168

UNIVERSITY OF CINCINNATI

May 14 1937

I hereby recommend that the thesis prepared under my supervision by Leon Shechter

entitled Oxidation-Reduction Indicators

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by:

E F Farnau

W. M. Burgess, Chairman.

Form 668—G. S. and Ed.—1M—7-31

OXIDATION-REDUCTION INDICATORS

A dissertation submitted to the

Graduate School
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

1937

by

Leon Shechter

B.S. in Ch.E. University of South Carolina 1933
M.S. University of South Carolina 1934

TABLE OF CONTENTS

19 Ag 37

INTRODUCTION

The determination of oxidation-reduction potentials, though a comparatively new field, is of utmost importance, both to the biologist and to the organic chemist.

In 1775 Lavoisier published his discovery of oxygen, and two years later disclosed as a result of his animal experiments that respiration and life depend closely on a balance between oxygen and the living tissues. From that time onward the biologist has been interested in the question of oxidation and (if I may be permitted to coin a word) "de-oxidation" or reduction.

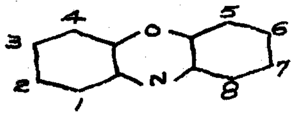
Many years elapsed ere Gillespie in 1920 published some data on electrode potentials induced by bacterial reduction. This publication suggested to Clark that here might be a means of obtaining quantitative information on reversible oxidation-reduction reactions.

Clark, on checking over and extending Gillespie's work, found that the electrode potentials observed under the conditions then employed were uncertain. In spite of this uncertainty remarkable relationships suggested themselves, but these could not be accepted without more rigid confirmation. It was evident that some improved method was necessary to check more carefully the electrode measurement of reduction intensity.

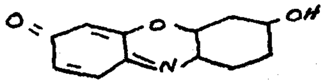
As a result a series of investigations was undertaken to determine the potentials of various dyes in equilibrium with their reduction products. Such systems were studied as would serve as indicators of this potential in much the same manner as hydrogen ion indicators serve for pH.

From this meager beginning Clark, Cohen, Conant, and others have developed the theory and use of a number of compounds as indicators of this oxidation-reduction intensity.

Among the classes of compounds studied have been indophenols[1] , azines, thiazines, and of late, oxazines. Of the three oxazines studied all have been substituted in the three and six position by amino or substituted amino groups.



We have decided to determine the potentials of resorufin, in which there are no amino groups, but in their place are hydroxyl and doubly bound oxygen groups.



Resorufin[2]

This compound is of interest because it permits a comparison with phenol indophenol, and because it displays a two color change on reduction, going from red to an intense blue or green on partial reduction, and thence to colorless.

DERIVATION OF EQUATIONS

1. Oxidation-Reduction Equilibria. In general any oxidation-reduction may be designated as follows:

$$[Red] \rightleftharpoons [Ox] + n[e]$$

The brackets indicate molar concentration. [Red] indicates the concentration of active reductant, [Ox] the concentration of active oxidant, n equals the number of electrons involved, [e] the concentration of electrons. The equilibrium state may be formulated by

$$\frac{[Ox][e]^n}{[Red]} = K$$

This equation applies generally; now let us apply it to two different systems, each involving the transfer of but one electron.

The equilibrium of one system may be formulated by

$$\frac{[Red]}{[Ox]} = \frac{[e]}{K}$$

Those of another system may be formulated by

$$\frac{[Red']}{[Ox']} = \frac{[e']}{K'}$$

If these two systems are brought together and allowed to interact until simultaneous equilibrium is established, the [e] of both are equal, for the mixed system can have but one potential. Hence

$$\frac{[Red]}{[Ox]} = \frac{K'}{K}\frac{[Red']}{[Ox']}$$

If the values of K' and K, or their ratio, are known it is possible to predict what ratio of a given oxidant and its reduction product could exist with a given ratio of the other oxidant and its reduction product. In the case where

$$\frac{[Red]}{[Ox]} = 1$$

the ratio $\frac{K'}{K}$ determines whether the second system is practically completely reduced, practically completely oxidized, or at some intermediate stage. By employing this means it would be possible to determine the relative oxidizing or reducing tendency of various systems.

In any two oxidation-reduction systems simultaneously in equilibrium, the term $[e]$ electron concentration or electron fugacity drops out.

2. <u>Electrode Potentials</u>. A noble metal contains free electrons; whenever an electrode of such a metal is placed in a solution containing an oxidation-reduction system, it acquires a charge, the electron concentration or charge increasing with increase in reduction intensity of the system.

Let the concentration of electrons in the metal be designated by $[e_m]$ and the electron concentration in the solution by $[e_s]$ The work necessary isothermally to transfer one faraday of electrons from concentration $[e_m]$ to $[e_s]$ is

$$W = RT \ln \frac{[e_m]}{[e_s]}$$

The work may be substituted by EnF:

$$EnF = RT \ln \frac{[e_m]}{[e_s]}$$

For n = 1,

$$E = \frac{RT}{F} \ln [e_m] - \frac{RT}{F} \ln [e_s]$$

For any one metal the concentration of electrons is constant; therefore

$$E = C' - \frac{RT}{F} \ln [e_s]$$

For the general equation of oxidation-reduction we have

$$[Red] \rightleftharpoons [Ox] + n[e]$$

$$\frac{[Ox][e]^n}{[Red]} = K$$

$$[e] = \sqrt[n]{\frac{K[Red]}{[Ox]}}$$

Substituting for $[e]$ in the above,

$$E = C' - \frac{RT}{F} \ln \left(\frac{K[Red]}{[Ox]}\right)^{1/n}$$

$$E = C' - \frac{RT}{nF} \ln \frac{K[Red]}{[Ox]}$$

$$E = C' - \frac{RT}{nF} \ln K - \frac{RT}{nF} \ln \frac{[Red]}{[Ox]}$$

On observing the foregoing equation, it becomes apparent that if E and T are measurable, the value of the ratio $\frac{[Red]}{[Ox]}$ can be determined, the other terms in the equation being constant for any one system: $C' + \frac{RT}{nF} \ln K$

For another system there would be another term which we shall designate by $C' + \frac{RT}{nF} \ln K_2$. The difference gives $\frac{RT}{nF} \ln \frac{K}{K_2}$ and this permits a determination of the ratio $\frac{K}{K_2}$. This ratio, as has already been pointed out, permits the classification and comparison of different oxidation-reduction intensities of various systems.

The determination of single electrode potentials such as E is impractical, for the method by which potentials are measured depends on the utilization of two half-cells. If we choose one of these as a standard and arbitrarily assign to it a zero potential we have now an excellent method of making comparisons of relative oxidation-reduction intensities. The standard chosen is the hydrogen electrode. This electrode is of platinized platinum dipping into a solution one normal with respect to hydrogen ion activity and having hydrogen at a pressure of one atmosphere bubbling over the electrode.

