XCIH. STUDIES ON XANTHINE OXIDASE.

VII. THE SPECIFICITY OF THE SYSTEM.

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THE study of the degree of specificity which an enzyme exhibits towards its substrate is always important for the proper understanding of the mechanism by which it acts. A number of systematic studies have been made on the specificity of certain hydrolysing enzymes, but up to the present no systematic study of any one oxidation-reduction enzyme appears to have been made from this point of view. This is possibly on account of the difficulty of separating these enzymes from one another. The xanthine oxidase however forms a very suitable example to choose for such an investigation, for, as Dixon and Thurlow [1924, 1] have shown, it can be prepared free from other enzymes from milk. The present paper describes a preliminary study of the specificity of this enzyme.

There is a fundamental difference to be considered between hydrolysing and oxidising enzyme systems. In hydrolysing systems we have, in addition to the catalyst, only one reactant to consider, namely, the substance hydrolysed (neglecting of course the water molecules which take part in the reaction). In this case we have only to consider the specificity of the enzyme catalyst towards this substrate. In the case of oxidising systems, however, we have, besides the catalyst, *two* reactants to consider. Before any reaction can occur in such systems there must always be present, in addition to the enzyme, both an oxidisable (reducing) substance and a reducible substance (or oxidising agent). In other words, we must have present simultaneously a hydrogen donator (hypoxanthine, etc.) and a hydrogen acceptor (oxygen, methylene blue, etc.).

The investigation of the specificity of an oxidation-reduction enzyme therefore falls naturally into two distinct sections. We have to determine how far the enzyme is specific on the one hand towards the hydrogen donator and on the other hand towards the hydrogen acceptor. In the case of the xanthine oxidase, therefore, we have first to determine how far other substances can replace hypoxanthine, using throughout a typical hydrogen acceptor such as methylene blue; and secondly we have to determine how far methylene blue can be replaced by other hydrogen acceptors, using throughout an efficient hydrogen donator such as hypoxanthine.

In the work which follows the xanthine oxidase used was prepared from milk partly by the method of Dixon and Thurlow [1924, 1], and partly by a later method which gives an extremely active preparation. It is hoped to publish details of this method shortly. Most of the experiments in which negative results were obtained were also repeated with whole milk, and the same results obtained.

THE HYDROGEN DONATOR.

In the experiments described in this section methylene blue was used throughout as the hydrogen acceptor, and various substances were tested to see whether they were capable of acting as hydrogen donators in presence of the oxidase.

Hypoxanthine and xanthine. The fact that in presence of the milk enzyme these purines rapidly reduce methylene blue was shown by Morgan, Stewart and Hopkins [1922], and the reaction was further studied by Dixon and Thurlow [1924, 2]. There can be no doubt that the same enzyme is responsible for the oxidation of both purines.

Aldehydes. The oxidation of aldehydes by methylene blue in presence of milk was shown by Schardinger [1902]. Schardinger used formaldehyde, but it appears that most aldehydes act in the same way. It is noteworthy, however, that glucose, lactose, etc., show no trace of reduction of methylene blue, even after many days with the most active enzyme preparation yet obtained.

The question as to whether the oxidation of aldehydes is brought about by the xanthine oxidase, or by a special aldehyde oxidase present together with this in the preparations used, is a difficult one. The problem has been discussed by Morgan, Stewart and Hopkins [1922] and by Dixon and Thurlow [1924, 2]. On the one hand, it seems unlikely that an enzyme so specific in other respects, as will be seen, should act upon such widely different substances as purine bases and aldehydes. On the other hand, a considerable amount of evidence was obtained by Dixon and Thurlow in support of the view that only one enzyme is concerned. I shall return to this point later.

Adenine. It was shown by Morgan, Stewart and Hopkins that adenine was able to reduce methylene blue very slowly in presence of milk. They believed that this was due to a gradual deamination of the adenine by traces of adenase with the production of hypoxanthine, which was then oxidised by the xanthine oxidase. Dixon and Thurlow [1924, 2], on the other hand, obtained some evidence that a direct oxidation of the adenine by the oxidase occurred. It cannot yet be stated definitely which view is correct.

It may be stated at once that the above-mentioned substances are the only ones I have found to be capable of acting as substrates for the xanthine oxidase.

It is, of course, necessary to use in all experiments a milk or enzyme preparation free from bacteria, since the bacteria themselves reduce methylene blue. For this reason one must always make sure by a control experiment that

the milk or oxidase solution does not reduce methylene blue by itself without the addition of a hydrogen donator. A failure to do this is apparently responsible for statements in the literature that many substances can act in a similar way to hypoxanthine in the milk oxidase system. Abelous and Aloy [1918, see also 1917], and Aloy and Valdiguie [1921] state that they found the following substances to reduce methylene blue in presence of milk or a preparation of the milk oxidase: all the aldehydes of the aliphatic and aromatic series; a large number of ptomaines; substances of the uric acid group—guanine, hypoxanthine¹, xanthine, alloxan; alkaloids—quinoline, morphine, cicutine, muscarine; toxalbumins—ricin, abrin; products of protein hydrolysis—proteoses, peptones, tryptophan; and various other substances belonging to the groups of ketones and amines (*e.g.* benzylamine). Unfortunately no experimental data were published, and it does not appear that control experiments were carried out. Since Abelous and Aloy's statement seemed to convey a totally wrong impression of the specificity of the milk oxidase, I have repeated most of their experiments, using very fresh milk and enzyme preparations free from bacteria; and, except in the cases previously mentioned and in the case of commercial peptone, which will be dealt with below, I have obtained completely negative results.

