LXV. THE SPECIFICITY OF
XANTHINE OXIDASE

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A DETAILED study of the question of identity of xanthine oxidase and the Schardinger enzyme was made in 1935 [Booth]. Most of the existing evidence in favour was confirmed while that against was shown either to have another explanation or to be insufficiently controlled. In addition several new lines of evidence were presented. The results of the investigation pointed strongly to identity though the hypothesis could not be regarded as rigidly proved. Since then other authors [Basu & Mukherjee, 1936; Dixon & Keilin, 1936; Lemberg *et al.* 1936; Michlin *et al.* 1936] have extended certain of the earlier evidence, each concluding in favour of identity. There remain however certain points in the literature against this view. An investigation of most of these is reported in this paper and yields no support for the separate enzyme theory. Considering the mass of evidence from the work of several authors there can be no doubt that xanthine oxidase activates aldehydes. Nothing short of preparation of each enzyme completely free of the other can be taken as proof of non-identity. The apparent suppression of one enzyme by an inhibitor is insufficient.

Because purines and aldehydes are both activated by the same enzyme, xanthine oxidase offers a case of dual specificity of unusual interest on two counts: firstly because all aldehydes hitherto tested are activated, while as Coombs [1927] showed, only 5 of some 17 purines which he and Morgan *et al.* [1922] tested are activated; and secondly on account of the chemical difference between purines and aldehydes. This specificity has been examined in detail in the present paper which includes: (*a*) an extension of the list of purines activated, with relative oxidation rates; (*b*) rates of oxidation of over 30 aldehydes; (*c*) a proof that cozymase is not activated; (*d*) particulars of experiments with substances related to aldehydes; (*e*) suggestions for the use of xanthine oxidase as a biochemical reagent for detecting aldehyde groups.

Methods

The Dixon & Kodama [1926] "whey" preparation of xanthine oxidase was made from Grade A milk. The pH was that of the $0.25M$ phosphate buffer, 7.2, in which the enzyme was dissolved, except that, when concentrated enzyme solutions were used the pH was lower on account of the buffering effect of the ammonium sulphate present in the preparation. A trace of octyl alcohol was added for prolonged experiments.

The "cozymase" preparation was made from yeast by the method of Myrback & Örtenblad [1935] and taken as far as the phosphotungstic stage. The last process was repeated and the resulting solution concentrated by low pressure distillation.

The anaerobic experiments were carried out in specially designed Keilin-Thunberg vacuum tubes. Unless stated differently, each tube contained 3 ml.

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buffered enzyme solution, 1 ml. $0·0005 M$ methylene blue, x ml. substrate and $1-x$ ml. water or another solution. Substrates commonly used were $0·2$ ml. $0·007 M$ hypoxanthine and $0·8$ ml. $0·02 M$ salicylaldehyde. The tubes were evacuated and washed out 3 times with nitrogen. In general the substrate was placed in the hollow stoppers and tipped into the enzyme mixture after 3 min. equilibration at $38°$. The anaerobic technique is rapid and convenient, but has certain limitations. The accuracy is probably greatest for reduction times of between 5 and 10 min. and these were obtained where possible. Even then the reduction times are liable to an error of $\pm 5\%$. In some experiments 80% reduction was taken as end point but the discrepancy between duplicates was greater than when complete reduction was taken as end point, on account of difficulty in matching the colours. The reduction velocity at the end was not markedly less than the mean. The times for complete reduction were therefore recorded.

The aerobic experiments were done in Warburg manometers, the substrate being tipped in from a side bulb after 15 min. equilibration. The purines were dissolved in alkali which may absorb CO_2 during manipulations. On tipping into the buffer after equilibration some of the CO_2 is liberated. Hence for total oxygen uptake with purines alkali was placed in the pots, although it must be avoided in the case of aldehyde oxidation. The temperature of the thermostat bath in all cases was $38°$.

ACTIVATORS AND ACCELERATORS

Effect of fat. Dixon & Thurlow [1924] found that boiled cream accelerated the reduction of methylene blue in presence of hypoxanthine but not in presence of acetaldehyde. Although they made no such claim this result might be quoted in favour of non-identity of the enzymes. Wieland & Rosenfeld [1930] found that a colloidal suspension of cholesterol had an accelerating effect on xanthine oxidase.

