XXXII. STUDIES ON XANTHINE OXIDASE.
XI. XANTHINE OXIDASE AND LACTOFLAVINE.

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UNTIL recently the view was held by many that molecular oxygen cannot react directly with the reducing systems of the cell but requires the intermediation of catalytic iron. Experiments on oxidase systems extracted from cells seemed to support this view. The succinic oxidase of muscle, for instance, as shown by Thunberg [1917] and others, will reduce methylene blue or oxygen in presence of succinate, but if a small amount of HCN is added all power of reducing oxygen is lost, while methylene blue is still reduced with the same velocity as before. This was interpreted as being due to the inactivation of the iron compound acting as an intermediary between the activated substrate and molecular oxygen.

The xanthine oxidase of milk and liver was however shown by Dixon and Thurlow [1925] to be unaffected by HCN, both with respect to methylene blue reduction and oxygen uptake. This system was therefore assumed to act in accordance with Wieland's theory of oxidation, in which molecular oxygen acts as a direct acceptor of the hydrogen of the substrate molecule.

The work of Warburg and Christian [1932] has shown the existence of another type of compound in cells which functions as an intermediary between the reducing systems and oxygen. This is a yellow pigment which does not contain iron or any other metal. It is rapidly reducible by the hexosemonophosphate dehydrogenase system isolated from several kinds of cells and is then reoxidised by molecular oxygen with the formation of H_2O_2; it can function catalytically even in the presence of HCN.

The recent researches of Kuhn *et al.* [1933] and Stern [1933] have shown that yellow pigments of this type, now known as flavines, occur in both milk and liver, and the possibility therefore arose that in the xanthine oxidase system prepared from these sources the oxygen reacted directly with the leuco-form of the yellow pigment and not directly with the substrate. The formation of H_2O_2 observed by Thurlow [1925] in this system might be accounted for by this reaction. If such were the case the xanthine oxidase could no longer be regarded as supporting the Wieland view.

It therefore became important to ascertain whether the uptake of oxygen by the xanthine oxidase system was dependent upon the presence of such yellow pigments.

This question could be settled experimentally in the following ways. (1) By complete removal of yellow pigment from xanthine oxidase preparations. This should abolish the power of taking up oxygen. (2) By addition of yellow pigment to pigment-free preparations, which should increase the rate of oxygen uptake. (3) By determining the ratio, rate of methylene blue reduction/rate of oxygen uptake, in a number of xanthine oxidase preparations prepared in different ways,

and comparing its value with the pigment content of the preparations. The smaller the pigment content, the larger should be the ratio. (4) By determining the power of the oxidase to reduce the yellow pigment.

Experimental methods.

Preparations of xanthine oxidase.

A. *Caseinogen preparation* [Dixon and Thurlow, 1924, 1].

The enzyme is precipitated together with the caseinogen and fat by half-saturation of the milk with ammonium sulphate. The fat is removed from the dried precipitate by thorough extraction with ether.

B. *Cream preparation* [Toyama, 1933].

Cream is separated from milk. The fat is dissolved away with ether, and the protein residue is dried in the desiccator.

C 1. *Whey preparation* [Dixon and Kodama, 1926].

Fresh milk is clotted by rennin, the enzyme precipitated from the whey by half-saturation with ammonium sulphate and the precipitate extracted with ether and dried.

C 2. *Charcoal-treated whey preparation* [Dixon and Kodama, 1926].

A solution of the C 1 preparation is treated twice with Merck's medicinal charcoal, and the enzyme is precipitated from the clear filtrate with ammonium sulphate. Most of the protein and all the yellow pigment are removed by this procedure.

C 3. *Kaolin-treated whey preparation* [Dixon and Kodama, 1926].

The enzyme is adsorbed from a solution of the C 1 preparation at p_H 5 with kaolin and subsequently eluted from the kaolin with 1 % Na_2CO_3. It is then precipitated as before with ammonium sulphate. This procedure also removes most of the protein and all the yellow pigment.

C 4. *Fuller's earth-treated whey preparation.*

Fuller's earth is known to adsorb the yellow pigment strongly and is used for its concentration from milk. A solution of the C 1 preparation is treated twice with fuller's earth (Boots). The filtrate contains practically all the enzyme, no yellow pigment and about half the original protein. The enzyme is precipitated as before with ammonium sulphate.