From the equation

$$H_2 \rightleftharpoons 2H^+ + 2e$$

we may write the equilibrium constant

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

Now the concentration of hydrogen is a function of the pressure, and therefore

$$\frac{[H^+]^2 [e]^2}{P} = K$$

Solving for $[e]$ and substituting in

$$E = C' - \frac{RT}{F} \ln [es]$$

we obtain

$$E_H = C_H - \frac{RT}{F} \ln \frac{\sqrt{P}}{[H^+]}$$

This equation is that which applies to the hydrogen electrode. On assuming the conditions of the standard of potential we have $P = 1$, $H \quad = 1$, and $E = 0$; therefore $C_H = 0$.

Let us now combine this standard cell with any oxidation-reduction half-cell

$$emf = E - E_H = C - C_H - \frac{RT}{nF} \ln \frac{[Red]}{[Ox]} + \frac{RT}{F} \ln \frac{\sqrt{P}}{[H^+]}$$

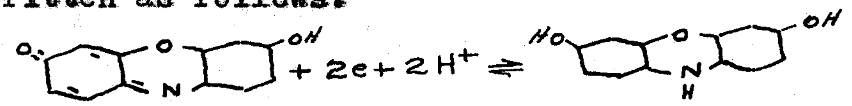
Since the reference cell is a standard cell, we have Peter's Equation:

$$E_h = E_o - \frac{RT}{nF} \ln \frac{[Red]}{[Ox]}$$

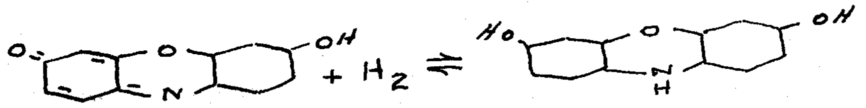
For the case where $\frac{[Red]}{[Ox]}$ can be fixed and the value of E_h measured, it is possible to calculate directly E_o. This value E_o is the same as would be obtained if $\frac{[Red]}{[Ox]}$ were set as one and the potential measured.

On plotting E_h versus percent reduction we obtain a curve the slope of which is determined by n -- the larger the n the smaller the slope. By plotting such a curve it is possible to predict the value of n for any unknown oxidation-reduction reaction.

3. *Derivation of Equation for Resorufin, Taking into Account Change of E_0 with pH.* The reduction of resorufin may be written as follows:



or



Let us consider the ionizable groups. First there is the oxidant: here we have an OH group which can ionize to give hydrogen ions. Second is the reductant: here we have the ionization of the two (OH) groups and that of the secondary nitrogen.

The various dissociations are as follows:

(A) $Ox \rightleftharpoons \bar{O}x + H^+$

(B) $H_3Red \rightleftharpoons H^+(\text{phenolic}) + H_2\bar{R}ed$

(C) $H_2\bar{R}ed \rightleftharpoons H^+(\text{phenolic}) + H\bar{R}\bar{e}d$

(D) $H\bar{R}\bar{e}d \rightleftharpoons \bar{R}\bar{e}\bar{d} + H^+(\text{amino})$

(A) represents the ionization of the phenolic group in the oxidant. Let K_1 equal the ionization constant.

(B) represents the ionization of the first phenolic group in the reductant. Let K_2 equal the ionization constant.

(C) represents the ionization of the second phenolic group in the reductant. Let K_3 equal the ionization constant.

(D) represents the ionization of the bridge nitrogen. Let K_4 equal the ionization constant.

Now setting up these constants, we have

(E) $$K_1 = \frac{[\overset{-}{\mathrm{ox}}][H^+]}{[\mathrm{ox}]}$$

(F) $$K_2 = \frac{[H^+][H_2\overset{-}{\mathrm{Red}}]}{[H_3\mathrm{Red}]}$$

(G) $$K_3 = \frac{[H^+][H\overset{--}{\mathrm{Red}}]}{[H_2\overset{-}{\mathrm{Red}}]}$$

(H) $$K_4 = \frac{[H^+][\overset{---}{\mathrm{Red}}]}{[H\overset{--}{\mathrm{Red}}]}$$

Peters' Equation as derived is

$$E_h = E_o + \frac{RT}{nF} \ln \frac{[\mathrm{ox}]}{[\mathrm{Red}]}$$

Let S_o equal the total concentration of oxidant in its various stages of ionization and S_r equal the total concentration of the reductant in its various stages of ionization. We have

$$E_h = E_o + \frac{RT}{nF} \ln \frac{S_o}{S_r}$$

Now

(I) $$S_r = [H_3\mathrm{Red}] + [H_2\overset{-}{\mathrm{Red}}] + [H\overset{--}{\mathrm{Red}}] + [\overset{---}{\mathrm{Red}}]$$

and substituting,

$$E_h = E_o - \frac{RT}{nF} \ln \frac{[H_3\mathrm{Red}] + [H_2\overset{-}{\mathrm{Red}}] + [H\overset{--}{\mathrm{Red}}] + [\overset{---}{\mathrm{Red}}]}{S_o}$$

Substituting in S_r the values obtained from (F), (G), (H), we get

$$S_r = \frac{[H_2\overset{-}{\text{Red}}][H^+]}{K_2} + \frac{[H\overset{--}{\text{Red}}][H^+]}{K_3} + \frac{[\overset{---}{\text{Red}}][H^+]}{K_4} + [\overset{---}{\text{Red}}]$$

Substituting further,

$$S_r = \frac{[\overset{---}{\text{Red}}][H^+]^3}{K_2K_3K_4} + \frac{[\overset{---}{\text{Red}}][H^+]^2}{K_3K_4} + \frac{[\overset{---}{\text{Red}}][H^+]}{K_4} + [\overset{---}{\text{Red}}]$$

Solving for the value of $[\overset{---}{\text{Red}}]$

$$[\overset{---}{\text{Red}}] = \frac{S_rK_2K_3K_4}{[H^+]^3 + K_2[H^+]^2 + K_2K_3[H^+] + K_2K_3K_4}$$

Now to derive the value of $[\text{Ox}]$

$$HOx \rightleftharpoons \overset{-}{O}x + H^+$$

$$S_o = [HOx] + [\overset{-}{O}x]$$

substituting from (E)

$$S_o = \frac{[\overset{-}{O}x][H^+]}{K_1} + [\overset{-}{O}x]$$

Solving for $[\overset{-}{\text{Ox}}]$ we get

$$[\overset{-}{O}x] = \frac{S_oK_1}{[H^+] + K_1}$$

Now substituting the values of $[\overset{---}{\text{Red}}]$ and $[\overset{-}{\text{Ox}}]$ into Peters' Equation, we get

$$E_h = E_o' - \frac{RT}{nF}\ln\frac{S_r}{S_o} + \frac{RT}{nF}\ln\frac{K_1\{K_2K_3K_4 + K_2K_3[H^+] + K_2[H^+]^2 + (H^+)^3\}}{K_2K_3K_4\{[H^+] + K_1\}}$$

Taking out the constant and combining with E_o, we get

$$E_h = E_o' - \frac{RT}{nF}\ln\frac{S_r}{S_o} + \frac{RT}{nF}\ln\frac{\{K_2K_3K_4 + K_2K_3[H^+] + K_2[H^+]^2 + [H^+]^3\}}{\{[H^+] + K_1\}}$$

The latter equation shows how the potential of the resorufin system varies with pH.

