CXXXIX. STUDIES ON XANTHINE OXIDASE.

VIII. THE OXIDATION-REDUCTION POTENTIAL OF THE OXIDASE SYSTEM.

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I. Introduction.

Since the publication of the valuable work of Biilmann and Lund [1921] on the oxidation-reduction potential of quinone derivatives and other organic substances, much attention has been given to the potentiometric study of the chemical affinity of oxidation-reduction reactions. In biological research, however, it is still in its infancy and its importance is not yet fully appreciated. The work described in the present communication was undertaken with the object of showing that the potentiometric method may be a valuable means for the study of biological oxidation systems.

It was decided to investigate a typical oxidation-reduction enzyme with a view to determining how far the potentiometric behaviour of such a system would resemble that of the (non-biological) oxidation-reduction systems so much studied from this point of view.

As a typical oxidation-reduction enzyme xanthine oxidase was chosen, owing to the ease of preparation of the enzyme in a purified form. Our present knowledge of this enzyme is due chiefly to the work done by Morgan, Stewart and Hopkins [1922] on its existence in milk, followed by the extensive studies of Dixon and Thurlow on the physico-chemical aspects of its action.

In presence of this enzyme hypoxanthine and xanthine are oxidised to uric acid by hydrogen acceptors such as molecular oxygen or methylene blue, etc. The reaction may be pictured as follows:

$$\underset{\text{Enzyme}}{E} + \underset{\text{Xanthine}}{X} + H_2O = EXOH_2$$

$$EXOH_2 + \underset{\substack{\text{Hydrogen}\\\text{acceptor}}}{A} = E + \underset{\text{acid}}{XO} + AH_2$$

in accordance with Wieland's theory of oxidation. The enzyme and its substrate first combine to form a complex of the type represented. The two hydrogen atoms of this complex are "active" and readily transferred to a hydrogen acceptor, as in the second equation. Many reducible substances can act as hydrogen acceptors in this system [Dixon, 1926], but those which have been most studied are molecular oxygen and methylene blue. In the first case the oxygen uptake can be measured by means of Barcroft's apparatus, and in the second case the reaction can be followed by the disappearance of the blue colour, using Thunberg's vacuum tubes.

II. Experimental technique.

The reaction vessel used was one of about 15 cc. capacity. It was tightly fitted with a rubber stopper with five holes, holding tubes for the gas inlet and outlet, the electrode, burette and agar salt bridge. The electrode consisted of a gold plate of 1 cm.^{2}. Platinum proved less satisfactory, owing to its slowness in following the change of the potential as the reaction proceeded. As the other half-element a saturated calomel electrode was used, which was connected with the reaction vessel by means of the agar salt bridge. The whole apparatus was immersed in a water thermostat kept at 18°. For the measurement of the potential the Unipivot type of potentiometer of the Cambridge Instrument Company was used. The readings were accurate to 1 millivolt.

III. Does hypoxanthine plus xanthine oxidase give a reduction potential?

Clark [1925] carried out an experiment on the Schardinger enzyme in fresh milk, which acts upon aldehydes in exactly the same way as the xanthine oxidase does upon xanthine (the two enzymes may be identical). He states that in presence of milk aldehyde becomes activated in such a way as to give a high reduction potential, which is not given by the aldehyde alone or by aldehyde plus boiled milk. The author, however, has been entirely unable to repeat these results, in spite of repeated attempts to do so[1]. This was the case both with fresh milk or with the purified preparation of the enzyme, and whether formaldehyde, acetaldehyde or hypoxanthine was used as hydrogen donator. Some experimental details are as follows.

5 cc. of a fresh milk, which had been tested and found to be very active with the methylene blue technique, were pipetted into the reaction vessel, and nitrogen gas, which had passed through two wash-bottles containing strong pyrogallol solution, bubbled through it. When the potential showed a steady value, 1 cc. of 1 % acetaldehyde, which was previously neutralised with sodium hydroxide, was introduced through the burette and the change of the potential was followed. Similar experiments with hypoxanthine were repeated, using 0·5 g. of the enzyme preparation obtained by the method of Dixon and Thurlow [1924, 1] or 0·002 g. of the enzyme purified by the method of Dixon and the author published elsewhere in this *Journal* [1926]. Each enzyme preparation was dissolved in 5 cc. of phosphate buffer solution ($p_H = 7·6$) and 1 cc. of hypoxanthine solution, containing 0·2 mg., was added instead of the acetaldehyde. The results of these measurements are illustrated in Fig. 1, where E_h is plotted as ordinate and time as abscissa.