D. *Sodium chloride preparation* (Dixon and Lemberg, unpublished).

The caseinogen and fat are precipitated from milk with sodium chloride, leaving the enzyme in solution, from which it is precipitated with ammonium sulphate. This gives a preparation of high activity containing a small amount of the yellow pigment. The complete details of the method will be published shortly.

Preparation of lactoflavine.

Ten litres of milk were acidified to p_H 4·6 with glacial acetic acid, and the caseinogen was filtered off. The filtrate was mixed with 500 g. of fuller's earth, which was filtered off after 1 hour and washed three times with 1 litre of distilled water and twice with 1 litre of ethyl alcohol. The lactoflavine was then eluted, following the procedure of Kuhn *et al.*, with a mixture of pyridine, acetic acid

and methyl alcohol. After centrifuging off the fuller's earth, the yellow solution was concentrated *in vacuo* to about 25 ml. and mixed with an equal volume of distilled water. The solution was then thoroughly extracted with ether, the yellow pigment remaining in the aqueous solution. The small precipitate which formed was filtered off. An equal volume of methyl alcohol was then added to the opalescent solution, causing the precipitation of a small amount of colloidal fuller's earth, which was filtered off. The yellow solution at this stage was perfectly clear and fluoresced strongly. It was then concentrated *in vacuo* to about 15 ml. and mixed with 10 volumes of acetone. The whole solution set to a gel, which could be easily broken up and filtered. The clear filtrate was again concentrated *in vacuo* to 15 ml., and phosphate solution (p_H about 7·4) was added until no more precipitate was formed. The precipitate was filtered off and the filtrate used in subsequent experiments. By colorimetric comparison with a lactoflavine solution of known concentration kindly given to us by Dr György it was found that the solution contained 50γ of lactoflavine per ml. The yield from 10 litres of milk was therefore 1 mg. of lactoflavine.

Detection of lactoflavine in xanthine oxidase preparations.

The dried enzyme preparations were extracted with small volumes of either methyl alcohol or pyridine, and the extracts were examined for greenish fluorescence in a strong beam of ultra-violet radiation, obtained from a mercury arc lamp with a nickel oxide filter. This test is exceedingly delicate, particularly when methyl alcohol is used, and 0·05γ of lactoflavine per g. of enzyme preparation can be detected with ease. In some cases aqueous solutions of the preparations were also tested for fluorescence.

Measurement of rate of reduction of methylene blue.

For the Thunberg technique to serve as a quantitative measure of the rate of reduction of methylene blue, the following conditions must be fulfilled. (1) The solutions must be completely freed from all traces of dissolved oxygen. (2) The substrate must not be mixed with the enzyme solution prior to evacuation of the tubes. (3) Temperature equilibrium must be attained by the tubes before the experiment begins.

The procedure adopted was as follows. Thunberg tubes of the Keilin type were used, and the hypoxanthine was placed in the hollow stopper. Four tubes were used for each determination. These were evacuated simultaneously with a water-pump, filled with oxygen-free nitrogen from a reservoir and allowed to come into equilibrium with the gas before being again exhausted. This process was twice repeated. The tubes were placed in a thermostat at 37°. The stoppers were worked in and the side-tubes filled with water in order to prevent air leakage. After three minutes the tubes were tilted simultaneously so as to mix the enzyme and substrate. By this procedure duplicates were found to agree to within 5 % in all cases.

The xanthine oxidase presents special difficulties in the determination of the rate of reduction of methylene blue, owing to the peculiar effects which are produced by changes of substrate concentration. Dixon and Thurlow [1924, 2] showed that over a certain range of hypoxanthine concentration the rate is maximum and independent of the concentration, but that with higher concentrations of substrate an inhibition ensues. A single determination, therefore, is insufficient to determine the true activity of the enzyme, and it is necessary in every case to work with two different concentrations of hypoxanthine, in order to ensure that the maximum rate is being measured.

The strength of the methylene blue solution used was determined by colorimetric comparison with a standard solution of a twice recrystallised specimen of pure medicinal methylene blue of known nitrogen and moisture content.

Measurement of rate of uptake of oxygen.

