The Chemistry of Xanthine Oxidase

4. THE PROBLEMS OF ENZYME INACTIVATION AND STABILIZATION*

By F. BERGEL and R. C. BRAY
Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W. 3

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It was stated in previous papers (Avis, Bergel & Bray, 1955; Avis, Bergel, Bray, James & Shooter, 1956; Avis, Bergel & Bray, 1956) that the enzymic activity of purified xanthine oxidase from cow's milk, like that of other enzymes, deteriorates in aqueous solution under a variety of mild conditions. Since then we recorded in a preliminary communication (Bergel & Bray, 1956) the stabilizing effects of sodium salicylate on this activity and mentioned briefly during a symposium the inactivation of the enzyme by certain metals (Bergel & Bray, 1958).

We have now extended our studies of loss and preservation of the catalytic power of xanthine oxidase in order to establish optimum conditions for storage and utilization of the enzyme in biological experiments (cf. Bergel, Bray, Haddow & Lewin, 1957; Haddow, de Lamirande, Bergel, Bray & Gilbert, 1958; Bergel, 1959). These studies should also help in elucidating more rapidly the chemical nature of the enzyme and in its separation from various closely related inactive compounds.

In this paper we demonstrate that at least three inactivation mechanisms, namely interaction with metals, photo-oxidation and gross denaturation, are operative, and that some of the losses of activity can be counteracted by a number of chemicals.

EXPERIMENTAL

Materials

Xanthine oxidase solutions. Samples of xanthine oxidase, prepared from seven different batches of buttermilk, were used; some were prepared by the method of Avis *et al.* (1955), the purification being taken to stage M5, M6 or M7, while others were prepared by ammonium sulphate treatment (cf. Haddow *et al.* 1958). The samples had E_{280}/E_{450} ratios ranging from 5·4 to 10·7 and activity/E_{450} ratios of from 20 to 90. The ratios E_{280}/E_{450} and activity/E_{450} are referred to as 'PFR' and 'AFR' in previous publications (Avis *et al.* 1955). However, most of the experiments, apart from the work on activation by ethylenediaminetetra-acetate (EDTA) (applicable to one enzyme preparation only), and those concerning photo-oxidation at 37°, were carried out on more than one sample with very similar results.

* Part 3: Avis, Bergel & Bray (1956).

Chemicals. These were generally of A.R. or laboratory-reagent grade. Water was glass-distilled and then passed down a deionizing column. Phosphate and acetate buffers were prepared according to Green (1933), 2-amino-2-hydroxymethylpropane-1:3-diol–maleic acid (tris–maleate) buffer was prepared according to Gomori (1955), and pyrophosphate buffer (pH 8) according to Avis *et al.* (1955). Crystallized bovine-plasma albumin was obtained from Armour Laboratories Ltd., Eastbourne, Sussex, 'Tween 80' (T.B. culture grade) from Honeywill and Stein Ltd., London, and reduced diphosphopyridine nucleotide (DPNH) and sodium *p*-chloromercuribenzoate (*p*CMB) from Sigma Chemical Co., U.S.A. 1:4-Naphthaquinol was prepared by reducing the quinone with zinc dust and dilute hydrochloric acid, filtering hot and leaving to crystallize; it had m.p. 175–182°. Peracetic acid was prepared by the method of Greenspan (1946).

Methods

Enzyme assays. The activity of xanthine oxidase solutions was generally measured by following uric acid formation spectrophotometrically at 295 mμ with xanthine as substrate and oxygen as electron acceptor. The conditions used (0·05M-pyrophosphate buffer, pH 8·2; approx. 0·1 mM-xanthine; 24°) were those of Avis *et al.* (1955). The solutions from the stability experiments were usually diluted about 100-fold in the assays. The diaphorase-like activity of xanthine oxidase (Table 5) was also measured spectrophotometrically, by following the disappearance of DPNH at 340 mμ with ferricyanide as electron acceptor, under conditions recommended by V. Massey (personal communication), namely 0·2M-acetate buffer, pH 4·6 and 24°. DPNH (final concn. 0·17 mM) was added immediately before the enzyme with 1·2 mM-potassium ferricyanide. Significant reaction occurred without enzyme, and corrections were made for the 'blank' rate.

Stability experiments. Samples of the xanthine oxidase solutions were diluted with the buffer or other reagents and stored for the appropriate time in a bath at the temperatures indicated. The solutions generally had volumes of about 0·2 ml. in 5 mm. × 50 mm. test tubes with rubber caps. The concn. of xanthine oxidase in the solutions was calculated from E_{450} (cf. Avis, Bergel & Bray, 1956). Xanthine oxidase activity was measured on samples taken immediately after making up the mixtures and then on further samples, after appropriate periods of incubation, and results were generally expressed as a percentage of the activity found immediately after mixing. For experiments on copper inactivation in the cold, the $CuSO_4$ was

added last, all solutions and pipettes being pre-cooled. For the experiment under nitrogen reported in Fig. 8*a* and *b*, the enzyme solution was stored in a shallow layer under the gas for 24 hr. in the cold, then filled into glass capillaries (melting-point tubes) in a glove cabinet filled with nitrogen. The tubes were sealed at each end inside the box with putty (Belco cellulose putty, I.C.I. Ltd.), then placed in a blackened test-tube immersed in the water bath. One capillary was removed and opened for the initial and each subsequent assay.

