Pyridoxal Phosphate as Coenzyme of Diamine Oxidase

By A. N. DAVISON

Roche Products Limited, Broadwater Road, Welwyn Garden City, Herts

(Received 24 February 1956)

Diamine oxidase is the enzyme responsible for the oxidative deamination of diamines such as cadaverine and putrescine. It is claimed that histamine is also a substrate for this enzyme, and that histaminase and diamine oxidase are one and the same enzyme (Zeller, 1951). This view is not, however, accepted by all workers (Kapeller-Adler, 1951).

The nature of the prosthetic group of histaminase or diamine oxidase has also not been clearly demonstrated. It has been suggested (Kapeller-Adler, 1949) that flavin-adenine dinucleotide (FAD) is associated with histaminase and is necessary for its activity (Zeller, 1951). Werle & Pechmann (1949) showed that diamine oxidase from plants could be activated by pyridoxal. In a preliminary note Sinclair (1952) presented certain evidence to show that pyridoxal phosphate is a coenzyme of histaminase, and suggested that the clinical signs of pyridoxine deficiency might be caused by an increased level of histamine; since, however, the concentration of histamine was found not to be raised, he later rejected this hypothesis concerning the lesions (Sinclair, 1953). Recently, in experiments on rats, he has shown that the level of histamine is significantly lower in deficiency of vitamin B₆, which is consistent with pyridoxal phosphate's being a coenzyme of histidine decarboxylase (Buxton, Mayer & Sinclair, 1956).

On the basis of work presented in this paper it now appears that pig-kidney diamine oxidase and histaminase are a single enzyme with pyridoxal phosphate as the coenzyme.

EXPERIMENTAL

Diamine oxidase. A partially purified enzyme extract was prepared from pigs' kidneys by the method of Born (1953).

Cysteine sulphinic acid decarboxylase. A chilled rat liver was homogenized in water (25%, w/v) and the homogenate was centrifuged at 8000 g for 10 min. at 0°. The supernatant was treated with (NH₄)₂SO₄ and then centrifuged at 15000 g for 10 min. The fraction obtained when the concentration of (NH₄)₂SO₄ was increased from 10 to 50% saturation was dialysed for 1–2 hr. against running tap-water; 1 ml. of dialysate was equivalent to 150 mg. of fresh liver.

Substrates and inhibitor. Cadaverine dihydrochloride (final concn. 0.04M), histamine phosphate (final concn. 0.025M) and cysteine sulphinic acid (final concn. 0.01M) were used as substrates. *iso*Nicotinic acid hydrazide (isoniazid, Rimifon) was used as an inhibitor.

Manometric estimations. The diamine oxidase preparation (3 ml.) was placed in the main compartment of a two-armed Warburg flask, 0.5 ml. of cadaverine and 0.5 ml. of inhibitor or water were placed in each side arm and 0.2 ml. of 2N-KOH was placed in the centre well of each flask. The flasks were gassed with O₂ for 6 min. and equilibrated for 10 min. before adding the inhibitor. The bath temperature was 37°. In all other respects the experimental methods previously reported for enzyme inhibition were used (Davison, 1956). Cysteine sulphinic acid decarboxylase activity was determined in 2 ml. of dialysate with addition of 0.067M Sørensen's phosphate buffer, pH 7.4, to give a final volume of 4 ml. The methods used were otherwise as previously reported (Davison, 1956).

RESULTS

Identity of histaminase and diamine oxidase. The findings of Zeller (1938) and Kapeller-Adler (1949), showing that there is no summation of the oxygen uptake of cadaverine or histamine when they are added together to diamine oxidase, has been confirmed. Further, enzyme activity, as measured by oxidation of either of the two substrates, is affected in the same way by heating (Table 1).

Attempted separation of apoenzyme and coenzyme. A partially purified diamine oxidase preparation was 50% saturated with ammonium sulphate, and the precipitate obtained after centrifuging at 15000 g for 10 min. was suspended in a little water. The suspension was dialysed for 70 hr. against distilled water at 4°. Attempts to activate this dialysate and also the original preparation by

Table 1. *Thermal inactivation of diamine oxidase*

Diamine oxidase (6 ml.) was heated for 10 min. at the stated temperature. After cooling in ice, the O₂ uptake at 37° of 2.8 ml. was determined with cadaverine (0.04M) and histamine (0.04M) as substrates.