The measurements were carried out in the Barcroft apparatus at 37° by the usual technique [Dixon, 1934]. The hypoxanthine was placed in "Keilin tubes" and mixed with the enzyme only after temperature equilibrium was attained. Air, and not oxygen, was used in the flasks, as control experiments showed that the rate was independent of the partial pressure of oxygen within wide limits. Any traces of CO_2 were absorbed by KOH-papers in the usual way. Here again experiments with different hypoxanthine concentrations must be carried out, as the effects of substrate concentration are similar to those observed with methylene blue.

Procedure.

Suitable amounts of dried enzyme powder were dissolved in $M/5$ phosphate buffer (p_H 7·77) and diluted so as to give convenient rates of reduction. Barcroft and Thunberg experiments were carried out simultaneously, and each experiment was always done in duplicate.

Experimental results.

The yellow pigment can be removed from the oxidase preparations by adsorption with charcoal, kaolin or fuller's earth, and also by dialysis. For instance, one adsorption by any of these adsorbents is sufficient to remove the pigment from the C 1 preparation so completely that no trace can be detected by the fluorescence test when the oxidase is subsequently precipitated and 5 g. of the dried precipitate extracted with methyl alcohol. Nevertheless, after three successive treatments with charcoal the enzyme is found to have lost none of its power of taking up oxygen, and treatment with kaolin or fuller's earth likewise leaves the rate of oxygen uptake unaffected. It is therefore clear that the oxygen uptake does not depend upon the presence of lactoflavine.

In order to determine whether the pigment influences the rate of oxygen uptake in any way, the ratio, rate of methylene blue reduction/rate of oxygen uptake, was determined for the various preparations of the oxidase and compared with their content of yellow pigment. The results are summarised in Table I.

Table I.

Preparation	Degree of concentration of oxidase	Presence of lactoflavine	M.B. reduced in 20 mins. (mm.^{3} O_2)	O_2 absorbed in 20 mins. (mm.^{3})	Ratio
Milk	1	+ + + +	76	125	0·61
A	30	+ + +	19	34	0·56
B	400	−	36	63	0·57
C 1	500	+ ?	230	328	0·70
C 2	2000	−	98	174	0·56
C 3	2000	−	111	181	0·61
C 4	1000	−	87	119	0·73
D	2000	+	195	322	0·61

The figures given in the second column of the table represent very approximately the degree of concentration given by the different methods of preparation, *i.e.* they give the number of units of enzyme in 1 g. of the solid compared with 1 g. of the original milk. A *minus* sign in the third column means that no trace

of lactoflavine could be detected in 5 g. of the solid by the procedure given above. The amounts of methylene blue reduced have been expressed in mm.^{3} of oxygen, on the assumption that one molecule of methylene blue is equivalent to one atom of oxygen, in order to be able to compare the two rates. The figures given in the fourth column represent the amounts which 2·5 ml. of enzyme solution would reduce in 20 minutes, calculated from the observed rate of reduction of 1 ml. of methylene blue (M/1500). The amounts of oxygen given in the fifth column are obtained from the initial linear portions of the oxygen uptake curves and represent the amounts absorbed by 2·5 ml. of the enzyme solutions in 20 minutes at the same temperature. Since the solutions of the various preparations of enzyme were arbitrarily diluted to different extents, so as to give convenient rates, the figures given in columns 4 and 5 give no index of the relative activities of the different preparations in the solid state and cannot be compared with one another.

It will be seen that the ratio of the two velocities remains remarkably constant, in spite of wide variations in the content of lactoflavine.

The addition of concentrated lactoflavine solution to preparations which had been freed from yellow pigment by dialysis or adsorption produced no increase in the rate of oxygen uptake, as shown in Tables II and III.

Table II.

Barcroft manometer	1	2	3	4
Dialysed oxidase (preparation A) (ml.)	2·5	2·5	2·5	2·5
Hypoxanthine (5 mg./ml.) (ml.)	0·2	0·2	0·2	0·2
Lactoflavine (0·00016 M) (ml.)	—	—	0·2	0·2
Water (ml.)	0·3	0·3	0·1	0·1
O_2 uptake in 20 mins. (mm.^{3})	38·0	37·4	35·7	36·6

Table III.

