CLXV. STUDIES ON XANTHINE OXIDASE. IX.

THE SPECIFICITY OF THE SYSTEM. II.

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One of the most remarkable phenomena connected with enzyme action is the very strict specificity of many enzymes for their substrates.

The oxidising enzymes have not yet been thoroughly studied from this point of view, and an enzyme which lends itself especially well to an investigation of this kind is the xanthine oxidase. The enzyme is easily obtained free from other enzymes and the purine group is particularly well adapted to this study because of the very large number of small changes that can be made in it.

Morgan, Stewart and Hopkins [1922] showed that this enzyme could activate both hypoxanthine and xanthine. They also showed that caffeine (1.3.7-trimethylxanthine), theobromine (3.7-dimethylxanthine), uric acid and guanine (2-aminohypoxanthine) were not activated by the enzyme, but they observed that adenine (6-aminopurine) was slowly oxidised under its influence. They ascribed the action on this last substance to a previous deamination of the adenine to hypoxanthine by some adenase that was present and the subsequent oxidation of the hypoxanthine to uric acid. Dixon and Thurlow [1924] thought, however, that it was probable that there was a direct action of the xanthine oxidase on the adenine and brought some evidence in support of this theory. Following up the above results Dixon [1926] investigated the action of the enzyme on 35 substances of physiological importance, but except for the above-mentioned substances and aldehydes the results were entirely negative.

The enzyme is a particularly interesting one because we can study separately the adsorption of the substrate on the enzyme and also the activation of the substrate by the enzyme, since Dixon and Thurlow [1924] have shown that those purines that are adsorbed inhibit the reduction of methylene blue by hypoxanthine or xanthine, owing to the formation of an interfering film on the surface of the enzyme. Activation of the purine can thus be detected by observing whether the methylene blue is reduced, and, on the other hand, adsorption can be studied by observing the effect of the purine on the velocity of the reduction of the dye by the xanthine.

Thus, by a study of a series of substituted purines, we may hope to determine what structure is necessary for (*a*) activation, and (*b*) adsorption of the substrate.

EFFECT OF STRUCTURE ON ACTIVATION.

The following purines were tested:

3-methylxanthine	3.8-dimethylxanthine
8-methylxanthine	1-methylguanine
9-methylxanthine	7-methylguanine
1.3-dimethylxanthine	1.7-dimethylguanine

and also benziminazole.

The whey preparation of Dixon and Kodama [1926] was used as a source of the enzyme and the results were confirmed using fresh milk. The experiments were carried out in Thunberg tubes in the usual way. The tubes were always evacuated, washed out with nitrogen and re-evacuated before being placed in the bath. The method used in the experiments was as follows.

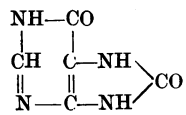
0·5 cc. fresh milk, 5 cc. buffer, 0·5 cc. methylene blue (1 in 1000) and 0·1 cc. of a solution of xanthine containing 5 mg. per cc. were placed in the bath at 40°; decoloration was complete in 120 secs. 2 cc. of the same milk, 3 cc. buffer, 0·5 cc. of the methylene blue and 0·1 cc. of a solution of the purine to be tested containing 5 mg. per cc. were also placed in the bath.

In no case of the above series of purines was there found to be any visible change of colour. Since a change of one quarter of the colour should be readily detected and the observations extended over 4 hours it is easily calculated that, if any of the purines were activated, the velocity of the reaction must have been less than one two-thousandth of that with xanthine.

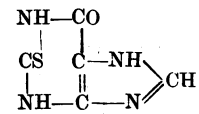
In the above experiment all the solutions of the purines were made up in the following manner. 50 mg. of each was accurately weighed into a 10 cc. graduated vessel. About 2 cc. of water was added and a drop of strong caustic soda. The vessel was agitated and when solution was complete water was added to the mark. Just before use very small quantities of strong hydrochloric acid were added until a cloudiness was apparent and this was just cleared by the addition of minimal amounts of dilute caustic soda. In this way the purines were obtained in a solution which was not alkaline enough markedly to affect the p_H of the reaction mixture.