It should be noticed that E_o has been converted into

E_o', another constant which takes in the value of $\ln \frac{K_1}{K_2 K_3 K_4}$

It is obvious from the last derived equation that the amount of change of voltage with pH depends on the degree of ionization of the various substituents on the benzene group. From past work[3] on oxazines it seems very probable that the dissociation of the bridge nitrogen in the reductant will be very small.

If the ionizations of the other groups are small the voltages of the indicator will change very little with pH. If large, the converse holds.

HISTORY OF RESORUFIN

Resorufin[4] was first prepared in 1872 by Weselsky. The method utilized involved the addition of nitric acid saturated with N_2O_3 to an ethereal solution of resorcinol.

The product was isolated, analyzed, and a structural formula proposed which eventually was proved incorrect. Weselsky named this new compound diazoresorufin.

Brunner and Kraemer prepared this same compound by reacting nitrobenzene with resorcinol and sulphuric acid. They proposed a new structure and a new name. The term diazoresorufin was supplanted by azoresorufin. The new structure proposed, however, was also incorrect.

It was not until Nietzki and his co-workers in 1889 published the result of their investigations that the true structure of this compound became known. The name was again changed to the one used today -- resorufin.

The latter investigators prepared and studied properties of a number of derivatives. Aside from this they proposed a structure for resazurin, a compound formed by oxidation of resorufin. It might be well to state that resazurin may be distinguished from resorufin in that it goes into solution in alkali with a blue color. This is of interest when compared with the experimental result obtained, in that resorufin turns blue on air oxidation of an alkaline solution.

SYNTHESES AND QUALITATIVE REACTIONS OF RESORUFIN

1. Preparation of Resorufin. This substance may be readily made by the following procedure.[5] A solution is made by dissolving resorcinol in concentrated sulphuric acid in the ratio of two parts of resorcinol to nineteen parts of acid. This is stirred until all the resorcinol goes into solution. Now add sodium nitrite, 0.8 to 3 parts for each part of resorcinol originally used. The solid nitrite is slowly added while the acid resorcinol mixture is rapidly stirred. On the first addition of nitrite the solution turns blue, possibly as a result of indophenol formation.

Upon continued addition of nitrite the solution heats up, the liberated heat being sufficient to help perform the reaction of ring closure. The addition of nitrite is slowly continued until the entire amount has been added. The reaction mixture is permitted to cool, whereupon it is poured into a large volume of water. Resorufin tends to become colloidal during this procedure, and rapid stirring appears to increase this colloidal tendency rather than diminish it.

It was found possible to precipitate the resorufin in large flakes by pouring the reaction mixture very slowly down the side of the beaker containing the water. Stirring was not used. After all the reaction product had been so added the precipitate was stirred, this procedure producing more easily filterable particles. The precipitate was washed with hot water and then dissolved in the least possible amount of dilute ammonia, then filtered and precipitated by addition of hydrochloric acid. The precipitate was filtered and well-washed with hot water. Before being used for potential measurements the resorufin was further purified as described under procedure.

Resorufin is insoluble in most solvents, a total of sixty substances being tried as recrystallizing agents without success. Resort was made even to liquid ammonia as a crystallizing agent, but without success.

2. Properties of Resorufin. A solution of resorufin was made in dilute hydrochloric acid, and powdered zinc was slowly added. The color changed from red to intense blue, probably due to meroquinone formation.[6]

That the blue-green color is not due to a complex formation between the metal ions and the resorufin was proved by Dr. John Weaver. Twenty-four different metal ions were used in conjunction with resorufin, the pH's being varied, and during none of these tests did any coloration of either solution or precipitate result.

An acid solution of resorufin may be reduced by stannous chloride, zinc, or sodium amalgam. Each of these reducing agents produces first a blue-green color, which is finally discharged to a pale straw yellow on adding excess reducing reagent. This reduced solution, on standing in air, is slowly re-oxidized , first to the blue-green coloration, and finally to the original red color of resorufin. Ferricyanide or dichromate immediately reoxidizes the leuco compound to the red form.

In alkaline solution resorufin is an intense rose red and possesses a golden fluorescence. On reduction with sodium hydrosulphite the color is completely discharged. No intermediate green or blue color is apparent. Resorufin is very rapidly re-oxidized to the colored compound on shaking in air, the reversal being accomplished in a few seconds. Air oxidation becomes slower as the acidity of the solution is increased. In strongly acid solutions the time for oxidation of the leuco compound rises, several hours frequently being required to bring about the return of the red color.

The color of resorufin varies with pH. A solution .0008 molar is a dull red-orange between pH's of 1 to 3; from pH 3 to 4 or 5 the color is salmon pink; for pH's 5 to 7 the red color becomes intensified until it is a light cherry red; in more alkaline regions the color intensifies.

Resorufin made alkaline with NaOH is unstable, as is evidenced by the observation that the cherry red color of this compound slowly changes, finally becoming a deep, clear blue. The transformation is more rapidly accomplished by bubbling air through the solution. This property of instability of resorufin takes place in all alkaline regions, the speed of the transformation decreasing with decrease of alkalinity.

This instability is further confirmed by electrode measurements, for drifts were so extremely rapid in the alkaline range that it was impossible to follow the change in potential with a potentiometer.

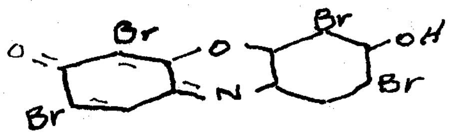
Perhaps this instability in alkaline regions could have been predicted from analogies with compounds such as para-amino phenol, hydroquinone, etc. The instability of these compounds in alkaline solution is well-known. After all, resorufin contains an OH group para to a nitrogen. It is reasonable to expect analogous behavior of this compound as compared with substances such as para-amino phenol, etc.

The blue color formed on air oxidation of resorufin on being acidified with hydrochloric acid becomes red. This color becomes green on addition of a reducing agent such as stannous chloride, and this color in turn may be converted to the original red on oxidation.

