as indifferent electroylte or in the preparation of the buffers were C. P. Reagent Grade chemicals.

Mercury

The mercury was first shaken with 10 per cent sodium hydroxide after which it was washed several times with distilled water. It was then shaken with 10 per cent nitric acid and again washed with distilled water followed by conductivity water. After drying, the mercury was distilled in vacuo and the distillate was stored in a clean glass bottle.

Apparatus

The electrical circuit

The electrical circuit used in this investigation for manually obtaining current-voltage curves is diagramed in

Figure 1. The circuit is similar to one described by Lingane and Kolthoff (1939). The parts labeled in Figure 1 are listed below.

R_1 , a radio rheostat of the "potentiometer" type having a resistance of 10 ohms

R_2 and R_4 , four-dial precision resistance boxes each of 9,999 ohms resistance

R_3 , a radio rheostat of the "potentiometer" type having a resistance of 1,000 ohms

S.C.E., a saturated calomel half-cell

i, the potentiometer plug-in connection for measuring the current flowing through the cell

E_a, the potentiometer plug-in connection for measuring the voltage applied to the electrolysis cell

E_c, the potentiometer plug-in connection for measuring the potential of the dropping mercury cathode against the saturated calomel half-cell

E_{an}, the potentiometer plug-in connection for measuring the potential of the anode (the quiet pool of mercury) against the saturated calomel electrode.

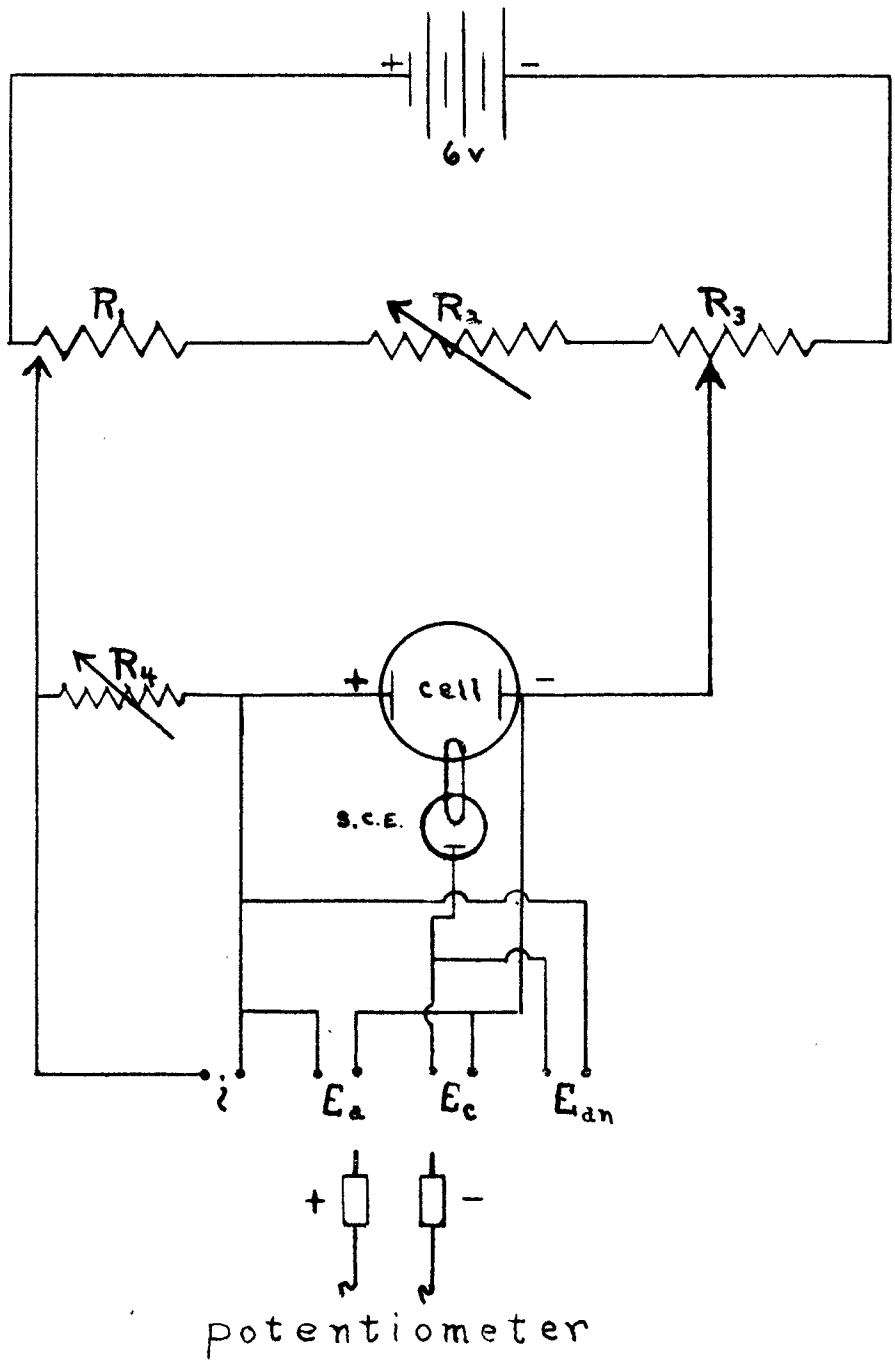


Figure 1 Apparatus

The dropping mercury electrode

The fabrication of a capillary suitable for use as a dropping mercury electrode is rather difficult because of the unusually small bore necessary to insure a slow enough drop-time. Kolthoff and Lingane (1939) prepared a capillary by drawing out the end of a 20 cm. length of Pyrex capillary tubing, of about 0.5 mm. internal diameter, until the internal diameter of the tip was about 0.03 to 0.04 mm. The constricted end was cut off at such a length that the drop-time in 0.1 M potassium chloride could easily be adjusted to 3 or 4 seconds by adjusting the pressure on the dropping mercury. Maas (1938) prepared an electrode by cementing a 2 to 3 cm. length of commercial thermometer tubing (internal diameter 0.03 mm.) into a wider glass tube which was connected to the mercury reservoir.

Preliminary measurements in this investigation were made employing a capillary which was prepared by drawing out a short length of Pyrex capillary tubing having an internal diameter of about 0.5 mm. Difficulty was experienced, however, in obtaining a capillary of uniform internal diameter which would give a drop-time that was sufficiently slow for quantitative polarography. The greater portion of the investigation was carried out using capillary tubing obtained from E.H. Sargent and Company, Chicago. The capillary tubing was made of lead-free Jena or Pyrex glass, or

the equivalent, having a bore diameter in the vicinity of 0.05 to 0.06 mm. The electrode was made from a 10 cm. section of this tubing, a single section being used for most of the work. When the capillary became dirty the dropping became erratic. It was possible to clean the dirty capillary with aqua regia.

The dropping electrode was attached to a mercury reservoir by means of a section of rubber tubing. This rubber tubing had been previously boiled in dilute alkali to remove some of the sulfur. The mercury reservoir was secured to a ring stand by means of a clamp and the pressure on the dropping mercury electrode could be adjusted by raising or lowering this clamp.

The cell assembly

The arrangement of the electrolysis cell is illustrated in Figure 2. The parts labeled in the diagram are listed below:

A, mercury reservoir, a mercury leveling bulb

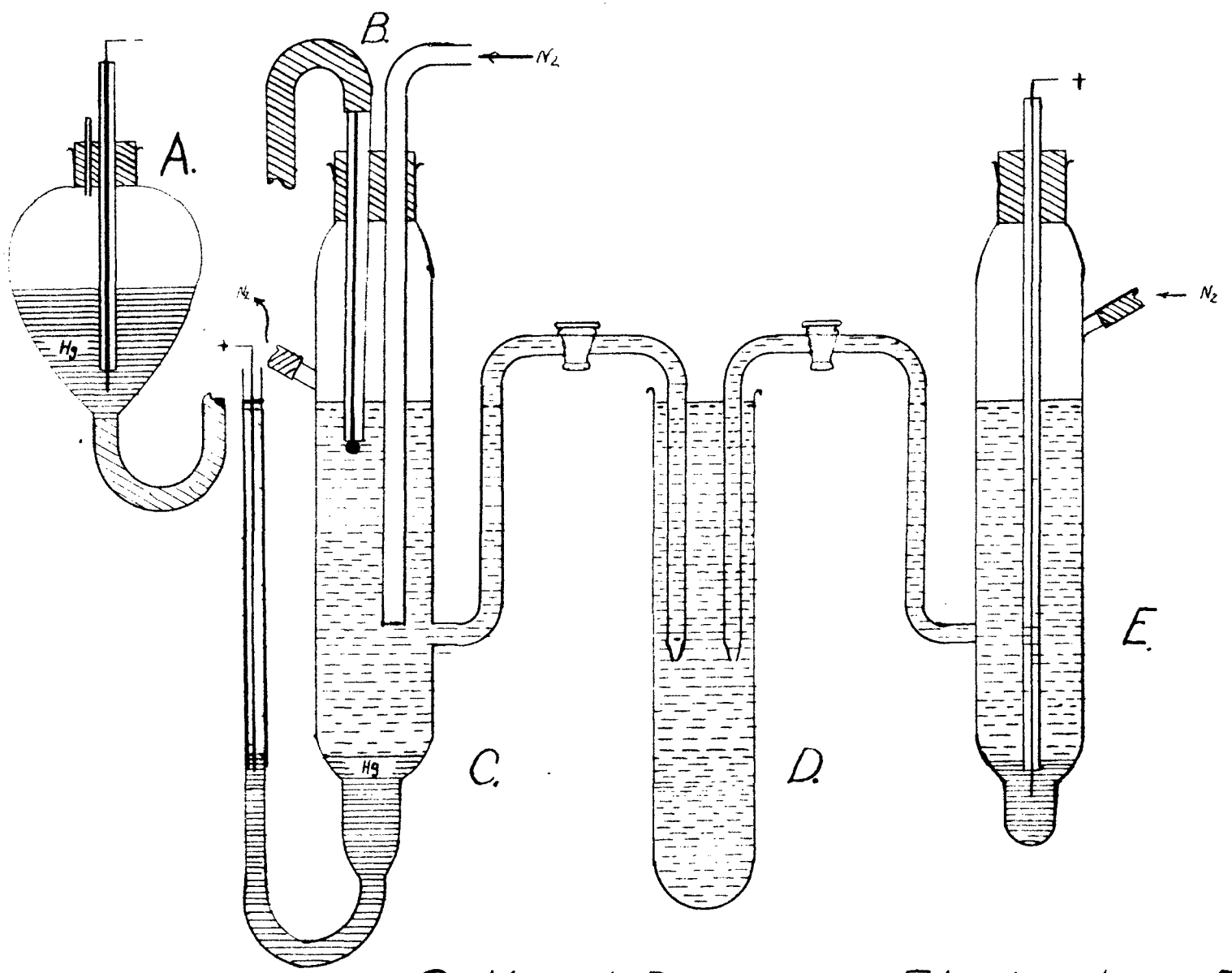
B, the dropping mercury electrode

C, large non-polarizable mercury electrode

D, salt bridge, saturated potassium chloride solution

E, saturated calomel reference electrode

The electrolysis cell was made by sealing an electrode connection onto the bottom of an ordinary calomel electrode vessel as shown in Figure 2. The top of the cell was closed



Cell and Dropping Electrode Fig. 2

by means of a two-hole rubber stopper through which were inserted the dropping mercury electrode and a nitrogen inlet tube.

The potentiometer

The potentiometer used was a Leeds and Northrup Student's Potentiometer having a voltage range from 0 to 2.3.

The galvanometer

The galvanometer employed in the potentiometer circuit as a null-point instrument was a Leeds and Northrup Pointer Type Galvanometer having a current sensitivity of 0.11 microamperes per mm. and a period of 3.5 seconds. A protective resistance of 40,000 ohms was connected in series with the galvanometer.

The thermostat

The cell assembly shown in Figure 2 was placed in a constant temperature water bath maintained at 25°C. ± 0.1°. In order to eliminate temperature gradients in the bath, it was stirred continuously by means of an electrical stirrer.

Methods

Procedure for obtaining current-voltage curves

The electrolysis cell was filled to a depth of about

3 cm. with clean mercury and a little calomel was placed on top of the mercury. It was found necessary to add the small amount of calomel in order to obtain stable readings during the first run after changing the quiet pool of mercury. The solution bearing the substance to be reduced and an indifferent electrolyte was then added to the cell. The solution was siphoned over into the arm which connected the cell to the salt bridge, and the ungreased stopcock on the arm was closed. This stopcock was kept closed during a run in order to retard diffusion across the salt bridge junction; however, a good connecting film of solution always existed around the stopcock.

After filling, the cell was placed in the water bath and allowed 15 or 20 minutes to attain the temperature of the bath. During this period, the dropping mercury electrode was inserted, and oxygen-free nitrogen was passed through the cell in order to remove the dissolved oxygen. Oxygen is reducible at the dropping mercury cathode; therefore, when the substance being investigated reduces at a potential more negative than oxygen, it is essential that the latter be removed before starting the analysis. The drop-time was maintained at four seconds throughout the course of the analysis by raising or lowering the mercury reservoir shown in Figure 2.

The source of the polarizing electromotive force was

a six-volt storage battery. The voltage applied to the electrolysis cell at the beginning of an experiment was approximately zero and was gradually increased by varying the resistance connected in series with the cell (see Figure 1). For this work it was desired to have the voltage increased in very small and uniform increments in order that the curve might more closely simulate a continuous curve. This uniform variation could not be produced easily by means of the "potentiometer" type rheostats, R_1 and R_3 of Figure 1, so a four-dial resistance box, R_2, was connected in series with R_1 and R_3. By means of this resistance box the applied voltage could be regulated as desired. During the first experiment on an electroreducible compound which had not been previously investigated, the applied voltage was increased very rapidly in order to determine approximately the reduction potential of the substance. Subsequent reductions were carried out by increasing the applied voltage in smaller more uniform increments over the "reduction range".

In most polarographic work in which the current-voltage curves are obtained manually, the current passing through the cell at any given applied voltage is determined by noting the amount of deflection of a sensitive galvanometer, usually a moving-coil mirror and scale instrument. A shunt is ordinarily employed to increase the current-measuring range of the sensitive galvanometer. The current through the cell

seldom exceeds 50 microamperes in ordinary polarographic analysis because the electroreducible or electroöxidizable substance must be present in extremely small quantities, 10^{-2} to 10^{-5} M, in order to produce an extreme state of concentration polarization.

The current values in this investigation were not obtained according to the method described above, but were obtained by measuring with a potentiometer the potential drop across a standard resistance placed in series with the cell. This method was chosen for two reasons: first, it is very precise and accurate, and second, a suitable galvanometer was not at hand. The method has the disadvantage of being time consuming.

At each successive setting of the resistance, R_2 in Figure 1, two measurements were made with the potentiometer. First, the potential drop across the standard resistance, R_4 in Figure 1, was determined with the potentiometer connected at i in Figure 1. The resistance, R_4, was set permanently at 7,000 ohms. Knowing the potential drop across the standard resistance, the current was determined by applying Ohm's law. Next, the potential of the dropping mercury electrode was measured against the saturated calomel half-cell by connecting the potentiometer at E_0, Figure 1.

The current increases as a mercury drop grows in size causing an oscillation of the galvanometer. If the period of the galvanometer is long in comparison with the time of forma-

tion for each drop, these oscillations are relatively small; on the other hand, if the period is equal to or faster than the drop-time, large current variations will be noted. Because of these oscillations the potentiometer was never perfectly balanced, but the balance point was taken to be the potential at which the galvanometer swung an equal number of divisions to the right and to the left of the galvanometer zero. The current measured at each setting of applied voltage was then an average current and not the current at any given instant during the life of a drop. This imperfect balancing made necessary the frequent checking of the working cell against the standard cell in the potentiometer circuit.

When a polarograph is used for obtaining current-voltage curves, it is common practice to use the quiet pool of mercury as the reference electrode. The potential of this quiet electrode, usually the anode, is measured against a standard electrode either before or after an analysis. During the course of the analysis only the voltage applied across the cell is measured. The potential of the dropping electrode in the above case is obtained by subtracting algebraically the potential of the quiet pool of mercury from the applied potential.

Some authors report half-wave potentials by merely giving the potential applied across the cell at the half-wave point.

Such values cannot be correlated with those obtained by direct reference to a standard electrode, since the potential of the anode is not a constant but varies considerably with the nature of the indifferent electrolyte used, the pH of the solution, and other factors. If the quiet pool of mercury is to be used as a reference electrode, its potential should be checked against some standard reference electrode at least before and after an analysis and preferably several times during the analysis.

By means of the circuit shown in Figure 1, it was possible to measure E_{an}, the potential of the quiet pool of mercury referred to the saturated calomel half-cell, at each setting of applied voltage. The algebraic subtraction of the potential of the quiet pool of mercury from the potential applied across the cell gave a value for the potential of the dropping electrode in close agreement with that obtained by measuring the potential of the dropping electrode directly against the saturated calomel half-cell. The dropping mercury electrode was the cathode throughout this investigation and its potential, hereinafter designated by E_c , was measured directly against a saturated calomel half-cell.

Procedure for obtaining half-wave potentials

To obtain the value of the half-wave potential from a current-voltage curve, a line was first drawn through the

residual current parallel to the ordinate. A second line then was drawn through the limiting current parallel to the first. Next a line was drawn parallel to and equidistant from the first two. The voltage at which this third line intersected the current-voltage curve was taken to be the half-wave potential. When a well defined limiting current was not obtained (see Figure 10), the half-wave potential was considered to be the inflection point on the curve. The inflection point was determined by plotting $\Delta I / \Delta E_c$ against E_c.

Determination of "t" and "m" in the Ilkovič equation

In order to use the Ilkovič equation (see equation 13) for calculating the diffusion current, the values of the different variables in the equation had to be known.

Ilkovič (1934), Kilthoff and Lingane (1939), and later Müller (1941) have demonstrated that the grams of mercury flowing from the capillary per second, m, is a constant at constant pressure and is almost independent of the applied potential. On the other hand, the drop-time, t, first increases, passes through a maximum at about -0.6 volt, and then decreases rapidly with increasing negative potential in accordance with the well-known electrocapillary curve for mercury. The product $m^{2/3}t^{1/6}$ was shown to be practically independent of the applied voltage provided the pressure on the

dropping mercury is maintained constant.

All of the limiting currents in this investigation which were treated quantitatively were determined at a constant drop-time of four seconds. This required that the weight of mercury flowing from the capillary per second be determined at the voltage at which the limiting current for each compound occurred. The purpose of the above procedure was to insure a drop-time at the limiting current which was within the range of quantitative polarography. The changes in m with applied voltage when the drop-time was held constant at four seconds are graphed on curve 2, Figure 3. Since the drop-time was constant, the pressure on the dropping mercury was different at each value of applied voltage for which a value of m was determined. The values in Figure 3 are applicable only to the capillary used for their determination.

In determining the values of m shown on curve 2, Figure 3, two small electrolysis cells were used. Both cells contained solutions of the same composition. The anode for both cells was a quiet pool of mercury. The dropping mercury cathode was placed in one cell and the drop-time and voltage applied across the cell were both carefully regulated. The dropping mercury cathode was then quickly transferred to the second cell which previously had been carefully weighed. After 100 drops of mercury had fallen at a constant voltage and drop-time, the cathode was removed and the cell again was weighed. The values of m obtained when the drops formed in a

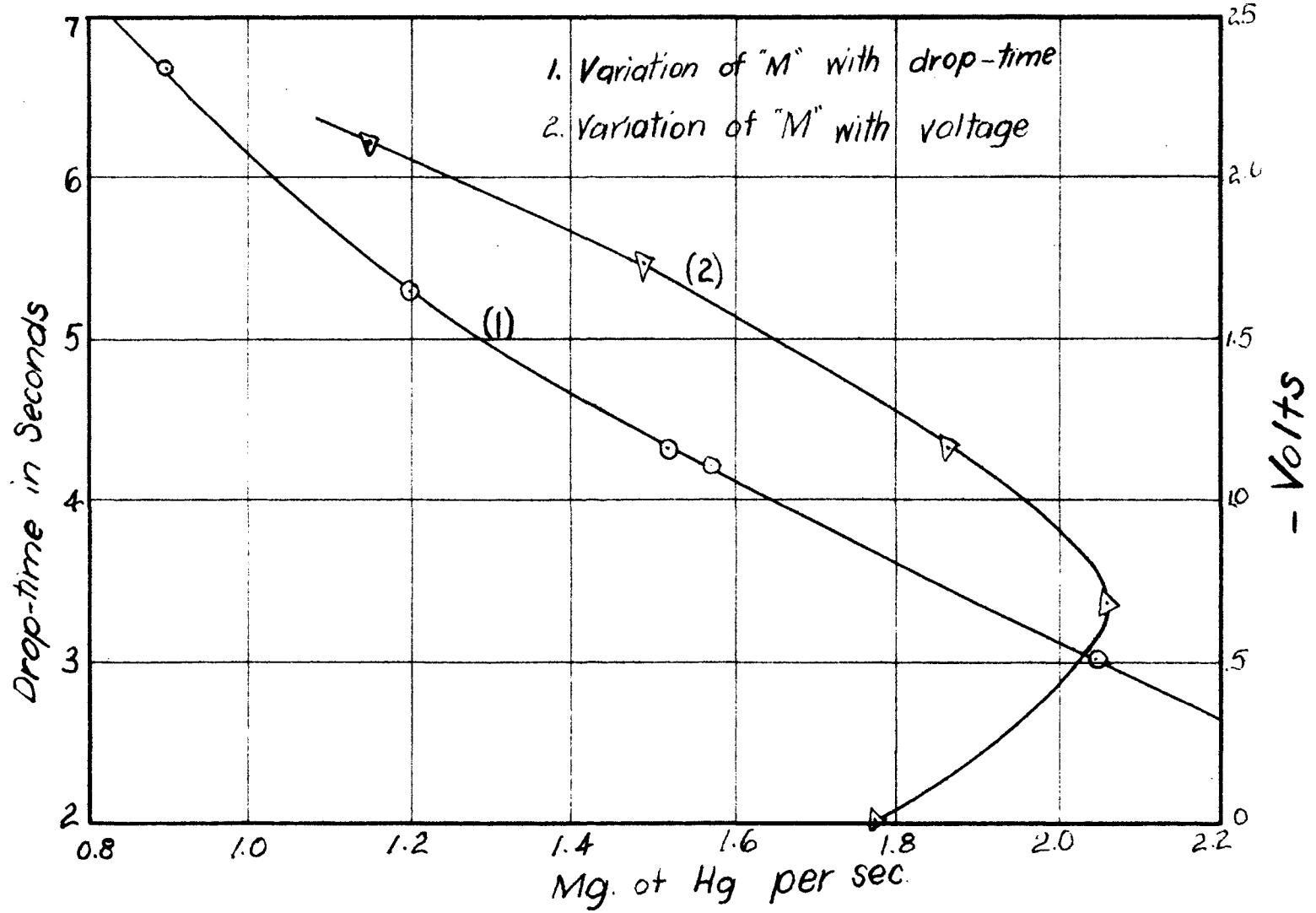


Figure 3. (1) Variation of m with drop-time with no voltage applied. (2) Variation of m with voltage at a constant drop-time of 4 seconds.

solution 2.5×10^{-3} M with respect to "ketose" were found not to be significantly different from those values obtained when the drops formed in 2.5×10^{-3} M sorbose, both solutions being 0.1 M with respect to lithium chloride.