My experiments were carried out as follows. The substances were made up in 1 % aqueous solution, and all the solutions were carefully adjusted to p_H 7.6 before use. 0.5 cc. of the solution was taken, together with 5 cc. of oxidase solution in p_H 7.6 phosphate buffer and 0.5 cc. of 1/5000 methylene blue. The experiments were carried out anaerobically in vacuum tubes as usual. If in any experiment no reduction had occurred even after many hours, the tube was opened and a small amount of hypoxanthine added in order to make sure that the enzyme had not been destroyed by the substance under investigation. In all cases a rapid reduction was then obtained, showing that none of the substances tried was toxic to the enzyme. The amount of substance taken was always considerably in excess of the amount of methylene blue used.

Guanine. This was prepared in the usual way from nucleic acid, and was recrystallised several times as the hydrochloride. No trace of reduction could be observed after many hours. (The reduction-time of the enzyme solution used was about two minutes with hypoxanthine.)

Alloxan. Three specimens were used. One was prepared by the oxidation

¹ A question of priority arises here. It has been thought that Morgan, Stewart and Hopkins [1922] were the first to detect the xanthine oxidase in milk, but it will be seen that Abelous and Aloy published their results four years earlier. Abelous and Aloy, however, merely include hypoxanthine and xanthine, together with aldehydes, in a list among a large number of substances which in actual fact do *not* reduce, as will be shown below. The reduction obtained with these other substances can apparently only have been due to the presence of bacteria, and this fact invalidates their experiments with the purine bases also. Priority therefore rests with Morgan, Stewart and Hopkins.

I am asked by Sir F. G. Hopkins to state that the observations of Morgan, Stewart and Hopkins were made quite independently of those of Abelous and Aloy.

of uric acid with bromine, another by the oxidation of uric acid with nitric acid, and the third by the oxidation of dialuric acid. The last two were twice recrystallised from water. No reduction could be observed with any preparation, even after two days. The presence of alloxan was found to slow down the reduction of methylene blue by hypoxanthine to some extent. This inhibition is probably due to the same cause as the inhibition by purines observed by Dixon and Thurlow [1924, 2].

Quinoline. This was dissolved in dilute HCl, a small amount of phosphate added as a buffer, and the solution brought to neutrality with dilute NaOH. By this means a clear solution was obtained. In presence of the oxidase no trace of reduction of methylene blue could be observed.

Morphine. This was a very pure preparation of the hydrochloride. Again no action occurred.

Ricin. This was a preparation "nach M. Jakoby" obtained from the "Vereinigte Chemische Werke Aktiengesellschaft, Charlottenberg." It was dissolved in phosphate buffer. Again the results of tests with the oxidase were entirely negative.

Tryptophan. This was a pure crystalline specimen prepared in this laboratory. Not the slightest reduction was obtained.

Ketones. Abelous and Aloy do not mention the nature of the ketones they used. Acetone certainly is not oxidised by the enzyme.

Benzylamine. A specimen of the hydrochloride obtained from British Drug Houses was used. Here again there was no action whatever.

Peptone. The case of peptone is especially important in view of certain statements in the literature. Abelous and Aloy, as stated above, include peptones in the list of substances capable of replacing hypoxanthine in the xanthine oxidase system. Aloy and Valdiguie [1921], however, state that, although commercial peptones reduce methylene blue in presence of the enzyme, the reducing substance can be extracted from the peptone by alcohol, and that after this extraction the peptone is left inactive. It is clear then that the effect is due to some substance present in the commercial peptone, and that the peptone itself can *not* replace hypoxanthine in the system. I have confirmed the observations of Aloy and Valdiguie, using both Savory and Moore's and Witte's peptone. I find however that, while the substance is extracted by ordinary 95 % alcohol, it is not extracted by absolute alcohol, or by ether.

Having regard to the method of preparation of commercial peptones, it seemed very probable that they would contain small amounts of hypoxanthine. To determine whether the activity of the commercial peptone was due to this the following procedure was adopted.

