5. *Rhodospseudomonas spheroides*, chick blood, yeast, and pig liver contain a heat-stable factor which stimulates coproporphyrin formation from porphobilinogen.

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The Chemistry of Xanthine Oxidase

6. VARIATIONS IN STABILITY AND THE PRESENCE OF AN INHIBITOR IN CERTAIN PREPARATIONS*

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It has been reported previously that the stability of xanthine oxidase samples varies from one preparation to another (Bergel & Bray, 1956, 1959). This paper reports results of a comparison of the properties of two preparations of the enzyme, one of which proved to be exceptionally unstable, whereas the other was rather stable. Kinetic data indicate that the stable preparation contained a competitive inhibitor of xanthine oxidase, the presence of which could account for the stability differences between the two samples.

MATERIALS AND METHODS

Xanthine oxidase solutions. These were prepared by methods described previously (cf. Bergel & Bray, 1959). Most of the experiments were carried out on two preparations, namely LXO-70 and LXO-116. The former was purified by the method of Avis, Bergel & Bray (1955) to stage M5 and had an activity/ E_{450} ratio of about 40; the latter was prepared by ammonium sulphate treatment (Haddow, de Lamirande, Bergel, Bray & Gilbert, 1958) and had an activity/ E_{450} ratio which fell from 50 to about 10

during the period over which experiments were carried out. The samples both had E_{280}/E_{450} ratios of around 10. They were stored at 0–5° as solutions containing about 1% of xanthine oxidase (calculated from E_{450}). Copper analyses were carried out by the method of Eden & Green (1940).

Chemicals and buffer solutions. These were generally the same as those used previously (Bergel & Bray, 1959). Catalase was obtained from General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A., and activity measurements indicated a purity of the order of 5%; soy-bean trypsin inhibitor was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Pyrophosphate buffer for the enzyme assays was prepared by mixing $\text{Na}_4\text{P}_2\text{O}_7$ and $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$; m-phosphate buffer, pH 5.8, contained 0.2M- Na_2HPO_4 and 0.8M- KH_2PO_4 ; the other phosphate buffers were diluted from this solution.

Enzyme assays and stability experiments. The methods employed were similar to those of Bergel & Bray (1959). However, the xanthine oxidase assays, apart from those in connexion with the stability tests, were carried out at 305 m μ , as solutions of xanthine have a lower absorption at this wavelength than at the one normally used, i.e. 295 m μ . At 305 m μ the value of $\Delta\epsilon$ for the conversion of xanthine into uric acid is about half of the corresponding figure at 295 m μ (cf. Avis, Bergel & Bray, 1956). In some cases it was found convenient to carry out assays in 0.5 or 4.0 cm.

* Part 5: Bray, Malmström & Vännegård (1959).

Table 1. *Stability of xanthine oxidase preparations LXO-70 and LXO-116*

Losses of activity (with xanthine as substrate and oxygen as electron acceptor) of the two samples were compared in a series of parallel experiments at a concentration of approx. 0.1% xanthine oxidase, under the conditions indicated. The buffer tris maleate is 2-amino-2-hydroxymethylpropane-1.3-diol-maleic acid (Bergel & Bray, 1959).

Temp.	Duration	pH	Buffer	Additives	Activity remaining (%)	
					LXO-70	LXO-116
0-5°	19 days	7.0	0.08 M-Tris maleate	—	94	69
0	24 hr.	7.0	0.08 M-Tris maleate	0.001 M-Ascorbic acid	92	86
56	3 hr.	5.8	0.8 M-Phosphate	0.03 M-Salicylate 0.01 M-EDTA	74	56
56	3 hr.	6.2	0.01 M-Phosphate	0.03 M-Salicylate 0.5 mg. of catalase/ml.	70	33
56	3 hr.	6.2	0.01 M-Phosphate*	0.03 M-Salicylate	47	19
					Mixture†: 40	

* Xanthine oxidase solutions were dialysed for 18 hr. in separate bags against the same buffer solution (approx. 1000 vol.) at 0-5° before the experiment; activities are expressed as percentages of that after dialysis.

† Mixed after dialysis; total xanthine oxidase concentration was as for experiments on separate batches.

spectrophotometer cells in place of the usual 1.0 cm. type. For assays at low substrate concentrations it was important to start taking readings as soon as possible after starting the reaction; when combined enzyme samples were assayed, the xanthine oxidase solutions were therefore mixed before they were added to the buffered xanthine solution.