Theory of stabilization by a competitive inhibitor. A competitive inhibitor might be expected to protect an enzyme by combining with the active centre to form a complex whose molecules decompose at a lower rate than do those of the free enzyme. If certain simplifying assumptions are made this effect can readily be calculated, and the theoretical curve in Fig. 9 for xanthine oxidase stabilization by salicylate was drawn from the equation given below, the values of the constants being obtained by trial and error. To obtain the equation it is assumed that the usual Michaelis conditions apply (i.e. the enzyme concentration is low) and that for both, the combined and free enzyme, the proportion which remains unchanged at the end of a period of heating is independent of their initial concentrations. It can then be shown that

$$A_c = A_\infty\left[\frac{1}{1+K/C}\right]+A_0\left[1-\frac{1}{1+K/C}\right],$$

where C is the concentration of the inhibitor and K is the dissociation constant of the complex that it forms with the enzyme. A is the fraction of the enzyme activity which remains at the end of the heating period. (This is measured after considerable dilution of the enzyme so that competitive inhibition does not interfere with the activity measurements.) The subscripts $_0$, $_c$ and $_\infty$ refer respectively to experiments carried out with the inhibitor at zero concentration, at concentration C and at a concentration sufficiently high to 'saturate' the enzyme.

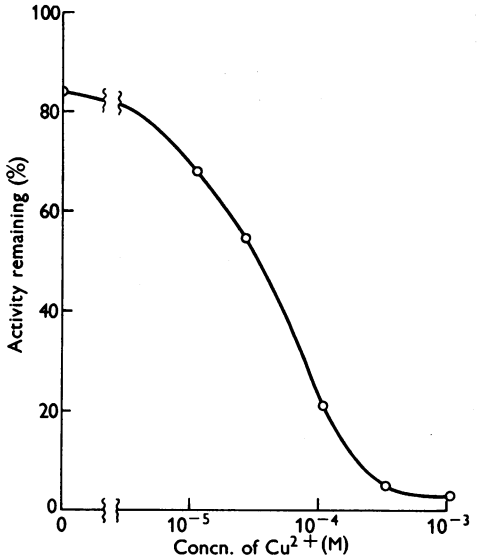

Fig. 1. Effect of various concentrations of cupric sulphate on xanthine oxidase. Samples were heated for 2·5 hr. at 37° in mM-acetate buffer, pH 5; activities are expressed as a percentage of that in an unheated sample without copper; the concentration of xanthine oxidase was 0·1%.

RESULTS

Inactivation by copper and other metal-containing reagents

We have reported before on inactivation of xanthine oxidase by copper, mercury, lead and silver salts (Bergel & Bray, 1958).

Philpot (1938) had already studied the effect of Cu^{2+} ions on xanthine oxidase, and an experiment carried out by us confirmed the sensitivity of our enzyme preparations to this metal (Fig. 1). As with hydrogen peroxide treatment and photo-oxidation at 37°, as described below, loss of enzymic

Table 1. *Loss of activity of xanthine oxidase in the presence of* Cu^{2+} *ions*

The solutions were incubated for 24 hr. at 0° with 0·1% xanthine oxidase and 0·1 mM-$CuSO_4$.

pH	Buffer (M)				EDTA (mM)	Activity remaining (%)
	Acetate	Tris–maleate	Pyrophosphate	Phosphate		
5·0	0·1	—	—	—	—	98
5·0	0·1	—	—	—	10	143
5·0	—	0·1	—	—	—	108
5·0	—	0·1	—	—	10	103
4·8	—	—	0·1	—	—	107
6·1	—	0·1	—	—	—	100
5·9	—	—	0·1	—	—	88
5·8	—	—	—	0·8	—	27
6·2	—	—	—	0·1	—	0
6·2	—	—	—	0·1	10	95
6·9	—	0·1	—	—	—	22
6·9	—	0·1	—	—	10	102
7·4	—	—	0·1	—	—	97
7·3	—	—	—	0·1	—	0
7·3	—	—	—	0·1	10	97
8·3	—	0·1	—	—	—	99
8·1	—	—	0·1	—	—	99

function in presence of copper took place without any visible change in the appearance of the clear reddish brown xanthine oxidase solutions, but obviously the activity/E_{450} ratio decreased. The reaction was found to be very sensitive to pH and to the nature and concentration of the buffers. This is illustrated by data shown in Table 1. Inactivation occurred only in the region of pH 6–7 at which denaturation at 56° was at a minimum (cf. Fig. 10). Pyrophosphate buffer or EDTA afforded virtually complete protection under all conditions tested, while with phosphate buffer the stability was greater at a high buffer concentration than at a low one. With the particular sample of xanthine oxidase used for these experiments (activity/E_{450} 20, E_{280}/E_{450} 7·4), EDTA in acetate buffer, at pH 5, produced a significant increase of activity over and above the activity of the sample before addition of the metal. This result was reproducible, but could not be observed with other samples of xanthine oxidase. The explanation for this may be that this enzyme concentrate had accidentally become contaminated with copper during dialysis against water with a high conductivity. That EDTA under suitable conditions reversed inactivation by added Cu^{2+} ions is shown in Fig. 2.

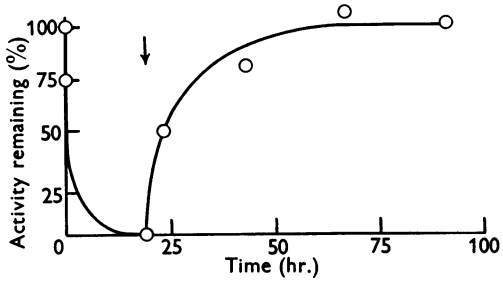

Fig. 2. Reversal of Cu^{2+} inactivation by EDTA. Xanthine oxidase (0·1%) in 0·1 M-phosphate buffer, pH 6·2, was treated at 0° with 0·1 mM-$CuSO_4$ at zero time. The first point represents the activity before adding Cu^{2+} and the second that immediately after the addition. After 19 hr., the sample was diluted by the addition of an equal volume of acetate buffer containing EDTA (final concentration 0·5 and 0·01 M respectively). The final pH was 5·0–5·2.

The protective effects of EDTA, sodium salicylate and sodium benzoate against copper inactivation at 5° and pH 7 are shown in Fig. 3: while salicylate and, to a lesser degree, benzoate reduced the rate of inactivation, EDTA prevented it completely. Salicylate, even at 37°, as shown in Table 2, could protect the enzyme completely against the effects of lower concentrations of copper.