200 g. of Savory and Moore's peptone were extracted in a Soxhlet apparatus with 95 % alcohol for a day. The alcoholic extract was carefully evaporated to dryness on the water-bath, leaving a small quantity of light-brown residue. This was extracted with ether, and the residue was dissolved in water. A

few drops of this solution reduced methylene blue actively in presence of xanthine oxidase. To this solution was then applied the ordinary method for the preparation of pure hypoxanthine from meat extract, as follows. The solution was made alkaline with ammonia, and ammoniacal silver nitrate was added: the resulting flocculent precipitate was filtered off and washed with dilute ammonia. The filtrate, after acidification, removal of the excess silver as chloride, and neutralisation, was found to be inactive. The precipitate was suspended in nitric acid of specific gravity 1.1, and dissolved by warming. On cooling a white crystalline precipitate separated out, resembling that usually obtained with hypoxanthine. This was filtered off and recrystallised from fresh nitric acid (sp. gr. 1.1). The pure white precipitate was then filtered off, suspended in a few cc. of water, and decomposed with H_2S . The silver sulphide was filtered off, the H_2S removed by aeration and finally by thorough shaking *in vacuo*, and the solution neutralised with dilute soda. A very small quantity of this solution was sufficient to reduce methylene blue in presence of xanthine oxidase, while control experiments without the oxidase showed that this reduction was not due to traces of H_2S remaining in the solution.

I think these facts may be said to show that the reducing properties of commercial peptones in presence of the xanthine oxidase are due to the traces of hypoxanthine they contain.

Aloy and Valdiguie give the following experiment as additional evidence that protein hydrolysis products can be oxidised by the milk oxidase. Fresh and boiled milk were digested separately with pancreatin at 37° for 12 hours. Both were then boiled and filtered, and portions of both filtrates were added to fresh milk and methylene blue. The methylene blue was reduced in the case of the milk which had been boiled before digestion, but no reduction occurred in the case where the milk was not boiled before digestion. They explain this result as follows. During digestion, oxidisable hydrolysis products are produced from the milk proteins. In the case of the fresh milk these are oxidised as soon as formed by the oxidase present in the milk and atmospheric oxygen. In the other case the initial boiling has destroyed the oxidase, and therefore the hydrolysis products do not become oxidised, and remain in solution. Now on adding fresh milk oxidase and methylene blue to both solutions one would naturally obtain a reduction in the latter case only.

An alternative explanation however is that the pancreatin itself contains hypoxanthine (as is extremely probable, considering its nature). When this is added to fresh milk the hypoxanthine will be rapidly oxidised by the oxidase, and hence there will be no reduction when the solution is ultimately tested with fresh milk and methylene blue. In the other case no xanthine oxidase is present, and thus the hypoxanthine will remain, and will account for the reduction finally observed. The digestion probably has nothing to do with the effects observed. I have tested two different samples of pancreatin with xanthine oxidase and methylene blue and found them both to reduce rapidly, although no reduction occurred in the absence of the xanthine oxidase. This

makes it very probable that the effects are in reality due to hypoxanthine and not to protein hydrolysis products.

The observations of Bach [1914] are probably to be explained in a similar manner. Bach found that "erepton," a preparation of very completely digested protein, was able to replace aldehyde in the milk oxidase system; and he explained this by supposing that the reduction was due to the actual formation of aldehydes from the amino-acids by means of the Strecker reaction. He was in fact able to obtain aldehydic substances by the distillation of erepton solutions. He admits, however, that the erepton solutions themselves contained no aldehydes, and that these were only formed during the process of distillation, so that his explanation appears to be unsatisfactory. It seems much more likely that the reduction in presence of milk was due, not to aldehydes, but to small amounts of hypoxanthine in the erepton, perhaps derived from the proteolytic enzyme preparations used in its production.

Sbarsky and Michlin [1925] state: "Als Sauerstoffakzeptoren für die Wirkung des isolierten Ferments können Aldehyde, Eiweissabbauprodukte, Xanthin und Hypoxanthin fungieren." The only evidence however adduced to show that protein breakdown products can act in this way is the fact that Liebig's extract of meat will reduce methylene blue in the presence of the xanthine oxidase. But this is to be expected, since Liebig's extract is so rich in hypoxanthine that it is the source from which this substance is usually obtained in the laboratory. (A careful reading of Sbarsky and Michlin's paper suggests, however, that it is just possible that by the term protein breakdown products they may mean hypoxanthine and xanthine, although the statement quoted does not give this impression.)

Summarising the facts, then, we may say that there appears to be no evidence that peptones, etc., can act as substrates for the xanthine oxidase; and that in cases where they have been obtained free from hypoxanthine they do not do so.

We now turn to the consideration of a number of substances which one might think would be more likely to be oxidised than most of those dealt with above, substances which are known to be oxidised in tissues by similar systems. Thunberg [1920] and others have demonstrated the oxidation of a number of substances by methylene blue in the presence of muscle and other tissues. These oxidations are brought about by enzymes of the same type as the milk oxidase. Experiments were therefore carried out to see whether the xanthine oxidase itself might not oxidise these substances to some extent.

Formate, acetate, lactate, succinate, citrate, glutamate. These were the more important substances found by Thunberg to be oxidised by muscle, in presence of which they reduce methylene blue with great rapidity. In presence of the xanthine oxidase, however, they all gave absolutely negative results, and not the slightest action could be observed. The resistance to oxidation of these substances by the xanthine oxidase is in marked contrast to the ease with which they are oxidised by the muscle enzymes. On the other hand, it is interesting to note that hypoxanthine and xanthine are not oxidised by

muscle, which contains only insignificant traces of the xanthine oxidase. (These traces are so small that they can only be detected by making a very concentrated extract of washed muscle in alkaline phosphate.) Thus it appears that none of the muscle enzymes can oxidise hypoxanthine, while the xanthine oxidase can oxidise none of the muscle donors tried.

