LXXV. STUDIES ON XANTHINE OXIDASE.

V. THE FUNCTION OF CATALASE.

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ALTHOUGH the enzyme catalase has been known for a considerable time, its biological function has never been definitely settled. It was first suggested by Loew [1902] that it had a protective action, and that its function was to prevent the accumulation in the cell of the hydrogen peroxide formed during the oxidation processes connected with cell respiration. It is well known that hydrogen peroxide is extremely toxic to most enzymes, and its presence in the cell, except in very small concentrations, would rapidly bring about their destruction. Loew suggested that the presence of catalase prevented this by keeping the peroxide concentration down to a low level. This view has been fairly generally held, but so far without having been established by definite experimental evidence except in the case of certain bacterial systems. McLeod and Gordon [1922] showed that pneumococci with a free supply of oxygen produced hydrogen peroxide in amounts sufficient to inhibit their growth; but if catalase was added they were able to grow much more vigorously than in its absence, so that Loew's view is thus supported at any rate as far as bacteria are concerned. In the present paper evidence is given to show that catalase does actually have a protective function in animal tissues.

In the second paper of this series [Dixon and Thurlow, 1924, 2] the results have been given of investigations on the xanthine oxidase by means of the methylene blue technique. Morgan, Stewart and Hopkins [1922] also carried out a number of experiments on this enzyme by measurements of oxygen uptake, and it was during an attempt to extend these investigations that the facts described in the present paper came to light.

.The preparation of xanthine oxidase previously described [Dixon and Thurlow, 1924, 1] was employed for the investigation. It is very suitable for the purpose, as it probably contains no other enzymes and shows no oxygen uptake in the absence of hypoxanthine, xanthine, etc. The oxygen absorption was measured by means of the Barcroft differential apparatus of the type used by Morgan, Stewart and Hopkins. The flasks of this apparatus were of a flattened form which exposed the fluid in a thin layer to the air above it so as to allow very rapid diffusion of oxygen into the liquid. Under these conditions the rate of oxygen absorption depends only on the rate at which it is used up by the enzyme, and is not limited by the rate of diffusion into

the liquid. In setting up each experiment solutions of the same composition were added to both flasks, but the hypoxanthine was added to one flask only. This ensured that each experiment was properly controlled. The experiments were in all cases carried out at a temperature of 40° and at p_H 7·6.

On investigating the effect of a change in the concentration of the enzyme on the rate of oxygen uptake, using a constant amount of hypoxanthine, it was found that the rate of the reaction was *not* directly proportional to the enzyme concentration, as was found to be the case with the methylene blue technique. Moreover, the total amount of oxygen taken up by the system was not always independent of the enzyme concentration. As long as the xanthine oxidase is present in excess the total oxygen uptake corresponds with the calculated amount necessary to oxidise the hypoxanthine (or xanthine) to uric acid. When, however, the concentration of enzyme used in the experiment is reduced, a point is reached where the oxygen uptake varies with the amount of enzyme taken. These facts can be seen from Fig. 1, where the results of a typical experiment are given.
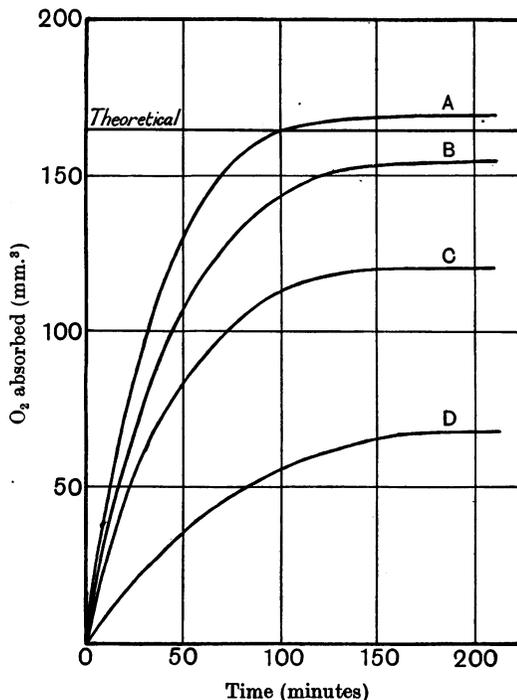

Fig. 1. Oxygen absorption by hypoxanthine with varying amounts of xanthine oxidase.
The solutions were made up as follows:

	A cc.	B cc.	C cc.	D cc.
Oxidase solution	2·0	1·5	1·0	0·5
p_H 7·6 buffer	0·5	1·0	1·5	2·0
Hypoxanthine (2 mg. per cc.)	0·5	0·5	0·5	0·5

The curves suggest that the enzyme is being gradually destroyed during the course of the reaction. In order to test whether this was the case the contents of the flasks of the Barcroft apparatus were transferred at the conclusion of the experiment to vacuum tubes, and tested anaerobically with methylene blue and a little fresh hypoxanthine. By comparing the reduction-time given by an enzyme solution which has brought about the oxidation of the hypoxanthine with that given by the solution in the control flask of the same apparatus it is possible to determine approximately how much of the enzyme has been destroyed during the oxidation. By this method it was found that in experiments B, C and D the enzyme had been totally destroyed during the oxidation, whilst in experiment A there had been considerable, though not complete, destruction. It follows therefore that in order to make sure that the oxidation of the hypoxanthine shall proceed to completion it is necessary to use an amount of enzyme sufficient to ensure that some of it shall be left undestroyed at the end of the reaction.

The experiment given in Fig. 1 was repeated, using milk instead of the xanthine oxidase preparation, and similar results were obtained. It is worth noting that the amounts of milk used by Morgan, Stewart and Hopkins in their experiments were not very greatly above the minimum required for complete oxidation of the hypoxanthine, so that it is almost certain that the greater part of the oxidase had been destroyed by the end of their experiments.

These facts show that it is almost impossible to interpret curves of oxygen absorption in terms of enzyme dynamics, since the concentration of enzyme is continuously changing during the progress of the reaction. The oxygen-absorption method is therefore not well suited to a quantitative investigation of the dynamics of systems of the type of the xanthine oxidase except for special purposes, and it seems that other methods, such as the methylene blue technique, are more likely to yield trustworthy results.

It can be shown from the methylene blue experiments given in the earlier paper [Dixon and Thurlow, 1924, 2] that the enzyme is not destroyed during the course of the reaction when methylene blue is substituted for oxygen. The effect seems only to occur in the presence of oxygen, and this fact at once suggests the possibility that hydrogen peroxide may be responsible for the destruction. It has been shown by Thurlow [1925] in the preceding paper of this series that hydrogen peroxide is formed during this reaction in appreciable quantities. The writer has found that xanthine oxidase is very sensitive to hydrogen peroxide, and is quickly destroyed even by quite small concentrations. It follows that the destruction of the enzyme during the reaction is indeed to be expected.

We have then in the xanthine oxidase a system, normally occurring in animal tissues, on which catalase should, if Loew's view is correct, exert a protective action. In order to investigate this question experimentally it is desirable to obtain a preparation of catalase which is free from xanthine oxidase and from oxidisable substances. Several methods of preparation were

tried, and the one which was found to give the most satisfactory results in this respect was that of Batelli and Stern [1904]. Catalase preparations made from liver by this method were found not to take up oxygen or reduce methylene blue either in the presence or in the absence of hypoxanthine.

The liver was minced and extracted with twice its own volume of water. The extract was mixed with two volumes of alcohol, and the resulting precipitate filtered off and dried. This precipitate was then extracted thoroughly with three volumes of water with shaking for some hours, and the extract after filtering was mixed with three times its volume of alcohol. The not very voluminous precipitate thus produced was filtered off and dried *in vacuo*. The preparation is readily soluble in water, and in the dry form retains its activity very satisfactorily.
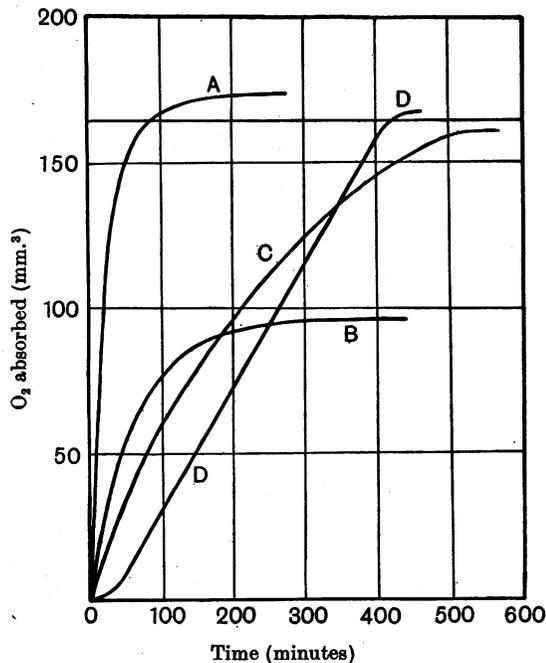

Fig. 2. Effect of addition of catalase.