Ascorbic acid at pH 7 markedly increased the rate of inactivation by copper, as can be seen in Table 3. EDTA cancelled this effect.

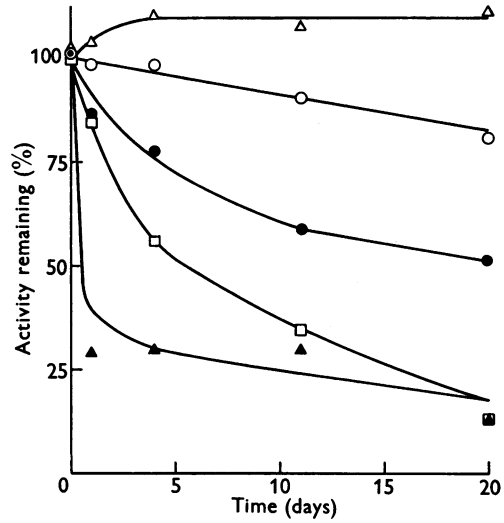

Fig. 3. Protection against copper inactivation at 5°. Xanthine oxidase (0·1%) in 0·04 M-tris–maleate buffer (pH 7·0) was treated with 0·1 mM-$CuSO_4$ (apart from control without copper). The samples were as follows: ○, control without copper or additive; △, 0·01 M-EDTA; ●, 0·01 M-salicylate; □, 0·04 M-benzoate; ▲, no additive.

Table 2. *Effect of salicylate on copper inactivation of xanthine oxidase*

The solutions were incubated for 24 hr. at 37° with 0·1% xanthine oxidase in 0·8 M-phosphate buffer, pH 5·8.

Concn. of $CuSO_4$ (mM)	Concn. of salicylate (mM)	Activity remaining (%)
—	—	53
—	1	76
0·03	—	14
0·03	1	77

Table 3. *Effect of ascorbate on copper inactivation of xanthine oxidase*

The solutions were incubated for 24 hr. at 0° with 0·1% xanthine oxidase in 0·1 M-tris–maleate buffer, pH 7.

Concn. of $CuSO_4$ (mM)	Concn. of ascorbic acid (mM)	Activity remaining (%)
0·1	—	43
0·1	1	4*
—	1	80
0·1	1 (EDTA 10 mM)	97

* Activity (%) before adding the reagents, rather than after, as the activity fell very rapidly on adding Cu^{2+} ions.

Table 4. *Effects of redox buffers on copper inactivation of xanthine oxidase*

The solutions were incubated for 3 hr. at 0° with 0·1% xanthine oxidase in 0·04M-tris–maleate buffer (pH 7), containing 0·04 mM-$CuSO_4$ and 0·3% of cetyltrimethylammonium bromide (Cetavlon) (to increase the solubility of the redox pairs).

Redox pair (each mM)	E'_0	Activity remaining (%) Without Cu*	With Cu†
Nil	—	105	64
Quinone–quinol	+0·28	112	57
Naphthaquinone–naphthaquinol	+0·04	107	26
Dehydroascorbic–ascorbic acid	+0·06	94	25

* Relative to control without $CuSO_4$ and Cetavlon.
† Remaining activity as percentage of that in previous column.

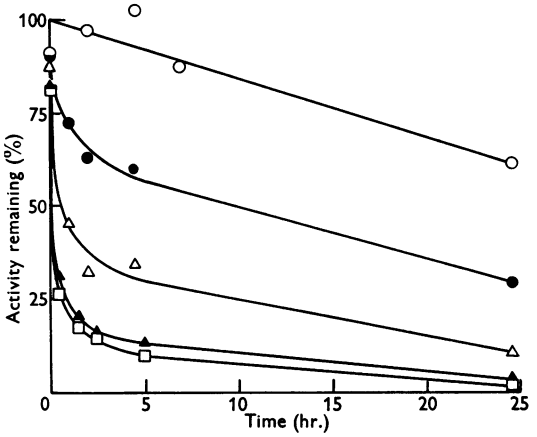

Fig. 4. Inactivation curves with varying concentrations of copper. Decomposition of xanthine oxidase (0·1%) at 37°, in 0·1-M-tris–maleate buffer, pH 6·9. Activities are expressed as a percentage of the extrapolated value at zero time for the control without copper; the first assay was carried out immediately after mixing at 20°. Concentrations of $CuSO_4$ were as follows: ○, nil; ●, 10 μM; △, 25 μM; ▲, 0·1 mM; □, 0·25 mM.

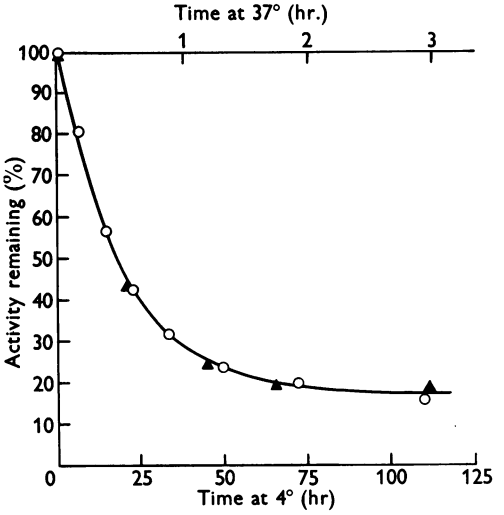

Fig. 5. Copper inactivation at 4° and 37°. Xanthine oxidase (0·1%) was incubated in 0·8M-phosphate buffer (pH 6), with 0·1 mM-$CuSO_4$. Activities are expressed as a percentage of those before adding copper. ○, Solution at 37°; ▲, solution at 4°. The activities of controls without copper were: 94% after 112 hr. at 4° and 87% after 3 hr. at 37°.