Barcroft manometer	1	2	3	4
Oxidase (preparation C 2) (ml.)	2·5	2·5	2·5	2·5
Hypoxanthine (5 mg./ml.) (ml.)	0·2	0·2	0·2	0·2
Lactoflavine (0·00016 M) (ml.)	—	—	0·2	0·2
Water (ml.)	0·3	0·3	0·1	0·1
O_2 uptake in 10 mins. (mm.^{3})	81·7	74·5	79·6	79·8

Table IV.

Thunberg tube	1	2	3	4
Oxidase (preparation A) (ml.)	1·0	1·0	1·0	1·0
Hypoxanthine (5 mg./ml.) (ml.)	0·2	0·2	0·2	0·2
Lactoflavine (0·00016 M) (ml.)	1·0	1·0	—	—
Methylene blue (0·00034 M) (ml.)	—	—	1·0	1·0
Reduction time	35 mins.	36 mins.	70 secs.	70 secs.

The power of the xanthine oxidase to reduce lactoflavine anaerobically was then tested by comparing the times of reduction of 60γ added lactoflavine and methylene blue in Thunberg tubes. The times given in Table IV are those for complete reduction as shown by complete disappearance of colour. It can be easily calculated that lactoflavine is reduced with a velocity of only 1/64th of that of methylene blue reduction.

Discussion.

The results given above show clearly that lactoflavine plays no part in the oxygen uptake by the xanthine oxidase. The rate of uptake is unaffected alike by complete removal and by addition of the pigment, and the ratio of the reaction rates with methylene blue and oxygen does not change with the pigment content.

An incredibly high catalytic activity would have to be assumed if traces of the yellow pigment, too small to be detected, are to account for the observed oxygen uptake. In preparation C 3, for example, certainly less than 0·05γ per g. was present. 10 mg. of the preparation, containing less than 0·0005γ of lactoflavine, were used in the experiment given in Table I, and took up 181 mm.^{3} in 20 minutes, or 9 mm.^{3} per minute. As 0·0005γ of lactoflavine is equivalent to 0·000015 mm.^{3} of oxygen, the lactoflavine would have to be reduced and oxidised 600,000 times per minute in order to account for the oxygen uptake, whereas Warburg and Christian found that the corresponding catalytic flavines of bottom yeast and lactic acid bacteria were reduced and oxidised only 24 and 30 times per minute respectively.

The observed rate of reduction of lactoflavine by xanthine oxidase also shows that it cannot account for the oxygen uptake. Table IV shows that an amount of lactoflavine corresponding to 1·8 mm.^{3} of oxygen was reduced in 35 minutes. In the same period this solution of oxidase would have taken up about 210 mm.^{3} of oxygen, so that it is clear that even this relatively large amount of pigment could only account for less than 1 % of the oxygen uptake.

Since Dixon and Thurlow showed that the xanthine oxidase differed from the majority of the oxidases, in that its ability to react with molecular oxygen was not inhibited by HCN, it has been regarded, in common with the other cyanide-stable respiration systems, as a case in which the oxygen reacted directly with the activated substrate molecules in accordance with Wieland's theory. Warburg and Christian [1933] have, however, shown that at any rate in some of these cases, which the haematin catalysis theory could not explain, the oxygen uptake could be accounted for by the action of catalytic flavines (which are resistant to cyanide), the oxygen reacting with these and not directly with the organic substrates.

Warburg and Christian assert that, just as the Wieland theory is wrong in all those cases in which the respiration is inhibited by HCN (for the oxygen then reacts, not with the substrate, but with the reduced form of a haematin), it is no less wrong in those (rarer) cases in which the respiration is not inhibited by HCN (for the oxygen then reacts, again not with the substrate, but with the leuco-form of the flavine); and they state that the idea of a direct reaction of molecular oxygen with the substrate must now be abandoned.

In the xanthine oxidase, however, we have an oxidase system whose oxygen uptake is due neither to haematin nor to flavine, but apparently to a direct reaction of oxygen with the activated substrate molecules.

Summary.

The ability of the xanthine oxidase of milk to react with molecular oxygen is not dependent upon the presence of lactoflavine. In this system the molecular oxygen probably reacts directly with the activated substrate molecules.

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