A number of other substances have previously been shown by other workers not to be oxidised by the xanthine oxidase.

Caffeine, theobromine, uracil, thymine, cytosine, histidine, uric acid. Morgan, Stewart and Hopkins found that none of these substances was able to bring about any reduction of methylene blue in presence of fresh milk, and I have confirmed these results, using preparations of the xanthine oxidase.

Amino-acids. Bach [1913] has shown that none of the following substances is oxidised by milk in presence of either methylene blue or nitrate as a hydrogen acceptor: glycine, tyrosine, alanine, serine, leucine, aspartic acid. He also obtained negative results with pyruvic and oxalacetic acids.

Summarising the results of this section we may then say that of about 35 substances of biological interest which have been examined only three, namely, xanthine, hypoxanthine and aldehydes (possibly also adenine to a slight extent), are oxidised by the milk oxidase.

THE HYDROGEN ACCEPTOR.

Here we find a very different state of affairs. So far from the enzyme exhibiting any specificity towards the hydrogen acceptor, it appears that the chemical nature of the acceptor is quite immaterial. Almost any reducible substance (oxidising agent) seems to be able to oxidise hypoxanthine in presence of the xanthine oxidase. In this section will be described the results of experiments with various substances as hydrogen acceptors, using hypoxanthine throughout as the hydrogen donor. Hypoxanthine was chosen because, of the three substrates of the oxidase, it is the one which gives the most rapid reduction. In all cases proper control experiments were carried out to make sure that neither the enzyme solution alone nor the hypoxanthine alone reduced the hydrogen acceptor, at any rate with a velocity sufficient to invalidate the experiment. It was found in certain cases that only a small concentration of the oxidising agent could be used, as higher concentrations destroyed the enzyme. It has already been shown by Dixon and Thurlow [1924, 1] that the xanthine oxidase is somewhat easily destroyed by oxidation.

Methylene blue. This has already been fully dealt with.

Thionine. The behaviour of thionine, which is methylene blue minus the four methyl groups, is precisely similar.

Guaiacum blue. It was frequently noticed, when testing the oxidase system for the presence of peroxide [Thurlow, 1925] with guaiacum tincture and peroxidase, that if the test-tube were allowed to stand for a short time the deep blue colour at first produced was decolorised, except for a layer at the surface of the liquid where it was exposed to the air. This was found to be

due to the reduction of the guaiacum blue by the xanthine oxidase hypoxanthine system. It is of interest in this connection to note that Mrs Callow [1926] found when testing bacteria for peroxidase that the blue colour produced in the test was similarly discharged, and she showed that this decoloration was due to those thermolabile agencies in the bacteria which reduce methylene blue.

Oxygen is of course the normal hydrogen acceptor when the reaction takes place *in vivo*. Morgan, Stewart and Hopkins [1922] studied the oxidation of both hypoxanthine and xanthine by oxygen and Wieland [1914] found that aldehyde was oxidised aerobically in presence of milk. Thurlow [1925] showed that in these reactions the oxygen becomes reduced to hydrogen peroxide, and Dixon and Thurlow [1925] showed that activation of the oxygen by iron, as assumed by Warburg [1923], is not necessary in this system.

Hydrogen peroxide. It was found by Thurlow [1925] that the hydrogen peroxide produced during the aerobic oxidation ultimately disappeared again as the reaction proceeded. This observation, taken in conjunction with the observation of Morgan, Stewart and Hopkins [see also Harrison and Thurlow, 1926] that the amount of oxygen used up was exactly equal to the amount theoretically necessary for the oxidation of the hypoxanthine to uric acid, must mean that the hydrogen peroxide is used up in the oxidation of hypoxanthine, becoming itself reduced to water. This is in harmony with the observation of Wieland [1921] that hydrogen peroxide can act as a hydrogen acceptor in catalytic dehydrogenation systems. It should be mentioned that hydrogen peroxide is unable to oxidise hypoxanthine in the absence of the xanthine oxidase as separate control experiments show. Moreover, hydrogen peroxide, except in very small concentrations, destroys the oxidase [Dixon, 1925], and under certain conditions one may get complete destruction of the oxidase while the solution still contains hypoxanthine and peroxide. In this case the peroxide does not disappear from the solution; even after many hours the strength of the peroxide test remains exactly the same. On the addition of more oxidase however the peroxide disappears.

It is interesting to note that in this reaction we have an instance of the mechanism which Wieland [1921] assumes to account for peroxidase action. This system is however certainly not a peroxidase, with which class of enzymes it has nothing in common. Peroxidases appear to act by quite a different type of mechanism.

Nitrate. It was shown by Dixon and Thurlow [1924, 3] that nitrate could oxidise hypoxanthine and xanthine in presence of the xanthine oxidase, becoming itself reduced to nitrite. It was shown that the nitrate behaved exactly like methylene blue in this system. Bach [1911] showed that aldehydes reduce nitrates in presence of milk.