In order to study this effect an enzyme preparation was dried in a vacuum desiccator, defatted by several extractions with ether, ground finely, again dried and extracted three times with washed, dried ether. The acceleration, by boiled cream, of methylene blue reduction with hypoxanthine was confirmed. But an acceleration with salicylaldehyde as substrate was also observed. The grease used for sealing the vacuum tubes had a similar effect. Hence the joke about the tap grease complicating the results has some foundation! Greaseless rubber-stoppered vacuum tubes were therefore used, and substrates added from cupsticks. Accelerations were observed with olive, linseed and paraffin oils, saponin and taurocholate. In all cases, including that of the boiled cream, the accelerations were under 20% and variable, being greater when the tube was shaken vigorously. Shaking aids emulsification and the result lends support to Dixon & Thurlow's theory that the effect is due to "the catalytic action of the large surface presented by the fat". Apart from this no complete explanation has yet been found, but one conclusion, germane to the present investigation, is clear, namely that fat has the same effect on both enzymes. Dixon & Thurlow's lack of effect with acetaldehyde can be explained as follows. The substrate concentration curve for acetaldehyde only reaches a maximum at high concentration. Although no figures are given, if we may judge from other experiments in the same paper, these authors used a concentration which was on the rising part of the curve. Acetaldehyde is soluble in most fats and oils; part of it was therefore removed from the aqueous enzyme phase by the emulsified fat. The lowering of the concentration of aldehyde in the aqueous phase annulled the effect of the fat. This explanation receives support from my experiments with salicylaldehyde. Boiled cream slightly accelerated the reduction time when the substrate was

present in optimal concentration ($0.003 M$) but retarded it when present in suboptimal concentration ($0.002 M$).

Boiled cream also accelerated the aerobic oxidation of hypoxanthine but the effect was barely outside experimental error—6 %.

Increase in activity of milk on standing. Wieland & Macrae [1930] found that when milk stands (with preservative) the xanthine oxidase, but not the Schardinger enzyme, activity increases and reaches a constant value on the third day. This I have been unable to repeat. Toluene and octyl alcohol were respectively added to two samples of milk which were stored at 2° and tested daily with hypoxanthine and salicylaldehyde. After 1 day methylene blue was reduced 5 % faster in each case but the increased activity was accounted for by development of a small "blank". On the third day both activities declined. Controls showed that the enzymes were not affected by the antiseptics.

Rennin. Dixon & Kodama [1926] found that their whey preparation had over 3 times the enzyme activity which could be accounted for by the activity of the original milk. They suggested that the rennin used in preparing the enzyme had liberated xanthine oxidase from an inactive precursor. A similar increase in Schardinger enzyme activity would provide evidence for identity. Unfortunately neither I nor the original authors have been able to repeat the observation. Milk does not contain an inhibitor which is removed during the preparation of the enzyme, for the activities of milk and enzyme preparation are exactly additive.

INHIBITORS

Substances related to substrates. Prof. H. O. L. Fischer kindly sent me some 6-amino-8-hydroxypurine. This purine inhibited both hypoxanthine and aldehyde oxidation considerably. The reduction times of methylene blue with hypoxanthine were: without aminopurine 8 min.; with $0.0003 M$ purine 25 min. With formaldehyde in optimal concentration the times were 8 and 130 min. respectively.

Several substances suspected as possible inhibitors of one enzyme system were found to inhibit neither. Again the results with both enzymes ran parallel. Among these substances were: 7-methylhypoxanthine, allantoin, pig serum, chloral hydrate, butylchloral hydrate, glucose, gluconate, propionate, octyl alcohol and trithioformaldehyde.
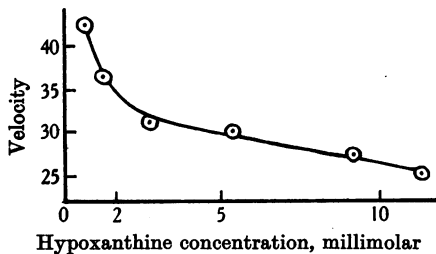

Fig. 1. Hypoxanthine concentration curve. Warburg manometric method. 38°. 2 ml. buffered enzyme solution pH 7.2 + 0.1 ml. catalase solution + 0.3 ml. hypoxanthine solution. The velocity is expressed as μl. oxygen per hour per 2.4 ml. solution.