The significance of the results is discussed later.

Two purines, other than hypoxanthine, xanthine and adenine, have been found which are activated by the enzyme, namely:



6.8-Dihydroxypurine



2-Thioxanthine

0·5 cc. fresh milk, 5 cc. methylene blue (1 in 1000) and 0·1 cc. of the solution of the purine containing 5 mg. per cc. were taken in each experiment.

	Time for complete reduction (secs.)
Hypoxanthine	120
Xanthine	225
6.8-Dihydroxypurine	250
2-Thioxanthine	350

From a consideration of their structure it might be expected that one molecule of the last two purines would reduce one molecule of methylene blue, since they would, in all likelihood, be oxidised to uric acid and thiouric acid respectively. To test this the following technique was devised and very clean-cut results were obtained. 0·15 cc. quantities of the solution of the purine to be tested (5 mg. per cc.) were accurately measured by means of a Pregl pipette into small test-tubes and these were introduced into large Thunberg tubes, which contained 10 cc. of milk and the various under-mentioned amounts of methylene blue. The Thunberg tubes were then evacuated several times, being washed out with nitrogen each time, and when this was complete the tubes were tilted so that the purine was mixed with the milk and the methylene blue, and the tubes were placed in the bath at 40° or 60°. It was found that, when using the 6.8-dihydroxypurine, 4·3 cc. of methylene blue (M/1000) were reduced very quickly, 4·5 cc. almost as quickly but that with 4·7 cc. a slight colour persisted for a considerable time. Thus the purine required about 4·6 cc. of the methylene blue and this is in good agreement with the theoretical figure of 4·9 cc. With the thioxanthine, 4·3 cc. was obtained for the titration instead of 4·45 cc. The agreement is excellent and the slight disparity is probably due to unavoidable traces of oxygen.

Xanthine oxidase prepared from ox spleen by the method of Morgan, Stewart and Hopkins was also capable of bringing about the oxidation of these purines. Using about 5 cc. of spleen, 0·5 cc. of methylene blue (1 in 1000) and 0·1 cc. of the purines (5 mg. per cc.) decoloration was complete in the following times.

At 40° (control without purine about an hour):

6.8-Dihydroxypurine	90 secs.
2-Thioxanthine	140

At ordinary temperatures (control without purine more than 8 hours):

Hypoxanthine	4·5 mins.	(4·8)
Xanthine	9	(9)
6.8-Dihydroxypurine	10	(10)
2-Thioxanthine	15	(14)

The numbers in the brackets are calculated from the figures given above when using milk, taking xanthine as the standard of reference. The satisfactory agreement of the velocity ratios shows that the same enzyme is responsible for the activation of all four purines. It is hoped to isolate the products of the oxidation of the 6.8-dihydroxypurine and the 2-thioxanthine.

Effect of structure on adsorption.

In order to determine which of the purines were adsorbed by the enzyme a series of experiments was carried out on each of them. Using a constant

amount of xanthine and adding varying quantities of the purine to be tested, inhibition curves were plotted for each purine and these are given graphically (Fig. 1). The exact description of the experiments is as follows. 2 cc. of buffer, 2 cc. of enzyme (2 % whey preparation), x cc. of the purine solution (5 mg. per cc.) and $(2 - x)$ cc. of water were placed in a series of Thunberg tubes. When the series was ready 0·1 cc. of xanthine solution (also 5 mg. per cc.) was quickly added to each, the tubes evacuated as quickly as possible, re-evacuated and placed in the bath at 40°. The time for the complete decoloration of each tube was noted and this was converted into speed by multiplying its reciprocal by 10,000, according to the procedure of Dixon and Thurlow, who also give other inhibition curves.

Discussion.