Dinitrobenzene. Lipschitz [1921] found that tissues were capable of reducing aromatic nitro-compounds, such as *m*-dinitrobenzene, which he found to be reduced to *m*-nitrophenylhydroxylamine. He repeated Thunberg's [1920]

work on the enzymes of muscle, using *m*-dinitrobenzene instead of methylene blue as the hydrogen acceptor, and obtained exactly the same results as Thunberg. It appears therefore that the tissue enzymes are able to work with dinitrobenzene as well as with methylene blue. Lipschitz used two routine tests for the reduction of the dinitrobenzene; first, the development of the yellow colour of the reduction product, which could be used for the colorimetric estimation of the reduction, since the dinitrobenzene itself is colourless; and secondly, the production of a strong red colour on the addition of alkali. On testing the xanthine oxidase I obtained similar results. To 5 cc. of oxidase solution containing 0.5 mg. of hypoxanthine was added about 0.1 g. of powdered *m*-dinitrobenzene, and the evacuated tube shaken at intervals. A yellow colour was quickly produced, which steadily deepened in intensity. On the addition of NaOH the solution turned deep red, but with a distinct purple tinge which may have been due to some other reduction product.

Picric acid. Abelous and Aloy [1903] have found that picric acid is reduced to picramic acid by certain reducing systems in tissue extracts. Tests were therefore carried out to see whether the xanthine oxidase system would behave similarly.

A few drops of dilute picric acid solution were added to 5 cc. oxidase solution containing 0.5 mg. of hypoxanthine and the solution was allowed to stand anaerobically. The deep orange-brown colour characteristic of picramic acid was rapidly developed, while in a similar control experiment without hypoxanthine there was no change whatever in the colour of the solution, which remained bright yellow.

Iodine. This also was found to act as a hydrogen acceptor in the oxidase system. One drop of approximately decinormal iodine solution was added to 5 cc. of enzyme solution with 0.5 mg. of hypoxanthine and a drop of starch solution. Side by side with this a control experiment was carried out containing the iodine, starch and oxidase, but with no hypoxanthine. Almost immediately the colour in the tube with hypoxanthine began to fade, and in a few minutes the solution was decolorised. At this time the control tube was apparently coloured as deeply blue as at first, and the contrast was very striking, but after a time the control tube also became decolorised, owing to the iodine attacking the protein of the enzyme preparation. The time taken for this decoloration, however, was about five times as long as that taken in presence of hypoxanthine, so that the results were quite convincing. A control experiment with iodine and hypoxanthine alone in the absence of the enzyme gave no decoloration of the iodine. Experiments made with bromine instead of iodine were inconclusive, as the bromine was too rapidly decolorised by the proteins present.

Permanganate. The results here were also quite definite. The enzyme was found to be easily destroyed by potassium permanganate, but by using only enough of the latter to give a quite definite pink colour to the solution this difficulty could be overcome. The experiments were carried out in the same

way as those with iodine. After a few minutes the pink colour in the tube with enzyme and hypoxanthine had completely disappeared, and a brownish cloud, presumably of manganese dioxide, had appeared, while the control tube containing enzyme only was still quite pink. The same changes were also observed in the control tube, but only after a period about three times as long as the decoloration-time with hypoxanthine. But by the time the control tube had become decolorised further changes had taken place in the tube containing hypoxanthine. The brownish cloud of manganese dioxide had apparently become further reduced by the system, and had redissolved, leaving the solution quite clear and colourless. A control experiment with hypoxanthine and permanganate in the absence of the oxidase showed that the permanganate was not reduced by the hypoxanthine alone.

*Alloxan*¹. It has been shown earlier in this paper that alloxan will not act as a hydrogen donator with the xanthine oxidase. It appeared possible, however, that it might act as a hydrogen acceptor in the oxidation of hypoxanthine by the xanthine oxidase, becoming itself reduced to dialuric acid. Now Bijlmann [1923] has shown that the alloxan-dialuric acid system is one which gives a definite oxidation-reduction potential, so that the reduction of alloxan can be investigated by the electrometric method. The experiment was carried out as follows. 5 cc. of a 1 % solution of the active enzyme preparation in p_H 7.6 phosphate buffer together with 1 cc. $M/100$ alloxan solution were placed in a reaction vessel of about 25 cc. capacity, and a stream of nitrogen was bubbled through in order to keep the solution stirred and to remove oxygen. The reduction potential was determined by means of a gold electrode immersed in the solution, connection being made with a standard calomel cell by means of an agar salt-bridge. The potential readings were accurate to 1 mv. A preliminary experiment showed that the potential given by alloxan was perfectly steady and not affected by the presence of the enzyme. The potential (relative to the normal hydrogen electrode) was initially + 288 mv., and after standing for a considerable time the reading was exactly (within 1 mv.) the same. Hypoxanthine solution containing 0.4 mg. was then added from a burette, when the following readings were obtained:

Time after addition of hypoxanthine (minutes)	E_h (mv.)	Time after addition of hypoxanthine (minutes)	E_h (mv.)
0	+ 288	5	+ 160
$\frac{1}{2}$	270	6	146
1	250	8	130
2	217	10	117
3	195	15	98
4	175		

The reduction of the alloxan is shown by the rapid change in the potential reading in the direction of higher reduction potential. To judge from some not very accurate measurements on the potential of alloxantin at this p_H , the last reading given corresponds to about 60 % reduction of the alloxan. It

¹ This and the following experiment were carried out jointly with Dr Keizo Kodama.

will be seen that only enough hypoxanthine was added to reduce about three-quarters of the alloxan, so that, as the reaction was still proceeding, although rather slowly, at the end of the experiment, the results are in approximate agreement with theory.