Originally one of the strongest pieces of evidence for identity was the inhibition of the Schardinger enzyme by uric acid anaerobically. Anaerobically hypoxanthine inhibits xanthine oxidase strongly, and the fall in the curve in Fig. 1 suggests that aerobically it also inhibits slightly. On the other hand

neither uric acid nor guanine inhibit during the aerobic oxidation of hypoxanthine, as shown by Dixon & Keilin [1936] using the caseinogen enzyme preparation. Similarly, using the whey preparation I have found that neither 0·0036 nor 0·001 M uric acid had any effect on the oxygen uptake rate with 0·0017 M hypoxanthine, even when added before the substrate. Uric acid does however inhibit the oxygen uptake rate with furfuraldehyde [Booth, 1935]. The fact that xanthine oxidase is unaffected aerobically by its most typical anaerobic inhibitor emphasizes the need for care in interpreting results obtained with inhibitors.

NOMENCLATURE

The suggestion is offered that the enzyme be called xanthine oxidase even when its aldehyde activity is being discussed. The name Schardinger enzyme is superfluous. The eventual classification of oxidases and dehydrogenases may require a renaming of xanthine oxidase but one name should still suffice.

PURINES AND XANTHINE OXIDASE

Dixon [1926] tested a large assortment of possible substrates with a xanthine oxidase preparation and found that, outside purines and aldehydes, none were activated. Coombs [1927] tested several purines with a whey preparation and divided them into groups:

(1) Activated and adsorbed;
(2) Adsorbed but not activated;
(3) Neither adsorbed nor activated.

His criterion for "adsorption" was inhibition of the enzyme when hypoxanthine was used as substrate for reduction of methylene blue. In the first group were placed only hypoxanthine, xanthine, 6:8-dihydroxypurine, 2-thioxanthine and adenine. In the second group were guanine, certain methylated purines and a dimethylated guanine. In the third group were other dimethylated purines. He concluded that adsorption and activation were dependent upon the completeness of the purine ring, that activation was prevented and adsorption lessened by methyl groups and that adsorption was increased by amino groups. Examination of the formulae shows that (a) methylation at 2, 6 or 8 positions would lessen (and in most cases prevent) oxidation, (b) any substituent (including methyl) in almost any other position, except possibly 7, would prevent enolization and (c) an amino group at 2 or 6 might aid enolization. Enolization may be a necessary condition for activation. It would be interesting to test deoxyxanthine, and purines with substituents other than methyl groups, particularly amino, in positions preventing enolization.

Prof. H. O. L. Fischer has kindly given me samples of four purines related to hypoxanthine. These are from the collection of purines prepared by Emil Fischer. The following three reduced methylene blue in presence of the active (but not in presence of boiled) enzyme

	Velocity
8-hydroxypurine	6
6-amino-2-hydroxypurine	3·5
6-amino-8-hydroxypurine	6

with the comparative velocities shown, the rate with hypoxanthine being taken as 100. That the reduction was not due merely to traces of impurities was shown by determining the total oxygen uptake. The 6-amino-8-hydroxypurine took up

$70 \mu l$. oxygen per mg. (mean of several runs with different initial substrate concentrations). The theoretical value for one atom of oxygen per molecule is $76 \mu l$. The rate of uptake with 6-amino-2-hydroxypurine in presence of a strong enzyme solution fell off gradually. After 8 hr. the oxidation was still proceeding slowly and a little under one atom of oxygen per molecule had been taken up. The course of the oxygen uptake with 8-hydroxypurine, determined at a series of initial concentrations (Fig. 2), is in two distinct stages. After the uptake of
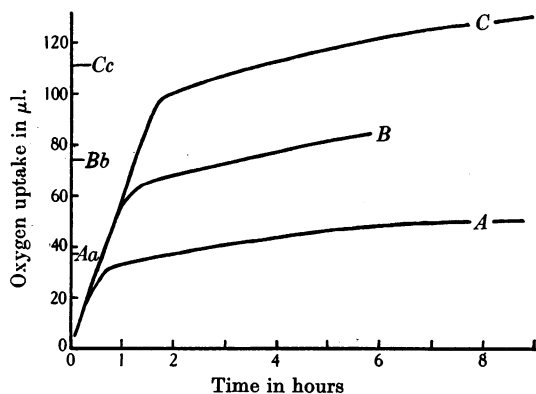