The solutions were made up as follows:

	A cc.	B cc.	C cc.	D cc.
Oxidase solution	2·0	0·5	0·5	0·5
Catalase solution	—	—	0·5	0·5
			(weak solution)	(strong solution)
p_H 7·6 buffer	0·5	2·0	1·5	1·5
Hypoxanthine (2 mg. per cc.)	0·5	0·5	0·5	0·5

In Fig. 2 is shown the effect on the oxygen uptake of the addition of catalase. Curves A and B show, as in Fig. 1, the course of the oxygen absorption with a fairly large and a small oxidase concentration respectively; curve C shows

the absorption with the same small amount of xanthine oxidase as in curve B, but with the addition of a relatively small quantity of catalase. It will be seen that the catalase has prevented, at any rate in large measure, the destruction of the oxidase, since the reaction ultimately proceeds to completion even with the small oxidase concentration, though of course at a slower rate than with the higher concentration in curve A.

At the conclusion of the experiment the solutions were tested as before with methylene blue *in vacuo* after the appropriate additions of hypoxanthine, in order to determine how much destruction of the oxidase had occurred. The results showed that in A about 90 % of the oxidase had been destroyed, whilst in B destruction had been complete. In C, however, only about 25 % had been destroyed, and the catalase thus shows a protective action which is very marked considering the small amount added. The same experiment has been repeated with several different preparations of xanthine oxidase and liver catalase, and similar results have invariably been obtained.

If stronger solutions of catalase are used, much more complete protection of the enzyme can be obtained. Curve D shows a typical curve obtained with an amount of catalase several times as great as that used in curve C, with the same amount of oxidase as in curves B and C. It is interesting to note that when the protection becomes more complete the curve of oxygen uptake becomes practically a straight line. When very strong catalase solutions are used, the curves always show a small "latent period" effect. The reaction begins very slowly, and does not attain its full velocity for 20–30 minutes. This effect, which can be seen to some extent in curve D, is at present unexplained.

It will be seen that initially the rate of oxygen uptake is definitely smaller when catalase is present than in its absence. This effect is quite independent of the "latent period," and can be seen in curve C as well as in curve D. Of course, complications are introduced by the fact that the oxidase concentration, when no catalase is present, soon falls, so that the velocity of the absorption falls below that given by the solutions containing catalase; but we may safely assume that during the initial parts of curves B, C and D the concentration of oxidase is roughly the same in all three cases. It is therefore legitimate to compare the velocities during the first part of the experiment. Thus it appears that catalase actually lowers the rate of oxygen uptake. This inhibition is, however, only apparent, and is to be expected. The catalase decomposes hydrogen peroxide in the following way $2H_2O_2 \rightarrow 2H_2O + O_2$, and there is thus an evolution of oxygen gas. The apparent volume of oxygen absorbed, as given by the reading of the Barcroft apparatus, is then the actual volume utilised by the xanthine oxidase *minus* the volume evolved by the catalase. Any catalase action is therefore bound to diminish the observed rate of oxygen uptake. This is probably the true explanation of the phenomenon, and there seems to be no reason to suppose that catalase has any direct action on the xanthine oxidase. The above action, of course, only affects the observed rate

of oxygen uptake; the amount of oxygen ultimately absorbed remains un-altered.

It was stated above that destruction of the oxidase occurred in milk as well as in solutions of the catalase-free caseinogen preparation. The amount of catalase contained in milk thus seems to be insufficient to protect the oxidase. The samples of milk used in the experiment, however, appeared to contain only a small amount of catalase, to judge from rough tests, and it is possible that a more average milk would not show so marked a destruction.

SUMMARY.

1. When the purine bases are oxidised by molecular oxygen in presence of xanthine oxidase as a catalyst, the oxidase is progressively destroyed during the course of the reaction. This fact makes the measurement of oxygen uptake unsuitable as a general method for the investigation of the dynamics of the enzyme.

2. The destruction is due to the hydrogen peroxide which is known to be formed during the reaction.

3. The destruction of the oxidase is prevented by the addition of catalase, which is thus shown to have a protective function in animal tissues.

The writer wishes to express his thanks to Sir F. G. Hopkins for his interest in the work, and also to the Royal Commissioners for the Exhibition of 1851 for the senior studentship held during the period of the investigation.

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