RESULTS

During ordinary storage in the refrigerator, xanthine oxidase preparation LXO-116 (in 0.01 M-phosphate buffer, pH 6.2, with 1 mM-salicylate) lost about 50% of its activity during 1 month, whereas with preparation LXO-70 (0.1 M-phosphate, pH 6.1, without salicylate) activity decreased at only about 5% per month. Data from a number of stability tests in which LXO-70 and LXO-116 were compared in parallel experiments are reported in Table 1; the latter sample lost activity at a higher rate than the former under all the conditions tried.

In an attempt to find differences other than of stability between the two xanthine oxidase samples, they were analysed for copper, this being a possible inactivating contaminant (Bergel & Bray, 1959). Both samples contained significant quantities of the metal, but the amounts were roughly equal, and corresponded to about 0.5 g. atom of copper/mole of xanthine oxidase in each case.

The effects of substrate and of enzyme concentration on the enzymic activity with xanthine as substrate and oxygen as electron acceptor were then studied (Fig. 1). It was found that the preparations differed markedly from one another, LXO-70 showing, at high enzyme concentrations, a decrease in specific activity, an increase in the xanthine concentration required for maximum activity and a broadening of the peak of the curve; these phenomena were not found with LXO-116. In dilute xanthine oxidase solution, LXO-70 gave maximum

Fig. 1. Effect of xanthine concentration on xanthine oxidase activity at 24° in 0.05 M-pyrophosphate buffer, pH 8.2. Relative specific activity = $k(\Delta E/\text{min.})$ (vol. in cell)/ (light path) (vol. of xanthine oxidase), where k is an arbitrary constant; different values of k were assumed for the different xanthine oxidase samples used in this experiment, but not for different dilutions of the same sample. Overall dilutions of xanthine oxidase were: $\blacktriangle$, 1 in 26; $\circ$, 1 in 260. All the curves are theoretical ones calculated by means of equation (9) of Hofstee (1955). The broken line was calculated with Hofstee's constants: K_s 2×10^{-5} , K_{ss} 10^{-3} , taking V_m 122 and assuming no inhibitor. The continuous lines were calculated with K_s 10^{-6} and K_{ss} 10^{-3} , assuming no inhibitor for LXO-116 and $[I]/K_i$ 50 and 500 for the upper and lower curves respectively for LXO-70; V_m 107 for LXO-116 and 150 for LXO-70.

activity at about 0.2 mM-xanthine, whereas for LXO-116 the corresponding value was about 0.05 mM.

Further data showing the decrease of the specific activity of LXO-70 at high enzyme concentrations with a low xanthine concentration are presented in Fig. 2. This figure also shows that addition of increasing amounts of LXO-70 to a fixed amount of LXO-116 resulted in a progressive decrease in the activity of the latter sample.

Experiments similar to those reported in Fig. 1 were carried out on a number of other xanthine oxidase samples at various stages of purification, including a buttermilk sample. In all cases the forms of the curves obtained were intermediate in shape between those given by LXO-70 and LXO-116. LXO-70 had been dialysed thoroughly about 9 months before the experiments reported above, but re-dialysis shortly before assay resulted in a sharpening in the peak of the curve, though the shape remained intermediate between its original form and that given by LXO-116. Dialysis of LXO-116 did not change the shape of its curve.

When samples of either LXO-70 or LXO-116 (without dialysis) were denatured by heating at 100° in 0.05M-pyrophosphate buffer, pH 8.2, and the supernatants were added to portions of LXO-116, some reduction of the activity of the latter sample occurred, the xanthine oxidase assay being performed at a low xanthine concentration.

The addition of trypsin inhibitor (final concentration approx. 0.1 mM) to a solution of xanthine oxidase did not result in any decrease in the activity of the enzyme.

Fig. 2. Effect of varying quantities of enzyme preparation LXO-70 on activity. Assays were carried out at 24° with 0.05 mM-xanthine in a total volume of 2.5 ml. with 0.05M-pyrophosphate buffer, pH 8.2. Δ, Relative specific activity of LXO-70 alone (calc. as in Fig. 1); ○, activity of a fixed amount of LXO-116 in the presence of added LXO-70, calculated as

$$\frac{(\text{Activity of mixture}) - (\text{activity of LXO-70 alone})}{(\text{Activity of LXO-116 alone})} \times 100.$$

The theoretical curve through the points was calculated by the use of equation (11) of Hofstee (1955), with K_s 10^{-6} , K_{ss} 10^{-3} and v 100, and taking values of $[I]/K_i$ appropriate to the dilution of LXO-70, on the same basis as for Fig. 1.

DISCUSSION

Kinetic experiments. The kinetic data are consistent with the presence of a competitive inhibitor of xanthine oxidase in LXO-70. Hofstee (1955) has studied the effects of substrate concentration on the activity of xanthine oxidase in the presence of competitive inhibitors; he could explain his results by equations obtained assuming the presence of two active sites in the xanthine oxidase molecule. The first of these was the primary substrate site; his inhibitors competed with xanthine for it and the Michaelis constant for xanthine at this site was K_s , 2×10^{-5} . Xanthine also combined with the second site, which may be the one normally used by the electron acceptor; the dissociation constant (K_{ss}) was 10^{-3} and this combination was the cause of the inhibition which occurs at high substrate concentrations.