Fig. 2. Oxygen uptake with 8-hydroxypurine in presence of xanthine oxidase (whey preparation) plus catalase. Warburg manometric method. 38°. *A*, with 0·44 mg. purine; *B*, 0·88 mg.; *C*, 1·3 mg. *Aa*, *Bb*, *Cc*, theoretical values for one atom of oxygen per molecule for *A*, *B*, *C*, respectively.

approximately one atom per molecule the rate fell to one-tenth of the initial rate. Presumably the first product is 2:8-dihydroxypurine which may be only very slowly oxidized; the alternative product—6:8-dihydroxypurine—is improbable since that is known to be oxidized as rapidly as xanthine [Coombs, 1927]. The suggestion is made that 2:8-dihydroxypurine may therefore be added to the list of purines activated by xanthine oxidase. These results, together with the initial relative oxygen uptake rates, are brought together in Table I. Hypoxanthine and adenine were also used with the same enzyme

Table I. *Oxygen uptake with purines*

Substrate	Relative initial rate	Atoms oxygen per molecule	Benedict's uric acid test
Hypoxanthine	100	2	+ +
Adenine	6	2	+
8-Hydroxypurine	6	1*	+
2:8-Dihydroxypurine	0·6		
6-Amino-8-hydroxypurine	7	1	+
6-Amino-2-hydroxypurine	4·5	Nearly 1	+

* The rate then fell off to one-tenth.

preparation and are included for comparison. In all cases the observations were discontinued within 9 hr. to avoid bacterial growth. The falling off in oxygen uptake was not due to destruction of the enzyme. This was demonstrated by methylene blue reduction experiments using the final contents of the Warburg pots as enzyme solution. Benedict's uric acid test was in each case positive with the oxidation product though not with the original substance. The test is, however, unspecific.

To test whether deamination by another enzyme precedes oxidation the two aminopurines were incubated anaerobically with the enzyme preparation in Thunberg tubes for various periods before tipping in methylene blue from the hollow stopper. In the case of the 6-amino-2-hydroxypurine a half-hour incubation had no effect but a 2 hr. incubation shortened the reduction time slightly. In the case of the 6-amino-8-hydroxypurine the reduction time was increased slightly by previous incubation. The results by this method being inconclusive ammonia was determined after incubation of each purine with the enzyme preparation. The latter was made by saturating whey with magnesium sulphate instead of half-saturating with ammonium sulphate (to minimize the initial ammonia content), drying the precipitate, defatting with ether, dissolving in phosphate buffer in the ordinary way and centrifuging. In presence of a 10 % solution of this preparation the two aminopurines and adenine each reduced methylene blue in a few minutes, proving that the preparation was enzymically very active. These three purines were incubated anaerobically each with aliquot portions of this enzyme solution. After 12 hr. the ammonia concentration, determined by the method of Parnas, was identical in all cases with that of the blank without added substrate. Similarly no ammonia was produced from adenine by 12 hr. aerobic incubation with the enzyme preparation. These purines are therefore assumed to be directly activated by xanthine oxidase. The non-production of ammonia from adenine confirms Dixon & Lemberg's [1934] finding that methylene blue was reduced by adenine even in presence of purified xanthine oxidase.

To complete the series there still remain to be tested one more probable substrate, namely 2-hydroxypurine, two more possible substrates, namely purine and deoxyxanthine, and the various thiopurines.

The fourth purine (from H.O.L.F.) was 7-methylhypoxanthine. It neither reduced methylene blue nor inhibited the reduction with hypoxanthine or aldehyde.