I have repeatedly found in control experiments that the oxidase plus hypoxanthine alone gives no reduction potential. A drift of a few (10–20) millivolts in either direction may be observed on the addition of the hypoxanthine, but there is no definite potential change either with hypoxanthine or aldehyde, or with fresh milk in place of the oxidase preparation.

Quinone. The quinone-quinol system also gives a definite oxidation-reduction potential, and the reduction of quinone can therefore be followed electrometrically in a similar manner. On testing 5 cc. of the enzyme solution with 1 cc. of $M/100$ quinone a slight drift of about 10 mv. in the potential reading occurred, but after a few minutes the potential had become steady; and after waiting a further period to make sure of this, 1 mg. of hypoxanthine was added. The readings were as follows:

Time after addition (minutes)	E_h (mv.)	Time after addition (minutes)	E_h (mv.)
0	+312	28	+87
4	286	29	65
8	278	30	50
12	272	35	32
20	255	60	32
26	224		

The reduction of the quinone is shown by the change in potential as before. The peculiar form of the reaction curve however, which has been observed in every experiment, requires further investigation.

Clark's "Reduction-potential Indicators." W. Mansfield Clark [1923–25] has recently introduced a series of reducible dyestuffs, belonging to the indophenol and indigo groups, whose oxidation-reduction potentials have been accurately measured; and he suggests their use as indicators for the determination of r_H^1 , just as the more familiar indicators are used to determine p_H . It was thought that by using these dyes as hydrogen acceptors in the xanthine oxidase system it might be possible to estimate the reducing power of this system. So far as I am aware this method has not been previously applied to any oxidation-reduction enzyme system.

The technique used was exactly the same as with methylene blue. The dyes² were made up in aqueous solutions equivalent in strength to the 1/5000

¹ The r_H scale introduced by Clark is a method of expressing the reducing power of a solution, strictly analogous to the p_H scale of acidity. For an explanation of the r_H notation reference must be made to Clark's papers, but the following brief remarks will not be out of place. By definition $r_H = -\log P$, where P is the pressure of hydrogen gas in a platinum surface in equilibrium with the solution. Thus a solution with a high reducing power has a small r_H value, and *vice versa*. In other words a substance with a low r_H is itself more difficult to reduce than one with a high r_H . At $r_H = 0$ the solution will evolve hydrogen gas spontaneously at the platinum surface; at $r_H = 41$ oxygen gas will be given off. The r_H is calculated from potential measurements.

² For these dyes I am much indebted to Dr and Mrs Needham. I understand that they were sent to Dr Needham by Professor Mansfield Clark.

methylene blue used previously, and the experiments were all carried out anaerobically at 14°. A 2 % solution of xanthine oxidase preparation in phosphate buffer solution was used, and the quantities were as follows: 1 cc. oxidase solution + 1 cc. p_H 7.6 phosphate buffer + 0.5 cc. of the dyestuff solution + 0.2 mg. hypoxanthine. The results of the experiments are given in the following table:

Indicator no.	Time for complete reduction	Approximate r_H range	No. of sulphonate groups
1	13 m.	7.2-11.2	2
2	50 m.	8.3-12.3	3
3	8 hrs.	10 -14	4
3a	{ 1 m. 35 s.	13 -17	0
4	{ 1 " 30 "	15.2-19.2	1
5	{ 2 " 20 "	15.4-19.4	1
6	{ 1 " 30 "	17.3-21.3	0
7	{ 1 " 10 "	17.7-21.7	0
8	{ 1 " 15 "	18.3-22.3	0
9	{ 55 "	19 -23	0

For convenience the indicators are referred to by numbers. Their names are as follows:

No.

1. Indigotine disulphonate.
2. Indigotine trisulphonate.
3. Indigotine tetrasulphonate.
- 3a. Methylene blue.
4. 1-Naphthol-2-sulphonate-2: 6-dichloro-indophenol.
5. 1-Naphthol-2-sulphonate-indophenol.
6. *o*-Cresol-2: 6-dichloroindophenol.
7. *o*-Cresol-indophenol.
8. 2: 6-Dibromophenolindophenol.
9. *o*-Chlorophenolindophenol.

The dyes are given in order of their reduction-potentials, no. 1 requiring the greatest energy to reduce it, and no. 9 being the easiest to reduce. The r_H range is given from 1 % to 99 % reduction, but of course the *useful* range is more limited.