Curve 1 in Figure 3 shows the variation of m with drop-time when no voltage was applied to the dropping mercury electrode. The drop-time was taken with a stop watch.

Method for obtaining diffusion coefficients

Herzog (1907) has shown the following relationship to hold for dilute solutions of non-electrolytes:

$$D = k\sqrt{\frac{1}{M.W.}} \qquad (23)$$

where D represents the diffusion coefficient, k is a constant, and M.W. is the molecular weight of the diffusing substance. Using known diffusion coefficients determined in the vicinity of 25°C., the constant has been found to be 8.9×10^{-5} when the diffusion coefficient is expressed in square centimeters per second. Some of the values in Table 1 have been determined from the above relationship; others were obtained from the International Critical Tables.

The values given in column four of Table 1 were obtained from the International Critical Tables, while those in column three were calculated by means of equation 23. Diffusion coefficients calculated by means of equation 23 were sufficiently accurate for use in this investigation.

Table 1

Diffusion Coefficients of Several Sugars and Polyhydric Alcohols at 25° C.

Compound	Molecular Weight	Diffusion Coefficient ($cm^2/sec. \times 10^6$)	
		Calculated	I.C.T.
Dihydroxyacetone	90	9.4	
Erythrulose	120	8.1	
Sorbose	180	6.6	
Tagatose	180	6.6	
"Ketose"	176	6.7	
Glycerol*	92	9.3	9.3
Arabinose*	150	7.3	7.9
Mannitol*	182	6.6	6.4

*Data given to show agreement between observed values and those calculated by means of equation 23.

Results

Preliminary investigations to check the accuracy of the apparatus and the methods employed

Reduction of cadmium ions. In order to check the accuracy of the apparatus and experimental methods employed in this investigation, it was desirable to study the reduction of some inorganic metal ion which had been previously investigated and whose half-wave potential is definitely established. The cadmium ion was chosen for this purpose because its

limiting current is at a voltage very close to the electrocapillary maximum for mercury and therefore gives no maximum on the current-voltage curve.

Cadmium chloride was used to prepare the standard solution of cadmium ions. The pure cadmium chloride was sublimed in order to obtain the salt completely free of water of hydration. A 0.0100 M solution of cadmium chloride was prepared from this salt. Enough of the standard solution was transferred by means of a pipette to a 100 ml. volumetric flask to make a solution 1×10^{-3} M. with respect to cadmium chloride. Five ml. of a 2.0 M potassium chloride solution were then added to the flask and the solution was diluted to 100 ml. In a similar manner, a solution 5×10^{-4} M with respect to cadmium chloride was prepared.

The current-voltage curves obtained using the two standard cadmium chloride solutions prepared above are shown in Figure 4. The half-wave potential obtained, -0.598 volt, was in excellent agreement with the value of -0.599 volt found by Lingane (1939), who also used 0.1 M potassium chloride as the indifferent electrolyte. A typical set of current-voltage data is given in Table 2.

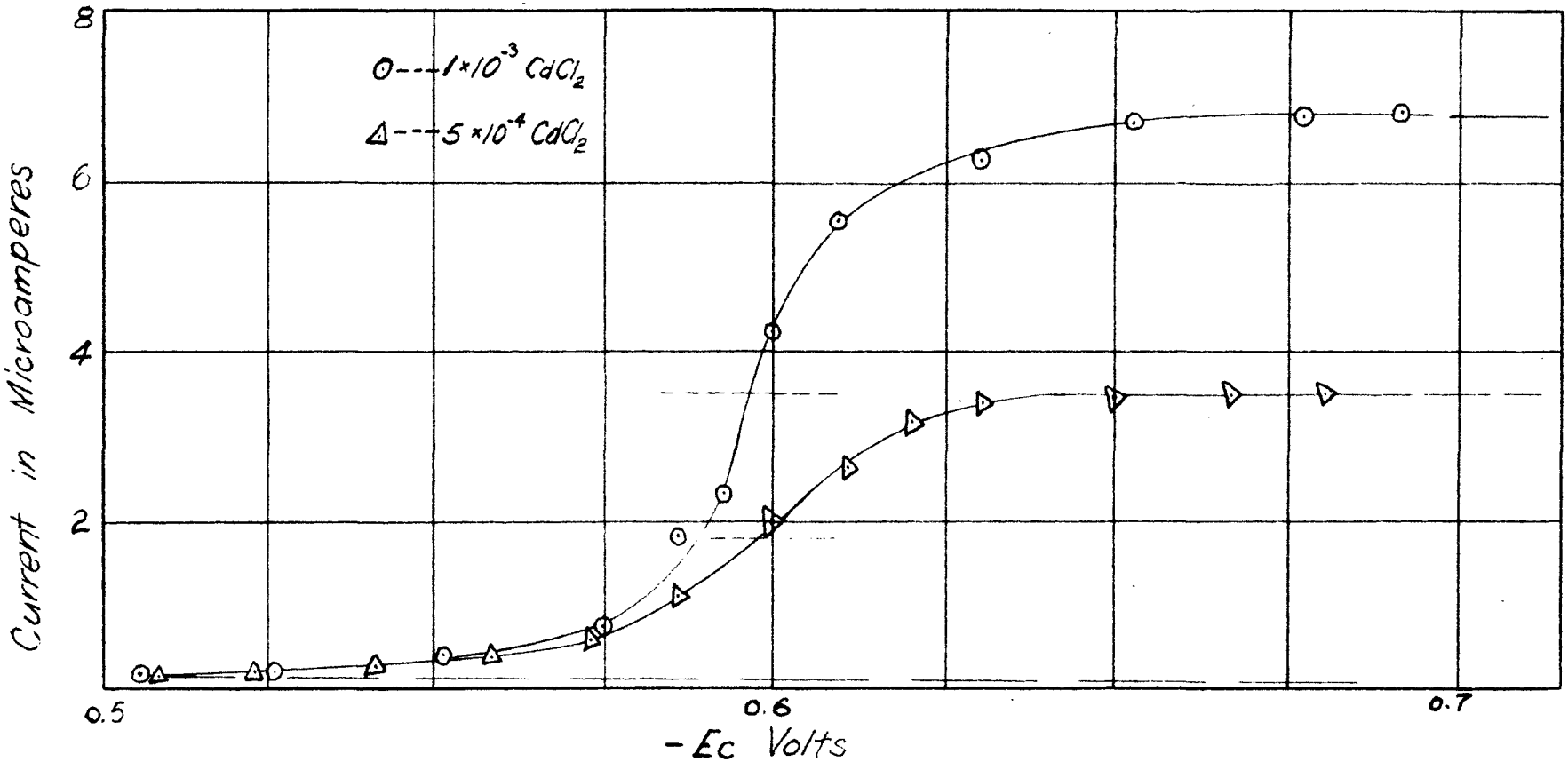


Figure 4. Current-voltage curves for the reduction of $CdCl_2$ in 0.1 M KCl; drop-time = 4 sec.; temp.=25°C.; reference electrode, saturated calomel half-cell.

Table 2

Current-Voltage Data for the Discharge of Cadmium Ions

Conc. = 1 x 10^{-3} moles per liter; drop-time = 4 sec.; temp. = 25°C.; indifferent electrolyte, 0.1 M KCl; R = 7,000 ohms.

Current (microamperes)	IR (volts)	$-E_c$ (volts)
0.06	0.0004	0.0143
0.07	0.0005	0.1620
0.10	0.0007	0.2465
0.16	0.0011	0.3390
0.16	0.0011	0.4212
0.16	0.0011	0.4703
0.16	0.0011	0.5055
0.21	0.0015	0.5261
0.40	0.0028	0.5506
0.76	0.0053	0.5750
1.80	0.0126	0.5856
2.34	0.0164	0.5930
4.23	0.0296	0.6002
5.50	0.0385	0.6090
6.24	0.0437	0.6295
6.67	0.0467	0.6527
6.84	0.0479	0.6581
6.84	0.0479	0.6765
6.86	0.0480	0.6907

The number of faradays of electricity required for the reduction of one mole of cadmium ions as calculated by means of the Ilkovič equation (equation 13) using experimental data was found to be in fairly good agreement with the theoretical value. This fact is shown in Table 3.

Table 3

Test of Equation 13 in the Discharge of Cadmium Ions

D*= 0.72 x 10^{-5} cm^2/sec.; m** = 0.00189 g./sec.; t = 4 sec.; temp. = 25°C.

Concentration (moles per liter)	I_d (microamperes)	n (theoretical)	n (calculated)
1 x 10^{-3}	6.6	2.00	2.11
5 x 10^{-4}	3.4	2.00	2.17

*The diffusion coefficient for cadmium ions at infinite dilution as calculated by Kolthoff and Lingane: Chem. Rev. 24, 30 (1939).

**This value of m was not taken from Figure 3 as the same capillary was not employed.

The meanings of the various designations used in Table 3 have been given previously in a discussion of the Ilkovič equation.

Maximum polarographic range for different buffers and electrolytes used in the investigation. The compounds investigated were reduced at different buffered pH values; therefore, it was necessary to perform a number of preliminary experiments in order to determine the maximum voltage at which each buffer could be used. Most of the standard buffer solutions were prepared according to directions given by Clark and Lubs (1916). After preparation of a buffer, its pH value was checked by measurement with a Cameron pH meter.

A series of potassium chloride-hydrochloric acid buffers was prepared covering the pH range 1.3 to 2.2. The buffer in this series having a maximal pH value, 2.2, could not be used to study reductions which occurred at a potential more negative than -1.3 volts because of the discharge of hydrogen ions.

Next, a series of potassium acid phthalate-hydrochloric acid and potassium acid phthalate-potassium hydroxide buffers was prepared covering the pH range 2.2 to 5.2. Enough potassium chloride was added to each buffer to make the total potassium ion concentration 0.1 M. The buffer of this series having the greatest pH value, 5.2, could not be used to investigate reductions which occurred at a potential more negative than -1.5 volts.

The voltages at which hydrogen was discharged in the case of two phosphate buffers is shown in Figure 5. At higher pH values the range was extended to more negative potentials; unfortunately, however, phosphate buffers do not exhibit much buffering capacity above pH 8.

In order to study reductions occurring at high pH values, potassium bicarbonate-potassium carbonate and boric acid-potassium hydroxide-hydrochloric acid buffers were employed. These are not primary acid buffers; therefore, according to Müller (1939[b]), they are of little value for buffering hydrogen ion concentrations around a dropping mercury electrode. They were used, however, at pH values for

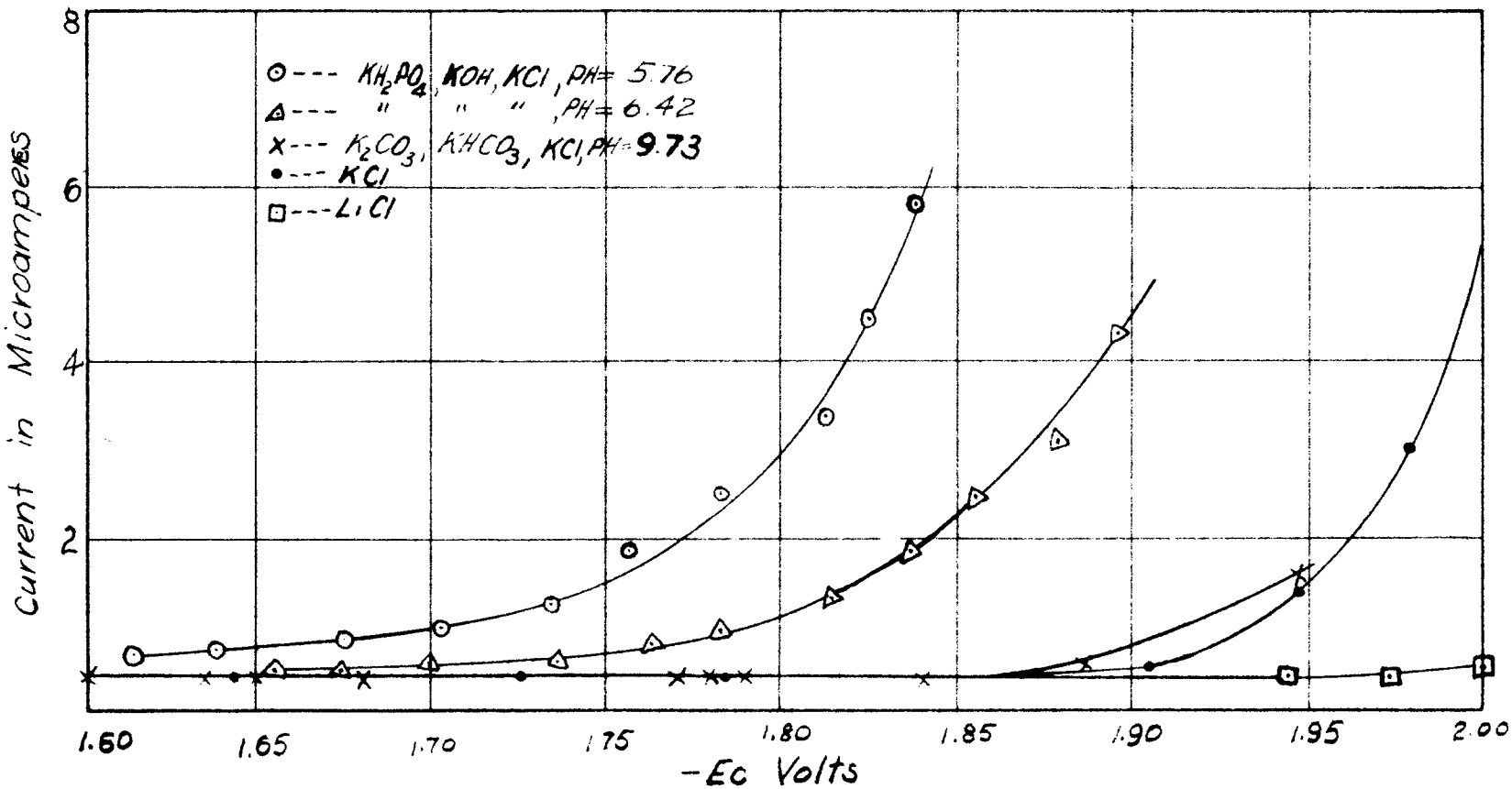


Figure 5. Current-voltage curves for various buffers and electrolytes; drop-time = 4 sec.; temp. = 25°C. LiCl and KCl curves are for 0.1 M solutions.

which a more suitable buffer could not be obtained.

Potassium chloride and lithium chloride were used as indifferent electrolytes for reductions performed in neutral unbuffered solutions. The current-voltage curves for these two electrolytes are shown in Figure 5.

Investigation of several ketones

Reduction of dihydroxyacetone at various concentrations in 0.1 M potassium chloride. A vacuum-dried sample of dihydroxyacetone was carefully weighed and transferred to a volumetric flask. The sample was dissolved and diluted to the mark with 0.1 M potassium chloride. Nitrogen was bubbled through the solution for about one hour to expel dissolved oxygen, as oxygen reacted slowly with the compound in solution. Solutions prepared according to the above directions were called "stock solutions". A fresh stock solution had to be prepared every two or three days since dihydroxyacetone solutions showed "aging effects" on standing. Samples for analysis were prepared by diluting the stock solution with 0.1 M potassium chloride employing standard volumetric equipment.

Current-voltage curves for three concentrations of dihydroxyacetone are shown in Figure 6. Many attempts were made to obtain more well-defined limiting currents by adding materials such as methyl red or gelatin but without success.

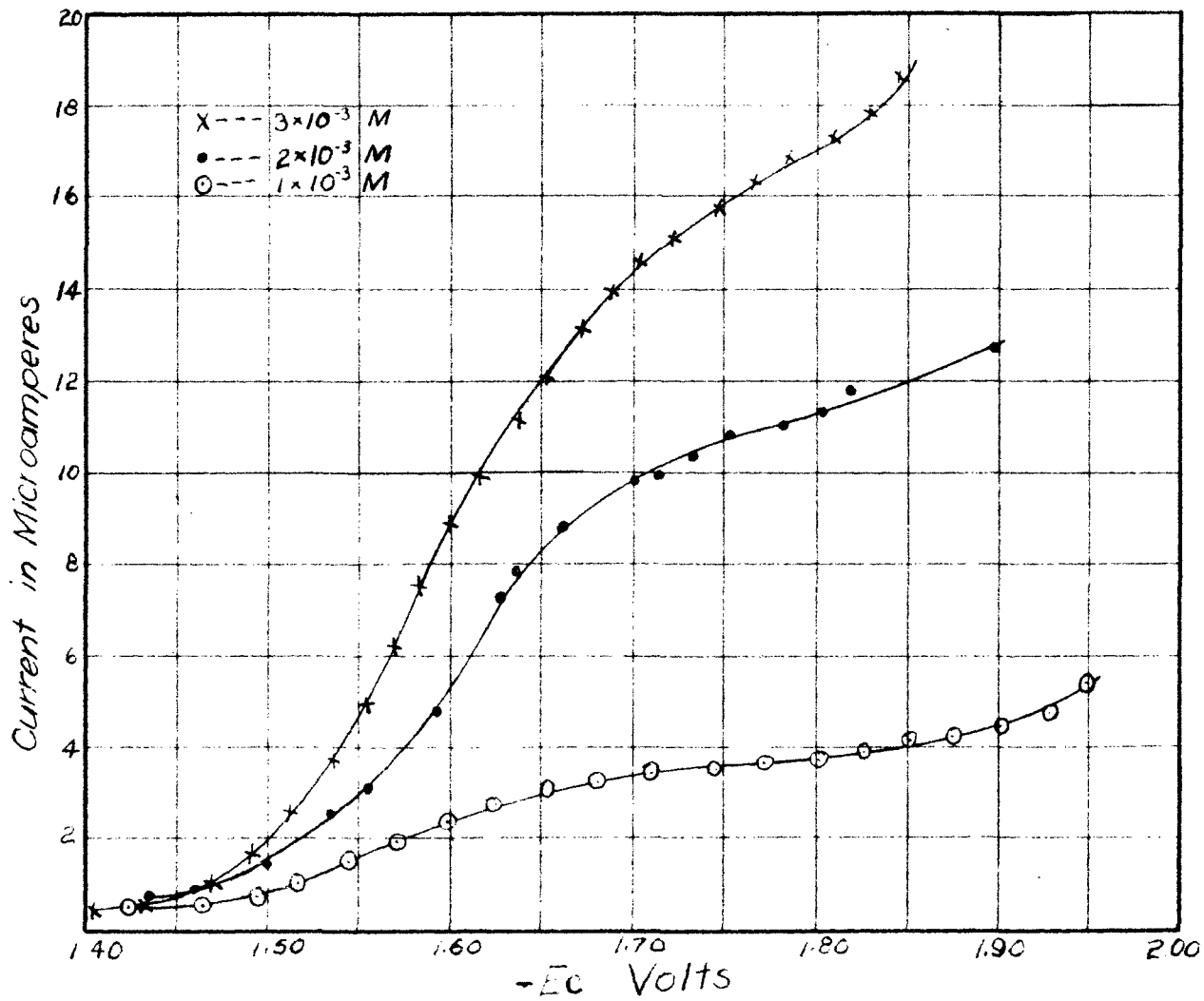


Figure 6. Current-voltage curves for the reduction of dihydroxyacetone at various concentrations; indifferent electrolyte, 0.1 M KCl; drop-time = 4 sec.; temp. = 25°C.