That the effect of ascorbic acid was due to reduction of the metal to the Cu^+ state, which must be therefore a more potent inactivator than the Cu^{2+} ion, was demonstrated in experiments with redox buffers (Table 4). Cu^{2+}/Cu^+ has $E'_0 + 0{\cdot}16$ (Anderson & Plaut, 1949). It is thus not surprising that dehydroascorbic–ascorbic acid and 1:4-naphthaquinone–1:4-naphthaquinol, both having lower and very similar redox potentials (cf. Anderson & Plaut), should produce a greater loss of activity, in contrast with benzoquinone–quinol, which would be expected to maintain the metal in its higher-valency state.

The kinetic aspects of the inactivation reaction by copper are illustrated in Figs. 4 and 5. Fig. 4, showing the effects of different concentrations of metal on the course of reaction, discloses the existence of rapid and slow phases. The extent of the former depended on the concentration of copper employed, while the latter was not influenced by the metal. A change of temperature from 37° to 4° reduced the rate of the first phase of reaction 40-fold (Fig. 5) without affecting the activity remaining at the end of this phase.

In order to confirm that inactivation by copper was taking place during the incubation period and not in the assay, the effect of varying the volume of the xanthine oxidase–copper mixture used in the assay, after 24 hr. incubation in 0·8M-phosphate (pH 6), with 0·1 M-$CuSO_4$, was studied. The activities were found to be the same whether 50-, 100- or 250-fold dilution was employed for the assay.

Treatment of xanthine oxidase with copper, like many other procedures for destroying the

purine oxidase activity of the enzyme (Avis, Bergel & Bray, 1956; Bergel & Bray, 1958), had no effect on the activity towards DPNH as substrate. This is illustrated by the data in Table 5; these were obtained by measuring the diaphorase-like activity with ferricyanide as electron acceptor, a procedure devised by V. Massey (personal communication) which gives considerably higher absolute activities than do other methods (cf. Avis, Bergel & Bray, 1956). In fact the absolute activities in the two assay systems in the experiment reported in Table 5 were approximately equal before the copper was added.

Table 5. *Effect of copper on diaphorase activity of xanthine oxidase*

The solutions were incubated for various times at 0–2°, with 0·1% xanthine oxidase in 0·06M-tris–maleate buffer (pH 7·0), with 0·1 mM-$CuSO_4$.

Time (hr.)	Xanthine–O_2 activity (%)	DPNH–$Fe(CN)_6^{3-}$ activity (%)
0·3	92	98
2·3	77	100
27	55	96
432	39	100

We have studied the effects of sodium *p*-chloromercuribenzoate (*p*CMB) in the absence of substrate, and obtained results which indicated a lower reactivity of crystallized xanthine oxidase towards this reagent than was observed by either Harris & Hellerman (1956) or Fridovich & Handler (1958*a*). No change in the visible colour of xanthine oxidase solutions resulted from our treatment with *p*CMB. The enzyme differed in this respect from succinic dehydrogenase (Massey, 1958). We have not been able to reverse *p*CMB inhibition of xanthine oxidase after prolonged incubation, with either EDTA or glutathione.

Fig. 6 shows that with our highly purified preparation of xanthine oxidase nine equivalents of *p*CMB (expressed as a number of SH groups reacting, calculated from $\Delta\epsilon_{250\ m\mu}$ $7{\cdot}6 \times 10^3$; Boyer, 1954) combined without causing any loss of activity of the enzyme. This contrasts with experiments by Fridovich & Handler (1958*a*) in which eight equivalents, in the absence of the substrate (as in our experiments), produced considerable inactivation.

Our results in Fig. 7 show that with a large amount of the organo-metal derivative ([*p*CMB]/[xanthine oxidase] = 105) substantial inactivation

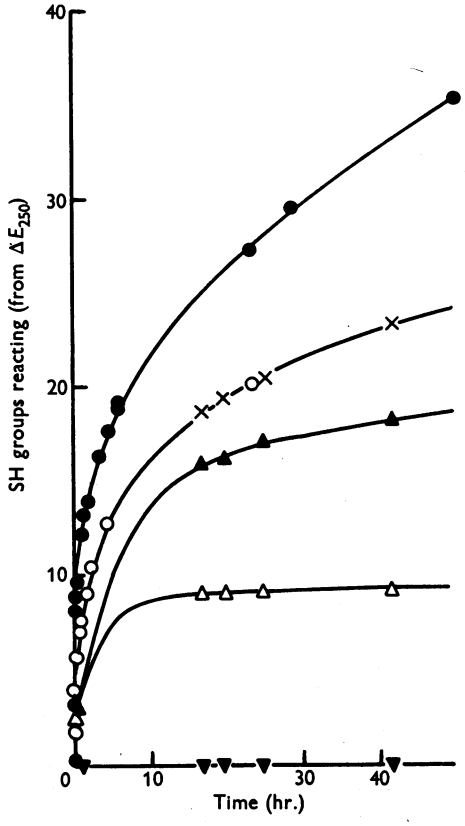

Fig. 6. Reaction of SH groups of crystallized xanthine oxidase with varying molar proportions of *p*CMB. A constant xanthine oxidase concentration of 0·075% (i.e. 2·5 μM) in 0·1M-pyrophosphate buffer (pH 8) was used at 24°. ▼, Xanthine oxidase alone; △, [*p*CMB]/[XO] = 9; ▲, [*p*CMB]/[XO] = 17; ○, ×, [*p*CMB]/[XO] = 26 (two expts.); ●, [*p*CMB]/[XO] = 105. Activity figures (relative to controls containing no *p*CMB, after 20–70 hr.) were respectively 100, 84, 46 and 4% at [*p*CMB]/[XO] ratios of 9, 17, 26 and 105.