The first important point which appears from these results is that the xanthine oxidase system completely reduces all the indicators; that is to say, its reducing power is so great that it lies beyond the region at present covered by indicators. Its r_H must be below 7.

The second point is the effect of sulphonic groups on the velocity of reduction of the dyes. It will be seen from the table that indicators 3a, 6, 7, 8 and 9, which are not sulphonated, are all reduced rapidly at about the same rate. On the other hand, 4 and 5, which contain one sulphonic group, are definitely slower; while 1, 2 and 3, which contain 2, 3 and 4 sulphonic groups respectively, show the very large effect on the reduction rate of more than one such group. It is to be noted that among the last three cases mentioned, no. 3, the one which, from reduction potential data, we know is the easiest of the three to reduce, has the slowest reduction velocity; while no. 1, the most difficult to reduce, is reduced quickest. This clearly shows that the effect is simply due to the sulphonic group. Since this group is strongly dissociated, each group will give one negative charge to the molecule, so that the effect may be one of charge merely.

The point is of importance in connection with attempts to determine the r_H of biological systems which are not in equilibrium by means of these indicators. For instance, J. and D. M. Needham [1925, 1926, 1, 2] have made micro-injections of these dyes into a large number of different cells in an investigation of the internal r_H of the living cell. They find that in the majority of the cells studied, 6, 7, 8 and 9 were reduced, while 1, 2, 3, 4 and 5 remained in the oxidised form. (Methylene blue was not used in most cases.) They point out that the change from reduced to oxidised does not correspond with the change from the indophenol to the indigo series, and they conclude that the change is not due to a change in the chemical structure of the dye. An examination of the list of dyes shows however that dyes 1-5 are all sulphonated, while 6-9 contain no sulphonic groups; so that it may be said that sulphonated dyes exist in these cells in the oxidised form, and non-sulphonated dyes in the reduced form. Now a dye which is only slowly reduced in a cell will be re-oxidised, as fast as it is reduced, by oxygen if this is present, or possibly by other hydrogen acceptors in the cell. In this case the dye will be present mainly in the oxidised form. On the other hand, if the dye is reduced more rapidly than it can be oxidised, it will be mainly in the reduced form. This would be equally true no matter whether the reduced or oxidised form of the dye were used initially.

The reducing power of the living cell is due, in large measure at any rate, to systems of the same type as the xanthine oxidase. A consideration of these facts then suggests that the apparent results obtained may depend not so much upon the r_H of the cell as upon whether the indicator used is sulphonated or non-sulphonated.

In this connection it is significant that the Needhams [1926, 2] found one cell, *Nyctotherus cordiformis*, which when deprived of oxygen reduced nos. 3 and upwards. Now in presence of oxygen this cell reduced only no. 6, and was unable to reduce 4 and 5. In other words, the apparent r_H of the cell rose just to the junction of the sulphonated with the non-sulphonated series. This result would seem to be due to an effect of the kind just pictured.

A further, somewhat different, criticism applies to the case where the oxidised form only is added initially. Unless the time of observation is very long the more slowly reacting indicators may not have reached their final stage by the end of the observation. For instance, if we were determining the r_H of the xanthine oxidase system with indicators 3 and 3a, and the observation only lasted a few minutes, we should say that as 3a was completely reduced the r_H must be about 13 or less, while as 3 remained completely oxidised the r_H must be above 14. Needham's observations generally lasted only a few minutes.

Methylene blue forms a very interesting case. As will be seen from the table, it is a non-sulphonated dye falling within the r_H range of the sulphonated dyes. If what has just been said is correct, we might possibly expect, in a biological system with a small r_H value, to find methylene blue (3a) reduced,

while 4 and 5 are oxidised, 6 again being reduced. It is a significant fact that J. and D. M. Needham [1925, p. 276] found that methylene blue was rapidly reduced¹ by the amoeba, while 4 was oxidised, 5 was partially reduced, and 6-9 completely reduced. 1-3 were not reduced [1926, 2]. Needham [1926, 1] also found that only the non-sulphonated dyes were capable of staining *Paracentrotus* egg cells.

Glutathione. The oxidised or disulphide form of glutathione was shown by Hopkins [1921] to act as a hydrogen acceptor for certain tissue systems, whose nature has up to the present not been determined. Muscle tissue, for instance, rapidly reduces the disulphide to the sulphydryl form, but it was found by Hopkins and Dixon [1922] that none of the better-known oxidation-reduction enzymes of muscle was able to effect this reduction. On testing the xanthine oxidase system it was found that this also was unable to reduce glutathione. Xanthine oxidase, hypoxanthine and oxidised glutathione solutions were placed in a vacuum tube and the mixture allowed to stand anaerobically for many hours, after which the nitroprusside test was applied to the solution. The test was absolutely negative. It appears from other considerations that the disulphide compound can only be reduced by systems having a very high reducing power, and it seems that the xanthine oxidase system has not a sufficiently low r_H to effect this reduction.

DISCUSSION.