Table 4 shows the relationship between the calculated and the observed values for the diffusion currents for dihydroxyacetone at various concentrations. The calculated values were determined using the Ilkovič equation (equation 13).

Table 4

Relation Between the Calculated and Observed Values for the Diffusion Current for Dihydroxyacetone at Various Concentrations

$\underline{D}$ = 9.4 x 10^{-6} $cm.^2$/sec. (Table 1); $\underline{m}$ = 0.00149 g./ sec. (Figure 3); $\underline{t}$ = 4 sec.

Concentration (moles/liter x 10^3)	Diffusion Current (microamperes)	
	Calculated $\underline{I_d}$	Observed $\underline{I_d}$
1.0	6.1	3.2
1.5	9.1	-
2.0	12.2	10.5
3.0	18.3	14.9

The terms used in Table 4 have the same meaning as given in the discussion of the Ilkovič equation. The diffusion currents in column three of this table were obtained by subtracting the residual current from the limiting current.

Reduction of Dihydroxyacetone in buffered and unbuffered

solutions. The half-wave potentials observed for the reduction of dihydroxyacetone in various buffered and unbuffered soltuions are given in Table 5. The pH value of each different solution was determined with a Cameron pH Meter after the sample had been prepared for analysis.

All reductions of dihydroxyacetone in buffer solutions more acid than pH 5 failed to produce a "cathodic wave". Plots of the data obtained showed continuous current-voltage curves due to the discharge of hydrogen ions.

The phosphate buffers were used over the pH range 5.7 to 7.7. The current-voltage curve obtained at pH 5.7 showed no limiting current, but only an inflection point which was considered to occur at the half-wave potential. The inflection point was determined in the manner demonstrated in Figure 10, that is, by plotting $\Delta I / \Delta E_c$ against E_c. At higher pH values better limiting currents were obtained for reductions of dihydroxyacetone in phosphate buffers.

The half-wave potentials obtained when lithium chloride was used as an indifferent electrolyte agreed closely with those obtained when potassium chloride was employed.

Table 5

Half-wave Potentials of Dihydroxyacetone in Various Buffered and Unbuffered Solutions

Concentration (moles/liter x 10^3)	Buffer or Electrolyte	pH	Half-wave Potential (-volts)
Drop-time 2 seconds or less			
1.0	0.1 M KCl	Neutral	1.615
0.50	0.1 M KCl	"	1.625
0.31	0.1 M LiCl	"	1.597
0.63	0.1 M LiCl	"	1.595
0.75	0.1 M KCl (KOH added)	8.2	1.615
0.50	KH_2PO_4,KOH,KCl	5.7	1.623
0.50	"	6.9	1.645
0.50	"	7.7	1.625
0.50	H_3BO_3,KOH,KCl	8.9	1.641
0.50	"	10.0	1.641
Drop-time 4 seconds			
1.0	0.1 M KCl	Neutral	1.590
2.0	0.1 M KCl	"	1.605
3.0	0.1 M KCl	"	1.590
1.0	KH_2PO_4,KOH,KCl	6.4	1.576
1.5	"	6.4	1.557
1.0	$KHCO_3$,K_2CO_3,KCl	9.7	1.578
1.5	"	9.7	1.567
2.0	"	9.7	1.573

Borate buffers were used in the study of the reductions of dihydroxyacetone over the pH range 8.0 to 10.0. The reduction in a borate buffer, pH 10, gave a limiting current which was found to decrease to one-half its initial value when the solution was allowed to stand for 12 hours. In order to determine whether this phenomenon was caused by the borate buffer or the high basicity of the solution, a reduction of dihydroxyacetone was carried out using a potassium carbonate-potassium bicarbonate buffer, pH 9.7. Again the

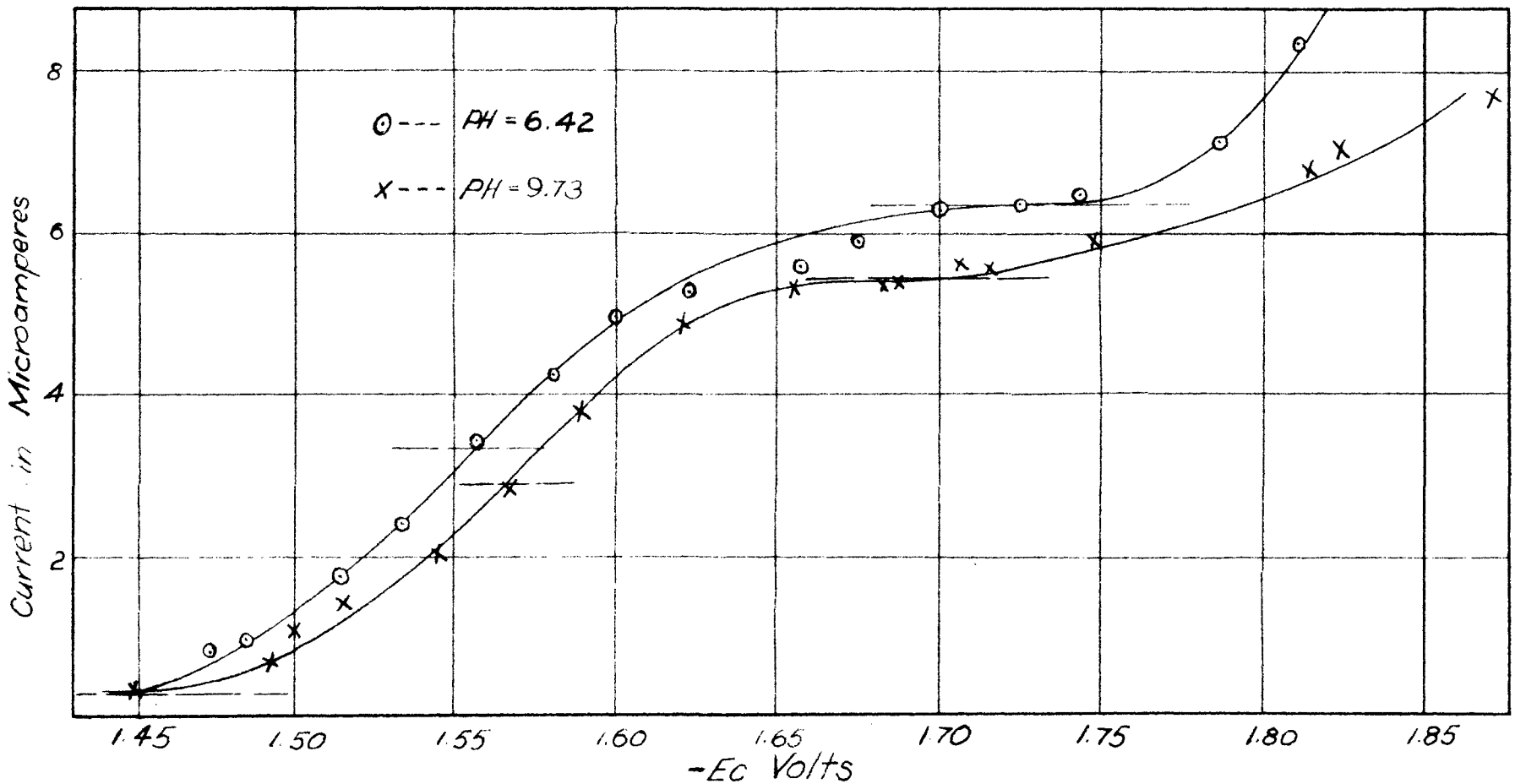


Figure 7. Current-voltage curves for the reduction of dihydroxyacetone in buffered solutions. pH 6.42, phosphate buffer; pH 9.73 carbonate buffer. Drop-time = 4 sec.; temp. = 25°C.

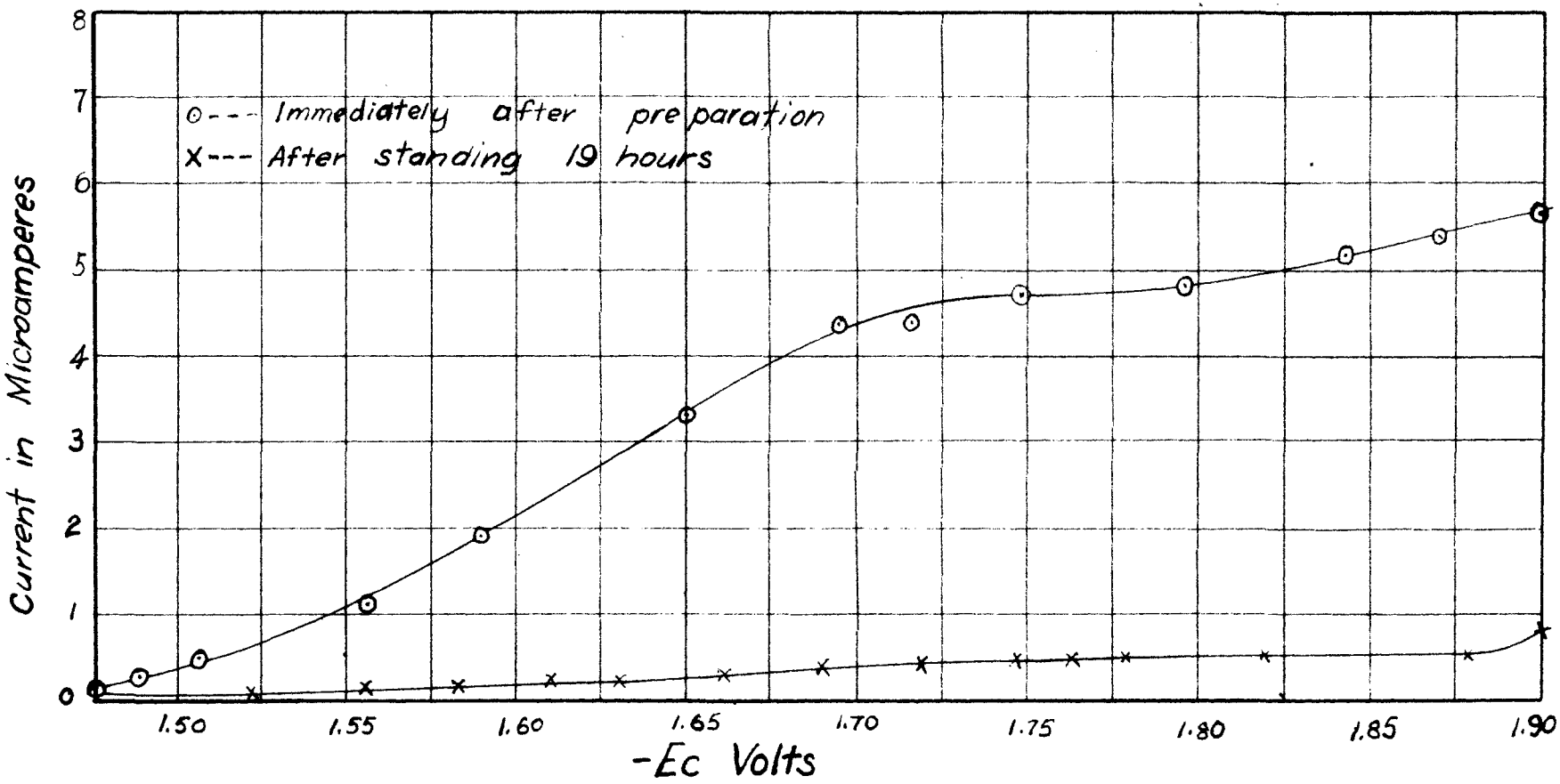


Figure 8. Current-voltage curves for the reduction of dihydroxyacetone in a carbonate buffer, pH 9.7. Drop-time = 2 sec.; temp. = 25°C.

limiting current decreased rapidly upon standing. After 19 hours the current-voltage curve showed no break (see Figure 8). At high pH values, then, dihydroxyacetone was rapidly decomposed or converted into a form which was no longer reducible.

Table 5 shows that no regular shift of half-wave potential with pH was observed. Figure 7 shows typical curves for the reduction of dihydroxyacetone at two different pH values.

Reduction of dihydroxyacetone in the presence of a bacterial juice prepared from Acetobacter suboxydans. Wieland and Bertho (1928) have demonstrated the presence of dehydrogenases in the oxidative dissimilation of ethyl alcohol by the acetic acid bacteria. These authors found it impossible to separate the enzymes from the bacteria in the case of Bacterium orleanense; consequently, dehydrogenation studies were carried out using suspensions of the bacteria in buffered solutions. Both oxygen and quinone were observed to function as hydrogen acceptors. Bertho (1930) later extended this work to include other acetic acid bacteria and other substrates.

Dehydrogenases also would be expected to play a role in the action of Acetobacter suboxydans on polyhydric alcohols; accordingly, in this investigation attempts were made to prepare a bacterial juice which would show dehydrogenase activity.

It was thought that if the reduction of dihydroxyacetone could be carried outin the presence of an "active" bacterial juice prepared from Acetobacter suboxydans, the enzyme might be adsorbed at the mercury/solution interface and there exhibit an effect on the reduction potential of the compound. Since most enzymes which function in hydrogen transfer are considered to be reversible oxidation-reduction systems, a further interesting possibility was that the bacterial juice prepared would show a "cathodic wave" characterizing some reversible enzyme system.

The bacterial juice employed was prepared according to a procedure similar to that given by Wiggert, Silverman, Utter, and Werkman (1940). The Acetobacter suboxydans was grown on a glycerol medium containing per 100 ml.: 6.0 grams of glycerol, 0.50 gram of yeast extract, and 0.30 gram of primary potassium phosphate. The bacteria were cultured at 28°C. in large mold flasks. Each flask contained only 300 ml. of medium in order to insure a large surface-volume ratio. After incubation for a period of four days the cells were separated from the broth by centrifugation in a Sharples supercentrifuge at about 35,000 RPM. The cells were washed with several 100 ml. portions of phosphate buffer (pH 6.4).

Six grams of washed cells, 18 grams of finely ground Pyrex glass, and enough phosphate buffer (pH 6.4) to form a thick paste were mixed intimately by means of a spatula. An 8 gram portion of the resulting paste was ground vigorously

in a well-iced mortar for four minutes. All such 8 gram portions were combined after grinding and extracted with 2 ml. of phosphate buffer (pH 6.4) for each 8 gram ground portion. The mixture was centrifuged for 15 minutes on a number 2 International Centrifuge at about 2,500 RPM. The supernate was clarified further by centrifugation in a Beams ultracentrifuge at about 175,000 RPM. The supernate from the latter centrifugation was used as the bacterial juice.

The bacterial juices prepared according to the above procedure did not show any activity by the methylene blue technique when either glycerol or sorbitol was used as a substrate.

Two ml. of the bacterial juice were diluted to 50 ml. with 1.5×10^{-3} M dihydroxyacetone in a phosphate buffer solution of pH 6.4. Reduction of dihydroxyacetone in the resulting solution gave a current-voltage curve (Figure 9) which showed no well-defined limiting current. The decomposition voltage, however, was shifted to a somewhat more positive value. Several other concentrations of the bacterial juice were used but no important variations in the shape of the current-voltage curves were obtained.

Reductions of dihydroxyacetone also were performed in the presence of the bacterial juice using a carbonate buffer of pH 9.7. Figure 10 shows the current-voltage curve obtained using a solution prepared by diluting 0.6 ml. of bacterial juice to 50 ml. with 1.5×10^{-3} M dihydroxyacetone in the carbonate buffer. Although a symmetrical curve was obtained, the

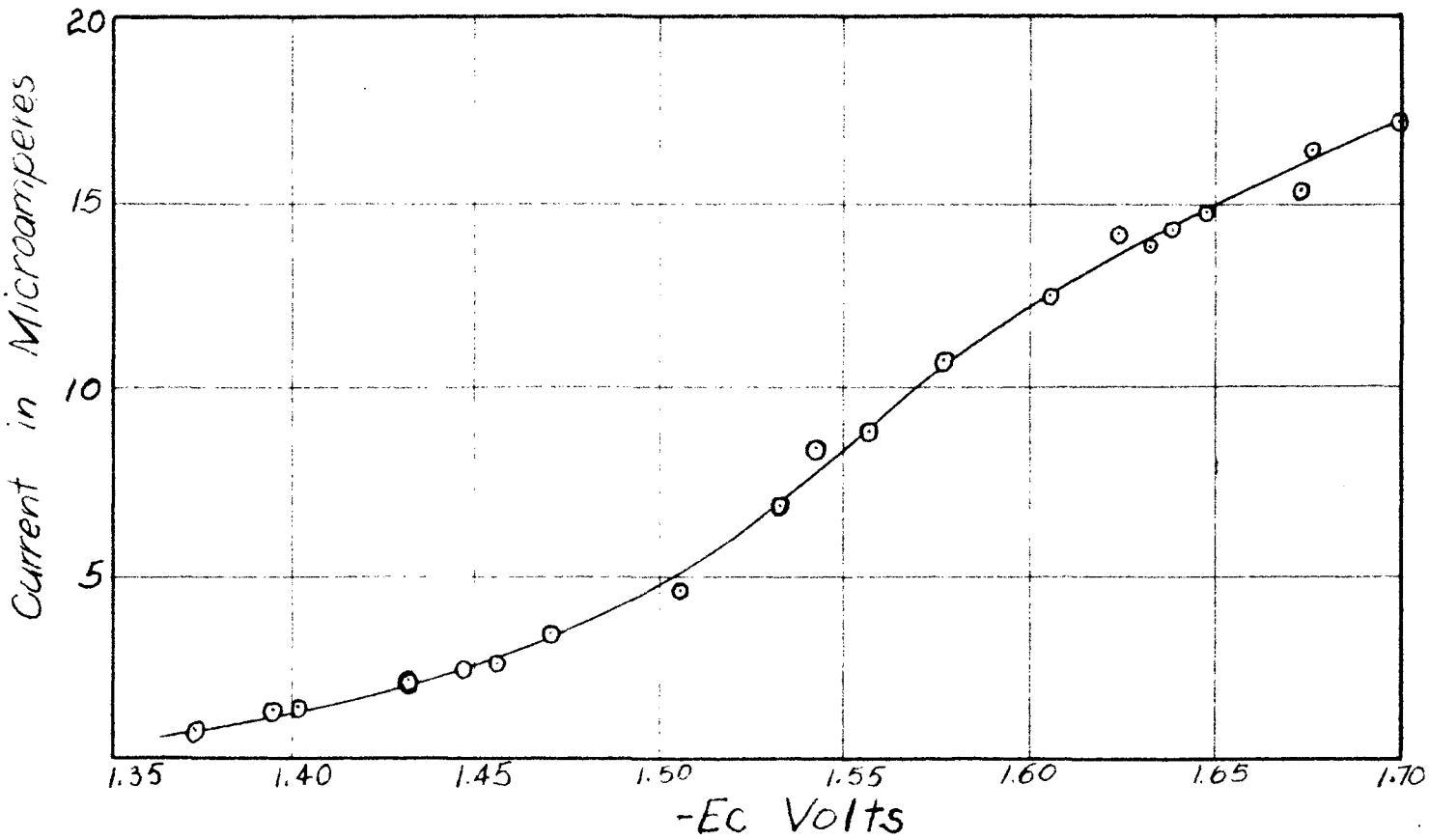


Figure 9. Current-voltage curve for the reduction of 1.5×10^{-3} M dihydroxyacetone in phosphate buffer, pH 6.4; 2.0 ml. of bacterial juice added; drop-time = 4 sec.; temp. = 25°C.