Temp.	Histamine		Cadaverine	
	O ₂ uptake (μl./min.)	Activity (%)	O ₂ uptake (μl./min.)	Activity (%)
37–50° (mean of 6)	0.9	100	3.33	100
55	0.95	105	3.93	118
60	1.05	117	4.23	126
70	0.79	88	3.14	94
72.5	0.42	47	1.42	43
75	0.18	20	0.51	15
77.5	0	0	0	0

incubation with 100 $\mu\text{g.}$ of pyridoxal phosphate/Warburg flask for 30 min. at 37° were unsuccessful. In another experiment, when diamine oxidase was incubated for 30 min. with deoxypyridoxine phosphate (10^{-3}M) at 37°, there was no inhibition of enzyme activity. It was therefore concluded that if pyridoxal phosphate was present as coenzyme it was very strongly bound to the apoenzyme.

Inhibition by isonicotinic acid hydrazide. Various concentrations of isonicotinyl hydrazide were incubated with diamine oxidase at 37° in Warburg flasks. At different times substrate (either cadaverine or histamine) was added and the residual enzyme activity determined. Similar experiments were carried out with cysteine sulphonic acid decarboxylase and isonicotinic acid hydrazide. Fig. 1 shows the inhibition produced when isonicotinic acid hydrazide ($5 \times 10^{-4}\text{M}$) was incubated with the enzyme solutions.

It will be seen that the rates of inhibition are similar for both enzymes. The rates of inhibition have been calculated from a separate graph where log percentage activity was plotted against concentration $\times$ time (this including all experiments with varying isonicotinic acid hydrazide concentration and incubation times). The approximate rate constant for each reaction, calculated from the slope of the line determined by the method of least

Fig. 1. Inhibition of diamine oxidase and cysteine sulphonic acid decarboxylase by isonicotinic acid hydrazide. Isonicotinic acid hydrazide ($5 \times 10^{-4}\text{M}$) was incubated with diamine oxidase or cysteine sulphonic acid decarboxylase for different times at 37° before adding cadaverine (x), histamine (Δ) or cysteine sulphonic acid (O). The method used is described in the text. Where several values have been obtained for each point the mean has been plotted.

squares, was 83 $\text{min.}^{-1}\text{l.mol.}^{-1}$ (cysteine sulphonic acid decarboxylase), 85 $\text{min.}^{-1}\text{l.mol.}^{-1}$ (diamine oxidase with cadaverine) and about 69.5 $\text{min.}^{-1}\text{l.mol.}^{-1}$ (histamine as substrate). Since the enzyme activity with histamine as substrate is low, this last value was subject to large errors.

It is of interest that incubation of $2 \times 10^{-3}\text{M}$ isonicotinyl hydrazide with monoamine oxidase for 20 min. at 37° resulted in only slight inhibition.

Reactivation by pyridoxal phosphate after inhibition by isonicotinic acid hydrazide. For this work diamine oxidase was prepared by adding cold acetone (about 4°) to a 50% (w/v) aqueous suspension of pig kidney, resuspending the insoluble residue in acetone and then following the method of Born (1953). The diamine oxidase was incubated with isonicotinic acid hydrazide (10^{-3}M) for 30 min. at 37°. The inhibited enzyme and a control were dialysed for 3 hr. at 4° against distilled water.

The effect of incubating the dialysed enzyme preparation with pyridoxal phosphate for 30 min. at 37° was examined. Fig. 2 shows that some reactivation occurs at the higher pyridoxal phosphate concentration. Similar reactivations could not be obtained by addition of pyridoxal or pyruvate (100 $\mu\text{g./flask}$).

It has been noted that diamine oxidase prepared by adding cold acetone (−5°) directly to dried-kidney slices cannot be easily reactivated by pyridoxal phosphate after treatment with isonicotinic acid hydrazide.

Fig. 2. Reactivation of diamine oxidase after inhibition by isonicotinic acid hydrazide. After inhibition by isonicotinic acid hydrazide and dialysis, the residual enzyme activity was determined with cadaverine as substrate (●). The effect of addition of pyridoxal phosphate (2.5 $\mu\text{g./ml.}$ and 25 $\mu\text{g./ml.}$) is indicated by x and O, respectively. A dialysed control with and without pyridoxal phosphate is shown by ▲.

DISCUSSION

Kapeller-Adler (1951) has suggested that histamine may be oxidized by a specific histaminase not identical with diamine oxidase. Her conclusions were based on different ratios of histamine and cadaverine oxidation by different enzyme sources and preparations of diamine oxidase. However, Born (1953) has shown that such differences in the oxidation of histamine and aliphatic diamines do not establish that two different enzymes are concerned unless differences in the secondary oxidation of the products of enzymic reaction are excluded. Competition experiments, inhibition data and substrate ratios of crude and purified preparations (Zeller, 1951), together with the present finding that oxidation of histamine and cadaverine by diamine oxidase shows the same sensitivity to thermal inactivation, are consistent with the conclusion that one enzyme in the pig-kidney preparation is responsible for oxidation of both histamine and aliphatic diamines.

