PRELIMINARY NOTES

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The system: hydroxylamine–oxygen–cupric ion. A model hydroxylase in the inactivation of ox and pig liver carboxylesterases

Hydroxylamine has long been known to be a powerful nucleophilic reagent and the hydroxylaminolyses of carboxylic acid esters[1], amides[2] and thiol esters[3] have been studied in detail. In investigations of the mechanism of action of hydrolytic enzymes, hydroxylamine has been used extensively as a water analogue[4]. However, hydroxylamine has been shown to inhibit a number of enzymes[5-7].

FISHBEIN AND CARBONE[8] investigated the effect of hydroxylamine on urease activity. The enzyme was 20% inhibited by 16 μM hydroxylamine in 30 min at 25°. Inhibition of sulphydryl enzymes by hydroxylamine has been ascribed (surprisingly) to non-specific oxidation of –SH groups by the reagent[9]. However, FISHBEIN AND CARBONE found no changes in free –SH concentration of the enzyme or of cysteine which was added to reverse the inhibition by hydroxylamine.

In this laboratory, the effect of hydroxylamine on the decarbamylation of dimethylcarbamoyl-carboxylesterase (ox liver) has been investigated[10]. In the absence of hydroxylamine, the decarbamylation constant is approx. $2 \cdot 10^{-5}$ sec^{-1} at pH 8 and 25°. Hydroxylamine was found to increase the rate of decarbamylation. Fig. 1 shows the effect of addition of 0.25 M hydroxylamine to the carbamoyl-enzyme. There was an initial rapid recovery of activity followed by a secondary loss. Cysteine (4 mM) protected the enzyme against this secondary loss of activity, as has also been found for the pig and sheep liver enzymes. Native enzyme also showed similar effects (Fig. 1).

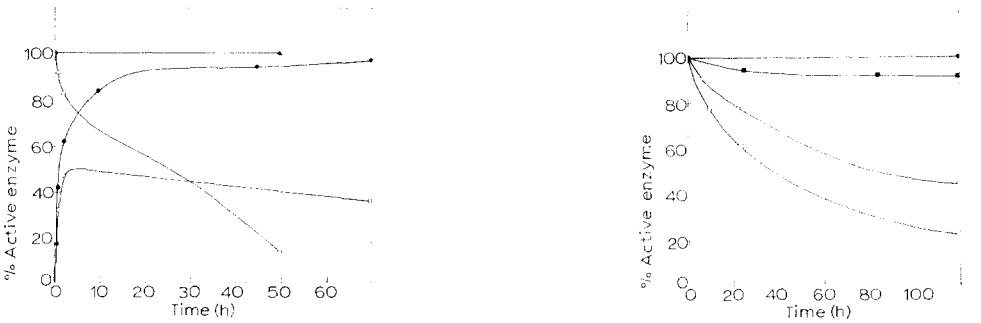

Fig. 1. The effect of hydroxylamine (0.25 M), and of hydroxylamine (0.25 M) *plus* cysteine ($4 \cdot 10^{-3}$ M), on the reactivation of dimethylcarbamyl-carboxylesterase and on the inactivation of native carboxylesterase (ox liver). The reactions were carried out in 0.01 M Tris buffer (pH 8.1) at 25° and activity was measured by a spectrophotometric assay using *o*-nitrophenyl butyrate as substrate. The solutions were not agitated. ○—○, dimethylcarbamyl-enzyme *plus* hydroxylamine; ●—●, dimethylcarbamyl-enzyme *plus* hydroxylamine *plus* cysteine; △—△, native enzyme *plus* hydroxylamine; ▲—▲, native enzyme *plus* hydroxylamine *plus* cysteine.

Fig. 2. The effect of Cu^{2+} ($8 \cdot 10^{-5}$ M) and of chelating agents on the activity of ox liver carboxylesterase in the presence of hydroxylamine (0.25 M). The reactions were carried out in 0.08 M Tris buffer (pH 8.6) at 25°. The solutions were not agitated. ●—●, hydroxylamine *plus* Cu^{2+} *plus* cysteine ($1 \cdot 10^{-2}$ M); ■—■, hydroxylamine *plus* Cu^{2+} *plus* EDTA ($1.7 \cdot 10^{-2}$ M); ○—○, hydroxylamine (*plus* adventitious copper) only; □—□, hydroxylamine *plus* Cu^{2+}.

The inactivation was shown to be irreversible, since activity could not be recovered upon dialysis.