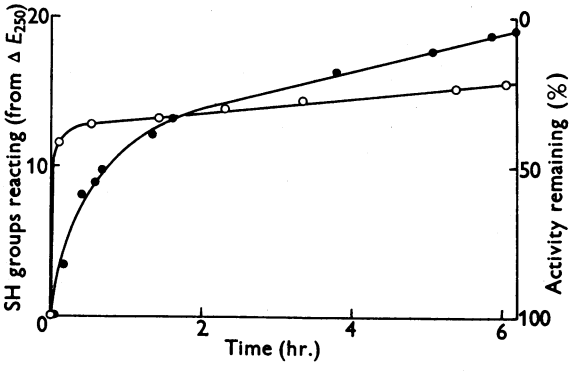

Fig. 7. Number of SH groups reacting and activity, with [*p*CMB]/[XO] = 105. The data on the number of groups reacting is the same as that in Fig. 6. The activity was calculated relative to a control without *p*CMB. The reaction mixture was diluted to a *p*CMB concentration of 10 μM for the activity measurements; the extent of dilution before assay was not critical. ○, Percentage activity (ordinate on right); ●, SH groups reacting.

occurred when the changes in extinction indicated that only a very few SH groups could have reacted.

Inactivation by hydrogen peroxide, peracetic acid and by photo-oxidation at 37°

Hydrogen peroxide and peracetic acid. It has been reported by Siegel & Weintraub (1952) that their relatively crude and dilute samples of xanthine oxidase were substantially inactivated by H_2O_2, even at concentrations of the reagent as low as 0·1 mM. In contrast, we have found that purified xanthine oxidase in relatively concentrated solution at 0–5° was much more resistant to the oxidizing agent. Table 6 shows that even with M-H_2O_2 the inactivation process took several hours, while 0·01 M-H_2O_2 had little effect unless the enzyme was diluted considerably. As the inactivation with M-H_2O_2 did not produce precipitation or change of colour, we have used this procedure to prepare an inactive form of xanthine oxidase (cf. Bergel & Bray, 1958). On the other hand, peracetic acid could not be used for this purpose because, although mM-solutions of the reagent gave rapid inactivation, this was accompanied by loss of colour, indicating a breakdown of the xanthine oxidase molecule.

Photo-oxidation at 37°. Although storage of enzyme solutions for two days at 37°, without access to air or under nitrogen or in complete darkness, caused some loss of their activity, both oxygen and light had a marked accelerating influence on the inactivation process (Table 7). This was not accompanied by any significant precipitation of denatured protein. With larger volume samples the fall in activity slowed down, or even stopped, after some hours unless the solutions were stirred to increase the access of air.

Table 6. *Effect of hydrogen peroxide on xanthine oxidase*

The solutions were incubated for various times at 0–5° with the reagent, in M-phosphate buffer, pH 6.

Concn. of H_2O_2 (M)	Xanthine oxidase (%)	Time (hr.)	Activity remaining (%)
0·01	0·4	53	92
0·01	0·04	53	75
0·01	0·004	53	7
1·0	0·4	—	84
1·0	0·4	1·5	38
1·0	0·4	19	0

Thermal denaturation at 56°

It has been mentioned above that xanthine oxidase solutions could lose their activity without precipitation or other changes in the physicochemical properties of the metalloflavoproteins (cf. Avis, Bergel, Bray, James & Shooter, 1956) but with a decrease in activity/E_{450} ratio. However, when inactivation was carried out at 56°, an arbitrarily chosen temperature high enough to achieve a speedy loss of activity, this process was largely one of denaturation. Thus when a sample of xanthine oxidase at a concentration of 0·9% was kept at this temperature in 0·1 M-phosphate buffer (pH 6) for 4·5 hr., considerable precipitation occurred and the suspension retained 57% of the original activity. The mixture was cooled and, in order to remove any free flavin derivatives, was dialysed at 5°, side by side with, as a control, a sample of the starting solution which had been diluted with buffer to give the same activity as the heated sample. After centrifuging, the supernatant from the heated and unheated samples had activity/E_{450} of 52 and 55 respectively. This meant that, while the overall activity of the heated sample had fallen by 43%, its activity/E_{450} ratio had decreased by only 6%. Thus almost all of the activity loss was attributable to gross denaturation, which was accompanied by removal of material absorbing at 450 mμ, either by centrifuging or by the dialysis.

The course of the inactivation reaction at 56° is shown in Figs. 8*a* and *b*. Under a variety of conditions the activity was found to fall rather rapidly by 20–35% on placing the solutions in the thermostat; after this the rate of loss of activity decreased, and subsequently the inactivation process followed unimolecular kinetics fairly closely. In

Table 7. *Loss of xanthine oxidase activity at 37°*

The solutions were incubated for 48 hr. under ordinary laboratory lighting conditions (except for the second experiment), with 0·6% xanthine oxidase, in M-phosphate buffer, pH 6.

Conditions		Vol. of xanthine oxidase (ml.)	Activity remaining (%)
Standard	With air above solutions	0·2	10
Wrapped in aluminium foil	With air above solutions	0·2	39
Standard	With air above solutions	1·0	29
No air space	With air above solutions	1·0	74
Oxygen	First bubbled through buffer, then passed over samples for rest of time	0·5	32
Nitrogen	First bubbled through buffer, then passed over samples for rest of time	0·5	85