The broken line in Fig. 1 was calculated for LXO-116 by using Hofstee's equation for xanthine oxidase in the absence of an inhibitor and taking his values for the constants; a good fit with the experimental data could not be obtained, whatever value of V_m was assumed. Other values of the constants were therefore tried, and it was found that if K_{ss} was left unchanged and K_s was taken as 10^{-6} , a much better fit was achieved. Accepting for the time being these latter values as the true constants for inhibitor-free xanthine oxidase, it was then assumed that an inhibitor was present in LXO-70. Hofstee's equation being used once again, various values of $[I]/K_i$ were tried until the best fit of the theoretical curves to the experimental data for this enzyme sample was obtained. As shown in Fig. 1, a satisfactory fit at both the xanthine oxidase concentrations at which assays were carried out was achieved. The value $[I]/K_i$ 1.3×10^4 for undiluted LXO-70, which was used in the calculations for Fig. 1, also fits further data on the activity of various concentrations of this xanthine oxidase batch at a fixed xanthine concentration (Fig. 2). Fig. 2 also shows that, on mixing the two enzyme samples, LXO-116 was inhibited to about the extent required on the basis of the above-mentioned theory.

The presence of an inhibitor in LXO-70 but not in LXO-116 thus gives, in so far as the experimental points in Figs. 1 and 2 fall on the theoretical curves, a satisfactory explanation of the results of the kinetic experiments. The discrepancy between the present value of K_{ss} , i.e. 10^{-3} , and that given by Hofstee (1955) could be explained by assuming that his enzyme contained an inhibitor similar to the one present in LXO-70. If his assay solutions had $[I]/K_i$ 19, the value of the 'apparent' Michaelis constant, $K_s (1 + [I]/K_i)$, would be equal to his value of 2×10^{-5} and his results would fall into line

with those presented above. Figures in the literature for the K_i value of xanthine oxidase vary widely (cf. Fridovich & Handler, 1958b), but the lowest recorded value of K_i , 0.9×10^{-6} (Lowry, Bessey & Crawford, 1949) is in good agreement with the present work. It therefore seems likely that the higher values recorded by a number of workers may have been an indication of the presence of inhibitors in their preparations. Two other points in the literature which could be explained on a similar basis are the variations of K_i from one xanthine oxidase preparation to another, reported by Booth (1938), and a flattening of the peak of maximum activity on the xanthine-activity graphs at high xanthine oxidase concentrations, which was observed by Dixon & Thurlow (1924).

Stability experiments. The differences of stability between LXO-70 and LXO-116, which remained in the presence of ethylenediaminetetra-acetic acid (EDTA) or of ascorbic acid, and the similarity of their copper contents suggested that trace-metal impurities were not responsible for the stability differences (cf. Bergel & Bray, 1959). Similarly, as catalase did not affect the relative stabilities the differences could not be due to hydrogen peroxide inactivation. The fact that mixing the batches resulted in a stability slightly nearer to that of LXO-70 than to LXO-116 (Table 1) suggested that the former might contain a protecting agent of some kind. Bergel & Bray (1959) showed that competitive inhibitors stabilized xanthine oxidase and, as the kinetic results discussed above showed that LXO-70 contained an inhibitor, it would have been reasonable to suppose that this was the protecting agent responsible for the stability differences. However, two difficulties remained to be explained before this hypothesis could be accepted. As shown in Table 1, the addition of an excess of salicylate, which is a known xanthine oxidase inhibitor-stabilizer, did not bring the stability of LXO-116 up to that of LXO-70. It is therefore necessary to postulate that the complex between the unknown inhibitor present in LXO-70 and the xanthine oxidase molecule is less sensitive to inactivation than is the salicylate-xanthine oxidase complex, which is in turn, of course, more stable than the free xanthine oxidase molecule. This assumption seems to be a plausible one as the inhibitor may be quite different chemically from salicylate and might combine with the active centre of xanthine oxidase in a different manner. Questions about the dialysability of the inhibitor are discussed below.