Resorufin is reduced by platinum catalyst and hydrogen in acid solution to give first a green solution and finally a completely colorless one. The catalyst was prepared by the method of Adams, as described in the collected volume of "Organic Syntheses I to X".

3. Bromination Products of Resorufin. Dissolve resorufin in NaOH and add bromine to the solution until the sol-

ution smells permanently of this substance and a brown precipitate starts to form. At this point warm the solution, filter the precipitate, wash with water, and dissolve with alcohol. Then distill off the alcohol, wash with water, dissolve in alcohol, and once again evaporate. The product is "Iris Blue", the tetrabrom derivative of resorufin.



This product forms a red, amorphous mass, which in alcohol forms a violet solution with blood-red fluorescence. At 100° it starts to decompose.

However, when the bromination is carried out by means of a mixture of KBr and $KBrO_3$, a compound differing in properties from Iris Blue is formed. The directions are:

Dissolve one gram of resorufin in 10 cc. of 6N NaOH, then dissolve 3.9 g. KBr and 1.1 g. $KBrO_3$ in a minimum amount of cold water. The solutions are mixed and 15 cc. of dilute acetic acid added drop by drop, the solution being kept cool. Allow it to stand one-half hour; evaporate down to half volume; and filter off the precipitate.

The crystals formed differ from those of the product formed by sodium hydroxide and bromine. On going into solution in alcohol they produce a blue-violet solution with an intense red fluorescence.

In six normal sodium hydroxide the green color which is produced is converted to yellow by sodium hydrosulphite. The crystals dissolved in concentrated sulphuric acid give a brownish solution possessing a slight yellow-green cast. In ammonium hydroxide this substance produces a green solution with a red-violet fluorescence. The reaction is unknown.

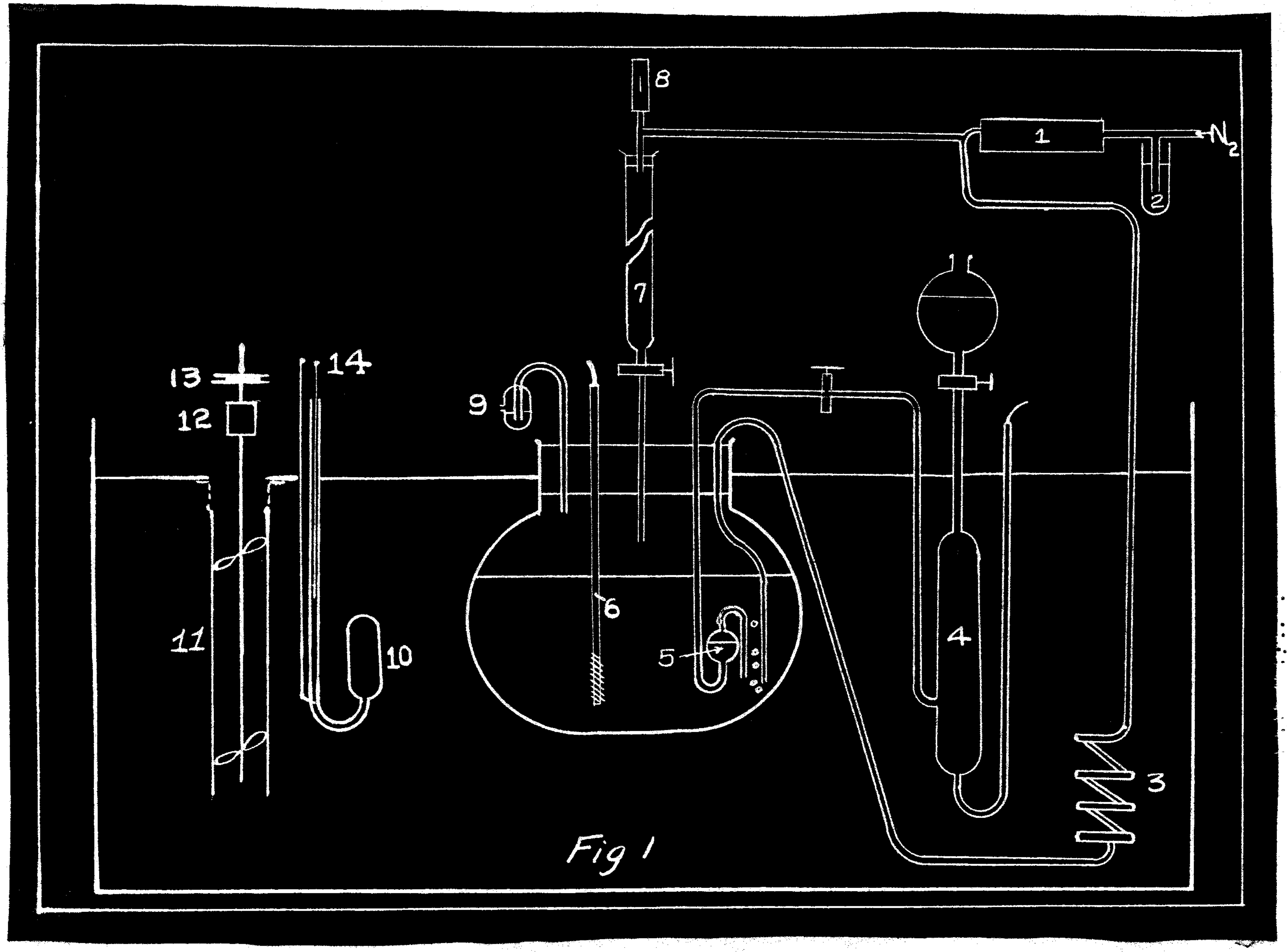


Fig 1

Key to Figure I

1. Pyrex tube containing copper
2. Mercury trap
3. Copper coil
4. Calomel cell
5. Goose neck
6. Electrodes (not shown in duplicate)
7. Burette
8. Bunsen valve
9. Mercury trap
10. Thermoregulator
11. Archimedes' lift
12. Rubber universal
13. Rubber-lined pulley
14. Leads to relay and heating coil

APPARATUS

The accompanying diagram depicts the apparatus used. The titration vessel was a 250 cc. soxhlet flask immersed to its neck in the thermostat, which was kept at the constant temperature of 30° ± .02° C. The heating unit was a nichrome resistance coil running through a pyrex tube filled with mineral oil, mineral oil being chosen because it did not tend to char on continued usage.

The heating coil was operated through a home-made thermoregulator completely filled with either ether or toluene. Either liquid proved satisfactory, ether being slightly better in that it possesses the greater coefficient of expansion and thereby tends to respond to a smaller variation in temperature.

The thermoregulator was connected to a relay (Precision Temperature Regulator, Model A) which operated on alternating current. This relay proved very satisfactory, since its make and break was extremely rapid and the tendency for arcing across the mercury surface was practically eliminated.