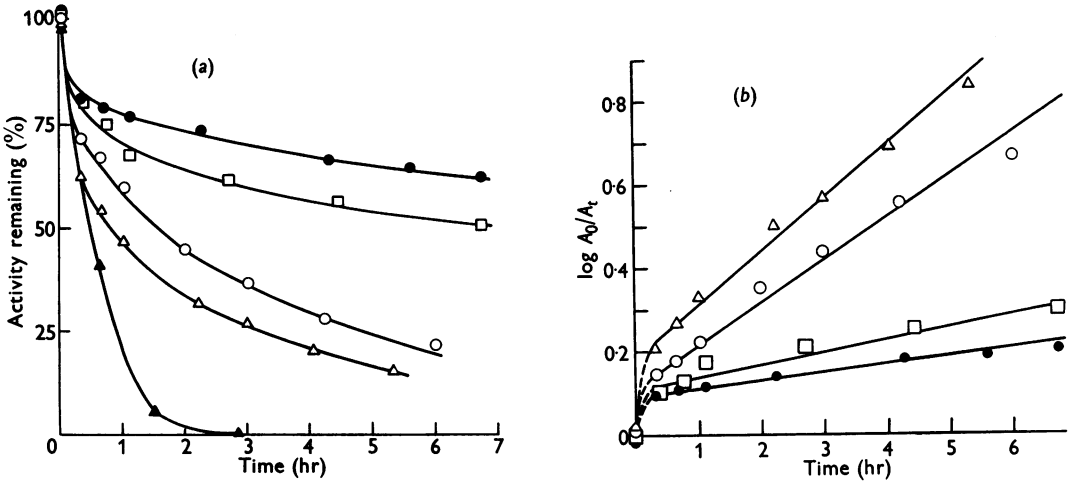

Fig. 8. Decomposition of xanthine oxidase at 56°. The concentration of xanthine oxidase was 0·4% in 0·1M-phosphate buffer (pH 6·0) containing 0·1M-NaCl. A_0/A_t = Activity at start/activity at time t. ○, In darkness, under N_2, with 0·01M-EDTA; all other samples were without exclusion of air and under ordinary laboratory lighting conditions; ▲, no additions; △, 0·01M-EDTA; □, 0·01M-EDTA and 0·05M-sodium benzoate; ●, 0·01M-EDTA and 0·01M-sodium salicylate.

Table 8. *Loss of xanthine oxidase activity at* 56°

The concn. of xanthine oxidase was 0·1% for the four experiments, which were carried out at different times on the same enzyme preparation.

Buffer	Additives	Activity remaining (%)
Incubated for 15 hr., at pH 7, with 0·03M-salicylate		
0·1M Phosphate	—	37
0·5M Phosphate	—	43
0·9M Phosphate	—	50
0·1M Phosphate	1·1M-$(NH_4)_2SO_4$	47
0·1M Phosphate	1·1M-NaCl	33
0·1M Phosphate	3·2M-NaCl	21
Incubated for 1 hr., at pH 6		
0·1M-Tris–maleate	—	74
0·1M-Acetate	—	67
0·1M-Phosphate	—	58
0·1M-Pyrophosphate	—	55
Incubated for 3 hr., at pH 7		
0·9M-Phosphate	—	32
0·9M-Phosphate	0·01M-Sulphosalicylate	29
0·9M-Phosphate	0·01M-Gentisate	32
0·9M-Phosphate	0·01M-*p*-Aminosalicylate	46
0·9M-Phosphate	0·01M-Benzoate	51
0·9M-Phosphate	0·01M-Salicylate	83
Incubated for 3 hr., at pH 7		
0·9M-Phosphate	—	24
0·9M-Phosphate	0·01M-EDTA	40
0·9M-Phosphate	0·01M-Salicylate	63
0·9M-Phosphate	0·01M-Salicylate; 0·01M-EDTA	77
0·9M-Phosphate	0·04M-Benzoate	51
0·9M-Phosphate	0·04M-Benzoate; 0·01M-EDTA	75

other experiments, which are not reported in detail, decomposition curves of the same general shape as those in Figs. 8*a* and *b* were obtained with a number of different samples of xanthine oxidase. The form of these inactivation curves remained unchanged at various concentrations of xanthine oxidase, although diluting the enzyme resulted in an increased rate of decomposition (cf. Fig. 11). Also partial inactivation at 56° of the sample, discussed at the beginning of this section, with subsequent dialysis and centrifuging, did not produce a disappearance of the characteristic initial rapid drop in the activity of the solution (cf. Figs. 8*a* and *b*).

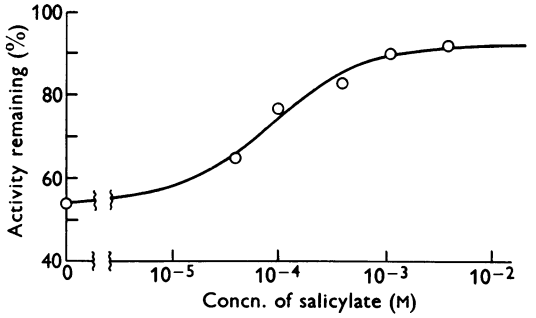

Fig. 9. Decomposition of xanthine oxidase with varying concentrations of sodium salicylate, during 1 hr. at 56°, in 0·9 M-phosphate buffer, pH 6. The theoretical curve through the experimental points was calculated assuming a dissociation constant for the xanthine oxidase–salicylate complex of 80 μM (see Experimental).

Table 8 shows the effect of inorganic salts and of various other compounds on the stability at 56°, at pH 6 or 7. The effects of salts were relatively small, though in the presence of sodium salicylate high concentrations of phosphate had a slight stabilizing effect, while sodium chloride seemed to decrease stability somewhat. Salicylate increased the stability at 56° substantially; sodium benzoate and *p*-aminosalicylate also had some effect, but sulphosalicylate and gentisate were without effect. EDTA had a slight protecting action and this was to some extent additive to that given by salicylate or benzoate.

The effect of varying the concentration of salicylate on the stability of xanthine oxidase is illustrated in Fig. 9, and that of pH in Fig. 10. The region of optimum stability was pH 6–8 in pyrophosphate buffer or in tris–maleate buffer, with or without salicylate. The latter had little stabilizing action in tris–maleate, though it was effective in pyrophosphate.

As expected, decomposition was more rapid in dilute solutions than in concentrated solutions of the enzyme (Fig. 11). Maintaining a constant protein concentration by adding albumin, or introducing a non-ionic detergent (Tween 80), did not affect the results of such experiments.