Nature of the inhibitor. The kinetic results give a value for $[I]/K_i$ for LXO-70, but not for K_i . However, it is possible to arrive at some idea of the magnitude of this constant by making an estimate of the value of $[I]$. The inhibitor is unlikely to be present in LXO-70 at above, say, 0.01M , and a

lower limit for it is set by the concentration of active xanthine oxidase in the sample, which is about $10\text{ }\mu\text{M}$. Hence the value of K_i probably lies between about 10^{-8} and 10^{-9} , and the inhibitor is thus quite a potent one, which would not be expected to be removed completely from the solution by ordinary dialysis. Although, as the kinetic results indicated, the concentration of inhibitor was substantially reduced by dialysis, it was apparently not completely eliminated; the stability experiments confirmed that a stabilizing factor remained in LXO-70 after dialysis. Thus all the experimental data could be explained satisfactorily on the assumption that the inhibitor and the stabilizer of LXO-70 were identical.

There is very little information about the chemical nature or the source of the inhibitor, but it seems that it must have been present in substantial amounts in LXO-70, and it may be significant that this sample had been subjected to prolonged storage at 5° . It was also apparently present in smaller quantities in a number of other xanthine oxidase preparations, including a buttermilk sample, whereas it was absent from LXO-116. (The reason for this absence is not clear, but it does not seem to have anything to do with the method used for the preparation of the sample, since other samples prepared in the same manner were much more stable.) Somewhat unexpectedly, however, an inhibitor was found in solutions obtained from LXO-116 after denaturation at 100° . It is possible that we may be concerned with more than one xanthine oxidase inhibitor, and it is perhaps tempting to speculate, in the absence of any direct evidence, that they may be peptides analogous to the well-known trypsin inhibitors (cf. Laskowski & Laskowski, 1954); however, their dialysability suggests a somewhat lower molecular weight. (Soybean trypsin inhibitor, which is capable of interacting with a number of proteolytic enzymes in addition to trypsin, was without inhibitory effect on xanthine oxidase.) If, as seems probable from these considerations, denaturation of the xanthine oxidase molecule can lead to the formation of an inhibitor, this could explain the break in the inactivation curves of xanthine oxidase at 56° observed by Bergel & Bray (1959). The rate of loss of activity would decrease as the inhibitor concentration increased and became unimolecular when sufficient of it was present to 'saturate' the remaining xanthine oxidase. Variations in the inhibitor concentration may account for many of the batch variations in xanthine oxidase stability which are not explained by previous work (Bergel & Bray, 1959).

It seems unlikely that any of the inhibitory effects reported in this paper could be due to the presence of 'inactive' xanthine oxidase molecules. Though not catalytically active, these might well

still be capable of substrate binding (cf. hypoxanthine binding by cyanide-treated xanthine oxidase, Fridovich & Handler, 1958*a*). However, in this case inhibition would be due to substrate-inhibitor rather than enzyme-inhibitor combination; this type of inhibition is important only when a sufficient number of inhibitor molecules are present to bind an appreciable proportion of the substrate molecules (Friedenwald & Maengwyn-Davies, 1954), and as the total xanthine oxidase concentration calculated from the E_{450} value did not exceed $1 \mu\text{M}$ in any of the assays, this type of inhibition may clearly be neglected in this work.

SUMMARY

1. The stabilities of two xanthine oxidase preparations have been compared under a variety of conditions; one of these preparations was found to be consistently more stable than the other.

2. Kinetic experiments indicated that the more stable sample contained a xanthine oxidase inhibitor; the presence of this could account for the observed stability differences.

3. Evidence bearing on the occurrence, formation and nature of the inhibitor are discussed briefly, and it is suggested that variations in its concentration may be responsible for certain discrepancies and anomalies in the properties of xanthine oxidase recorded in the literature.

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Arteriovenous $^{14}\text{CO}_2$ Differences and the Pentose Cycle in the Cow's Udder

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There is a large body of evidence that the pentose cycle occurs in the mammary gland. This evidence is based: (1) on studies of the conversion of different types of labelled glucose into CO_2 (Abraham, Hirsch & Chaikoff, 1954; Glock, McLean & Whitehead, 1956; Duncombe & Glascock, 1956; Abraham, Cady & Chaikoff, 1957; Black, Kleiber, Butterworth, Brubacher & Kaneko, 1957); (2) on measurement of glucose 6-phosphate and 6-phosphogluconate dehydrogenases (Glock & McLean,

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1953, 1954); (3) on the detection of some of the intermediate compounds of the pentose cycle in mammary tissues (Peeters & Debackere, 1956); and (4) on the determination of the distribution of ^{14}C in the 6 carbons of the glucose and galactose moieties of lactose (Schambye, Wood & Kleiber, 1957; Wood, Gillespie, Joffe, Hansen & Hardenbrook, 1958*a*; Wood, Joffe, Gillespie, Hansen & Hardenbrook, 1958*b*). However, Duncombe & Glascock (1956) have indicated that the direct oxidative pathway may not be as important in the udder of ruminants (sheep) as in the rat mammary