Cozymase and Xanthine Oxidase

Dixon & Lemberg [1934] showed that the reduction of methylene blue by certain nucleosides in presence of the undefatted whey preparation was really due to hypoxanthine which had been split off by nucleosidases. When the xanthine oxidase preparations were freed from these hydrolysing enzymes the reduction no longer occurred. A preparation of "cozymase" similarly reduced methylene blue in presence of crude—though not in presence of purified—xanthine oxidase, but these authors did not determine whether cozymase itself or some other substance in the preparation was concerned here or whether cozymase was functionally inactivated. Reports that xanthine oxidase inactivates cozymase have since appeared. Lipmann [1935] found that a xanthine oxidase preparation inactivated cozymase aerobically but not anaerobically. Andersson [1936] reported that the Schardinger enzyme inactivated cozymase both as coenzyme of alcoholic fermentation and as coenzyme of dehydrogenation. Reinvestigation has produced the following results.

"Cozymase" was incubated anaerobically with a strong xanthine oxidase preparation. After 15 min. the mixture was boiled and tested. The solution failed as coenzyme for the lactic acid dehydrogenase system prepared from pig heart by the method of Green & Brosteaux [1936]. It also failed to promote fermentation by dialysed yeast juice. The eight necessary controls were done (e.g. cozymase was found to be functionally active after being boiled with boiled xanthine oxidase preparation) and the experiments were repeated with other

preparations. There cannot be any suitable hydrogen acceptor in either the enzyme or "cozymase" preparations for no uric acid is produced by incubation of the enzyme with hypoxanthine or "cozymase", and oxygen is excluded. Hence the process is not oxidative and the cozymase-inactivating enzyme is evidently a nucleosidase.

The xanthine oxidase preparation (with its contained nucleosidases) can be a useful reagent in coenzyme studies. Certain systems, for instance α-glycerophosphate, succinic and other dehydrogenases appear not to require a coenzyme though that may be only on account of separation difficulties. If such dehydrogenase is not inhibited by the xanthine oxidase preparation and does not itself behave as a substrate fo₤ the reduction of methylene blue in presence of the latter, it is unlikely that a coenzyme of the nucleoside type is concerned.

Harrison [1933] showed that xanthine oxidase (milk preparation) reacted with glucose dehydrogenase, except when the latter was protected by bisulphite, and concluded that the activating group on the glucose enzyme is an aldehyde. This result now loses some of its significance, for the coenzyme would be inactivated by a nucleosidase in the milk preparation.

ALDEHYDES ACTIVATED BY XANTHINE OXIDASE

The original substrate used by Schardinger [1902] was formaldehyde. Since that time other aldehydes have been used, particularly acetaldehyde, and now it is generally assumed, though not proved, that all aldehydes are activated by the one enzyme. The specificity towards aldehydes has therefore been examined in detail.

The aldehydes in Table II reduced methylene blue in presence of the enzyme preparation from milk. At least all of those marked † have been used by other workers. Those marked * have also been shown to take up oxygen in presence of the enzyme. Comparative reduction rates (taking the hypoxanthine rate as 10,000) are given, but these must be accepted as only approximate for various reasons, some of which are as follows. (a) Certain aldehydes, e.g. formaldehyde, o-phthalaldehyde, destroy the enzyme. (b) Most aldehydes are only slightly water-soluble: with these the rates might be dependent on the degree of emulsification, amount of shaking and other factors. (c) As the substrate concentration curves are in general not flat several concentrations should be used and the maximum rate recorded: the experimental technique however is then unjustifiably complicated except with the reasonably soluble aldehydes. Curves were obtained for 7 of the more soluble, which are indicated by ‡. In all other cases a drop of the liquid or 2 mg. of the solid was placed in a Thunberg tube stopper and vigorously shaken with the enzyme-dye mixture at the end of the usual 3 min. incubation. The aldehydes were used as fresh as possible; liquids of doubtful purity were redistilled. Because the range of velocities is great the enzyme and methylene blue concentrations were varied considerably. In most cases a control was performed with boiled enzyme: no reduction in 24 hr. is indicated by 0 and a slow reduction time by S; when the reduction was comparable with that for the unboiled enzyme the ratio of the two rates is shown. In the last case a test was made with protein-free buffer: if the methylene blue was reduced at all this is indicated by B. The pH of the solutions was always below 7·3. Propaldehyde and isobutaldehyde were also activated but the reduction rates were not compared with a standard. A test could not be made with o-phthalaldehyde as it reacted with the protein of the enzyme preparation, producing a black substance. Methylglyoxal and n-valeraldehyde have been shown by Dixon & Lutwak-Mann [1937] to be activated by xanthine oxidase.