The results given in the preceding sections bring out clearly the main point of this work, namely, that the xanthine oxidase exhibits a remarkable specificity as regards the hydrogen donator (the substance oxidised), but none at all as regards the hydrogen acceptor (oxidising agent). The system would be a perfect example of clean-cut specificity were it not for the fact that aldehyde can act as the hydrogen donator in addition to the purine bases. Neglecting aldehyde for the moment, however, we can say that the enzyme is in the highest degree specific towards the hydrogen donator. In the purine series itself this is very marked: hypoxanthine and xanthine appear to be the only substances oxidised, and the introduction of amino or methyl groups into the molecule is sufficient entirely to prevent the enzyme acting on it. It is hoped to carry out a further and more detailed investigation of the purine substances from this point of view. Again, outside the purine series, the oxidase was found to be without action on any of the considerable number of substances tested, although many of these are known to be readily oxidised by other enzymes of a similar type to the xanthine oxidase.

It should be pointed out here, in connection with these negative experiments, that the technique used was capable of detecting extremely slow reactions. With the solutions used, the time for complete reduction of the methylene blue by hypoxanthine was about two minutes. As the tubes were

¹ Too much stress must not be laid on this, as they were later unable to repeat this result [1926, 2], but it appears that Clark has also observed this anomaly with methylene blue [Needham, 1926, 2, p. 389].

allowed to stand for 6–8 hours in most cases, and in some cases for much longer than this, and as a reduction of one-fifth of the dye is observed with ease, it follows that if these substances are oxidised at all they are oxidised at a velocity less than one two-thousandth of that of hypoxanthine.

With regard to the case of aldehyde, it cannot be maintained that it is definitely established that the oxidations of aldehyde and purines are due to the same enzyme. If they are not, then *both* enzymes must be remarkably specific. On the other hand, there appears to be a fairly strong balance of evidence in favour of identity, although a certain part of this can be explained away by supposing that we have two different enzymes adsorbed on the same colloid.

There appears, however, to be no need to suppose that a specific catalyst is necessary for the activation of aldehyde. Bredig and Sommer [1909] have shown that aldehydes are activated even by a non-specific catalyst like colloidal platinum, a catalyst which I have found to be quite unable to activate hypoxanthine. I found a platinum sol which rapidly reduced methylene blue in presence of aldehyde to show no reduction whatever when hypoxanthine was substituted for aldehyde. Thus it appears that hypoxanthine requires a specialised catalyst for its activation, while aldehydes are easily activated and do not necessarily require a special catalyst. It is quite certain that there are other enzymes which will oxidise aldehydes, but which are altogether without action on hypoxanthine. Aldehydes are also activated by amino-acids, as well as by a number of inorganic catalysts. It must be remembered that aldehydes are very easily oxidisable substances, even in the absence of catalysts, and in this respect they are quite unlike substances such as hypoxanthine and succinic acid which are extremely difficult to oxidise in the absence of their respective enzymes.

With regard to the hydrogen acceptor, it is quite clear that no specificity at all can be observed. It is only necessary to mention a few examples, *e.g.* methylene blue, oxygen, nitrates, permanganate, iodine and alloxan, to show that the exact chemical nature of the substance seems to be immaterial. One might almost say that any oxidising agent, which does not destroy the enzyme, will bring about the oxidation of hypoxanthine in presence of the xanthine oxidase.

The facts brought out in this work are in harmony with, and give further support to, the view that the oxidase activates the hydrogen donator and not the acceptor.

It is interesting to compare this state of affairs with the case of the peroxidase system, where it is the hydrogen acceptor (the peroxide) which is activated. Here we find the opposite result, and there appears to be little or no specificity towards the hydrogen donator. Guaiacum, benzidine, quinol, *p*-phenylenediamine, naphthol, nitrites, leucomalachite green, formates and many other substances are oxidised; in fact what applies to the hydrogen acceptor in the case of the xanthine oxidase applies here to the hydrogen donator.

How far the results obtained with the xanthine oxidase apply also to the other enzymes of the same type is a question about which little can be said at present. The observation of Lipschitz that dinitrobenzene gave throughout the same results as methylene blue with muscle tissue is good evidence that the hydrogen acceptor is in general unspecific. There is good evidence also that the hydrogen donator is specific, although the degree of specificity is as yet undetermined.

SUMMARY.

1. Using the methylene blue technique, out of 35 substances which have been tested, the only ones which have been found to be oxidised by the xanthine oxidase of milk are: hypoxanthine, xanthine, aldehydes, and possibly adenine to a slight extent.

2. The following can act as hydrogen acceptors in oxidising hypoxanthine in presence of the xanthine oxidase: methylene blue, thionine, guaiacum blue, all Clark's "reduction-potential indicators," oxygen, hydrogen peroxide, nitrates, dinitrobenzene, picric acid, permanganate, iodine, alloxan, quinone.

3. The oxidase shows a high degree of specificity towards the hydrogen donator, but none at all towards the hydrogen acceptor.

4. Certain facts are described which show that caution must be exercised in the interpretation of results obtained by the application of Clark's oxidation-reduction indicators to biological systems.

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