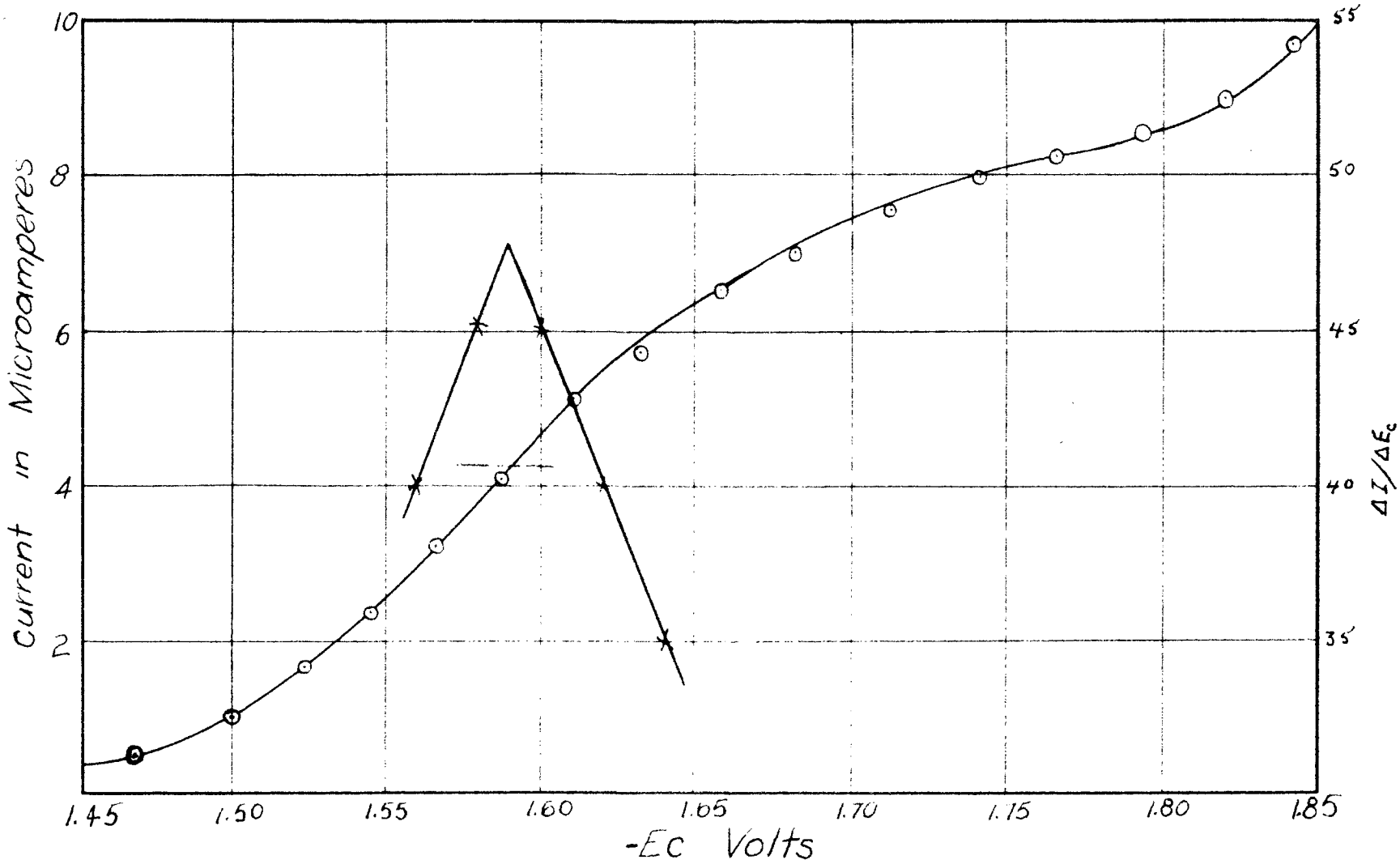


Figure 10. Current-voltage curve for the reduction of 1.5 x 10^{-3} M dihydroxyacetone in carbonate buffer, pH 9.7; 0.6 ml. of bacterial juice added; drop-time = 4 sec.; temp. = 25°C.

limiting current could not be determined easily. The half-wave potential taken as the inflection point on the curve was found to be -1.59 volts, a value which agreed closely with the values in Table 5. The method of determining the inflection point is given in Figure 10. When more concentrated solutions of the bacterial juice were used, current-voltage curves were obtained which showed no limiting current.

The only observed effect of the bacterial juice in the above experiments seemed to be a decreased hydrogen overvoltage on mercury.

Reduction of l-erythrulose in 0.1 M lithium chloride. The reductions of erythrulose were carried out using the diluted fermentation liquor. The composition of the meso-erythritol medium used to produce the erythrulose is given in the section of this thesis dealing with materials.

After incubation of the inoculated medium for six days at 28°C., the fermentation liquor was analyzed using the polarographic method. Preliminary investigations showed that dilution of 1.00 ml. of the fermentation liquor to a volume of 200.0 ml. with 0.1 M lithium chloride gave a convenient concentration for analysis. Figure 11 shows two current-voltage curves obtained for reductions carried out using the diluted fermentations. The higher curve, dilution 1:200, is almost twice the height of the lower curve, dilution 1:400; therefore, the magnitude of the limiting current appears to be

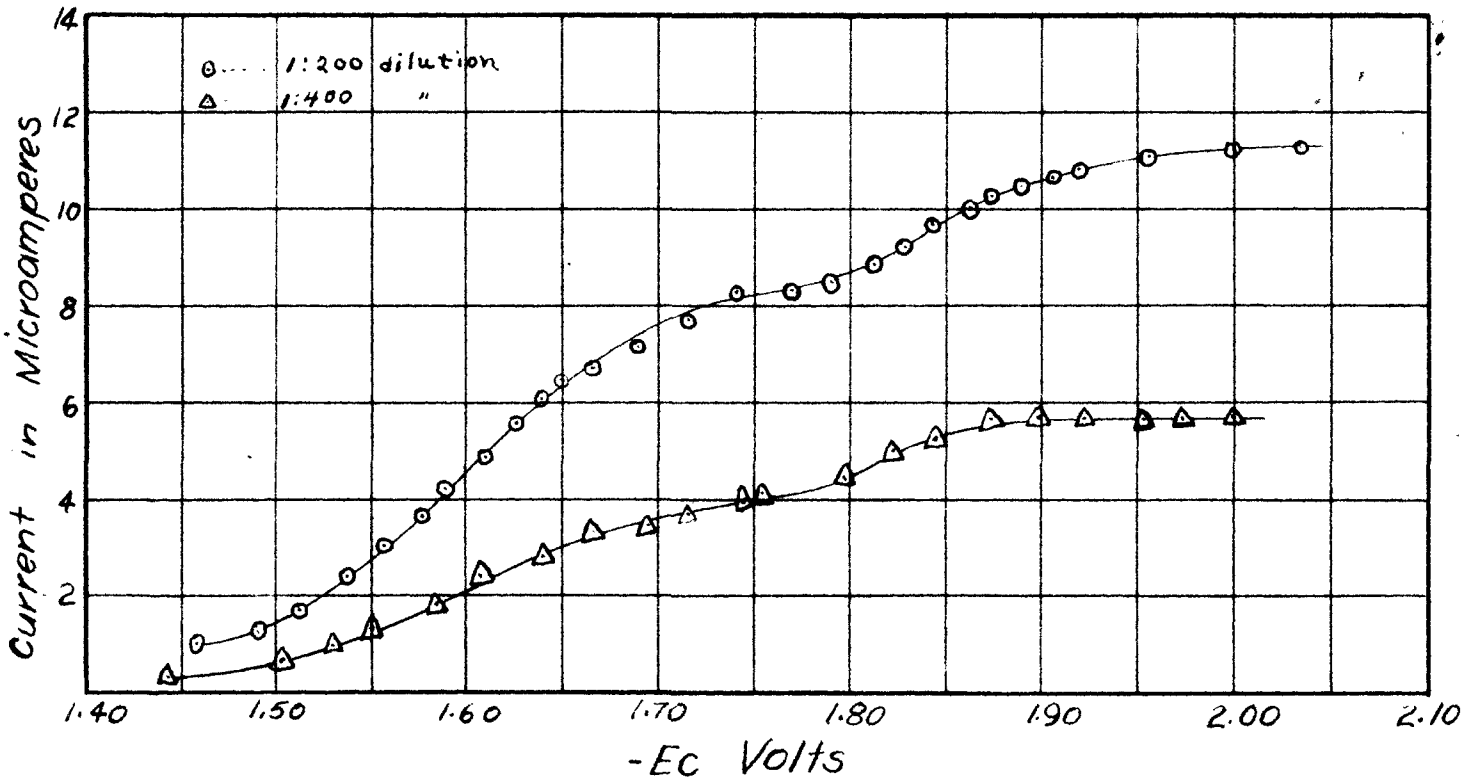


Figure 11. Current-voltage curves for the reduction of erythrulose in a six-day meso-erythritol fermentation; indifferent electrolyte, 0.1 M lithium chloride; drop-time = 4 sec.; temp. = 25°C.

directly proportional to the concentration of reducible material.

Using a medium of the same composition as the one used above, Whistler and Underkofler (1938) obtained 96 per cent yields of erythrulose after a seven-day period of fermentation. The yield was determined by means of the Shaffer-Hartmann sugar titration method. On the basis of a 96 per cent yield the diffusion current value for erythrulose at 1:200 dilution calculated by means of the Ilkovič equation was found to be 6.1 microamperes. Figure 11 shows that the current-voltage curves formed two distinct "waves", the first being somewhat higher than the second. Either the first "wave" was due to the reduction of erythrulose and the second to some other reducible material formed in the medium, or the erythrulose formed two "waves" in the presence of the bacteria and enzymes introduced from the fermentation liquor, thereby indicating a two-step reduction. Assuming the first "wave" to be due to the reduction of erythrulose, the observed value for the diffusion current at 1:200 dilution was 7.2 microamperes. This value is higher than the calculated value given above.

The half-wave potential for the first "wave" was -1.60 volts while that for the second was around -1.85 volts. Averaging these two values gave a half-wave potential of -1.72 volts.

Reductions carried out employing the sterilized, diluted, and unfermented medium showed only a residual current passing through the electrolysis cell up to an applied voltage of -2.0.

Reduction of l-sorbose at various concentrations in 0.1 M lithium chloride. A vacuum-dried sample of pure sorbose was accurately weighed and dissolved in 0.1 M lithium chloride to form a standard stock solution of the compound. Nitrogen was bubbled through the solution for about an hour to expel dissolved oxygen. The sorbose solutions did not show an "aging effect" as markedly as did the dihydroxy acetone solutions mentioned earlier; consequently, the stock solutions could be kept for longer periods. Samples employed in analysis were prepared by diluting the stock solution with 0.1 M lithium chloride.

Very smooth current-voltage curves showing well-defined limiting currents were obtained for the reduction of sorbose in 0.1 M lithium chloride. Figure 12 shows curves for three different concentrations of sorbose while Figure 13, a plot of diffusion current against molarity, shows the linear relationship between the diffusion current and the concentration of the reducible compound. Table 6 demonstrates the relation between the diffusion currents calculated by means of the Ilkovič equation and those determined experimentally.

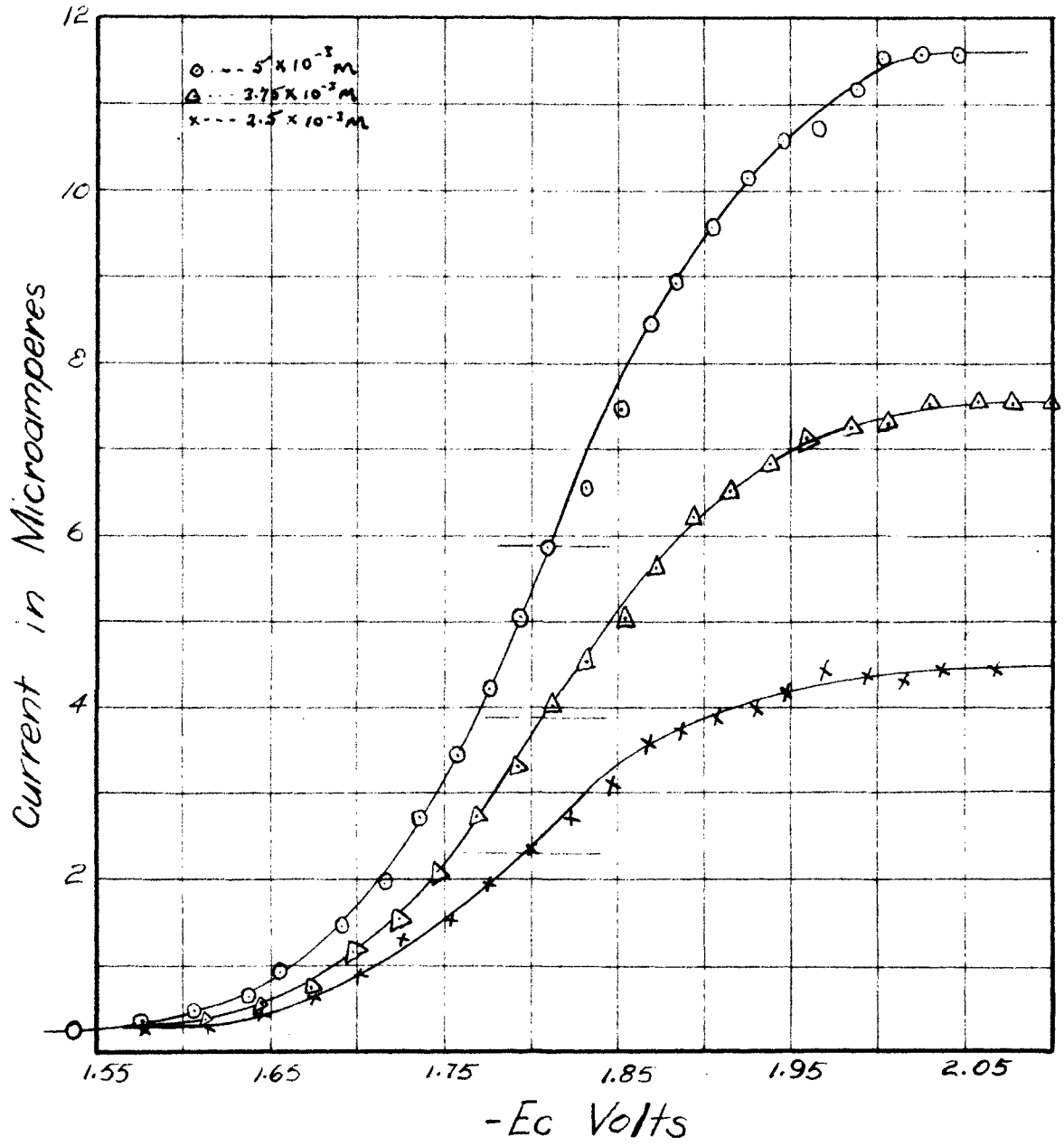


Figure 12. Current-voltage curves for the reduction of sorbose at various concentrations in 0.1 M lithium chloride; drop-time = 4 sec.; temp. = 25°C.

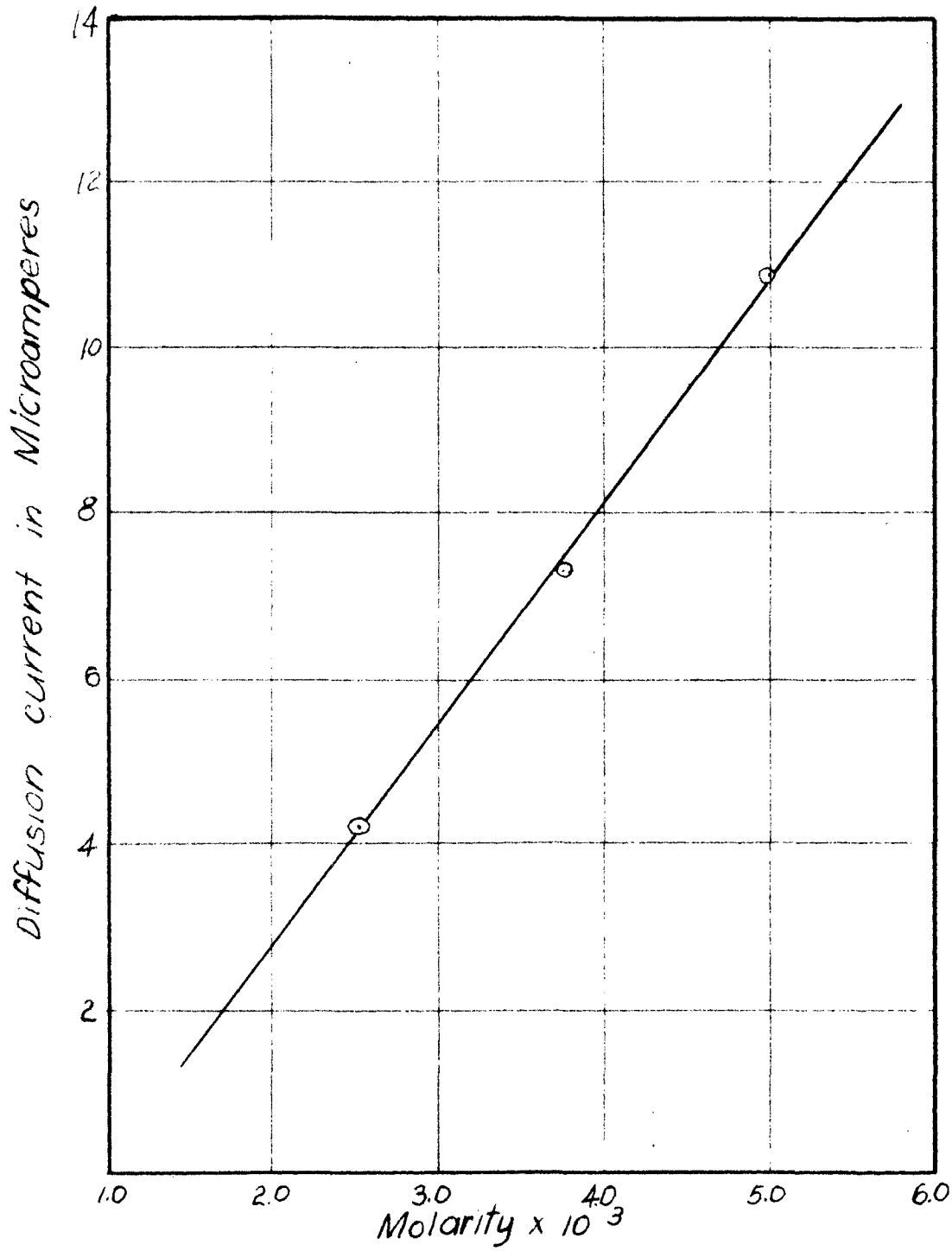


Figure 13. Relationship between diffusion currents and concentration of sorbose.

Table 6

Diffusion Currents of Sorbose at Various Concentrations

$D = 6.6 \times 10^{-6}$ cm.^{2}/sec. (Table 1); $m = 0.00125$ g./sec. (Figure 3); $t = 4$ sec.; temp. = 25°C.

Concentration (moles/liter x 10^3)	Diffusion Current (microamperes)	
	Calculated	Observed
2.50	11.3	4.2
3.75	17.0	7.3
5.00	22.7	10.9

Reduction of l-sorbose in 0.1 N lithium carbonate. Unfortunately, buffers containing potassium ions could not be used in studying the reduction of sorbose since the discharge of these ions began at a potential corresponding to the limiting current of sorbose. Because no suitable buffer could be obtained, lithium carbonate was chosen to produce alkaline solutions for the reduction of this compound. The effect of changes in acidity of the solution on the half-wave potential of sorbose is shown in Table 7.

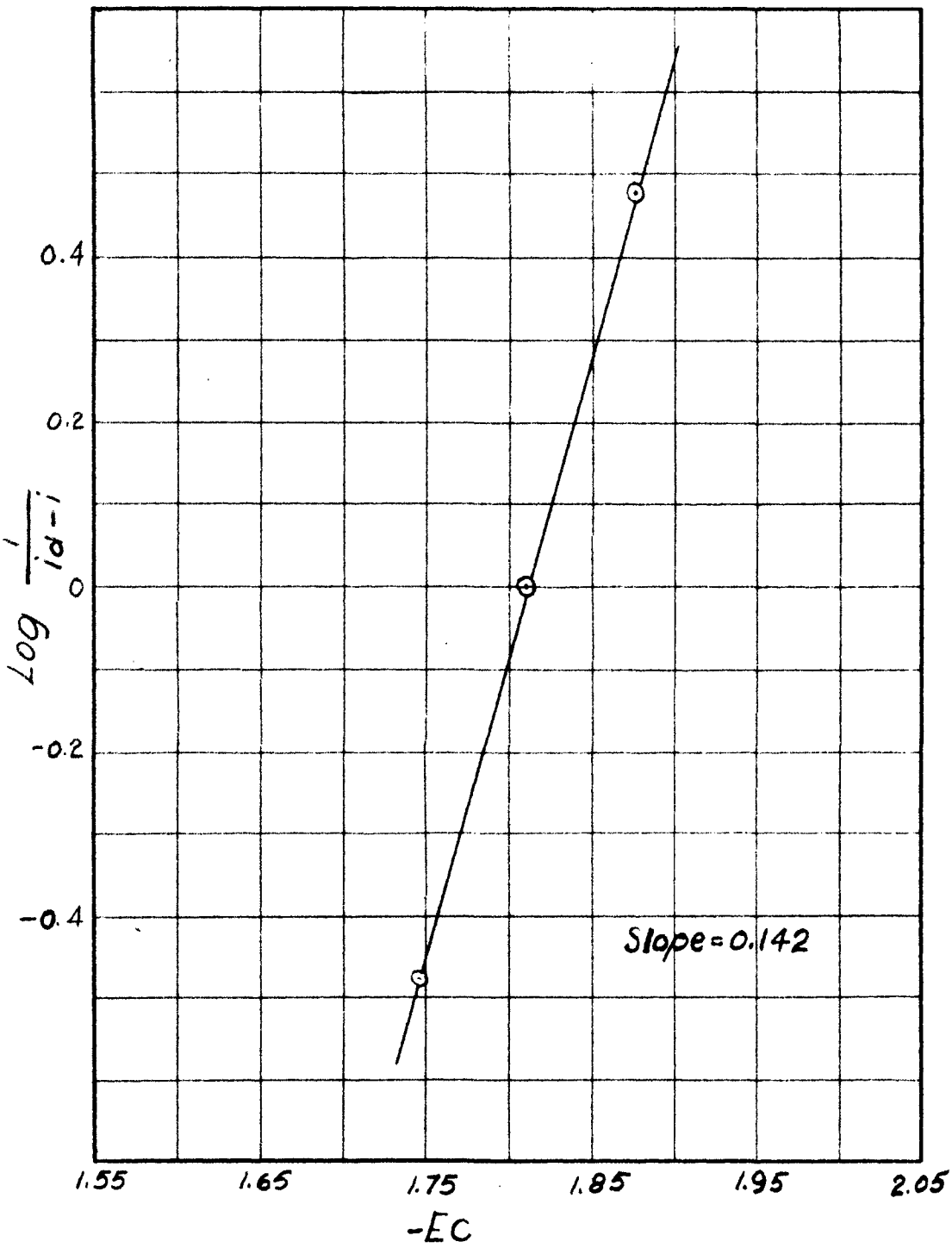


Figure 14. Relation of log $\underline{I}/\underline{I}_d - \underline{I}$ to $\underline{E}_c$ in the case of the reduction of 5.0×10^{-3} M sorbose in 0.1 M lithium chloride.