Chemically, there is little evidence to suggest that hydroxylamine acts as an oxidising agent near neutrality, and this makes the proposal of SCHEUCH *et al.*[9] exceedingly unlikely. While the mechanism of oxidative decomposition of hydroxylamine has not been studied rigorously, MOEWS AND AUDRIETH[11] and ANDERSON[12] have reported that the reaction is catalysed by a low concentration of Cu^{2+} (100 μM).

Fig. 2 shows the effects of added Cu^{2+} and metal chelating agents on ox liver carboxylesterase activity in the presence of hydroxylamine. It is clear that low levels of Cu^{2+} (80 μM) catalyse the inactivation and that chelating agents protect the enzyme in the presence of Cu^{2+} and hydroxylamine. In independent experiments, it has been shown that the inactivation was not produced by various reported stable oxidation products of hydroxylamine: nitrite, nitrous oxide or hyponitrite.

We have found that the system, hydroxylamine–Cu^{2+}–oxygen behaves as an hydroxylating system, and will for example, slowly produce dihydroxyphenylalanine

TABLE I

EFFECT OF THE MODEL HYDROXYLATING SYSTEM, HYDROXYLAMINE–Cu^{2+}–OXYGEN ON OX AND PIG LIVER CARBOXYLESTERASES AND ON α-CHYMOTRYPSIN

Flasks (25 ml) containing the reactants listed below, in a total volume of 5.25 ml of 0.38 M phosphate buffer (pH 7.6), were gently agitated at 25° to facilitate oxygen uptake, the gas being periodically supplied by flushing. Final concentrations (unless otherwise specified): $CuSO_4$ (Cu^{2+}) $1.2 \cdot 10^{-4}$ M; hydroxylamine, 0.25 M; EDTA, $1.9 \cdot 10^{-4}$ M; hydroquinone, $3 \cdot 10^{-4}$ M. Residual activity was determined periodically by spectrophotometric assay of aliquots against *o*-nitrophenyl butyrate as substrate for the esterases and *p*-nitrophenyl acetate for α-chymotrypsin. The appropriate corrections were made for catalysis by hydroxylamine.

Enzyme	*System*	*Residual activity (%)*			
		2 h	*4 h*	*8 h*	*24 h*
Ox liver carboxylesterase (0.51 mg/ml)	Cu^{2+}–hydroxylamine–O_2	45	7.5	6.7	—
	EDTA–hydroxylamine–O_2	102	98	94	—
	Cu^{2+}–O_2	101	99	84*	—
	Cu^{2+}–hydroxylamine**	99	—	92	76
	Cu^{2+}–hydroxylamine–O_2***	32	6.3	—	—
	Cu^{2+}–hydroxylamine–O_2–hydroquinone***	32	6.8	—	—
Pig liver carboxylesterase (1.52 mg/ml)	Cu^{2+}–hydroxylamine–O_2	63	57	10	9
	EDTA–hydroxylamine–O_2	97	99	96	100
	Cu^{2+}–O_2	99	89	94	89
α-Chymotrypsin (1.1 mg/ml)	Cu^{2+}–hydroxylamine–O_2	—	—	89	—

* The reason for this aberrant result is unknown. Values of 93% and 96% were observed in similar experiments.

** The reaction was carried out with no agitation, with Cu^{2+}, $8 \cdot 10^{-5}$ M.

*** The Cu^{2+} concentration in these experiments was $8 \cdot 10^{-5}$ M.

derivatives from both tyrosine and *N*-acetyl-L-tyrosineamide. The dihydroxyphenylalanine derivatives were estimated by the colour test due to ARNOW[13] and ROWBOTTOM[14], standardised against dihydroxyphenylalanine. Exposure of ox and pig liver carboxylesterases to the system, hydroxylamine–Cu^{2+}–oxygen, results in rapid

inactivation. That the reaction requires all three components is shown in Table I. α-Chymotrypsin showed little loss of activity under the same conditions.

In independent experiments, we have shown that the system, hydrogen peroxide –Cu^{2+} (refs. 15, 16) behaves similarly. Both systems, therefore, could give rise to the same hydroxylating species[17–19].

It appears unlikely that the inactivation of the carboxylesterases involves free radicals, because the inclusion of hydroquinone ($3 \cdot 10^{-4}$ M) had no effect on the loss of activity of ox liver carboxylesterase in the presence of hydroxylamine, Cu^{2+} and oxygen (Table I).

These model systems and their effects on these and other enzymes, *e.g.* carboxypeptidase A, continue under investigation with a view to establishing which amino acid residues are modified.

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