The bath was set at 30° by a standard thermometer, the range of temperature being determined with a Beckmann. Thirty degrees centigrade was chosen in order that this work might be comparable to that of Clark and Cohen.

Since excellent stirring is necessary in a bath requiring accurate thermal regulation, use was made of two brass Archimedes lifts set at suitable positions in the bath. The stirrers were driven by a quarter horsepower motor, the speed of which was regulated by a transformer.

A soxhlet titration vessel was equipped with a six-hole stopper. Two of them served as inlets for duplicate electrodes, a third as an inlet for nitrogen, the fourth as a nitrogen outlet, the fifth as a means of connection for the saturated calomel half-cell, and the last for the tip of the measuring burette.

1. Preparation, Use, and Care of Electrodes. Electrodes were used in duplicate to insure equilibrium conditions throughout the solution. The method of preparation was as follows: the platinum electrodes were of heavy wire offering a total surface of about one square centimeter. To the end of this wire was fused an inch-and-a-half length of fine platinum wire by means of an oxygen blowtorch. The fine end was carefully welded into soft glass tubing, care being taken to make good seals and to anneal them well. A thick seal is not to be recommended for uniform annealing becomes impossible and the electrode frequently develops microscopic cracks and thereby leads to erratic reading. The electrodes, without mercury, are placed in a beaker of cold water and the temperature raised to the boiling point.[7] This was allowed to boil some ten minutes and then permitted to cool. If cracks develop, water seeps into the

tube and they are thereby detected. If such is the case the electrode must be remade.

The platinum wire was wrapped about the glass tube, cleaned in alcoholic KOH to remove grease, and then treated with aqua regia. For final cleansing the electrodes were filled with mercury and immersed in fresh cleaning solution prepared from C. P. reagents, heated to 125° C., and permitted to stand until cool. When ready for use, the electrodes were removed, washed well with distilled water, rinsed in ethyl alcohol, and air dried for a period of twenty minutes.

Gold electrodes were all plated on platinum which had been prepared as above, care being exercised to cleanse the wire thoroughly before deposition of the plate. A solution of potassium auric cyanide was used. To prepare this solution, dissolve 0.7 g. of auric chloride in 50 cc. of water, precipitate the gold with dilute ammonia, taking care to avoid an excess. Filter, wash rapidly, and dissolve immediately with KCN solution, containing 1.25 g. of KCN in 100 cc. of water. Boil free of ammonia. The solution of potassium auric cyanide is now ready for use. Make the electrode which is to be plated the cathode, and using a gold anode plate for three minutes at six volts and a current density of 0.2 amps per square centimeter.

As an added precaution electrodes were occasionally heated in an alcohol flame. This procedure must be carefully undertaken, however, for cracks frequently develop.

2. Equipment and Method of Nitrogen Purification. Nitrogen bubbling through the system accomplished two purposes: it stirred the buffered dye solution, and it produced an inert atmosphere which prevented oxidation of the partially reduced dye.

Since commercial tank nitrogen contains from .35 to .50% oxygen as an impurity, it is obvious that all traces of this material must be removed scrupulously, for even slight traces cause marked discrepancies in results. Oxygen tends to seep through rubber connections, and for that reason as little rubber tubing as possible was employed in making connections. Where this was absolutely necessary, heavy gum pressure tubing was used. This was tightly wired and coated either with bakelite varnish or Khotinsky cement.

Two methods for removing oxygen were employed. One was an alkaline solution of pyrogallol prepared in the following proportions: 18% KOH, 15% pyrogallol, and 67% water. This mixture was used at the outset of the research, and when frequently prepared proved passably satisfactory. However, it possesses the disadvantage of being messy to handle, and tends, on getting old, to liberate carbon monoxide.

The final method used, and by far the most convenient, was essentially that recommended by Clark. For my purposes a meter length of combustion tubing, one inch in diameter, was filled with tightly rolled C. P. copper gauze. This was placed in a muffle furnace and maintained at a temperature between 400° and 500° C. The nitrogen, on passing through, loses its oxygen content by formation of cuprous oxide, and passes out completely pure.

At the completion of a six hour run it was found that one-third of the copper had been oxidized. The tank nitrogen was disconnected and a cylinder of hydrogen used to replace it. In a very few minutes the copper oxide was completely reduced and the gauze was once more bright and ready for further use in the removal of oxygen.

The exhaust nitrogen from the titration vessel passed through a bubbling trap containing mercury, thus preventing atmospheric oxygen from diffusing back into the system.

3. Equipment and Method of pH Determination. The equipment for hydrion determination was a modification of the well-known Hillebrand electrode. The glass bubbling apparatus was inserted in a rubber stopper which fitted in a straight-walled vessel containing the buffer solution. A second hole in the stopper served as an inlet for the calomel half-cell. A third contained an outlet tube which just dipped into water. This addition was used, for it prevented back diffusion of oxygen. This setup obviated the possibility of inaccurate pH measurements due to the presence of oxygen in the system.

Hydrion measurements were made on the pure buffer diluted in the ratio of 5 cc. of water to 50 cc. of buffer. The dye was not present in the solution, the assumption being made that the hydrogen ion concentration is not appreciably altered by the presence of the dye. Since the final concentration of resorufin was never greater than .00008 M, this assumption can be safely made.

A number of hydrogen electrodes were most carefully prepared as recommended by Clark.

Hydrogen was supplied from a cylinder of electrolytic gas and purified by passing through KOH solution to remove carbon dioxide, through cotton to remove entrained moisture, and finally through a tube of heated copper wire to remove oxygen.

The hydrogen electrode and attached saturated calomel half-cell were immersed in the constant temperature bath, corrections were made for barometric pressure and temperature in calculating the pH. Whenever there was any doubt as to the accuracy of an electrode it was immediately discarded. When everything was functioning smoothly, hydrogen electrodes reached equilibrium in fifteen minutes, and maintained constancy for periods of one-half hour or more.

4. The Saturated Calomel Half-Cell. The design of this electrode may be seen in the diagram. The half-cell was very carefully prepared from specially purified materials. The purification of mercury was accomplished by stirring the material for many hours with dilute nitric acid. This served to dissolve foreign metals. Frequent change of the

nitric acid is to be recommended, for an equilibrium is set up between the foreign metal and the mercury in solution, and at this point the acid no longer purifies.

When the mercury no longer tended to remain in individual droplets the treatment was halted, the nitric acid decanted, and the residue well-washed with distilled water, dried, and finally distilled twice by Hullet's method. The final distillation in vacuo recommended by Clark was not made.