Table 7

Half-wave Potentials of Sorbose in Various Unbuffered Solutions

Concentration (moles/liter x 10^3)	Indifferent Electrolyte	pH	Half-wave Potential (- volts)
2.50	0.1 M LiCl	Neutral	1.800
3.75	"	"	1.807
5.00	"	"	1.807
3.75	0.1 N Li_2CO_3	10.7	1.848
5.00	0.1 N Li_2CO_3, LiCl	10.5	1.830

From an inspection of the calculated and observed diffusion current values in Table 6, it is evident that if the Ilkovič equation can be applied to the reduction of sorbose at the dropping mercury cathode, then the value for the number of faradays required per mole is more likely one than two. If the reductions represented in Figure 12 are illustrative of reversible processes, the slope of the straight line obtained by a graph of log $\frac{I/I_d - I}{}$ against E_c should be 0.0295 at 25°C. for $n = 2$ or 0.059 for $n = 1$.

Table 8 shows the values of E_c at different heights on a current-voltage curve illustrating the reduction of 5.0 x 10^{-3} M sorbose in 0.1 M lithium chloride.

Figure 14 shows the straight line obtained when log $\frac{I/I_d - I}{}$ was plotted against E_c. The slope was found to be 0.142.

Table 8

Relation of $\underline{I/I_d - I}$ to $\underline{E_c}$ for the Reduction of 5.0×10^{-3} M Sorbose in 0.1 M LiCl

$\underline{I/I_d}$	$\underline{E_c}$ (- volts)	$\underline{I/I_d - I}$	log $\underline{I/I_d - I}$
0.25	1.745	0.333	- 0.47756
0.50	1.806	1.000	0.00000
0.75	1.881	3.000	+ 0.47712

Reduction of d-tagatose at various concentrations in 0.1 M lithium chloride. The production of tagatose by Acetobacter suboxydans has not been reported; therefore, strictly speaking, this compound does not belong in the series studied in this investigation. However, it is highly probable that d-talitol would be oxidized by Acetobacter suboxydans since it has the "proper configuration" for fermentation. Lack of a reported fermentation is probably due to the scarcity of d-talitol.

Heyrovský and Smoleŕ (1932) have reported sorbose and fructose to be reducible at -1.80 volts in neutral solution. This investigation has confirmed the reduction value of the former compound. Since tagatose is an isomer of sorbose and fructose it too would be expected to have a half-wave potential value around -1.80 volts.

Figure 15 shows the current-voltage curves for the re-

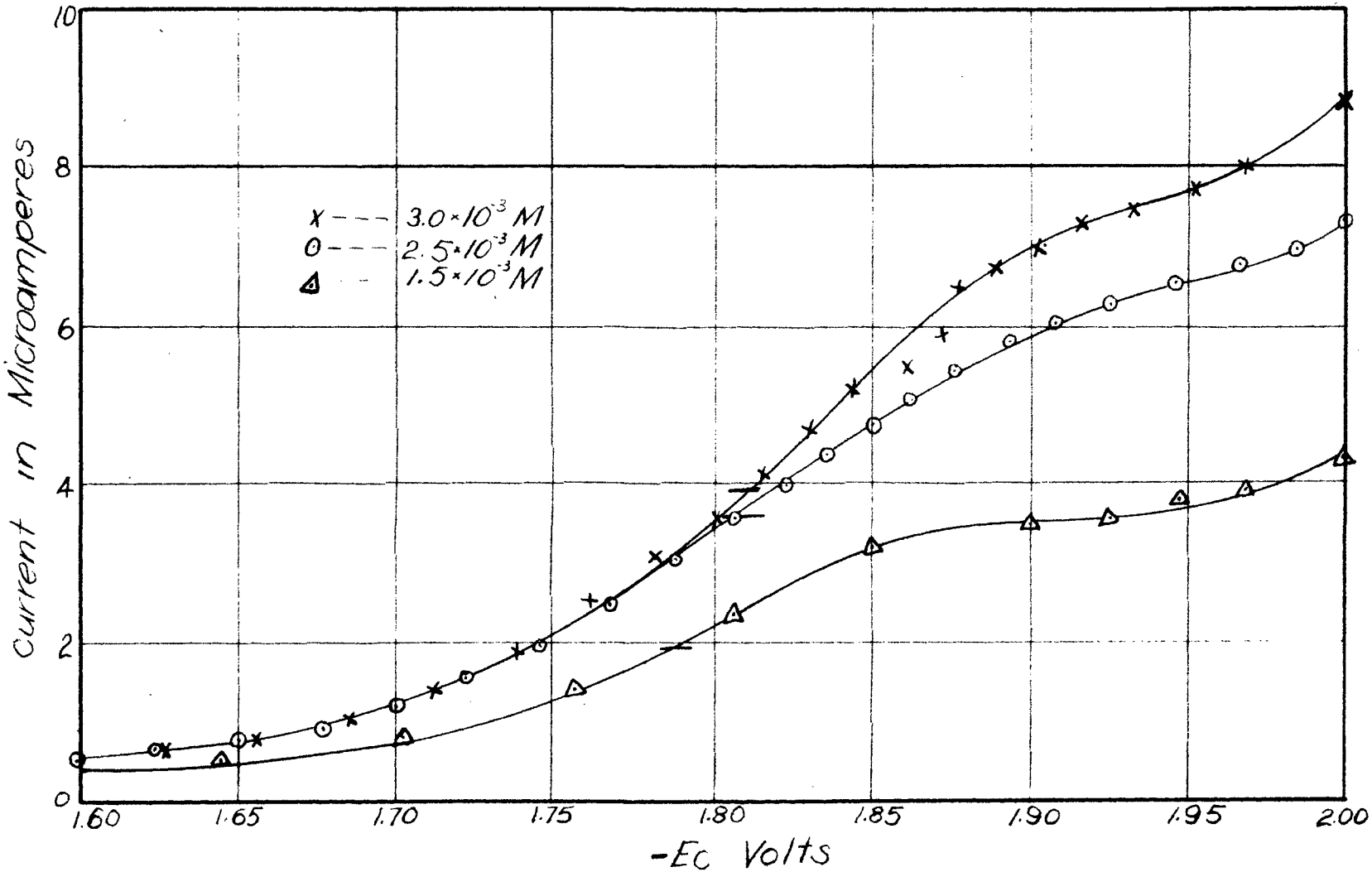


Figure 15. Current-voltage curves for the reduction of tagatose at various concentrations in 0.1 M lithium chloride; drop-time = 4 sec.; temp. = 25°C.

duction of tagatose at three different concentrations. The curves are not the smooth S-shaped type obtained with sorbose. Moreover the relationship between the diffusion current and concentration of tagatose does not appear to be a linear one; however, this is probably due to the fact that the limiting currents were difficult to determine.

Table 9

Half-wave Potentials and Diffusion Currents for the Reduction of Tagatose at Various Concentrations in 0.1 M LiCl

Concentration (moles/liter x 10^3)	Half-wave Potential (- volts)	Diffusion Current[1] (microamperes)	
		Calculated	Observed
1.50	1.790	6.8	3.1
2.50	1.806	11.3	6.1
3.00	1.809	13.6	7.2

[1] The values of m, t, and D for tagatose are the same as those for sorbose.

Reduction of "ketose" at various concentrations in 0.1 M lithium chloride. Previous investigations in this laboratory by Dunning, Fulmer, Guymon, and Underkofler have disclosed that the action of Acetobacter suboxydans upon the cyclic polyhydric alcohol i-inositol produced an oxidation product which analysis showed to be principally a diketo-i-

inositol. Although a full identification of the ketose compound has not been completed, results to date uphold the originally assigned formula.

The reduction of this ketose compound at the dropping mercury electrode was of particular interest inasmuch as the above authors had postulated that i-inositol and its oxidation products might exist in reversible oxidation-reduction systems. This postulate was offered to shed light upon the function of i-inositol as Bios I. It was hoped that this investigation might demonstrate a reversible oxidation-reduction system as had been proposed. In addition, it was further thought that a polarographic reduction of the ketose compound might offer additional evidence for the correctness of the assigned formula.

A standard stock solution of the "ketose" was prepared in 0.1 M lithium chloride. Solutions employed in the reduction investigations were prepared by diluting the stock solution with 0.1 M lithium chloride. Current-voltage curves for four different concentrations of "ketose" are illustrated in Figure 16. The strictly linear relationship between diffusion currents and concentration of "ketose" is shown in Figure 17. In Table 10 are presented data showing the relation between the experimentally determined diffusion currents and the diffusion currents calculated by means of the Ilkovič equation assuming four electrons to be required for the reduction of each molecule of "ketose".

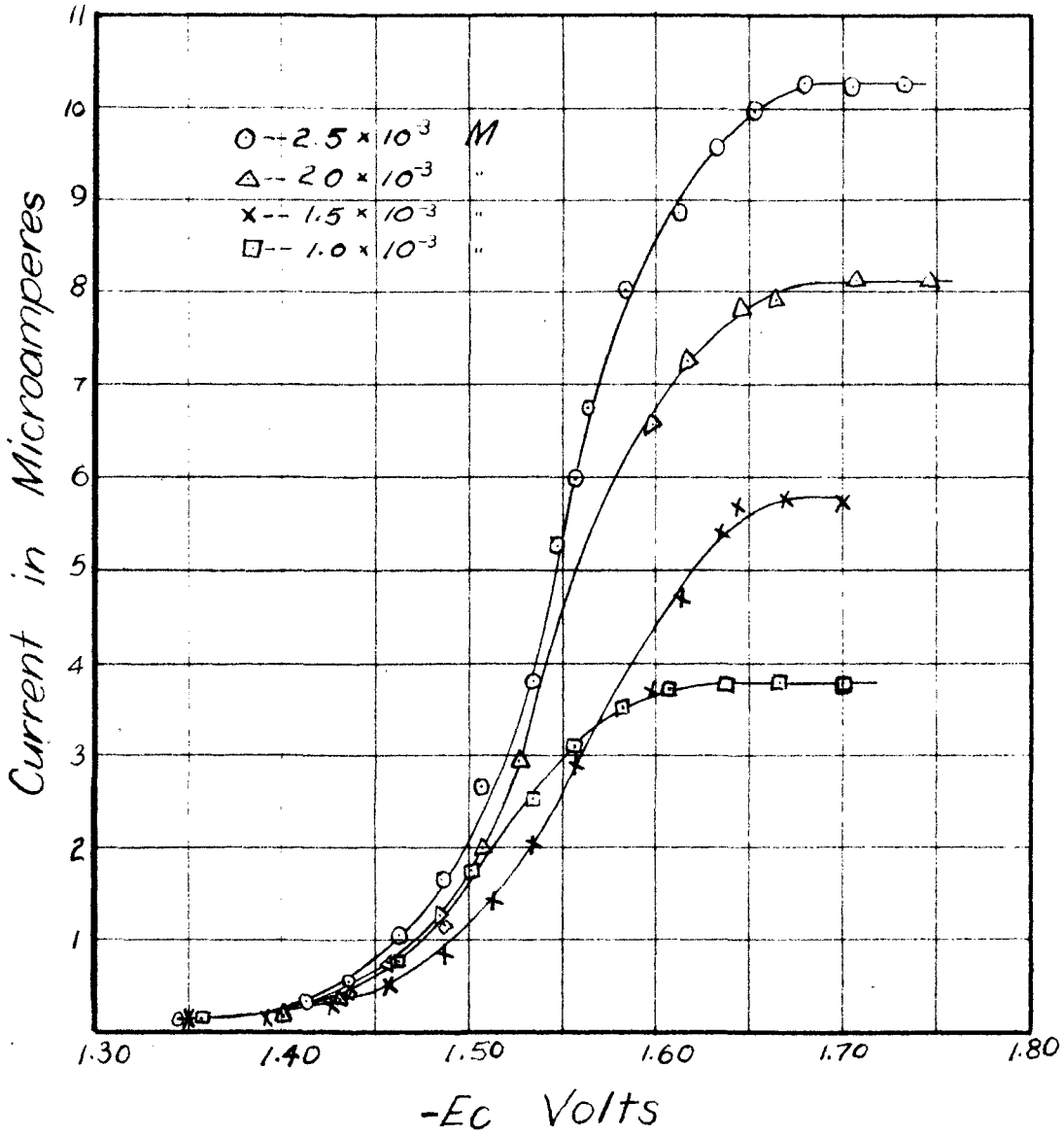


Figure 16. Current-voltage curves for the reduction of "ketose" at various concentrations in 0.1 M lithium chloride; drop-time = 4 sec.; temp. = 25°C.

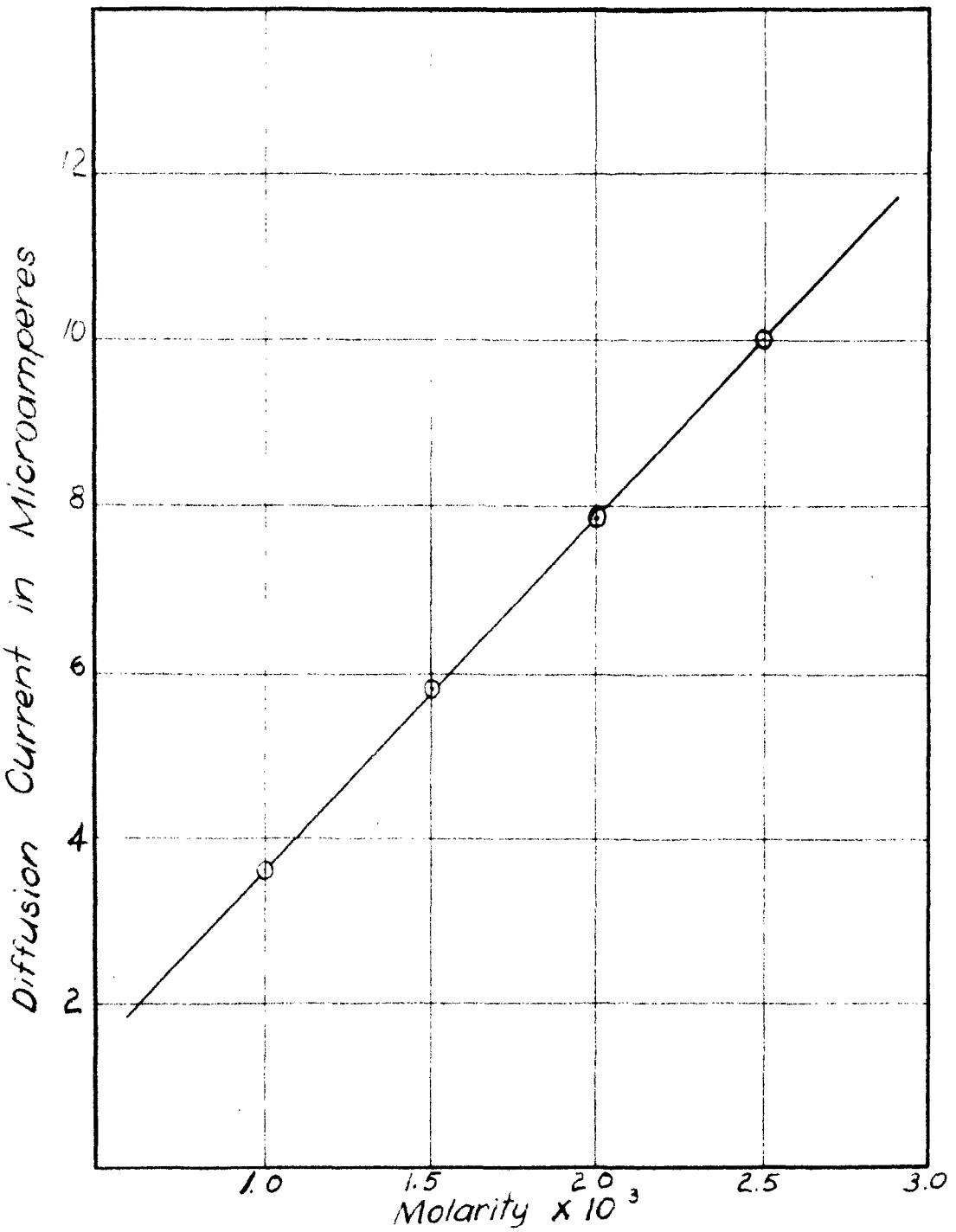


Figure 17. Relation between diffusion currents and concentration of "ketose".

Table 10

Diffusion Currents for the Reduction of "Ketose" at Various Concentrations in 0.1 M LiCl

D = 6.7 x 10^{-6} $cm.^2$/sec. (Table 1); m = 0.00153 g./ sec. (Figure 3); t = 4 sec.; n = 4; temp. = 25°C.

Concentration (moles/liter x 10^3)	Half-Wave Potential (- volts)	Diffusion Current (microamperes)	
		Calculated	Observed
1.0	1.521	10.5	3.6
1.5	1.550	15.7	5.8
2.0	1.555	20.9	7.8
2.5	1.545	26.2	10.0

Table 10 shows a marked discrepancy between the observed and calculated values for the diffusion currents. It was interesting to note, however, that with a 2.5 x 10^{-3} M sorbose solution, assuming n = 2, the ratio of the calculated to the observed diffusion current was 2.69, while the same ratio for a 2.5 x 10^{-3} M "ketose" solution, assuming n = 4, was found to be 2.62. When values of log $I/I_d - I$ were plotted against E_c in the case of reduction of 2.5 x 10^{-3} M "ketose", a slope of 0.083 was obtained.

Reduction of "ketose" in various buffered and unbuffered solutions. In order to determine the shift of half-wave potentials with pH for "ketose", the compound was reduced in a

series of phosphate buffer solutions. Figure 18 shows current-voltage curves for the reduction of "ketose" in phosphate buffers at three different pH values. The shift of the half-wave potential with pH was observed to be small but in a regular order. The curve for the reduction at the highest pH value showed signs of breaking into two separate "waves" around an applied potential of -1.52 volts.

Reductions attempted in the more acid range using potassium chloride-hydrochloric acid and potassium acid phthalate-potassium hydroxide buffers showed no characteristic "ketose wave" because of the discharge of hydrogen ions.

Current-voltage curves for "ketose" in 0.1 M potassium chloride showed very pronounced maxima which could be almost completely eliminated by the addition of several drops of dilute methyl red solution (see Figure 19). The half-wave potential obtained in 0.1 M potassium chloride did not check with the value obtained in 0.1 M lithium chloride (see Table 11). A small amount of this difference in half-wave potentials may be accounted for by the fact that 0.1 M lithium chloride has a higher specific resistance than 0.1 M potassium chloride; therefore, a larger IR correction is necessary in the former case (see equation 21).

The current-voltage curve for the reduction of "ketose" in a potassium carbonate-potassium bicarbonate buffer, pH 9.8,

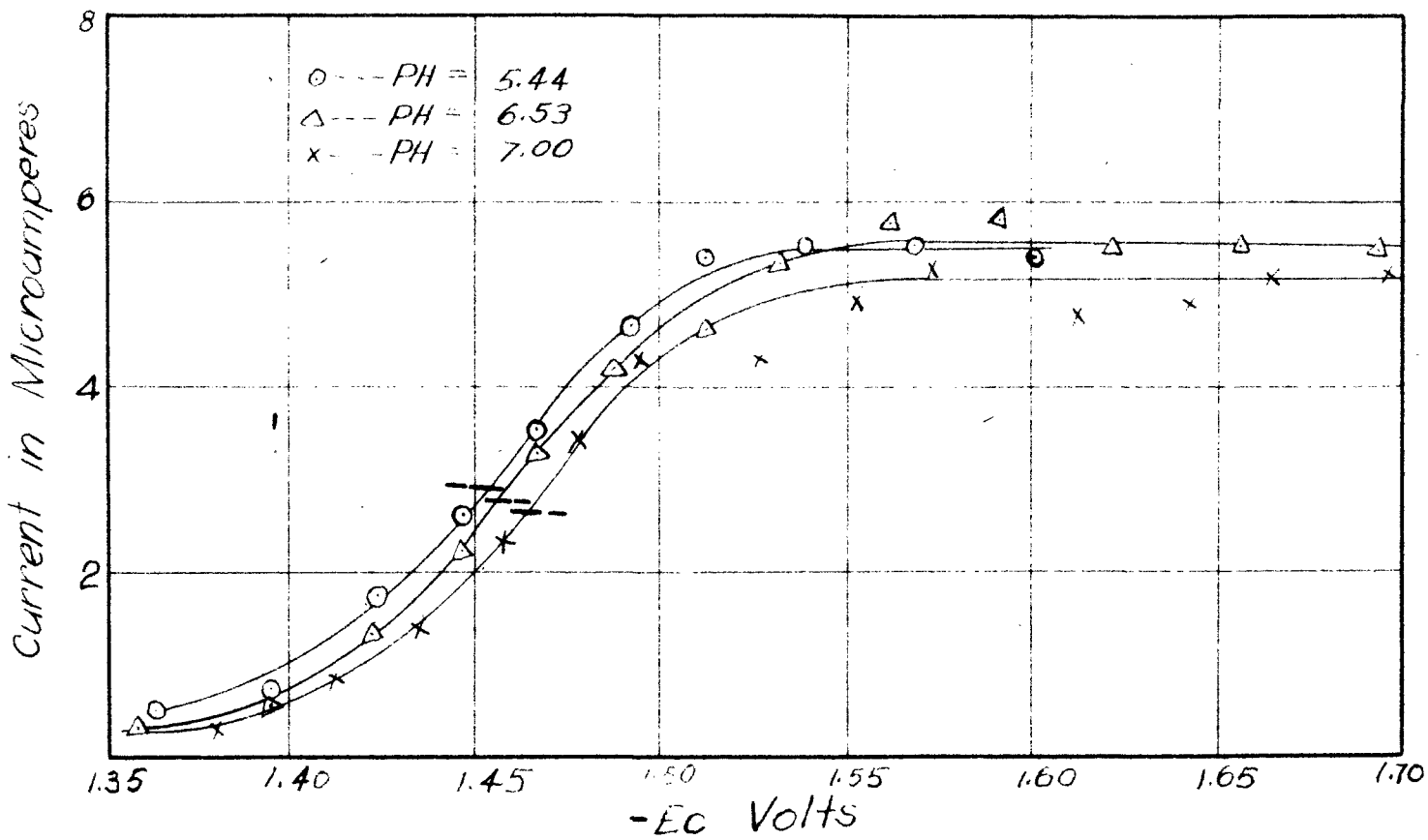


Figure 18. Current-voltage curves for the reduction of "ketose" in KH_2PO_4, KOH, KCl buffers; drop-time = 4 sec.; temp. = 25°C.