The inhibition of diamine oxidase by carbonyl reagents (Zeller, 1951; Werle, Schauer & Hartung, 1955) has indicated that a carbonyl group such as that of pyridoxal phosphate is necessary for enzyme activity. Determination of the rate of inhibition by isonicotinic acid hydrazide of enzymes has been shown to be a useful means of detecting the presence of pyridoxal phosphate as coenzyme, particularly when it is tightly bound to the apoenzyme (Davison, 1956). It has been shown that the rates of inhibition of an established pyridoxal phosphate-containing enzyme, cysteine sulphinic acid decarboxylase (Bergeret, Chatagner & Fromageot, 1955; Hope, 1955), and diamine oxidase (histaminase) are similar. Also pyridoxal phosphate will reactivate diamine oxidase after inhibition by isonicotinic acid hydrazide. It is possible that pyridoxal phosphate has reactivated the enzyme by a reaction between pyridoxal phosphate and the isonicotinic acid hydrazide bound to the (unknown) carbonyl group of the enzyme. However, the finding that neither pyridoxal nor pyruvate can reactivate the inhibited enzyme makes it more likely that the added pyridoxal phosphate reactivates by a combination with the apoenzyme. This supports the earlier work of Werle & Pechmann (1949) and Sinclair (1952). It is known that diamine oxidase requires the presence in the substrate of two basic groups (Blaschko, 1953), one of which is a free amino group (Zeller, 1951). It may therefore be deduced that diamine oxidase possess an acidic group (perhaps conferring

substrate specificity) and pyridoxal phosphate (for reacting with the free amino group) at its active centre. This possibility does not exclude the participation of flavin-adenine dinucleotide at some stage in the enzyme reaction.

It is of interest that Tabor, Tabor & Rosenthal (1954) have demonstrated that amine oxidase (spermine oxidase) from ox plasma is inhibited by carbonyl reagents and is progressively inhibited by isonicotinic acid hydrazide at a rate comparable with that found in our experiments with cysteine sulphinic acid decarboxylase. It was suggested that pyridoxal phosphate may function as coenzyme for this enzyme. Liver monoamine oxidase differs from this enzyme, not only in substrate specificity but also in not being inhibited in a similar way by isonicotinic acid hydrazide.

SUMMARY

1. Evidence is given which is consistent with the identity of histaminase and diamine oxidase of pig kidney.

2. It is concluded that pyridoxal phosphate is probably a coenzyme for diamine oxidase (histaminase).

My thanks are due to Dr A. L. Morrison for his interest in this work, to Mr R. F. Denman for his skilled help and to the directors of Roche Products Limited for permission to publish this work. I am also indebted to Dr H. M. Sinclair for his criticism of the manuscript.

REFERENCES

- Bergeret, B., Chatagner, F. & Fromageot, C. (1955). *Biochim. biophys. Acta*, **17**, 128.
- Blaschko, H. (1953). *Brit. med. Bull.* **9**, 146.
- Born, G. V. R. (1953). *Brit. J. Pharmacol.* **8**, 42.
- Buxton, J., Mayer, A. & Sinclair, H. M. (1956). *J. Physiol.* **131**, 17 P.
- Davison, A. N. (1956). *Biochim. biophys. Acta*, **19**, 131.
- Hope, D. B. (1955). *Biochem. J.* **59**, 497.
- Kapeller-Adler, R. (1949). *Biochem. J.* **44**, 70.
- Kapeller-Adler, R. (1951). *Biochem. J.* **48**, 99.
- Sinclair, H. M. (1952). *Biochem. J.* **51**, x.
- Sinclair, H. M. (1953). *Proc. Nutr. Soc.* **12**, 94.
- Tabor, C. W., Tabor, H. & Rosenthal, S. M. (1954). *J. biol. Chem.* **208**, 645.
- Werle, E. & Pechmann, E. von (1949). *Liebigs Ann.* **562**, 44.
- Werle, E., Schauer, A. & Hartung, G. (1955). *Klin. Wschr.* **33**, 562.
- Zeller, E. A. (1938). *Helv. chim. acta*, **21**, 1645.
- Zeller, E. A. (1951). In *The Enzymes*, **2**, pt. 1, p. 544. Ed. by Sumner, J. B. & Myrback, K. New York: Academic Press Inc.