The calomel used for the cell was prepared electrolytically from purified mercury by the method of Lipscomb and Hulett[8], modified somewhat for our purposes. Platinum electrodes were used and the HgCl which formed was scraped off the mercury surface by means of a flattened rod. Mercurous chloride prepared in this manner, aside from being pure, offers the advantage of being intimately mixed with mercury. This is an essential feature in preparing cells of reproducible voltage.

5. The Measuring Burette. The burette was the same as those usually used, except that a long piece of heavy-walled capillary tubing was fused to the bottom of the stop cock. The lower end was drawn down to a nozzle, and by this means measured quantities of reducing solution were introduced into the titration vessel. A layer of benzene one to two centimeters thick served to prevent air oxidation of the reducing agent. Aside from this an added precaution was taken, in that the burette was equipped with a bunsen valve through which flowed a constant stream of nitrogen.

6. Electrical Equipment. Voltages were measured by a Leeds & Northrup student's potentiometer, and a ballistic galvanometer (Leeds & Northrup, Cat. No. 2420-C) was employed to determine the null point. So sensitive was the instrument that it permitted the use of a closed stop cock in the KCI bridge of the calomel cell.

To insure accuracy in the millivolt readings, measurements were recorded to a tenth of a millivolt. Our potentiometer cannot be relied on for such accuracy, but since only three-place accuracy was desired finally, it served very well.

The standard of potential was a saturated Weston cell, calibrated by comparison with a Bureau of Standards cell.

In our measurements corrections were not made for change of voltage in the standard with temperature.

A four-volt storage battery supplied the working current for the potentiometer.

BUFFERS

1. Composition of Buffers.

1.	250	cc. M/5 NaCl,	250	cc. M/5 HCl,	000	cc. H_2O
2.	250	"	125	"	125	"
3.	250	"	30	"	220	"

4.	250	cc. M/5 Citric,	50	cc. M/5 NaOH,	450	cc. M/5 NaCl,	250	H_2O
5.	250	"	125	"	375	"	250	"
6.	250	"	210	"	290	"	250	"
7.	250	"	300	"	200	"	250	"
8.	250	"	400	"	100	"	250	"
9.	250	"	500	"	0	"	250	"
10.	208	"	500	"	0	"	292	"
11.	185	"	500	"	0	"	315	"

12.	250	cc. M/5 Na_2HPO_4,	230	cc. M/5 HCl,	520	cc. H_2O
13.	250	"	190	"	560	"
14.	250	"	145	"	605	"
15.	250	"	100	"	650	"
16.	250	"	40	"	710	"
17.	250	"	15	"	735	"

18.	250	cc.M/5 H_3BO_3,	10	cc.M/5 NaOH,	490	cc.M/5 NaCl,	250	cc.H_2O
19.	250	"	16	"	484	"	250	"
20.	250	"	30	"	470	"	250	"
21.	250	"	55	"	445	"	250	"
22.	250	"	80	"	420	"	250	"
23.	250	"	160	"	340	"	250	"
24.	250	"	240	"	260	"	250	"
25.	250	"	260	"	240	"	250	"

2. <u>Preparation and Purification of Buffers</u>. Materials employed in buffers were all carefully purified. Boric acid, sodium chloride, di-sodium phosphate, citric acid were all recrystallized five times from doubly distilled water.

Sodium hydroxide, free of carbon dioxide, was prepared by the method of Carnog.[9]

Titanous chloride[10] was prepared by the method of Polidori as described by Clark and Cohen.

PROCEDURE AND CALCULATIONS

1. Preparation of Sample. Resorufin prepared by the previously described method was put in solution in dilute ammonia and filtered. Thereupon HCl was added till the dye was completely precipitated. The suspension was heated, filtered through a Buchner, washed eight or ten times with water, placed on a porous plate and dried. The dry material was very finely powdered, placed in a soxhlet, and extracted with absolute alcohol for a period of three days. The alcohol was evaporated and the purified resorufin used for emf measurements.

2. Preparation of Stock Solution. A stock solution of resorufin, approximately .0008 molar, was prepared by weighing out the calculated quantity of purified dye. This was put into solution by warming and any undissolved material was filtered on a fritted filter. The dye solution was used in the proportion of 5 cc. of dye to 50 cc. of buffer.

3. Experimental Procedure for Voltage Measurement. The buffer was made up in the proportions represented in the table, deoxygenated and protected by a layer of C. P. benzene. One hundred and fifty cubic centimeters of buffer were introduced into the titration flask and 15 cc. of stock dye solution added.

The calomel half-cell was set up and flushed with saturated KCl HgCl solution. The pure buffer, without dye content, but diluted in the ratio of 50cc. to 5 cc. of water, was drawn up into the goose-neck of the salt bridge by sucking on a rubber tube attached to the KCl reservoir of the calomel electrode. If care was taken in the procedure, a sharp line of demarcation was discernable in the wide portion of the goose-neck. Since saturated KCl was employed as a bridge, correction for liquid junctions was neglected.

A definite amount of $TiCl_3$ was now rapidly weighed out and introduced into a hundred cubic centimeters of buffer solution, to which had been added ten cubic centimeters of water. By careful manipulation and by using a protecting layer of benzene it was possible to use as little as .02 grams of $TiCl_3$ for a reduction. It is essential that as small an amount as possible of this material be used for it tends to hydrolise and produce HCl with a consequent change of pH.

The reducing solution was introduced into the burette and protected by a 2 cm. layer of benzene. Aside from this a bunsen valve connected by a T-tube inserted in the top of the burette was flushed with nitrogen. The two methods of protection served to protect the reducing solution from atmospheric oxidation.

The electrodes were inserted; the titration vessel was stoppered, immersed in the bath, and clamped. Purified nitrogen was now blown through at a rate of 400 - 600 cc.

per minute, and this was continued for one hour to insure the complete removal of oxygen, both from the buffer and from the atmosphere within the flask.

At the expiration of this period a measured volume of reagent was introduced and the equilibrium potential recorded. Frequently a drift in potential was noted. In such instances the plateau value was accepted as the true reading.

It was found that the duplicate electrodes checked more closely in the flat portion of the curve than they did near the end point. For near the end point even minute differences in concentration produced relatively large differences in voltage.

During the titration the volume of reducing agent added was plotted against the e.m.f., the end point being taken as the point of steepest slope in the titration curve. The end point once located permitted the calculation of the relative concentrations of oxidant and reductant:

$$\frac{[Red]}{[Ox]} = \frac{V}{V_e - V}$$

where V_e equals the volume corresponding to the end point, and V the volume introduced at the time of e.m.f. measurement. From this ratio, E_0 can be calculated from Peters' Equation.

For resorufin, $n = 2$. This fact may be determined by the slope of the titration curve. Aside from this, calculations based on the assumption that $n = 2$ agree very well with the experimentally obtained curves.