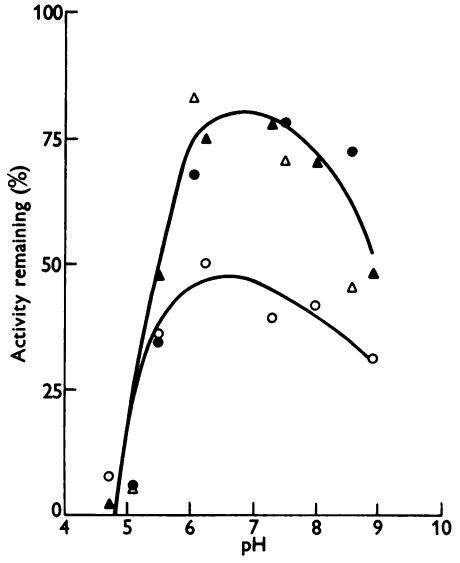

Fig. 10. Effect of pH on decomposition of xanthine oxidase during 1 hr. at 56°. ○, 0·1 M-Pyrophosphate buffer; ▲, 0·1 M-pyrophosphate with 0·03 M-salicylate; △, 0·1 M-tris–maleate buffer; ●, 0·1 M-tris–maleate buffer with 0·03 M-salicylate.

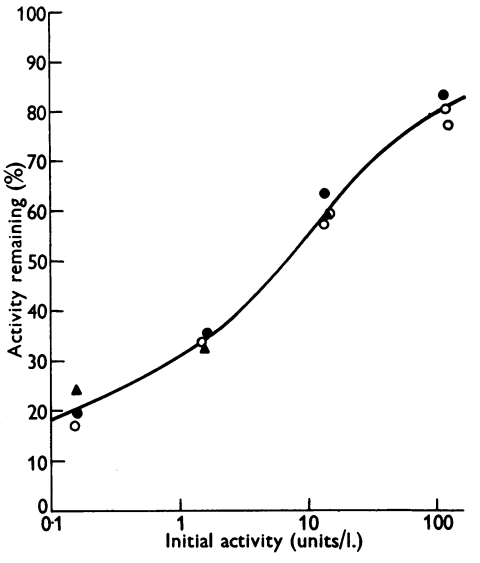

Fig. 11. Effect of enzyme concentration on decomposition during 1 hr. at 56° in 0·1 M-pyrophosphate buffer, pH 8. ○, No additions; ▲, solutions containing 1% of albumin; ●, solutions containing 0·05% of Tween 80. (For definition of activity units, see Avis *et al.* 1955.)

DISCUSSION

Metal inactivation

The experiments carried out with copper give no definite information on the groups of the xanthine oxidase molecule which interact with the metal. However, the facts that the inactivation is reversible under some conditions, at least, by EDTA, and that the Cu^{+} ion is a more potent inactivator than the Cu^{2+} ion, suggest a reaction with a sulphur-containing group of the enzyme. Harris & Hellerman (1956) have proposed, after experiments with *p*CMB and other reagents, that xanthine oxidase has SH groups essential for its activity. Fridovich & Handler (1958*a*, *b*) found that *p*CMB inactivated the enzyme much more rapidly in the presence than in the absence of hypoxanthine, and from this and other results concluded that the active site of xanthine oxidase may contain a metal (Fe or Mo) mercaptide group which was converted by the substrate into an SH group and the reduced form of the metal (cf. Bray, Malmström & Vänngård, 1959). In our experiments with crystallized xanthine oxidase and *p*CMB there was very little correlation between inhibition and the apparent number of SH groups blocked. It seems that either the reagent was inactivating by reaction with a non-sulphur-containing group (cf. Rabinovitch & Barron, 1955) or, if one accepts the presence of a metal mercaptide, that high concentrations at least of *p*CMB could compete with the metal of the enzyme for the sulphur in the absence of the substrate. In the latter case, the mechanism of copper reaction with xanthine oxidase was possibly analogous to that with *p*CMB. Consequently the failure to attain complete interaction with copper under some of our conditions (Figs. 4, 5) might have been due to an equilibrium state between active enzyme with iron or molybdenum mercaptide groups and inactivated enzyme containing copper mercaptides.

The effects of the inorganic salts and other reagents on copper inactivation probably reflected changes in the availability of the inactivating metal ion which was partially removed by 'complexing' or chelation.

Whereas in our experiments ascorbic acid alone caused only slight inactivation (Table 3), Feigelson (1952) reported on the inhibitory effect of this vitamin on his xanthine oxidase concentrate. In view of our own observations and those by Mapson (1946) on urease and Ito & Abe (1953) on β-amylase, it seems logical to conclude that the phenomenon reported by Feigelson was due to reduction of traces of copper present in his sample to Cu^{+} ions, which then inactivated his xanthine oxidase preparation.

Oxidative inactivation

Inactivation by hydrogen peroxide, formed during the enzymic reaction, has been commented on previously (e.g. Dixon, 1925).

The mechanism of inactivation by added hydrogen peroxide in our experiments may have been analogous to that occurring at 37°, which was essentially a photo-oxidation. In both these reactions the enzyme lost its activity, but remained almost entirely in solution without any substantial decrease in E_{450}. Other workers have reported on the inactivation of xanthine oxidase in homogenates or crude preparations by oxygen (Stadie & Haugaard, 1945; Mann & Quastel, 1946) and by light (Siegel & Weintraub, 1952). The photo-oxidation of a number of other enzymes has been studied in some detail by Polis & Shmukler (1953), Weil, James & Buchert (1953) and Weil & Seibler (1955). We found no evidence suggesting that the photo-oxidation process could be reversed *in vitro*, though reversal *in vivo* may be possible (cf. Haddow *et al.* 1958). Whether photo-oxidation liberates any of the co-factors from xanthine oxidase or reduces the firmness of their binding to the protein is a question which has not yet been investigated.

The temperature coefficient of a photo-oxidation reaction would be expected to be lower than that of a thermal denaturation; hence it was not surprising that at 56° the latter reaction, which was accompanied by precipitation of the enzyme protein and by liberation of the flavin, predominated.