The results obtained in the experiments on the effect of structure on activation show in a striking manner the high degree of specificity which obtains in this system. The introduction of a single methyl group in either ring is sufficient entirely to prevent any activation, although the inhibition curves show that the enzyme still adsorbs the compounds, but not so strongly as the unsubstituted xanthine. Dixon [1926] showed that the introduction of an amino-group in the 2 position was also sufficient completely to prevent any action, and the introduction of an amino-group in the 6 position (adenine) reduced the velocity to one-fiftieth of that of xanthine.

The fact that 6.8-dihydroxypurine and xanthine are both converted to uric acid at approximately the same rate shows that the two stages of the oxidation take place independently of one another. Particularly interesting is the case of 2-thioxanthine, in which the place of one of the atoms of oxygen in xanthine is taken by an atom of sulphur. As far as the enzyme is concerned, it seems that the sulphur behaves practically in the same way as the oxygen. There is, however, a slight difference in the speed of oxidation—the sulphur compound being oxidised a little more slowly, but this may be due to the molecule not being adsorbed to the same extent.

In the inhibition experiments it seems that the purines can be divided into three classes:

(*a*) those strongly adsorbed:

Hypoxanthine	6.8-Dihydroxypurine
Xanthine	Guanine
3-Methylxanthine	1-Methylguanine
Uric acid	7-Methylguanine
Adenine	1.7-Dimethylguanine

(*b*) those adsorbed to only a small extent:

8-Methylxanthine	Alloxan
9-Methylxanthine	

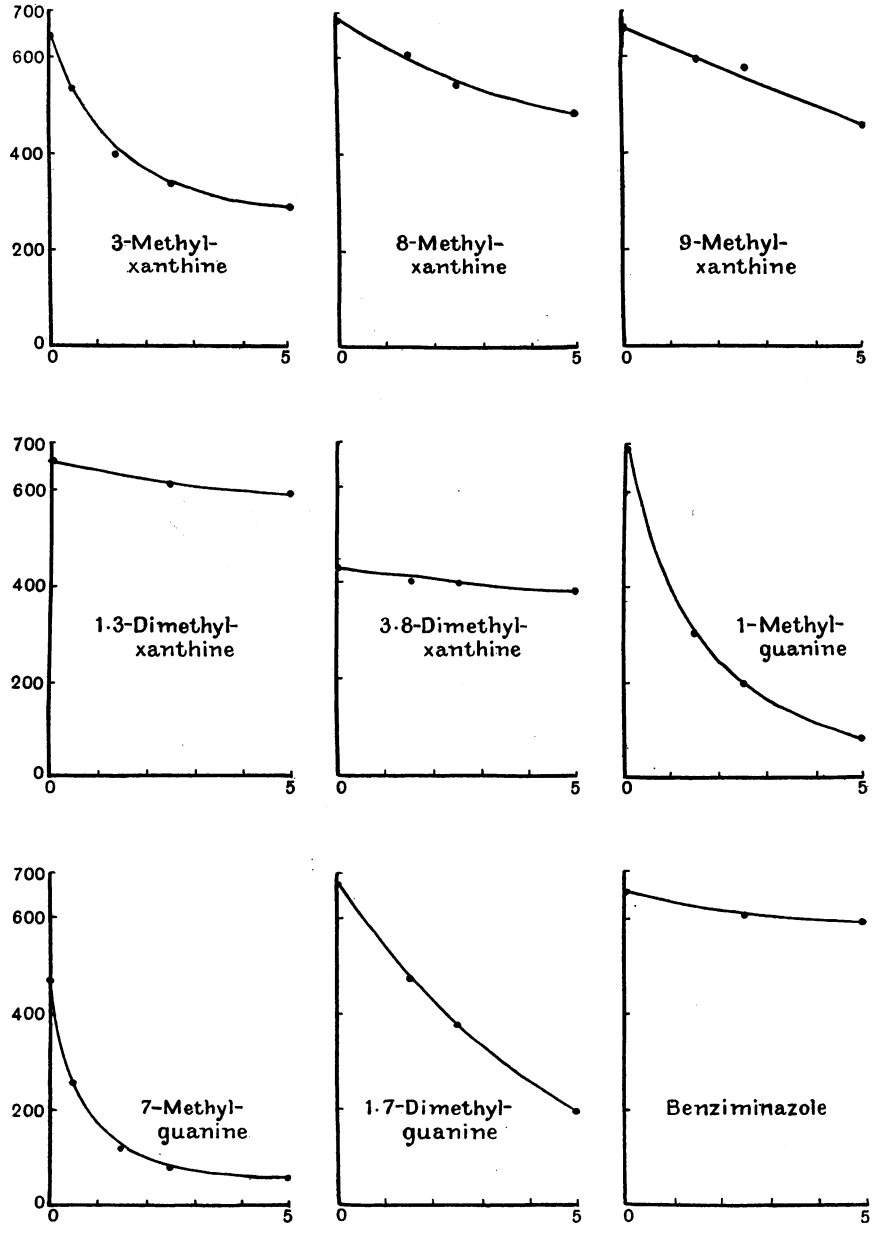


Fig. 1.

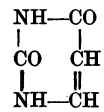
Ordinates represent reaction velocity. Abscissae give mg. of purine added

(*c*) those not absorbed, or only to a very slight extent:

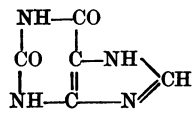
Benziminazole	Thymine
1.3-Dimethylxanthine	Cytosine
3.8-Dimethylxanthine	Uracil
Caffeine (1.3.7-trimethylxanthine)	Histidine
Theobromine (3.7-dimethylxanthine)	

(This table also includes the results of other workers.)

It is clear that neither the pyrimidine nor the iminazole ring can by itself cause adsorption of the enzyme—it seems necessary to have the complete purine structure. The pyrimidines are not adsorbed, neither is an iminazole compound such as histidine, and the same is true of benziminazole which contains a benzene ring instead of the pyrimidine ring which is present in the purines. On the other hand, the iminazole ring seems to be very necessary for adsorption and this is very clearly demonstrated by a comparison of uracil



Uracil



Xanthine

and xanthine, because, whereas the latter is strongly adsorbed the former is not.

The introduction of a methyl group, particularly in the iminazole ring, tends to prevent adsorption. Thus in the methylxanthines we have much less adsorption than with xanthine itself. The effect of methyl groups in preventing adsorption is very plainly shown when more than one is introduced—these compounds are only adsorbed to a very small extent.

The introduction of an amino-group strongly favours adsorption, so much so as to overpower the effect of the methyl groups. In the guanines the monomethyl compounds are adsorbed much less than guanine itself but even when two methyl groups are present adsorption is very marked, whereas in the case of the xanthines two methyl groups almost completely prevent adsorption.

It seems likely from the above results that the attachment of the substrate to the enzyme is due partly to the iminazole ring and also partly to the structure of the 1.6-position. The data at present available, interesting and suggestive as they are, are insufficient to afford a basis for a complete theory of the mechanism of adsorption, but it is hoped to complete the above series of experiments so that a satisfactory theory can be evolved.

Summary.

1. The action of the xanthine oxidase on a series of substituted purines has been studied with a view to determining the effect of structure of the purine on (*a*) its activation, (*b*) its adsorption by the oxidase.

2. The enzyme was able to activate only two of these purines, namely; 6.8-dihydroxypurine and 2-thioxanthine.

3. These two purines take up one atom of oxygen for each molecule during their oxidation by this system.

4. The enzyme is very specific, the introduction of a methyl group into the pyrimidine or iminazole ring being sufficient entirely to prevent activation.

5. The complete purine skeleton—the two-ring structure—is necessary for adsorption. The introduction of an amino-group strongly favours adsorption; and the introduction of methyl groups, particularly in the iminazole ring, tends to prevent adsorption.

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