As will be seen from Fig. 1, the fresh milk and the caseinogen preparation gave only a slight change in potential on the addition of acetaldehyde and hypoxanthine respectively, while the purified preparation of the

[1] Dr M. Dixon has also been unable to repeat Clark's results.

same activity as 5 cc. of the fresh milk gave a still smaller change with these hydrogen donators. Possibly some reducible substance exists in the fresh milk which gives this slight potential when reduced.

In no case has any potential change of the order given by Clark been observed with fresh milk or with the enzyme preparations[1]. Occasionally, however, curves similar to Clark's have been obtained when a not very fresh milk was used, but these potential changes were obviously due to bacteria, and were *not* dependent on the presence of aldehyde. It is significant that the final potential reading in these experiments, which was the same as that obtained by Clark after inoculating the milk with bacteria, was identical with the final value given by Clark for the Schardinger system; this fact suggests that the effects he observed may possibly have been due to bacteria.

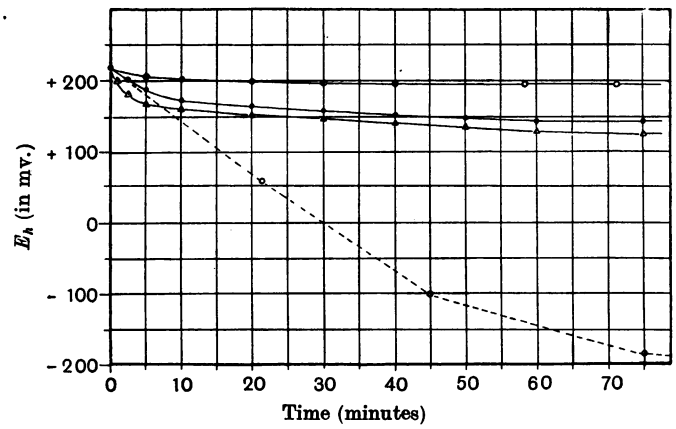

Fig. 1. The reduction potential of xanthine oxidase + hypoxanthine.

–○——○–	Purified oxidase preparation + hypoxanthine.	p_H 7·6. 14°
–●——●–	Caseinogen preparation + hypoxanthine.	p_H 7·6. 14°
–△——△–	Fresh milk + aldehyde.	p_H 7·0. 14°
--○----○	Clark: Fresh milk + aldehyde	p_H 7·0. 37°

It is, moreover, clear that the potentials observed by Clark cannot represent the true reducing potential of the enzyme system; for it follows from a consideration of the curve given by him that the apparent reduction potential only reaches a value theoretically sufficient to begin to reduce methylene blue about 30 minutes after addition of the aldehyde, whereas actually under similar conditions the reduction begins at once and may in fact be complete in under 1 minute.

IV. The oxidation-reduction potential in presence of a hydrogen acceptor.

This potentiometrically inactive system (xanthine oxidase + hypoxanthine or aldehyde), however, exerts its full reducing power when it comes in contact with reducible substances. This is the extremely interesting and characteristic feature of biological oxidation-reduction systems, namely that, while giving

[1] Experiments carried out at 37° gave results similar to those at 14°.

no reduction potential themselves, they are yet able to bring about the reduction of a large number of hydrogen acceptors. The following experiments show the effect on the potential of the presence of various hydrogen acceptors.

(*a*) *The potential in presence of oxygen.*

2 mg. of the purified enzyme dissolved in 6 cc. of the phosphate buffer ($p_H = 7{\cdot}6$) were placed in the reaction vessel and air bubbled through. When the potential had attained a steady value, 1 cc. of hypoxanthine solution containing 0·2 mg. was introduced from the burette and the change of the potential was followed. The results are shown in the following diagram (Fig. 2).