On the data sheets the E_o's are calculated from a number of points near the central portion of the curve, and averaged to give a value accepted as the true E_o' for the system. Observations near either extremity are neglected, for they are more readily subject to change from variation in pH, temperature, or determination of the end point.

Throughout all calculations the sign of the electrode was used. The value .2426 volts was taken as the potential of the saturated calomel electrode at 30° C.

DATA

Working Equation:

$R = 8.315$ joules/deg.

$T = 303.14°$ K

$n = 2$

$F = 96500$ coulombs

$$E_o = E_h \quad .03006 \log$$

TABLE I

Titration of approximately .00008 M Resorufin in buffer #1, pH 1.036, with titanous chloride solution. T = 30° C.

Vol. $TiCl_3$ used	$\frac{\text{Red}}{\text{Ox}}$	E E.M.F. of cell	E_h Electrode potential	E_o
.30	.0227	.2587	.501	.452
1.10	.0886	.2036	.446	.414
1.60	.1344	.1878	.430	.404
2.10	.1843	.1755	.418	.396
3.10	.2982	.1590	.402	.386
4.10	.4360	.1473	.390	.379
5.10	.607	.1355	.378	.372
6.10	.824	.1222	.365	.362
7.10	1.11	.1092	.352	.353
9.62	2.48	.0683	.311	.323
12.70	15.88	.0225	.265	.301
13.10		.0011	.244	
13.20		.0054	.237	
13.30		.0160	.226	
13.40		.0249	.218	
13.50		.0356	.207	
13.60		.0403	.202	
13.70		.0448	.198	

TABLE II

Titration at 30° C. of approximately .00008 M Resorufin in buffer #9, pH 5.015, with titanous chloride solution.

Vol. $TiCl_3$ in cc.	$\frac{\text{Red}}{\text{Ox}}$	E E.M.F. of cell	E_h Electrode potential	E_o
1.76	.212	.1782	.421	.401
3.76	.597	.1610	.404	.399
4.76	.899	.1594	.402	.401
5.76	1.34	.1541	.397	.401
6.76	2.05	.1495	.392	.401
7.76	3.375	.1439	.386	.402
8.26	4.59	.1354	.378	.398
8.76	6.75	.1313	.374	.399
8.96	8.15	.1258	.368	.395
9.16		.1173	.360	
9.36		.1162	.359	
9.56		.1113	.354	
9.76		.1015	.344	
9.96		.0899	.3325	

End Point 9.96 cc.
Average E_o = .3995

TABLE III

Titration of approximately .0008 M Resorufin in buffer #10, pH 5.610, with titanous chloride solution. T = 30°.

Vol. $TiCl_3$ in cc.	$\frac{Red}{Ox}$	E E.M.F. of cell	E_h Electrode potential	E_o
.5		.186	.429	
1.5	.1515	.177	.420	.395
2.50	.281	.172	.415	.398
3.50	.443	.167	.410	.399
4.50	.652	.162	.405	.399
5.52	.938	.158	.401	.400
6.50	1.328	.151	.394	.398
7.50	1.922	.143	.386	.395
8.50		.133	.376	
9.50		.121	.364	
10.50		.104	.347	
10.70		.094	.337	
10.80		.092	.335	
11.00		.085	.328	
11.20		.083	.326	
11.40		.076	.319	

End Point 11.40 cc.
Average E_o = .3977

TABLE IV

Titration of approximately .00008 M Resorufin in buffer #11, pH 6.110, with titanous chloride. T = 30° C.

Vol. $TiCl_3$ in cc.	$\frac{Red}{Ox}$	E E.M.F. of cell	E_h Electrode potential	E_o
2.00	.1052	.1892	.4318	
4.00	.235	.1734	.4160	.396
5.00	.313	.1665	.4091	.394
6.00	.400	.1640	.4066	.395
7.00	.500	.1594	.4010	.392
8.00	.616	.1564	.3990	.393
9.00	.750	.1554	.3980	.394
10.00	.910	.1499	.3925	.392
11.00	1.10	.1491	.3917	.393
12.00	1.334	.1443	.3869	.391
16.00		.1200	.3626	
16.80		.1116	.3542	
17.60		.1024	.3450	
18.00		.0962	.3388	
18.40		.0923	.3349	
18.80		.0848	.3274	
19.20		.0762	.3188	
19.60		.0739	.3165	
20.00		.0639	.3065	
20.40		.0567	.2993	
20.80		.0512	.2938	
21.28		.0387	.2813	

End Point 21.00 cc.
Average E_o = .393

DISCUSSION OF ERRORS

The determination of oxidation-reduction potentials, by the method employed, is subject to several sources of error.

A. The assumption that the concentration ratio of reductant to oxidant is identical with the activity ratio is not absolutely correct, even though the dye is highly diluted.

B. Another error results from the drift of potential with time. This drift is extremely rapid in all alkaline ranges with the compound studied -- so much so that measurements were impossible.

C. The exact determination of the end point is exceedingly difficult and permits a range of one to two per cent in its estimation. As a result, E_0 calculations made from data at either the beginning or the end of the titration do not give constant values. For this reason, points near the central portion of the titration curve were chosen in determining E_0.

D. The voltages of oxazines vary with dilution. This has been shown by Preisler and Cohen.[3]

E. The assumption that the reducing solution is of the same pH as the dye solution is not strictly accurate, for titanous chloride cannot be prepared free of excess hydrochloric acid. This acid content will produce a slight change in the buffer's pH.

DISCUSSION OF RESULTS

Table I was given in order that one might see how resorufin acts on being titrated in a solution containing a chloride buffer. On examining the data one observes that the E_0 calculated is not a constant as predicted by theory. It is obvious that something is happening to the resorufin in the titration vessel. From the results it would seem that a portion of the oxidant, over and above that required by the amount of reducing solution added, is being removed.

There occur three possible explanations as to the reason for this variation, namely: meriquinone formation, salting out of an insoluble resorufin salt, or decomposition of the oxidant.

Attempts were made to calculate the E_0 values on the assumption that a meriquinone, composed of one molecule of oxidant to one molecule of reductant, combined to give a complex. This attempt gave E_0 values which checked no better than those based on the assumption that $n==2$.

If meriquinone formation occurs, it is by no means necessary that the complex formed be composed of oxidant and reductant in the ratio of one mole to one. For a more complete discussion of this type reaction, see reference (1,IX).

That meriquinone formation does occur is quite probable, for it should be remembered that resorufin, on being reduced in high acid solution, undergoes an intermediate blue-green color.

The salting out of insoluble resorufin is also a distinct possibility, for the solubility of the hydrochloride is extremely low. It may be that the variation of E_0 can be explained in this manner.

There is the possibility that the oxidant decomposes in the acid region. However, it should be noted that there is no experimental reason to confirm this assumption.