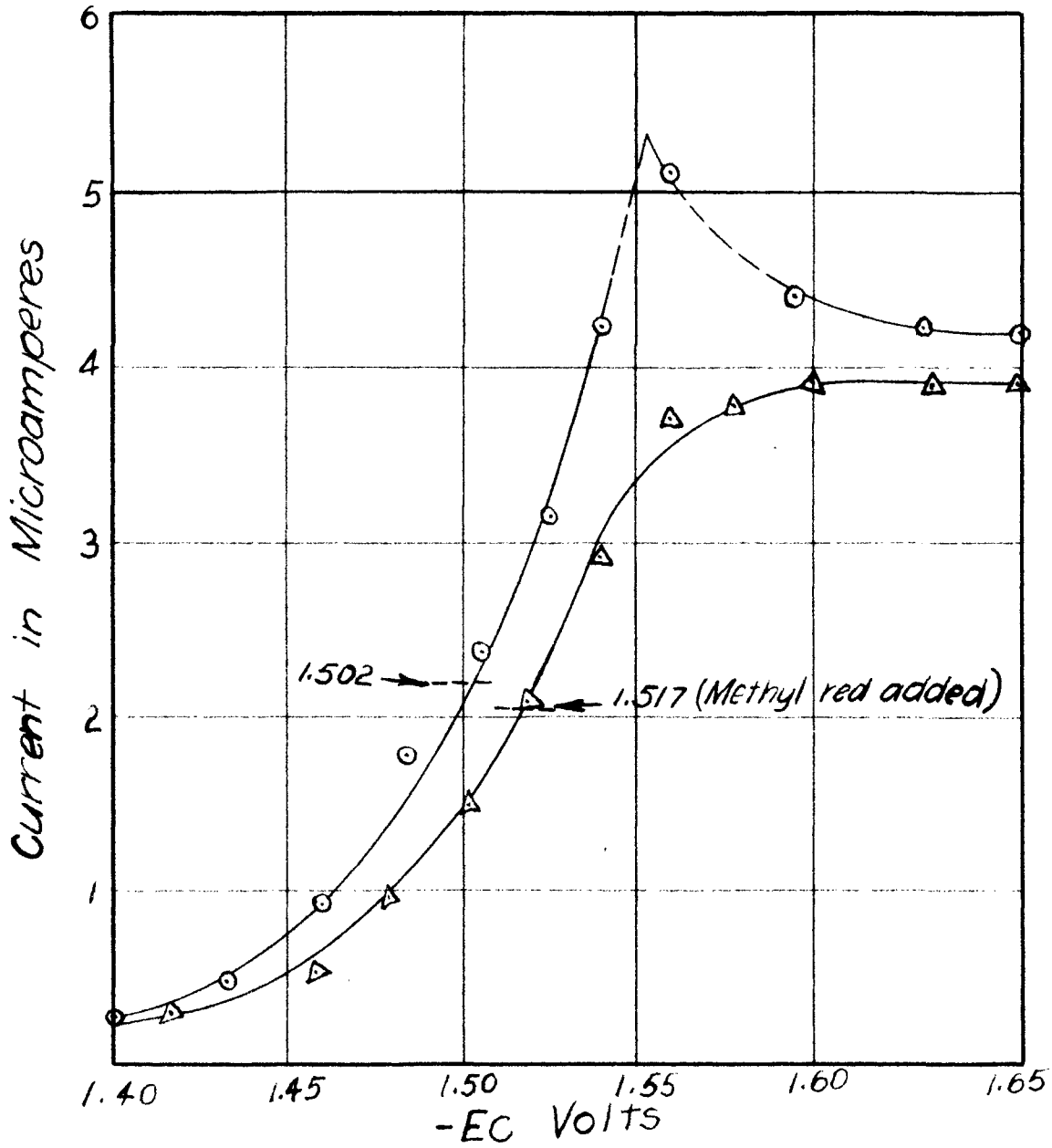


Figure 19. Current-voltage curve for the reduction of 1.0×10^{-3} M "ketose" in 0.1 M KCl; drop-time = 4 sec.; temp. = 25°C.

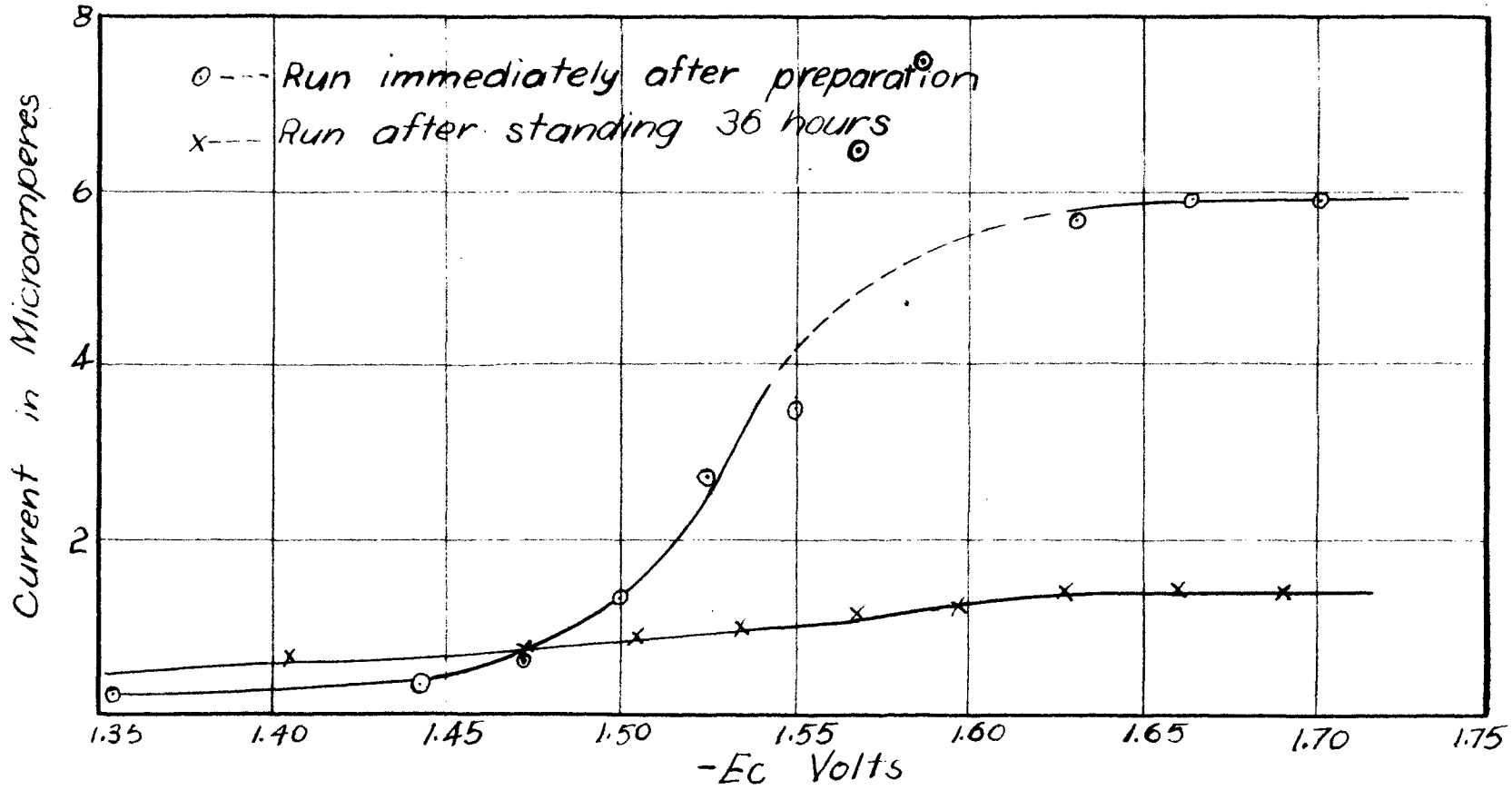


Figure 20. Current-voltage curves for the reduction of "ketose" in K_2CO_3, $KHCO_3$, KCl buffer, pH 9.82; drop-time = 4 sec.; temp. = 25°C.

is illustrated in Figure 20. It was observed that solutions of "ketose" in the carbonate buffer gave no "reduction wave" after standing for a period of 36 hours. This observation was similar to the one made in the case of dihydroxyacetone. In the above case the change was due probably to decomposition of the "ketose" in the strongly basic solution. "Ketose" formed a clear solution in the carbonate buffer, pH 9.8, which changed to a yellow color after standing about 12 hours. This yellow color immediately disappeared upon admitting oxygen to the flask.

The current-voltage curve (Figure 20) for the reduction of "ketose" in the carbonate buffer showed a maximum similar to the one obtained when the reduction was carried out in 0.1 M potassium chloride.

In Table 11 are presented half-wave potential values of "ketose" in various buffered and unbuffered solutions.

Table 11

Half-Wave Potentials of "Ketose" in Several Buffered and Unbuffered Solutions

Buffer or Electrolyte	pH	Half-Wave Potential (- volts)
0.1 M LiCl	Neutral	1.550
0.1 M KCl	"	1.498
KH_2PO_4, KOH, KCl	7.00	1.465
" " "	6.53	1.457
" " "	5.44	1.454
K_2CO_3, $KHCO_3$, KCl	9.82	1.532

Polarographic investigations of a mixture of two ketones.

Reduction of "ketose" and l-sorbose contained in the same solution. The half-wave potential values for the reduction of a series of ketose compounds under different experimental conditions have been presented in the foregoing section of this thesis. The values obtained, arranged in order of increasing negative potential, are as follows: "ketose", -1.550 volts; dihydroxyacetone, -1.596 volts; erythrulose, -1.72 volts (an average of the two half-waves obtained); sorbose, -1.807 volts; and tagatose, -1.807 volts.

If the potentials given in the above series have any thermodynamic significance, then "ketose", the oxidized form of a system more positive in the series, should oxidize sorbitol, the reduced form of a system lower in the series, provided a suitable catalyst is present. The work of Adkins and Cox (1938) in the equilibration of alcohols and ketones using aluminum t-butoxide as a catalyst has been referred to previously.

Before oxidation of sorbitol by "ketose" could be attempted, it was necessary to show that "ketose" could be detected quantitatively in the presence of sorbose and vice versa. Figure 21 shows current-voltage curves obtained when 0.1 M lithium chloride solutions containing both "ketose" and sorbose were analyzed. The half-wave potential and

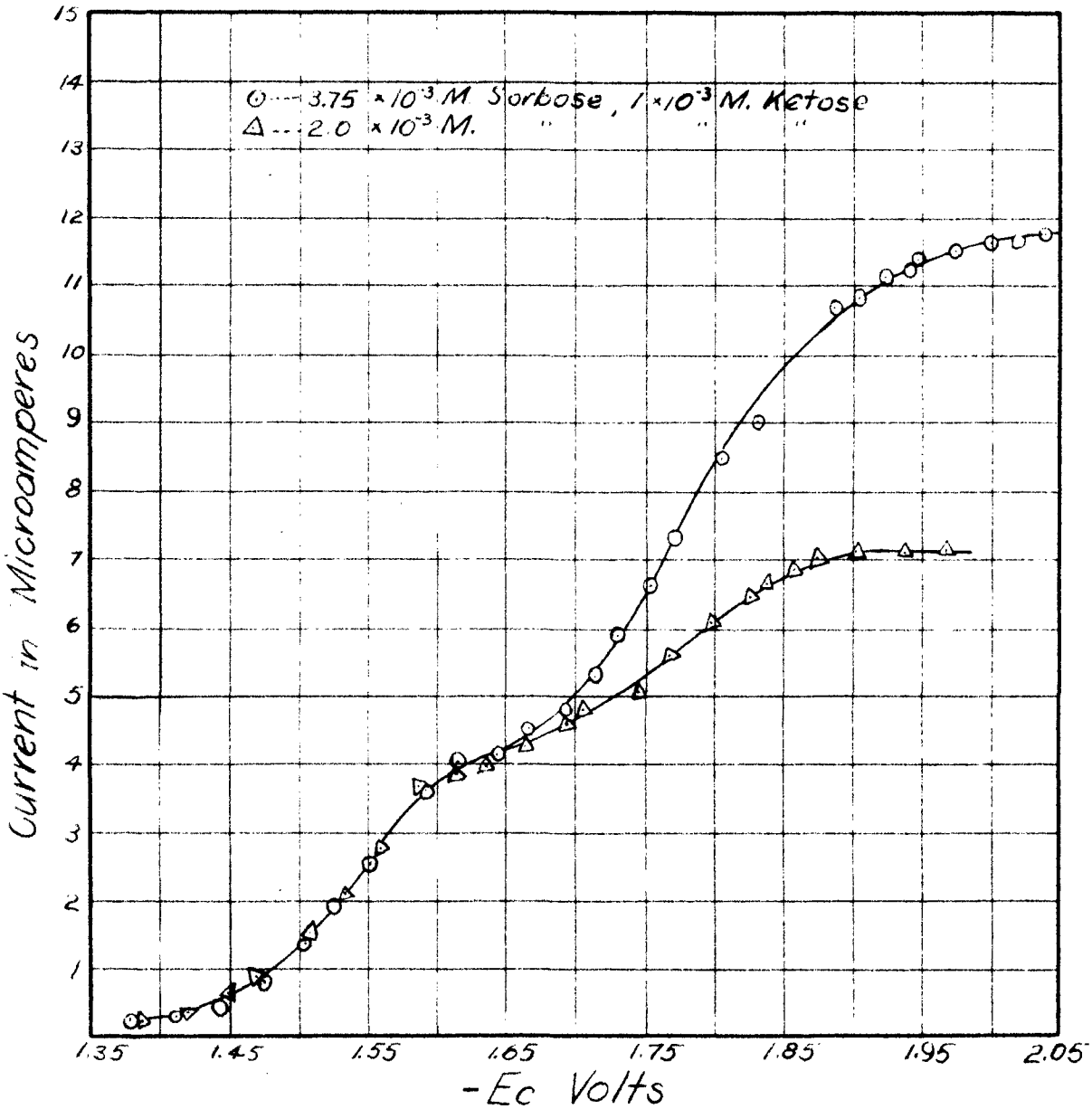


Figure 21. Current-voltage curves for the reduction of mixtures of "ketose" and sorbose in 0.1 M lithium chloride; drop-time = 4 sec.; temp. = 25°C.

diffusion current of each of the two compounds was unaffected by the presence of the other.

Effect of "ketose" upon sorbitol in the presence of palladium black. It was thought that palladium black might be employed as a catalyst in the equilibration of "ketose" and sorbitol since this catalyst has been found to be active in a great many dehydrogenation processes.

The palladium black was prepared in the following manner. A good grade of washed asbestos was suspended in a very dilute alkaline glucose solution. A small amount of a dilute solution of palladium chloride was added and hydrogen was bubbled through the solution whereupon instantaneous reduction of the palladium chloride occurred, the finely divided palladium black being deposited on the asbestos fibers. The palladium-coated asbestos was washed several times with hot water and finally was suspended in distilled water. It was demonstrated to be quite active in the reduction of methylene blue. This material was tried as a catalyst in the equilibration mentioned above.

A solution 0.01 M with respect to both sorbitol and "ketose" was prepared and nitrogen was passed through the solution in order to expel dissolved oxygen. Palladium black was then added and the system was allowed to stand in a nitrogen atmosphere for a period of four hours. At the end of this time the solution was filtered away from the

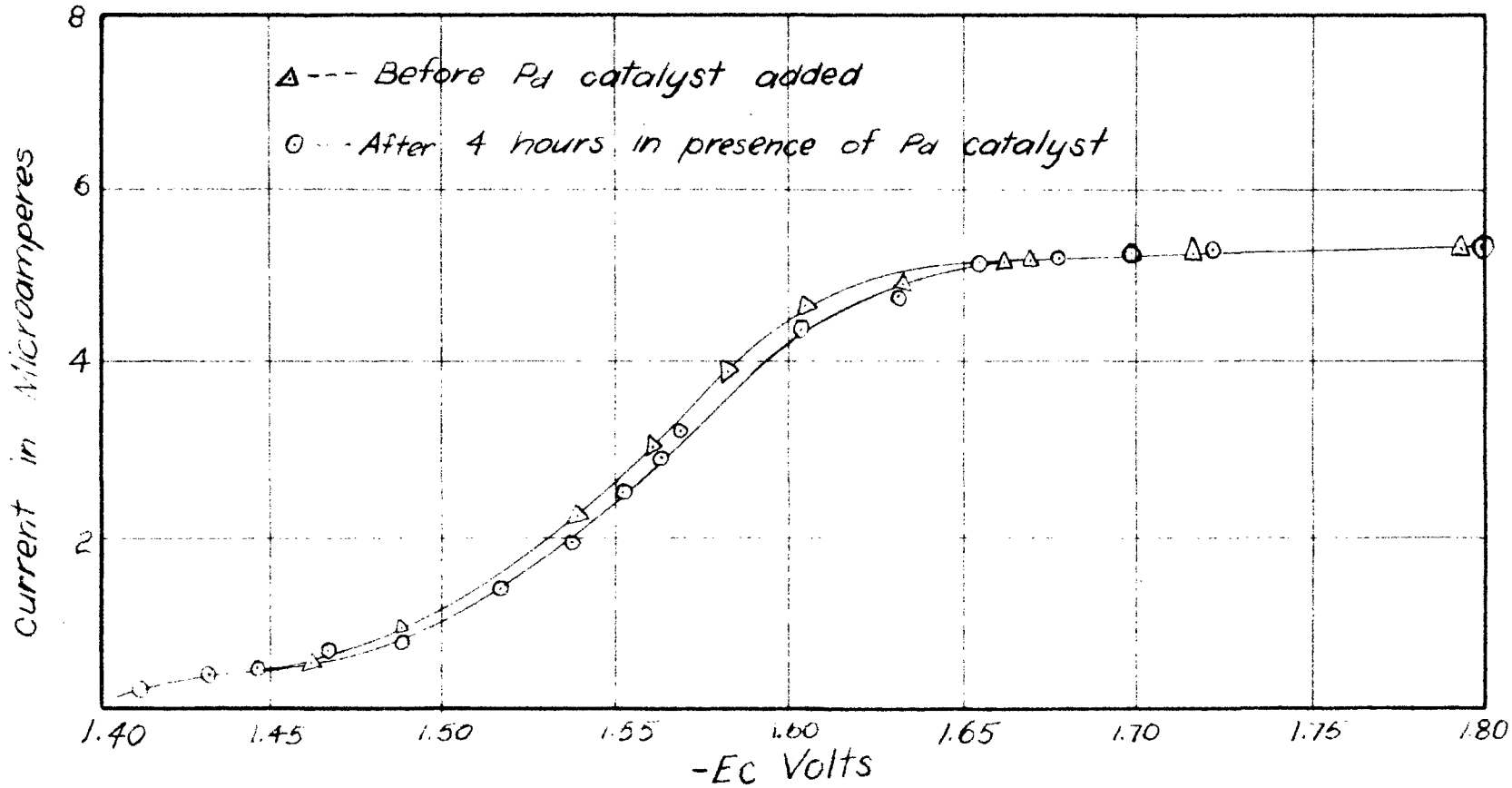


Figure 22. Current-voltage curves for the reduction of a "ketose" -sorbitol mixture in 0.1 M lithium chloride before and after the addition of a palladium-black catalyst; drop-time = 4 sec.; temp. = 25°C.