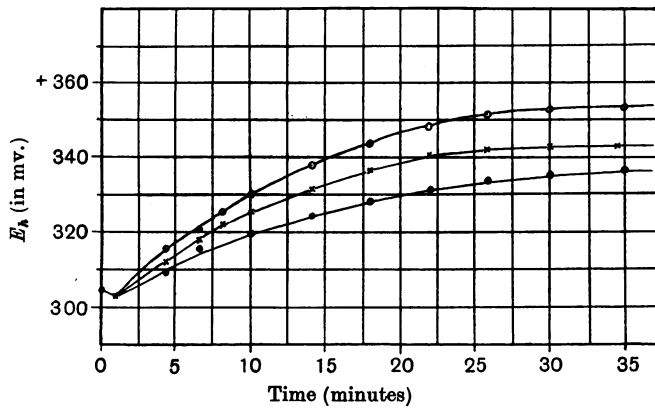

Fig. 2. The oxidation-reduction potential in presence of air.
-○——○- 4 mg. oxidase + 0·5 mg. hypoxanthine
-×——×- 2 mg. ,, + 0·5 mg. ,,
-●——●- 2 mg. ,, + 0·2 mg. ,,

As shown in the above figure, the potential shifts in the direction of higher oxidation potential on the addition of hypoxanthine when oxygen is present. This effect might be expected from the results of Thurlow [1925], who showed that in this system hydrogen peroxide is formed, which will give an oxidation potential.

It will be interesting to compare this with the following results, which were obtained by adding pure dilute hydrogen peroxide solution of known strength to a solution of 2 mg. of enzyme in 5 cc. of phosphate buffer. By this means we may obtain some idea of the amount of hydrogen peroxide formed.

The results obtained are given in Fig. 3, where the observed potentials are plotted against the logarithm of the volume (cc.) of hydrogen peroxide added. 1 cc. of the solution used contained 0·00118 mg. H_2O_2.

The curve is obviously a straight line, and can be represented by the equation

$$E_h = K + a.\log C,$$

where E_h is the observed potential reduced to the normal hydrogen scale, C is the concentration of hydrogen peroxide, and K and a are constants,

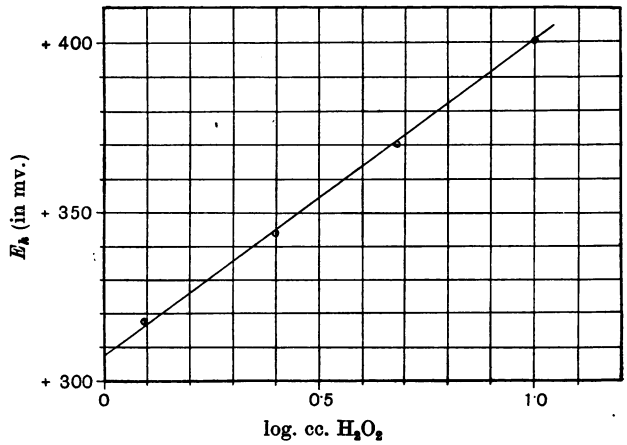

Fig. 3. The oxidation potential of H_2O_2.

which in this case have the values of 308 and 93 mv. respectively. From this equation we can calculate, from the values of E_h observed in the experiments of Fig. 2, the amount of hydrogen peroxide produced in that experiment, as in the following table.

Table I. *The amount of hydrogen peroxide produced.*

Xanthine oxidase mg.	Hypoxanthine mg.	E_h mv.	Corresponding amount of H_2O_2 mg.	Theoretical amount mg.
2	0·2	+337	0·0236	0·0625
2	0·5	+343	0·0279	0·125
4	0·5	+354	0·0365	0·125

The theoretical value is calculated on the assumption that one molecule of hypoxanthine gives rise to two molecules of hydrogen peroxide. The actual amount of hydrogen peroxide found is, however, smaller than this, owing to the fact that the peroxide molecules may react further with activated hydrogen atoms from the hypoxanthine, as follows: $H_2O_2 + 2H = 2H_2O$. This reaction was shown to occur by Thurlow.

(*b*) *The potential in presence of methylene blue.*

As already mentioned, xanthine oxidase plus hypoxanthine does not give any reduction potential to account for the reduction of methylene blue; it is a well-known fact, however, that the dye is rapidly reduced. Now the oxidised and reduced forms of the dye, as shown in detail by Clark [1925], give a reversible oxidation-reduction potential, which can be calculated (for a given p_H) by Peters' equation:

$$E_h = E_k - \frac{RT}{nF} \log \frac{[\text{Red}]}{[\text{Ox}]},$$

where E_h R, T, n and F have their usual significance, and E_k is a constant characteristic of this particular oxidation-reduction equilibrium. [Red] and [Ox] are the concentration of the reduced and oxidised forms of the dye respectively. By means of this equation the percentage reduction of the methylene blue can be calculated from the potential value.