Table II. *Aldehydes activated by xanthine oxidase*

Aldehyde	Methylene blue reduction rate	Boiled enzyme
Acetaldehyde†*‡	7250	S
Aldol	700	
Anisaldehyde	2800	0
Benzaldehyde	8000	0
n-Butaldehyde	60	0
Cinnamaldehyde	4100	0
Crotonaldehyde	70	S
Cuminaldehyde	580	0·3 B
Decanaldehyde	80	0
Duodecanaldehyde	20	0
Dimethyl-*p*-aminobenzaldehyde†	40	
Formaldehyde†*‡	9000	0
Furfuraldehyde*‡	7500	0
Glyceraldehyde‡	140	0·5
Heptanaldehyde	240	S
o-Hydroxybenzaldehyde (salicylaldehyde)*†‡	6000	0
m-Hydroxybenzaldehyde	8000	0 +
p-Hydroxybenzaldehyde	13000	0
β-Indolealdehyde	140	0
o-Methylbenzaldehyde	1500	0
o-Methoxybenzaldehyde	210	0
o-Nitrobenzaldehyde	20	0
Octylaldehyde	2	0
p-Phthalaldehyde	130	0
Phenylacetaldehyde	80	0·7
Phenylglyoxal	20	S
Phenylpropaldehyde	240	0·2 B
Piperonal†*‡	5600	0
Protocatechuicaldehyde	100	
*iso*Valeraldehyde	5	0
Vanillin†*‡	1050	0

The reduction rates of methylene blue were additive with formaldehyde and piperonal in suboptimal concentrations and competitive in optimal concentrations. This confirms the accepted view that only one enzyme is concerned in the activation of these two aldehydes. But as formaldehyde (the first member of the series) differs more from piperonal than the rest differ from each other it may be taken that all are activated by the same enzyme. Further, since these are representative of such widely differing chemical types it seems probable that all aldehydes would be activated. In contrast to the effect on purines, methylation does not prevent activation of aldehydes. Evidently xanthine oxidase could be used, provided that suitable controls were performed, as a biochemical reagent for testing for the presence of an aldehyde group.

SUBSTANCES RELATED TO ALDEHYDES

A number of substances related to aldehydes has been tested: chloral hydrate, butylchloral hydrate, paraldehyde, glucosone and mannose. Although a very strong xanthine oxidase solution was used no reduction of methylene blue was observed in 10 hr. Neither chloral hydrate, butylchloral hydrate, glucose nor gluconate affected the reduction rates using hypoxanthine or salicylaldehyde, even after previous incubation with the enzyme; hence these substances do not combine with the enzyme.

SUMMARY

1. Evidence against the identity of xanthine oxidase and the Schardinger enzyme is investigated but not substantiated. The conclusion is reached that xanthine oxidase has now been proved identical with the Schardinger enzyme, and the suggestion is offered that the latter name be discontinued.

2. Four purines have been added to the existing list of 5 activated by xanthine oxidase and the rates of their oxidation determined: 8-hydroxypurine, 6-amino-2-hydroxypurine, 6-amino-8-hydroxypurine and 2:8-dihydroxypurine. The direct activation of the two aminopurines has been proved, and that of adenine has been confirmed, by demonstrating that previous deamination does not occur.

3. Cozymase is destroyed by a nucleosidase but not by xanthine oxidase.

4. A list of 35 aldehydes activated has been compiled.

5. Substances related to aldehydes—mannose, glucosone, gluconate, chloral hydrate, butylchloral hydrate, paraldehyde—do not combine with the enzyme.

6. It is concluded that in addition to purines all true aldehydes—but only true aldehydes—are activated.

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