Stabilization by competitive inhibitors and metal-binding agents

The phenomenon of protection of enzymes by competitive inhibitors may be general and has been studied in detail with D-amino acid oxidase by Burton (1951), Vietch & McComb (1956) and Klein (1957). Sodium salicylate was a competitive inhibitor of xanthine oxidase with K_i 0·135 mM (Bergel & Bray, 1956). The similarity between this value and the dissociation constant for the postulated xanthine oxidase–salicylate complex, as calculated from the stability experiment at 56° reported in Fig. 9, namely 0·08 mM, might be taken as evidence that the salicylate did in fact stabilize by combining with the enzyme. However, the temperature, pH and salt concentration at which the two sets of data were obtained were substantially different; also there is some uncertainty as to the exact Michaelis constant of xanthine oxidase. An alternative possibility is that salicylate might stabilize, particularly in the experiments at 56°, by chelating trace-metal impurities; but the observation that this compound still had a beneficial effect even in the

presence of the much more powerful chelator, EDTA (Figs, 8*a*, *b*; Table 8), provided additional evidence that some of the effects of salicylate were due to enzyme–stabilizer interaction. On the other hand, in the experiments on copper inactivation, it is probable that salicylate was acting predominantly by chelation (i.e. inactivator–stabilizer interaction), as it could with advantage be replaced by EDTA under these conditions (Fig. 3). In the studies reported by Bergel & Bray (1956), salicylate stabilized the enzyme at 37°, but EDTA was without effect. The latter result indicates that trace-metal inactivators were absent in those early experiments; it thus seems that, as in thermal denaturation at 56°, salicylate stabilized the enzyme against photo-oxidation at 37° by combination with it.

The other two substances found to stabilize the enzyme in the present experiments were sodium benzoate and *p*-aminosalicylate. The latter has been reported by Di Fonzo (1952), to be an inhibitor of xanthine oxidase, and we have established the inhibitory action of benzoate, with K_i 1·5 mM measured under the conditions used previously and assuming the same K_s (Bergel & Bray, 1956). Thus the action of these two substances was probably entirely analogous to that of sodium salicylate, though the higher dissociation constant of the benzoate meant that it had to be employed in higher concentrations than salicylate to obtain comparable stabilization (Table 8). The anti-copper action of benzoate of course depended, not on chelation, but possibly on a low dissociation of the copper benzoate [cf. silver benzoate (Bjerrum, Schwarzenbach & Sillén, 1957)]. Sulphosalicylate and gentisate had no effects in these experiments, although we reported previously (Bergel & Bray, 1956) on their low stabilizing action at 37°.

Fried (1958) has reported that inorganic phosphate could exert both protecting and restoring actions on xanthine oxidase activity. Our experiments at 56° (Table 8) confirmed that phosphate exerted a slight protecting action, and preliminary experiments suggested that it might have a greater effect on the reaction at 37°, though this finding awaits confirmation.

Kinetics of thermal inactivation

The reason why the inactivation reaction at 56° did not follow strictly unimolecular kinetics is not clear. Prostatic acid phosphatase at 50–56° (London, Wigler & Hudson, 1954) and carboxypeptidase at 45–55° (Labouesse, 1957) have both been reported to decompose according to unimolecular kinetics. The data in Figs. 8*a* and *b*, however, suggest that the characteristic break in the curves was not due to a superimposition of photo-oxidation or metal inactivation on the thermal-denaturation reaction. A possible explanation of the phenomenon relates to the formation of an unknown inhibitor–stabilizer during denaturation of xanthine oxidase (R. C. Bray, unpublished work).

Surface denaturation seemed unimportant in the decomposition of xanthine oxidase at 56°, as albumin and a non-ionic detergent, which have been found to protect enzymes sensitive to surface denaturation at low concentrations, afforded no protection (Fig. 11) (cf. Labouesse, 1957; Tsuboi & Hudson, 1955).

Conditions for purification, storage and utilization

In the handling and storage of solutions of xanthine oxidase, one has to guard against all three inactivation mechanisms, namely inactivation by trace-metal contaminants, photo-oxidation and gross denaturation. In addition to these reactions, it is also apparently possible for the flavin to be split off without precipitation of the apo-enzyme under certain conditions (cf. Morell, 1952), though we have not studied this reaction. The evidence presented above shows that it should be possible to obtain some control over the three reactions by suitable additions of chelating agents and competitive inhibitors, and by protecting solutions of xanthine oxidase from oxygen and light.

Sodium salicylate is a particularly useful stabilizer, as it combines chelating and inhibitory properties. The value of its K_i is such that above concentrations of about mM it gave maximum stabilization (Fig. 9), while below concentrations of about 0·1 mM it did not cause significant inhibition in the ordinary activity assays. Enzyme solutions, stabilized by salicylate, could thus be assayed without prior dialysis, dilution alone being adequate to reverse inhibition.

As mentioned by Bergel & Bray (1956) it is convenient to add salicylate to xanthine oxidase samples during purification and storage. Batches of xanthine oxidase have been prepared and crystallized by the original method (Avis *et al.* 1955) with mM-stabilizer present at all stages, with good results. The conditions generally chosen for storing the enzyme have been as an approx. 1% solution just above freezing-point in M-sodium and potassium phosphate buffer (pH 6), containing 0·03M-sodium salicylate. Although these conditions might perhaps be improved on, according to the results presented above, they have generally given xanthine oxidase solutions which have lost activity at a rate of less than 5% per month, while in some cases activity has remained unchanged for more than two years.

SUMMARY

1. The loss of activity of solutions of xanthine oxidase has been studied under a variety of conditions in the presence of copper salts, *p*-chloromercuribenzoate, hydrogen peroxide and at 37° and 56°.

2. At least three mechanisms by which activity is likely to be lost have been distinguished. These mechanisms are heavy-metal inactivation, photo-oxidation and gross denaturation.

3. The effect of reducing agents (especially ascorbic acid) on inactivation by copper has been studied and is discussed in relation to the reported inhibitory action of ascorbic acid on xanthine oxidase.

4. Chelating and 'complexing' agents protected against metal inactivation.

5. Competitive inhibitors protected against photo-oxidation and gross denaturation.

6. Sodium salicylate (which may function both as an inhibitor and a chelating agent) has been found to be a particularly useful protecting agent during manipulation and storage of xanthine oxidase.

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