Since the oxidase-hypoxanthine system by itself gives no potential, as has been shown in the previous section, it may be expected that the potential observed anaerobically in presence of methylene blue will simply be due to the latter, so that the percentage reduction of the dye at any instant may be determined by measuring the potential given by the system.

In order to see whether this was actually the case the following experiment was carried out, in which the methylene blue was reduced step by step by adding successively small known amounts of hypoxanthine. After each addition the potential was determined after sufficient time had elapsed to allow the reaction between the hypoxanthine and the methylene blue to be completed and the potential to become steady. As one molecule of hypoxanthine reduces two molecules of methylene blue, as shown by Morgan, Stewart and Hopkins [1922], the percentage reduction of the methylene blue could be calculated from the amount of hypoxanthine added.

5 cc. of phosphate buffer solution ($p_H = 7{\cdot}6$) and 0·5 cc. of methylene blue (1 : 5000) were introduced into the reaction vessel, and nitrogen gas was bubbled through. After the potential showed a steady value, which was usually attained in a few minutes, a solution of 2 cc. of enzyme solution (4 mg.) + 2 cc. hypoxanthine solution (0·4 mg.) + 4 cc. distilled water was delivered from the burette 0·1 cc. at a time. At the various stages of reduction the potential was read when it became constant.

For comparison a similar experiment was carried out under the same conditions using standard titanous chloride solution to reduce the methylene blue instead of the hypoxanthine.

The two series of results are compared in Fig. 4 with the curve calculated from Peters' equation above.

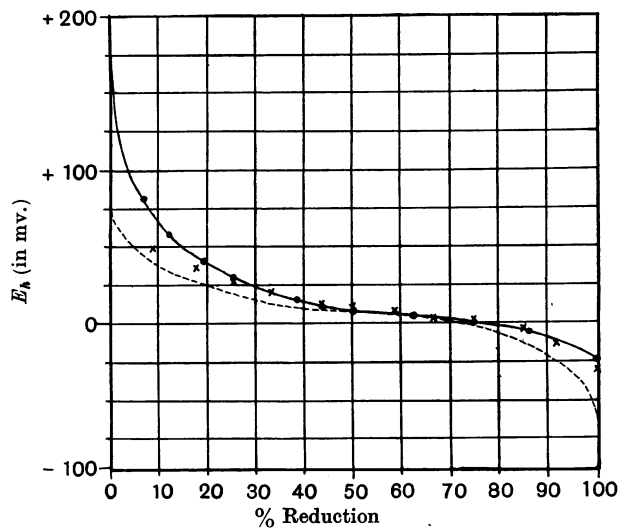

Fig. 4. The reduction of methylene blue by hypoxanthine and by $TiCl_3$.

-●——●- Xanthine oxidase + hypoxanthine. -×——×- $TiCl_3$. - - - - - - Theoretical.

The good agreement of the curves shows that xanthine oxidase plus hypoxanthine behaves in a precisely similar way to the inorganic reducing agent. The slight divergence between the experimental and theoretical curves is probably due to traces of oxygen in the nitrogen gas and in the solutions. These would tend to make the actual percentage reduction values lower than the calculated, and would have the effect of displacing the experimental curves to the right.

Besides methylene blue the oxidation-reduction indicators described by Clark and his co-workers [1923, 1, 2, 3, 4] can be reduced by the xanthine oxidase plus hypoxanthine, as shown by Dixon [1926]. The author has studied the potential of this system in presence of the following dyes. In all cases excess of hypoxanthine was used, and ample time was allowed for the reaction to proceed to completion. The final r_H values have been calculated in the usual way from the potential measurements according to the equation

$$r_H = 2\left(\frac{F.E_h}{RT} + p_H\right),$$

and compared with those from Clark's data for 99 % reduction.

Table II.

Dye	Final E_h mv.	r_H Found	r_H Clark
o-Chlorophenol-indophenol	+128	19·9	19·4
p-Cresol-indophenol	+107	19·1	18·5
1-Naphthol-2-sulphonic acid-indophenol	+ 12	15·6	15·6
Methylene blue	− 32	14·0	13·6

Though the final r_H is a little higher than that found by Clark in most cases, this may be due to traces of oxygen. It appears from these results that the final observed potential depends simply upon the dye, being the potential given by the reduced form in each case.