Numerous other titrations were attempted in the various buffers containing chloride ion. On no occasion could constant E_0's be found. It was considered unnecessary to furnish additional examples.

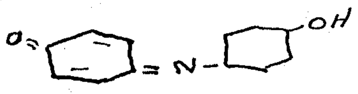
Tables II, III, and IV show titrations of resorufin, in non-chloride buffers, carried over ranges of pH in which the compound appears to act as a true oxidation-reduction indicator. It should be noticed that the E_0 varies but slightly with change of pH. This may be explained in that the ionizations of the hydroxyl groups are extremely slight. For a more complete understanding see the derivation of the theoretical equation for resorufin.

Attempts were made to titrate resorufin in alkaline ranges, using $Na_2S_2O_4$. These efforts were abortive, for drifts were so extremely rapid that readings could not be made.

Drifts of potential in this alkaline solution occurred even before addition of reducing solution.

Judging by the blue coloration resulting from air oxidation of the alkaline solution, it would seem that resorufin is converted into resazurin.[11]

Let us compare resorufin with phenol indophenol, which has the formula



It is noticed that phenol indophenol differs from resorufin in that it does not possess a bridge oxygen.

It is of interest to consider the change in ionization produced by the bridge oxygen present in resorufin. Phenol indophenol varies appreciably in E_0 with change in pH, while resorufin varies but little. This indicates that the ionizations of the characteristic groups in indophenol are diminished by substitution of the oxygen bridge.

SUMMARY

Reactions of resorufin have been qualitatively studied.

Oxidation-reduction potentials of this compound were measured and found to be most suitable at pH's of from 5 to 7.

• • • •   • • •

BIBLIOGRAPHY

(1) I. Clark, W. Mansfield, Introduction, 38, 433 (1923)

II. Clark & Cohen, An Analysis of the Theoretical Relations between Reduction Potentials and pH, 38, 666, (1923)

III. Clark & Cohen, Electrode Potentials of Mixtures of 1-Naphthol-2-Sulphonic Acid Indophenol and the Reduction Product, 38, 933 (1923)

IV. Sullivan, Cohen, & Clark, Electrode Potentials of Indigo Sulphonates, Each in Equilibrium with its Reduction Product, 38, 1669 (1923)

V. Cohen, Gibbs & Clark, Electrode Potentials of Simple Indophenols, Each in Equilibrium with its Reduction Product, 39, 381 (1924)

VI. Cohen, Gibbs, & Clark, A Preliminary Study of Indophenols: (A) Dibromo Substitution Products of Phenol Indophenol; (B) Substituted Indophenols of the Ortho Type; (C) Miscellaneous; 39, 804 (1924)

VII. Gibbs, Cohen, & Cannan, A Study of Dichloro Substitution Products of Phenol Indophenol, 40, 649 (1925)

VIII. Clark, Cohen, & Gibbs, Methylene Blue, 40, 1131 (1925)

IX. Clark, Cohen, & Gibbs, A Potentiometric and Spectrophotometric Study of Meriquinones of the p-Phenylene Diamine and Benzidine Series, Sup. No. 54 (1926)

X. Cannan, Cohen, & Clark, Reduction Potentials in Cell Suspensions, Sup. No. 55, (1926)

XI. Phillips, Clark, & Cohen, Potentiometric and Spectrophotometric Studies of Bindschedler's Green and Toluylene Blue, Sup. No. 61 (1927)

XII. Clark, Cohen, & Sullivan, A Note on the Schardinger Reaction, Sup. No. 66 (1927)

XIII. Gibbs, Hall, & Clark, Preparation of Indophenols Which May be Used as Oxidation-Reduction Indicators, Sup. No. 69 (1928)

XIV. Hall, Preisler, & Cohen, Equilibrium Potentials of 2, 6-Dibromobenzenone Indophenol-2'-Sodium Sulphonate, 2, 6-Dibromobenzenone Indophenol-3'-Sodium Sulphonate, 2, 6-Dichlorobenzenone Indo-2'-Chlorophenol, and 2, 6-Dimethylbenzenone Indophenol, Sup. No. 71 (1928)

XV. Cohen & Phillips, Potentiometric Studies of the Amino Indophenols: Phenol Blue, m-Toluylene Diamine Indophenol, and o-Toluidine Indophenol, Sup. No. 74 (1929)

(2) Nietzki, Dietze, & Mäckler, Ber., 22, 3020 (1889)

(3) Cohen & Preisler, Studies on Oxidation-Reduction, XVI The Oxazines: Nile Blue, Brilliant Cresyl Blue, Methyl Capri Blue, and Ethyl Capri Blue, Sup. No. 92 (1931)

Vellinger, Arch. Physique Biol., 7, 113 (1929)

Rapkine, Struyk, & Wurmser, C. R. Soc. Biol., 100, 1020 (1929)

Rapkine, Struyk, & Wurmser, J. Chim. Phys., 26, 340 (1929)

(4) Weselsky, P., Ann., 162, 273 (1872)

Weselsky & Benedikt, Monatsh., 1, 886 (1880; 5, 605 (1884)

Brunner & Kraemer, Ber., 17, 1847 (1884); 17, 1867 (1884); 17, 1872 (1884)

Kraemer, Ber., 17, 1875 (1884)

Nietzki, Dietze, & Mäckler, Ber., 22, 3020 (1889)

Nietzki & Mäckler, Ber., 23, 718 (1890)

(5) Ehrlich, J. Prakt. Chem., 139, 113 (1934)

(6) Clark, Cohen, & Gibbs, Studies on Oxidation-Reduction, A Potentiometric and Spectrophotometric Study of Meriquinones of the p-Phenylene Diamine and the Benzidine Series, Sup. No. 54 (1926)

(7) I. Lange, Handbook of Chemistry, p. 695, Handbook Publishers Co., Inc., (1934)

II. Morgan, Lammert, & Campbell, J. Am. Chem. Soc., 53, 454 (1931); 53, 597 (1931); 53, 2154 (1931)

(8) Lipscomb & Hulett, J. Am. Chem. Soc., 38, 21 (1916)

Richards, Z. Physik. Chem., 24, 39 (1897)

Richards & Archibald, Z. Physik Chem., 40, 385 (1902)

Sauer, Z. Physik. Chem., 40, 385 (1902)

Lewis & Sargent, J. Am. Chem. Soc., 31, 362 (1909)

(9) Cornog, J. Am. Chem. Soc., 43, 2573 (1921)

(10) Polidori, Z. Anorg. Allgem. Chem., 19, 306 (1899)

(11) Eichler, J. Prakt. Chem., 141, 91 (1935)

ACKNOWLEDGMENT

The author is indebted to Dr. E. F. Farnau for the conception of this problem, and for his aid in bringing it to a successful conclusion.