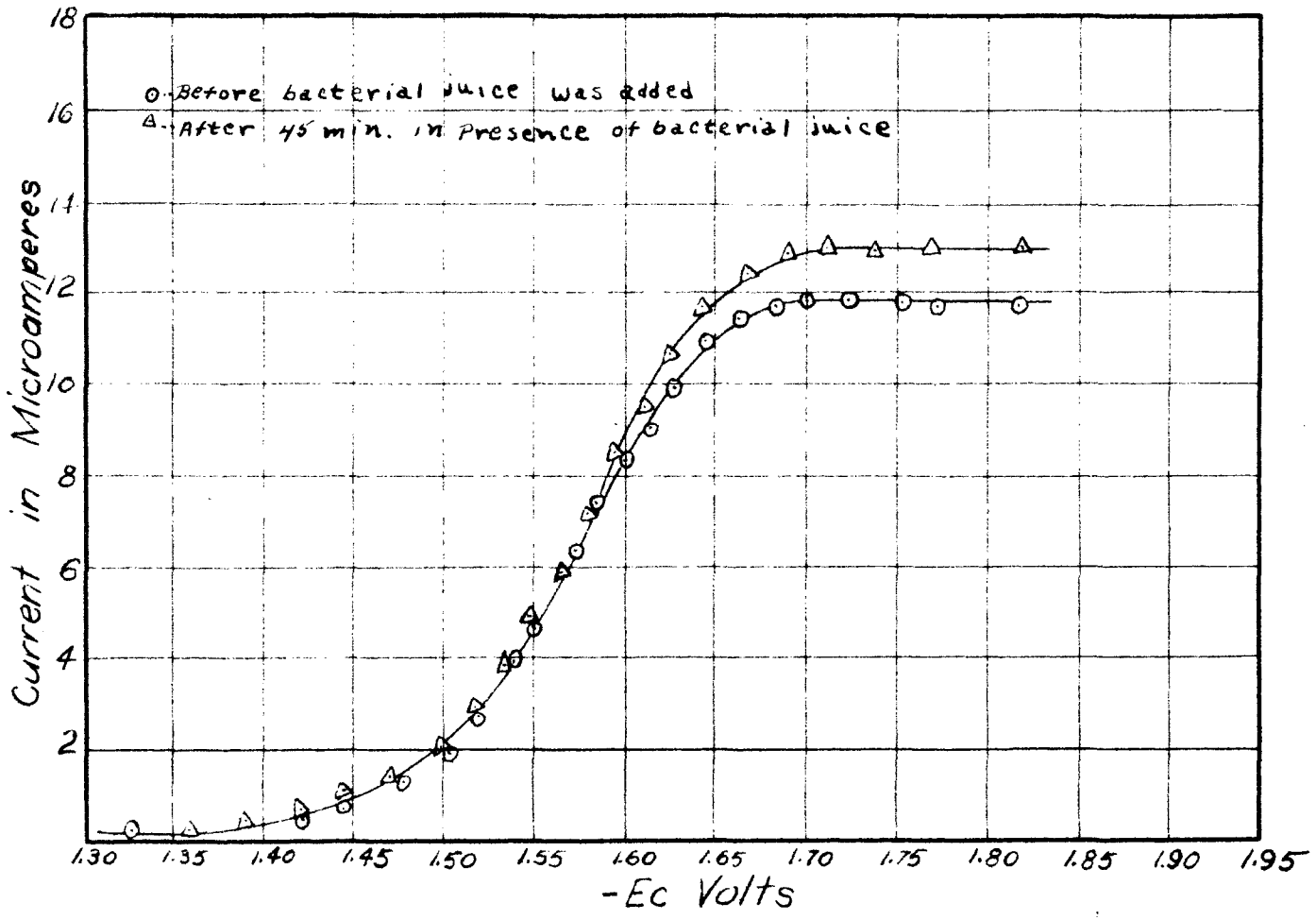


Figure 23. Current-voltage curves for the reduction of a "ketose" -sorbitol mixture in 0.1 M lithium chloride before and after the addition of a bacterial juice prepared from Acetobacter suboxydans; drop-time = 4 sec.; temp. = 25°C.

palladium black while maintaining a nitrogen atmosphere. The resulting solution was diluted 1:25, enough lithium chloride being added to make it 0.1 M with respect to this salt.

Figure 22 shows that no sorbose was formed from sorbitol by the action of "ketose" in the presence of palladium after a four-hour period.

Effect of "ketose" upon sorbitol in the presence of a bacterial juice prepared from Acetobacter suboxydans. The experiment discussed above was repeated using a bacterial juice from Acetobacter suboxydans as a catalyst in place of palladium black. The bacterial juice was prepared according to directions given previously. Figure 23 shows that again negative results were encountered.

The above experiments do not prove, however, that the oxidation of sorbitol by "ketose" is impossible. A suitable catalyst might not have been present in the bacterial juice. As the bacterial juice used was shown to be not highly active, too much significance should not be attached to the results obtained.

Relative fermentability of i-inositol and sorbitol by Acetobacter suboxydans. The fact that sorbose could be detected in the presence of "ketose" suggested the possibility of carrying out a fermentation for the purpose of determin-

ing the order in which i-inositol and sorbitol are fermented by Acetobacter suboxydans. It was thought that there might be some correlation between the order of fermentation of the various polyhydric alcohols and the polarographic half-wave potentials of the corresponding oxidation products.

An i-inositol-sorbitol fermentation was carried out employing a medium which contained 3.0 grams of i-inositol, 3.0 grams of sorbitol and 0.5 gram yeast extract per 100 ml. of solution. The medium was sterilized for 30 minutes at 15 pounds steam pressure. Inoculations were made by adding 1 ml. of an active culture of Acetobacter suboxydans to each 50 ml. of medium in 250 ml. Erlenmeyer flasks. The culture used as the inoculum was grown on a medium having a composition the same as that used in the fermentations to be investigated. The stock culture used in the initial transfer was taken from a 5 per cent glycerol, 0.5 per cent yeast extract agar slant.

The course of the fermentation was followed polarographically, a separate flask being taken for analyses performed at each time interval. As it was necessary to dilute the fermentation liquor for most of the analyses, preliminary experiments had to be run to determine the shape of the current-voltage curves at different dilution ratios when no oxidation products were present. Enough lithium chloride was

added in each case to make the final solution 0.1 M with respect to this salt. It was found that substances reducing at potentials more negative than -1.50 volts could not be detected in the undiluted fermentation medium due to the discharge at this voltage of some substance present in large quantities. The substance discharged was probably hydrogen. The depolarizing material was evidently a constituent of the yeast extract for, when the undiluted medium was treated with Norite, filtered, and the filtrate analyzed, no discharge was observed until a voltage of -1.80 was reached. "Ketose", then, could be determined in the undiluted fermentation liquor but not sorbose.

The first analysis run at the end of three hours using a fermentation liquor which had been filtered through Norite showed a "ketose wave" around -1.50 volts but this was probably due to the small amount of "ketose" added in the initial inoculum. Due to the discharge of hydrogen around a value of -1.70 volts no sorbose could be detected. Subsequent fermentations analyzed at various time intervals were diluted to give concentrations of reducible material which could be analyzed conveniently. Figure 24 shows current-voltage curves for the fermentations at the end of four different periods during the first day. The dilution ratio used to obtain these curves was 1:10 (1 ml. of fermentation liquor to make 10 ml. of solution). Norite was not added.

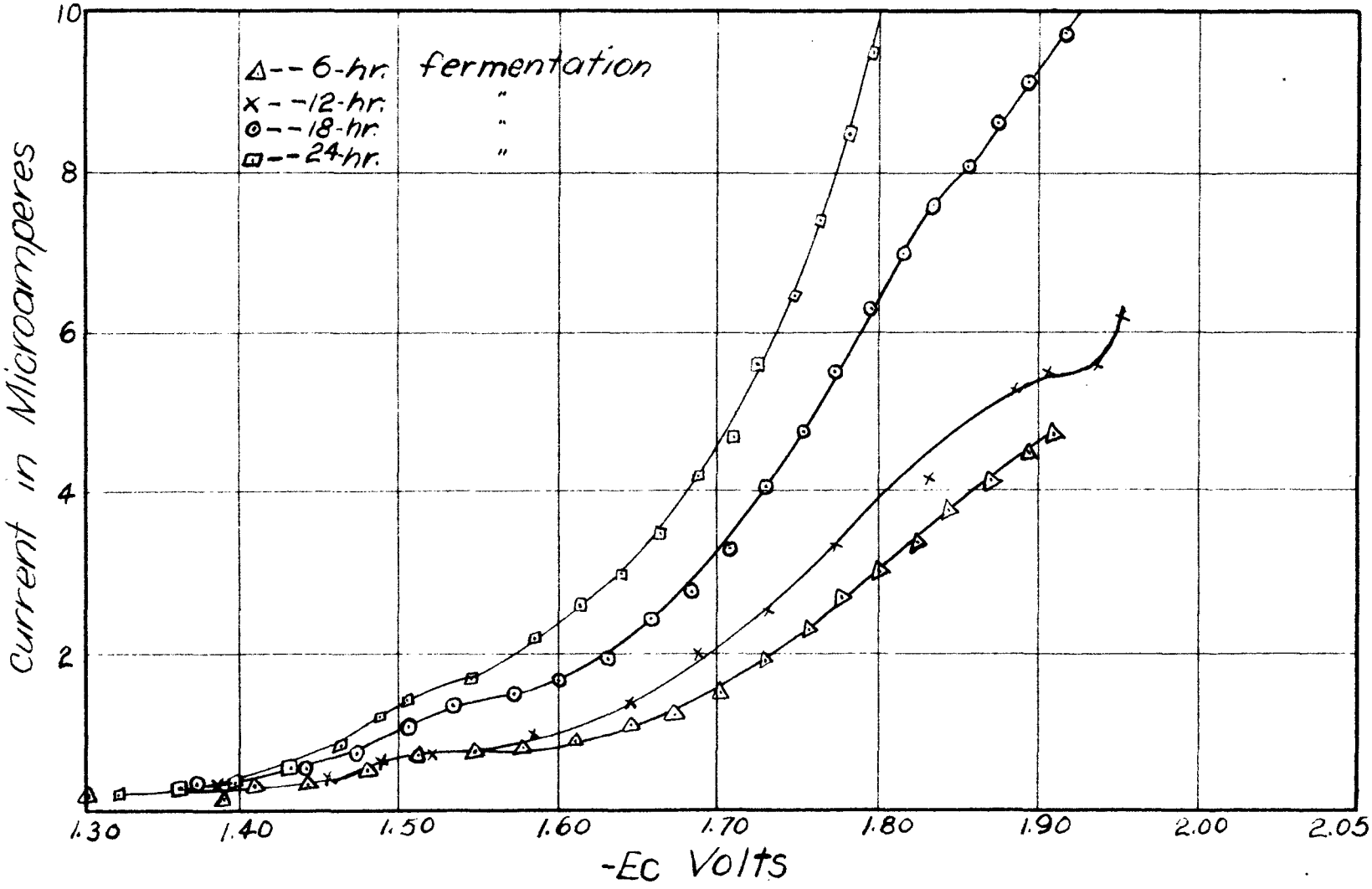


Figure 24. Current-voltage curves for an Acetobacter suboxydans fermentation of an i-inositol-sorbitol medium after various time intervals; dilution ratio I:10; drop-time = 4 sec.; temp. = 25°C.

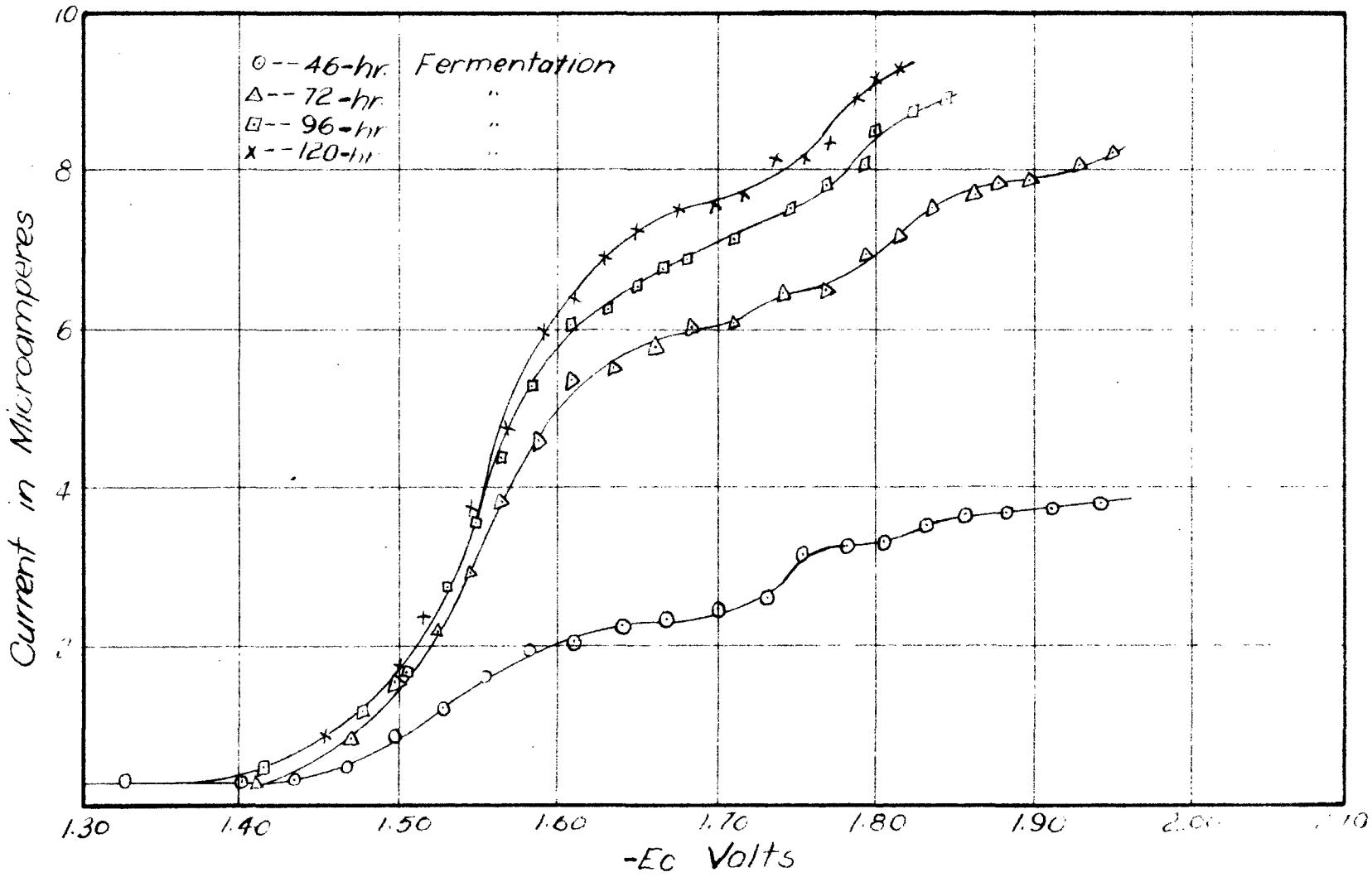


Figure 25. Current-voltage curves for an Acetobacter suboxydans fermentation of an i-inositol-sorbitol medium after various time intervals; dilution ratio 1:100; drop-time = 4 sec.; temp. = 25°C.

The concentration of "ketose" began to build up rapidly after the first day. Figure 25 shows current-voltage curves for the i-inositol-sorbitol fermentation at the end of 46, 72, 96, and 120 hours, respectively, when a dilution ratio of 1:100 was used. Sorbose could not be detected easily due to a simultaneous discharge of hydrogen or some other material. Since at equal concentrations the "wave" height for "ketose" is approximately twice as high as that for sorbose, the detection of the latter compound is more difficult in very low concentrations.

Many analyses were run at various time intervals and dilutions but it was impossible from the data obtained to determine which of the above two polyhydric alcohols was attacked first. The rate of formation of "ketose" could be followed readily, but, although a sorbose break was evident even during the early stages of the fermentation, no quantitative significance could be assigned to the data obtained in this region of the current-voltage curves. Until more data are accumulated and the analytical technique more highly refined, any statement made concerning the relative fermentability of i-inositol and sorbitol by Acetobacter suboxydans would have to be made with much reservation.

Table 12 shows the per cent yields of "ketose" after different time intervals as determined by the polarographic method. The diffusion currents taken from curves in Figures 24 and 25 were interpreted in terms of molarity using

the curve shown in Figure 17.

Table 12

Yields of "Ketose" from the Action of *Acetobacter suboxydans* on an *i*-Inositol-Sorbitol Medium After Various Time Intervals

Time (hours)	Per Cent "Ketose"
6	3
12	3
18	5
24	6
46	36
72	82
96	91
120	107

The limiting current for "ketose" showed no further increase after 120 hours. The 107 per cent yield of "ketose" reported at the end of the fermentation is easily understood when it is considered that the fermentation was diluted in the ratio of 1:100 in order to perform the analysis.

DISCUSSION

As pointed out by Müller (1939[b]), the fact that the polarographic half-wave potential of a reducible organic compound shifts regularly with pH does not necessarily mean that the total reduction process is a reversible one. Such shifts might be due to the selective measurement at the dropping mercury electrode of a reversible step in a reduction which is on the whole irreversible. The change in half-wave potential with pH cannot be used as an absolute criterion of reversibility, but systems which involve hydrogen ions and do not show regular pH effects may be classed definitely as irreversible.

The change in $\underline{E_o'}$ with pH for an organic oxidation - reduction system in which no cations are created by hydrogenation (for example, the reductions studied in this investigation) may be represented by formula 24.

$$E_o' = E_o + \frac{RT}{F}\ln(H^+) \qquad (24)$$

or at 25°C.

$$E_o' = E_o - 0.059\ pH \qquad (25)$$

Since, in well-buffered solutions, the half-wave potential is equal to the potentiometrically established $\underline{E_o'}$ for simple reversible organic oxidation-reduction systems, $\underline{E_{1/2}}$ can be

substituted for $\underline{E_o'}$ in equation 25.

In the cases investigated, the half-wave potentials should have shifted 0.059 volt toward more negative values for each unit change in pH. Inspection of Table 5 shows that in the case of dihydroxyacetone the changes actually were very slight and irregular. It appears that altering the buffer or indifferent electrolyte has a greater effect than does varying the pH. Table 7 shows that the half-wave potential of sorbose varied with pH in the expected direction, but the magnitude of the shift did not conform with equation 25. As the reductions of sorbose were carried out in unbuffered or poorly buffered solutions, the pH values in the body of the solutions might not have been identical with those at the electrode/solution interface where the reductions occurred. Figure 17 shows that the change in half-wave potential with change in pH for "ketose" was small but in the expected direction.

The data show that the reductions studied in this investigation are irreversible processes under the conditions at which they were performed. The half-wave potentials are probably more negative than the true $\underline{E_o'}$ values for the systems considered. The accuracy of this statement might be tested by calculations which could be made from thermal data employing the third law of thermodynamics. The thermal method of obtaining $\underline{E_o}$ values has the advantage of not being

restricted to perfectly reversible systems. Unfortunately, however, sufficient thermal data are not available to make possible the accurate calculation of the free energy of hydrogenation of the compounds investigated.

Enough thermal data are available for the sorbose-sorbitol system to make possible an $\underline{E_o}$ calculation based on a few reasonable assumptions. Oppenheimer and Stern (1939) have tabulated the value of $\Delta F^{\circ}_{\underline{298}}$ for the hydrogenation of glucose to form sorbitol as -7,000 calories. Assuming the free energy of hydrogenation of sorbose to be the same as that of glucose and employing the relationship $\Delta F^{\circ} = -nF\underline{E_o}$, $\underline{E_o}$ for the sorbose-sorbitol system is found to equal +0.152 volt.

The only obstacle to the direct calculation of the free energy of hydrogenation of sorbose is lack of heat capacity data for sorbose; all other necessary thermal data are available. By assuming the molal entropy of sorbose to be equal to that of glucose, a value which is accurately known, the free energy of hydrogenation of sorbose calculated by means of the third law of thermodynamics is found to equal -1,930 calories at 25° C. From this value $\underline{E_o}$ for the sorbose-sorbitol system is found to be +0.042 volt.

Since both of the above calculations are based on some assumptions, the $\underline{E_o}$ values obtained only roughly approximate the true value. Either of the two calculated values,

however, is sufficiently accurate to show the large discrepancy between the $\underline{E_o'}$ value for the sorbose-sorbitol system and the polarographic half-wave potential. The half-wave potential of sorbose at pH 7 is -1.807 volts referred to the saturated calomel half-cell. Referred to the standard hydrogen electrode this value is -1.561 volts. Substituting the latter value for $\underline{E_o'}$ in equation 25 gives an $\underline{E_o}$ of -1.148 volts (pH 0). From the foregoing, it is apparent that the sorbose-sorbitol system is either irreversible or else too electromotively sluggish to establish an equilibrium potential at the dropping mercury electrode.

It is extremely doubtful that electromotively sluggish systems can be equilibrated at a dropping mercury electrode in the presence of an enzyme since the period during which equilibrium must be established is very short, about 4 seconds. Previous investigations of electromotively sluggish systems (for example, the isopropyl alcohol-acetone system) have shown that a period of several hours often is required for equilibrium to be attained even in the presence of very active enzyme preparations. Nevertheless the polarographic method still should prove to be applicable to enzyme studies. If suitable dehydrogenases were present, it should be possible to establish equilibrium between two sugars and their corresponding polyhydric alcohols. The rel-

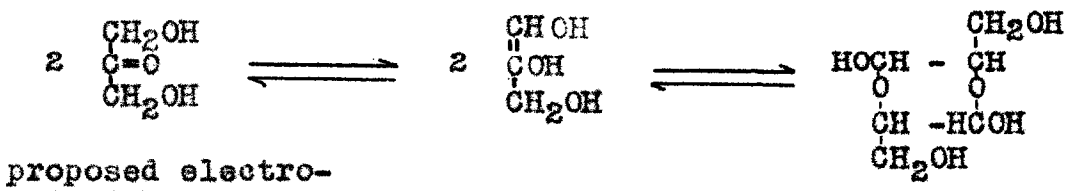
ative amounts of the two sugars present at equilibrium could be determined polarographically. Knowing the concentrations of the polyhydric alcohols and sugars present at the beginning of an experiment, and the concentrations of the sugars present at equilibrium, the equilibrium constant for the reaction could be calculated. From the equilibrium constant data, the different sugars could be arranged in the order of their oxidizing intensities. Baker and Adkins (1940) have already adapted this method to a study of ketones other than sugars employing aluminum t-butoxide as a catalyst. There is every reason to believe that the method can be adapted to enzyme equilibrated systems.