It will be clear from the results which have been given that the potentiometric method gives us a useful means of following the course of the reduction of methylene blue by the oxidase system. In Fig. 5 is given a typical series of potential-time curves illustrating this reaction. The experiments were carried out anaerobically as before.

It will be seen that the potential at first follows the S-shaped curve of Fig. 4, indicating a constant-velocity reduction; the velocity then falls off quite suddenly at the end of the reaction, giving a sharp break in the curve at a point corresponding to the disappearance of the colour. This is in agreement with the observations of Dixon and Thurlow [1924, 2], who found by means of a colorimetric method that the reduction proceeded at a constant rate until the reaction was practically complete, the velocity only falling off just at the end. It will be noticed that the reaction velocity is proportional to the enzyme concentration, as Dixon and Thurlow state.

From the fact that the sharp break in the curves corresponds with the instant at which reduction is complete it follows that the potentiometric

method can be used for the exact determination of the reduction-time, this being the interval between the addition of the hypoxanthine and the occurrence of the break. As a matter of fact, when the activity of the enzyme to be tested is strong, there is no advantage in this method over the widely used vacuum-tube method, except that the personal error in judging the last nuance is eliminated. When the enzyme solution is weak, however, and half an hour or more is required for complete reduction, this method may be valuable, for the initial change of E_h is a function of the enzyme concentration. The details of experiments on this point will be published later.

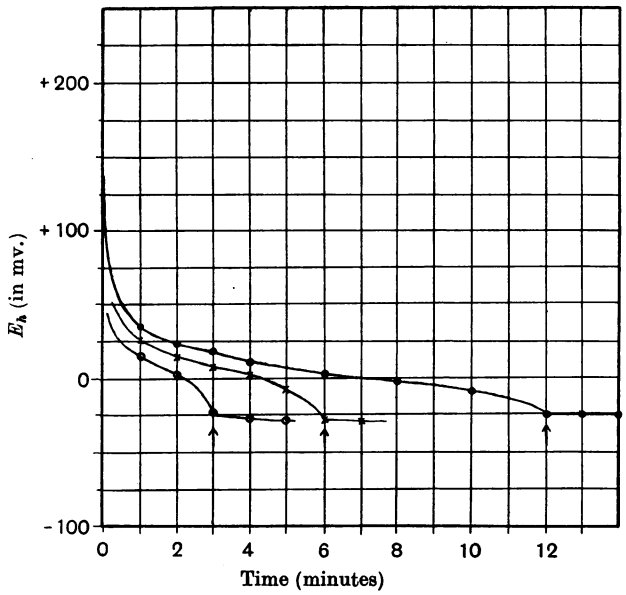

Fig. 5. E_h-time curve with different enzyme concentrations.
-●——●- 1 mg. enzyme -×——×- 2 mg. enzyme
-○——○- 4 mg. enzyme
Reaction system: 5 cc. phosphate buffer (p_H 7·6)
1 cc. enzyme solution, containing 1, 2 or 4 mg.
0·5 cc. methylene blue (1:5000)
1 cc. hypoxanthine, containing 0·2 mg.
The arrow indicates the point where colour disappeared.

Another application of the method is the qualitative and quantitative test as to whether a potentiometrically active substance is reduced by the enzyme or not, especially when the reduction of this substance is not accompanied by a colour change. For instance, the reduction of alloxan to alloxantin can be followed by this method.

Summary.

1. Hypoxanthine and aldehyde do not give reduction potentials when activated by xanthine oxidase.

2. If methylene blue be added to this system a reduction potential is given; this is, however, merely the potential of the dye itself.

3. The course of the reduction of methylene blue by this system, and the reduction-time, can be determined by potential measurements.

4. In the presence of oxygen xanthine oxidase plus hypoxanthine give a potential. This is due to the formation of hydrogen peroxide as shown by Thurlow. The amount of hydrogen peroxide formed has been determined by potential measurements.

5. The oxidation-reduction indicators of Clark are reduced by xanthine oxidase and hypoxanthine, and the reduced forms then give their respective reduction potentials.

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