Inspection of the observed and calculated values for the diffusion currents of the different ketose compounds studied in this investigation shows that the discrepancies are quite marked. The differences are too great to be attributed to possible errors in the methods of procedure. The errors occurring in determining the values of m, D, and C in the Ilkovič equation each should not have exceeded 5 per cent. Furthermore, it is illogical to assume that the Ilkovič equation does not apply in the case of reductions carried out in this investigation. This equation was derived on the basis of theoretical considerations and it has been tested experimentally in a number of inorganic reductions.

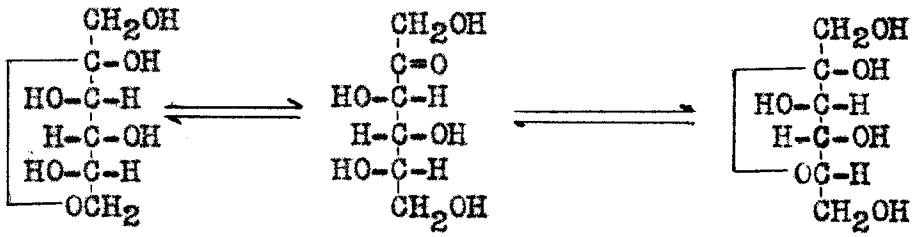
The discrepancies mentioned above are more likely explained by the existence of several isomeric forms of the ketose compounds in solution, at least one form of which is not reducible at the dropping mercury electrode.

Heyrovský and Smolér (1932) reported that the ketonic group in sorbose and fructose appeared to take up two electrons at high temperatures but that at ordinary room temperature the reduction was incomplete. These authors suggested that the ketoses (sorbose and fructose) were present in solution in two tautomeric forms one of which was easily electroreducible. High temperatures were reported to favor the formation of the easily reducible form. Brigl and Schinle (1933, 1934) have obtained direct evidence, based on changes in rotation with temperature, for the high degree of tautomerism displayed by aqueous solutions of fructose.

In accordance with the modern concept of ketose configurations, the postulated electroreducible and "non-reducible" forms of dihydroxyacetone and sorbose may be represented as follows:



proposed electro-reducible form of dihydroxyacetone



proposed electro-reducible form of sorbose

The term "non-reducible" as used above only implies that the substance is not electroreducible within the polarographic range.

The data obtained in this investigation indicate that a large per cent of the electroreducible form of dihydroxyacetone was present in neutral aqueous solutions. In strongly basic solutions the reduction "wave" almost completely disappeared. This was due probably to conversion of the easily reducible keto form of dihydroxyacetone to the more difficultly reducible enol form. Supporting evidence that the enol form of dihydroxyacetone is the difficulty reducible form is given in the fact that ascorbic acid, a compound having a double-bond structure somewhat similar to that in the enol form of dihydroxyacetone, is not reducible at the dropping mercury cathode. The six-membered dioxane-type ring shown above could have been formed from either the keto or enol form of dihydroxyacetone.

Experiments showed that the "non-reducible" furanose

and pyranose forms of sorbose must have been present in relatively large proportions in neutral or alkaline solutions.

The observed value for the diffusion current of erythrulose was slightly greater than the calculated value. This would indicate that all of the compound was present in a reducible form. However, since the reductions of erythrulose were carried out in the fermentation liquor instead of in pure solutions, quantitative interpretations of the data should be made with reservation. The tetrose sugars cannot exist in the monomolecular form as a pyranose ring, but only as the smaller furanose ring. The tetrose, erythrulose, would not be expected to form a ring at all. None of the tetrose sugars has been obtained in a crystalline form. In view of the above discussion concerning "non-reducible" ring formation in sugar solutions, a polarographic investigation of pure erythrulose, erythrose, and threose should prove very informative.

The existence of isomeric forms of course is not the only possible explanation for the discrepancies occurring between the calculated and observed values for the diffusion currents. The end products assumed to be formed in the reductions investigated might not have been identical with those actually formed, since the reduction mechanism was not known with certainty. The amount of reduced material formed during a polarographic reduction is extremely

small; consequently, the characterization of this material would be very difficult. Only speculation on the nature of the reduction reactions can be given. The expected reduction products in the cases studied would be the corresponding polyhydric alcohols; however, other products might have been formed (for example, pinacols).

Due to the adaptation of the polarographic method to such a wide variety of problems, its place in biological chemistry cannot be questioned. The method is particularly suitable as a means of detecting micro quantities of electroreducible or electroöxidizable compounds both quantitatively and qualitatively and herein lies its chief value to the biochemist.

SUMMARY AND CONCLUSIONS

1. The polarographic method has been employed in the investigation of the reduction of dihydroxyacetone, erythrulose, sorbose, tagatose, and "ketose" at the dropping mercury cathode. The current-voltage data were determined manually. The half-wave potentials for the reduction of the compounds in 0.1 M lithium chloride, referred to the saturated calomel half-cell and arranged in order of increasing negative potential, are as follows: "ketose", -1.55 volts; dihydroxyacetone, -1.59 volts; erythrulose, -1.60 volts (a second break was observed, half-wave, -1.85 volts); sorbose, -1.81 volts; and tagatose, -1.81 volts. The half-wave potential of sorbose agrees favorably with the value found by Heyrovský and Smolér (1932). The compounds listed above, with the exception of tagatose, were produced by the action of Acetobacter suboxydans upon the corresponding polyhydric alcohols. Considering the compounds investigated in this thesis along with other compounds formed by the action of Acetobacter suboxydans which have been investigated polarographically (such as, fructose, galactose, acetaldehyde, propionaldehyde, acetone, hydroxyacetone, acetylmethylcarbinol, and diacetyl), there appears to be no direct relationship between the polarographic half-wave potential of the compound and the ability of the organism to

form the compound.

2. The effect of pH on the half-wave potentials of dihydroxyacetone, sorbose and "ketose" has been found to be less than expected from theoretical considerations. The reductions are irreversible although in the case of "ketose" there might exist a reversible step, the nature of which is at present unknown. The E_o for the sorbose-sorbitol system calculated from thermal data does not agree with the half-wave potential for the reduction of sorbose (pH=0). Further evidence that the equation of the polarographic wave does not hold in the reduction of sorbose and "ketose" is given by the fact that plots of E_c against log $I/I_d - I$ do not give straight lines with slopes equal to RT/nF. The slope in the case of sorbose was 0.142 while that in the case of "ketose" was 0.083 as compared to theoretical values of 0.0295 and 0.0148 respectively.

3. Dihydroxyacetone has been reduced in the presence of a bacterial juice prepared from Acetobacter suboxydans but no effect on the half-wave potential was observed. However, it is suggested that the polarographic method may be employed in certain enzyme-catalyzed equilibrium studies.

4. The diffusion currents observed for the different compounds studied have been found to be less than those calculated by use of the Ilkovič equation. Explanations for these discrepancies have been presented.

5. In buffered basic solutions (at pH of about 10) the dif-

fusion currents for "ketose" and dihydroxyacetone have been observed to decrease with time. After standing for a period of less than a day these two compounds showed no reduction "wave". Furthermore, "ketose" developed a bright yellow color after standing several hours in a carbonate-bicarbonate buffer solution (pH=9.8). The yellow color disappeared immediately when the solution came in contact with air.

6. "Ketose" has been found not to give two separate reduction "waves" as is characteristic of several diketones; however, the reduction "wave" for two ketonic groups could easily overlap. At equimolar concentrations the diffusion current for "ketose" was approximately twice as great as that for sorbose probably indicating the presence of more reducible groups in the former case. The reduction of "ketose" in 0.1 M potassium chloride gives a steep maximum on the current-voltage curve.

7. The diffusion currents for sorbose and "ketose" have been shown to be strictly proportional to the concentration of these compounds. Furthermore "ketose" and sorbose may be analyzed quantitatively in the same solution.

8. Dihydroxyacetone, erythrulose, and "ketose" may be detected in diluted fermentation liquors without the removal of proteins. The use of the polarographic method in following the course of formation of reducible materials in a fermentation has been demonstrated.

LITERATURE CITED

Adkins, H., and Cox, F. W. 1938. Relative oxidation-reduction reactivities of ketones and aldehydes and applications in synthesis. J. Am. Chem. Soc., 60:1151-59.

Baker, R. H., and Adkins, H. 1940. Oxidation potentials of ketones and an aldehyde. J. Am. Chem. Soc., 62:3305-14.

Ball, E. G. 1937. Studies on oxidation-reduction. XXIII. Ascorbic acid. J. Biol. Chem., 118:219-39.

Barmore, M. A. 1929. Electrometric studies on pyruvic acid, lactic acid, and glyceric aldehyde. Unpublished Thesis. Library, Stanford Univ. Original not seen. Cited by Müller, O. H. Cold Spring Harbor Symposia Quant. Biol., Vol. VII: p.71 (1939).

Barron, E. S. G., and Hastings, A. B. 1934. Studies on biological oxidations. III. The oxidation-reduction potential of the system lactate-enzyme-pyruvate. J. Biol. Chem., 107:567-78.

Bertho, A. 1930. Zur Kenntnis des Essigferments. Ann., 474:1-64.

Borsook, H., and Schott, H. F. 1931. The rôle of the enzyme in the succinate-enzyme-fumarate equilibrium. J. Biol. Chem., 92:535-57.

Brigl, P., and Schinle, R. 1933. Kohlenhydrate, (XV. Mitteil.): Über Benzoyl- und Benzalderivate der Fructose. Ber., 66:325-30.

Brigl, P., and Schinle, R. 1934. Tetrabenzoyl-fructofuranose (Vorläuf. Mitteil.). Ber., 67:127-30.

Cantor, S. M., and Peniston, Q. P. 1940. The reduction of aldoses at the dropping mercury cathode: Estimation of the aldehydo structure in aqueous solutions. J. Am. Chem. Soc., 62:2113-21.

Clark, W. M., and coworkers. 1928. Studies on oxidation-reduction. I-X. U. S. Pub. Health Service. Hygienic Lab. Bull. 151.

Clark, W. M., and Lubs, H. A. 1916. Hydrogen electrode potentials of phthalate, phosphate and borate buffer mixtures. J. Biol. Chem., 25:479-510.

Conant, J. B. 1926. The electrochemical formulation of the irreversible reduction and oxidation of organic compounds. Chem. Rev., 3:1-40.

Conant, J. B., Alles, G. A., and Tongberg, C. O. 1928. The electrometric titration of hemin and hematin. J. Biol. Chem., 79:89-93.

Conant, J. B., and Scott, N. D. 1928. A spectrophotometric study of certain equilibria involving the oxidation of hemoglobin to methemoglobin. J. Biol. Chem., 76:207-22.

Coolidge, T. B. 1932. Cytochrome and yeast iron. J. Biol. Chem., 98:755-64.

Cox, F. W., and Adkins, H. 1939. Relative oxidation potentials of ketones. J. Am. Chem. Soc., 61: 3364-70.

Dunning, J. W., Fulmer, E. I., Guymon, J. F., and Underkofler, L. A. 1938. The growth and chemical action of Acetobacter suboxydans upon i-inositol. Science, 87:72-3.

Fulmer, E. I., Dunning, J. W., Guymon, J. F., and Underkofler, L. A. 1936. The effect of the concentration of sorbitol upon the production of sorbose by the action of Acetobacter suboxydans. J. Am. Chem. Soc., 58:1012-13.

Herzog, R. O. 1907. Diffusion von Kolloiden. I., Z. Elektrochem., 13:533-41.

Heyrovský, J. 1923. Electrolysis with a dropping mercury cathode. Part I. Deposition of alkali and alkaline earth metals. Phil. Mag., 45:303-15.

Heyrovský, J. 1934. A polarographic study of the electrokinetic phenomena of adsorption, electro-reduction, and overpotential displayed at the dropping mercury cathode. (Actualités scientifiques et industrielles, No. 90.). Hermann et Cie., Paris.

Heyrovský, J. 1936. Polarographie. In W. Böttger's Die physikalischen Methoden der chemischen Analyse, Vol. 2, pp. 260-322. Akademische Verlagsgesellschaft, Leipzig. Original not seen. Cited by I. M. Kolthoff, and J. J. Lingane, Chem. Rev., 24:2 (1939).

Heyrovský, J., and Ilkovič, D. 1935. Polarographic studies with the dropping mercury electrode. Part II. The absolute determination of reduction and depolarization potentials. Collection Czechoslov. Chem. Commun., 7:198-214.

Heyrovský, J., and Shikata, M. 1925. Researches with the dropping mercury cathode. Part II. The polarograph. Rec. trav. chim., 44:496-98.

Heyrovský, J., and Smoleŕ, I. 1932. Polarographic studies with the dropping mercury kathode. Part XXX. The electroreduction and estimation of fructose and sorbose. Collection Czechoslov. Chem. Commun., 4:521-30.

Hohn, H. 1937. Chemische Analysen mit dem Polarographen. Julius Springer, Berlin.

Ilkovič, D. 1934. Polarographic studies with the dropping mercury kathode. Part XLIV. The dependence of limiting currents on the diffusion constant, on the rate of dropping and on the size of drops. Collection Czechoslov. Chem. Commun., 6:498-513.

Ilkovič, D. 1936[a]. Polarographic studies with the dropping mercury electrode. Part III. The cause of maxima on current-voltage curves. Collection Czechoslov. Chem. Commun., 8:13-34.

Ilkovič, D. 1936[b]. Polarographic studies with the dropping mercury electrode. Part IV. The measurement of the polarization capacity. Collection Czechoslov. Chem. Commun., 8:170-77.

Ilkovič, D., and Semerano, G. 1932. Polarographic studies with the dropping mercury kathode. Part XXV. Increased sensitivity of micro-analytical estimations by a compensation of current. Collection Czechoslov. Chem. Commun., 4:176-80.

Kerr, R. W. 1940. The relative reducing potential of starch. J. Am. Chem. Soc., 62:2735-8.

Kodíček, E., and Wenig, K. 1938. Polarographic estimation of vitamin c. Nature, 142:35.

Kolthoff, I. M., and Lingane, J. J. 1939. The fundamental principles and applications of electrolysis with the dropping mercury electrode and Heyrovský's polarographic method of chemical analysis. Chem. Rev., 24:1-94.

Lehmann, J. 1934. Zur Kenntnis biologischer Oxydations-Reduktionspotentiale. Biochem. Z., 274:321-28.

Lewis, G. N. 1938. Acids and bases. J. Franklin Inst., 226:293-313.

Lingane, J. J. 1939. Thermodynamic significance of polarographic half-wave potentials of simple metal ions at the dropping mercury electrode. J. Am. Chem. Soc., 61:2099-2103.

Lingane, J. J., and Kolthoff, I. M. 1939. Fundamental studies with the dropping mercury electrode. I. The Ilkovič equation of polarographic diffusion currents. J. Am. Chem. Soc., 61:825-34.

Maas, J. 1938. Polarographic studies with the dropping mercury kathode. LXXI. An empirical study of the factors of the diffusion current. Collection Czechoslov. Chem. Commun., 10:42-53.

Michaelis, L. 1930. Oxidation-reduction potentials. (Monographs on Experimental Biology, No. 2.). J. B. Lippincott, Philadelphia.

Michaelis, L. 1935. Semiquinones, the intermediate steps of reversible organic oxidation-reduction. Chem. Rev., 16:243-86.

Michaelis, L., and Schubert, M. P. 1938. The theory of reversible two-step oxidation involving free radicals. Chem. Rev., 22:437-70.

Müller, O. H. 1939[a]. Oxidation and reduction of organic compounds at the dropping mercury electrode and the application of Heyrovský's polarographic method in organic chemistry. Chem. Rev., 24: 95-124.

Müller, O. H. 1939[b]. Polarographic investigations of reversible and irreversible oxidations and reductions at the dropping mercury electrode. Cold Spring Harbor Symposia Quant. Biol., Vol. VII;59-73.

Müller, O. H. 1940[a]. Oxidation-reduction potentials measured with the dropping mercury electrode. III. Polarographic study of quinhydrone in buffered and unbuffered solutions. J. Am. Chem. Soc., 62:2434-41.

Müller, O. H. 1940[b]. Applications of the dropping mercury electrode for the detection of intermediate radicals. Annals of the New York Academy of Sciences, Vol. XL:91-110.

Müller, O. H. 1941. The polarographic method of analysis. III. Fundamentals of quantitative analysis. J. Chem. Education, 18:172-77.

Müller, O. H., and Baumberger, J. P. 1937[a]. Oxidation-reduction potentials measured with the dropping mercury electrode. Part I. Studies with quinhydrone and estimation of limits of measurements. Trans. Electrochem. Soc., 71:169-80.

Müller, O. H., and Baumberger, J. P. 1937[b]. Oxidation-reduction potentials measured with the dropping mercury electrode. Part II. Polarographic investigation. An introduction to a new method. Trans. Electrochem. Soc., 71:181-94.

Müller, O. H., and Baumberger, J. P. 1939. The keto-enol tautomerism of pyruvate ion studied polarographically. J. Am. Chem. Soc., 61:590-96.

Needham, J., and Needham, D. M. 1925. The hydrogen-ion concentration and the oxidation-reduction potential of the cell-interior: a micro-injection study. Proc. Roy. Soc. (London), Ser. B, 98: 259-86.

Oppenheimer, C. and Stern, K. G. 1939. Biological oxidation. Nordemann Pub. Co. Inc., New York.

Parks, G. S., and Huffman, H. M. 1932. The free energy of some organic compounds. The Chemical Catalog Co., New York. Am. Chem. Soc. Monograph No. 60.

Quastel, J. H., and Whetham, M. D. 1924. The equilibria existing between succinic, fumaric, and malic acids in the presence of resting bacteria. Biochem. J., 18:519-34.

Reichstein, T., and Bosshard, W. 1934. d-Tagatose, Diaceton-d-tagatose und d-Tagaturonsäure. Helv. Chim. Acta., 17:753-61.

Schmidt, O. 1938. Untersuchungen über das Hämoglobin-Methämoglobin-Redoxsystem. Biochem. Z., 296: 210-74.

Shibata, K. 1935. Cytochrom und Zellatmung. Ergeb. Enzymforsch., 4:348-64.

Shikata, M., and Shoji, K. 1927. Applications of the polarographic method. Part III. Application to the microanalysis of reducible substances in fermentation products. Mem. Coll. Agr. Kyoto Imp. Univ., 4:75-91.

Stotz, E., Sidwell, A. E., Jr., and Hogness, T. R. 1938. The spectrophotometric determination of the equilibrium in oxidation-reduction systems; the potential of cytochrome c. J. Biol. Chem., 124: 11-23.

Thunberg, T. 1925. Das Reduktions-Oxydationspotential eines Gemisches von Succinat-Fumarat. Skand. Arch. Physiol., 46:339-40.

Tomeš, J. 1937. Polarographic studies with the dropping mercury kathode. Part LXIII. Verification of the equation of the polarographic wave in the reversible electro-deposition of free kations. Collection Czechoslov. Chem. Commun., 9:12-21.

Underkofler, L. A., and Fulmer, E. I. 1937. The production of dihydroxyacetone by the action of Acetobacter suboxydans upon glycerol. J. Am. Chem. Soc., 59:301.

Warburg, O. 1928. Über die katalytischen Wirkungen der lebendigen Substanz. Julius Springer, Berlin.

Whistler, R. L., and Underkofler, L. A. 1938. The production of l-erythrulose by the action of Acetobacter suboxydans upon erythritol. J. Am. Chem. Soc., 60:2507-8.

Wieland, H. 1922. Über den Mechanismus der Oxydationsvorgänge. Ergeb. Physiol., 20:477-518.

Wieland, H., and Bertho, A. 1928. Über den Mechanismus der Oxydationsvorgänge. XV. Das Wesen der Essigsäure-Gärung. Ann., 467:95-157.

Wiggert, W. P., Silverman, M., Utter, M. F., and Werkman, C. H. 1940. Preparation of an active juice from bacteria. Iowa State Coll. J. Sci., 14: 179-86.

Winkel, A., and Proske, G. 1936. Über die elektrolytische Reduktion organischer Verbindungen an der Quecksilber-Tropfelektrode (II. Mitteil.). Ber., 69: 1917-29.

Wishart, G. M. 1923. On the reduction of methylene blue by tissue extracts. Biochem. J., 17:103-14.

Wurmser, R. 1930. Oxydations et réductions. Les Presses universitaires de France, Paris.

Wurmser, R., and Filitti-Wurmser, S. 1936. Sur l'équilibre entre l'alcool isopropylique et l'acétone en présence d'alcooldéshydrase. Potentiel d'oxydoréduction du système -CHOH- $\rightleftharpoons$ -CO- . J. chim. phys., 33:577-86.

Wurmser, R., and Filitti-Wurmser, S. 1938. Le potentiel d'oxydoréduction du cytochrome. Compt. rend. soc. biol., 127:471-73.

Wurmser, R., Mayer, N., and Crépy, O. 1936. Le potentiel d'oxydoréduction de la réductone. J. chim. phys., 33:101-10.