The Inactivation of Pea-Seedling Diamine Oxidase by Peroxidase and 1,5-Diaminopentane

By J. M. HILL

Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts.

(Received 23 March 1967)

1. The rate of oxidative deamination of 1,5-diaminopentane by pea-seedling extracts, which contain diamine oxidase [diamine-oxygen oxidoreductase (deaminating), EC 1.4.3.6], was increased by adding pyridoxal or pyridoxal phosphate. 2. Evidence was obtained that pyridoxal does not activate the apoenzyme of diamine oxidase, but prevents the inactivation of the enzyme. 3. This inactivation only occurred when 1,5-diaminopentane was the substrate and depended on a second thermolabile factor in the extract besides the diamine oxidase. 4. Purified diamine oxidase, when catalysing the oxidation of 1,5-diaminopentane, was rapidly inactivated in the presence of peroxidase. 5. The inactivation was prevented not only by pyridoxal and pyridoxal phosphate but also by several unrelated compounds including α -oxoglutarate, catechol and *o*-aminobenzaldehyde. 6. It is suggested that peroxidase catalyses the further oxidation of the product of the oxidative deamination of 1,5-diaminopentane to a compound that inactivates diamine oxidase. 7. The results diminish the relevance of previous evidence that plant diamine oxidase contains pyridoxal phosphate.

Many amine oxidases, including the diamine oxidases of plant and animal tissues (Werle & Pechmann, 1949; Kenten & Mann, 1952; Zeller, 1938), and the spermine oxidase and benzylamine oxidase of plasma (Hirsch, 1953; Bergeret, Blaschko & Hawes, 1957), are inhibited by carbonyl reagents. Zeller (1938), who first demonstrated that pig-kidney diamine oxidase was inhibited by cyanide, attributed this inhibition to the presence of a carbonyl compound in the prosthetic group of the enzyme. Several of these enzymes have now been purified; the plasma amine oxidases (Yamada & Yasunobu, 1962*a*; Buffoni & Blaschko, 1964) and the plant diamine oxidases (Mann, 1961; Werle, Trautschold & Aures, 1961) are pink and have absorption maxima in the range 470–510 m μ ; most contain copper (Yamada & Yasunobu, 1962*b,c*; Buffoni & Blaschko, 1964; Mann, 1961; Hill & Mann, 1962; Mondovi, Rotilio, Finazzi & Scioscia-Santoro, 1964; Yamada, Odachi & Ogata, 1965). Evidence has been presented strongly suggesting that the carbonyl component of the plasma amine oxidases is pyridoxal phosphate (Yamada & Yasunobu, 1963; Blaschko & Buffoni, 1965), and by analogy it has been assumed that all amine oxidases inhibited by carbonyl reagents contain pyridoxal phosphate (Blaschko & Buffoni, 1965).

Werle & Pechmann (1949) observed that the oxygen uptake of dialysed clover-seedling extracts

in the presence of 1,5-diaminopentane (cadaverine) was increased by the addition of pyridoxal. It was therefore suggested that pyridoxal phosphate is a component of the prosthetic group of plant diamine oxidase, and, as hydrogen peroxide was produced during the reaction, it was further suggested that the enzyme also contains FAD. Goryachenkova (1956) reported increases in both oxygen uptake and ammonia formation when pyridoxal phosphate and FAD were added to dialysed clover-seedling extracts in the presence of 1,6-diaminohexane. However, Werle & Hartung (1956) found that not only pyridoxal and pyridoxal phosphate but also several apparently unrelated compounds including polyphenols, α -oxoglutarate, cysteine and ascorbic acid activated the oxidative deamination of 1,5-diaminopentane by undialysed clover-seedling extracts. The activation is therefore unspecific, and the results provide no evidence that pyridoxal phosphate is present in plant diamine oxidase. In spite of this, the work of Werle & Pechmann (1949) is still quoted as evidence for the presence of pyridoxal phosphate in plant diamine oxidase (Suzuki, 1966). The present work, which forms part of a wider study into the nature of the carbonyl component in plant diamine oxidase, re-examines the observations of Werle & Pechmann (1949) and Werle & Hartung (1956). Pea-seedling extracts and purified diamine oxidase preparations were used and an

alternative explanation for the effect of pyridoxal is offered.

MATERIALS AND METHODS

Pea-seedling extracts. Pea seedlings (*Pisum sativum* var. Fill-basket, from Sutton and Son Ltd., Reading) were grown in sand in the dark for 9 days at about 20°. The seedlings were washed free from sand, and ground with 0.2 M-phosphate buffer, pH 7.0 (0.5 ml./g. of seedlings), and acid-washed sand in a chilled mortar. The paste was squeezed through cotton cloth and the extract diluted with water so that 1 ml. of extract corresponded to 1 g. of tissue.

Enzymes. Pea-seedling diamine oxidase was purified as described by Hill & Mann (1964). The assay was based on the initial rate of uptake of O₂ by reaction mixtures containing diamine oxidase, catalase (25 µg.), 10 mM-1,4-diaminobutane and 67 mM-phosphate buffer, pH 7.0, in a total volume of 3 ml. One unit of diamine oxidase is defined as the amount that catalyses the oxidation of 1 µmole of 1,4-diaminobutane/min. at 25°, giving an initial rate of uptake of O₂ of 11.2 µl./min. Horseradish peroxidase was prepared by the method of Kenten & Mann (1954) as far as the second fractional precipitation with ethanol. Its activity was measured as described by Hill & Mann (1966). The specific activity (units/mg.) of the preparation used was 2200 and the haemin content was 0.89%. Crystalline ox-liver catalase (EC 1.11.1.6) was purchased from the Sigma (London) Chemical Co. Ltd. (London, S.W. 6) and L-glutamate dehydrogenase (EC 1.4.1.2) from C. F. Boehringer Corporation (London) Ltd. (London, W. 5).

Chemicals. 1,4-Diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, 1,10-diaminodecane, L-lysine methyl ester, spermidine, pyridoxal phosphate, pyridoxine and pyridoxamine were bought from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.), and NADH from C. F. Boehringer Corporation (London) Ltd. *o*-Aminobenzaldehyde was prepared by the reduction of *o*-nitrobenzaldehyde with ferrous sulphate and ammonia (Smith & Opie, 1955). α -Triperideine and isotriperideine were prepared as described by Schöpf, Komzak, Braun & Jacobi (1948). All other chemicals were bought either from Hopkin and Williams Ltd. (Freshwater, Essex) or from British Drug Houses Ltd. (Poole, Dorset).

Buffers. Phosphate buffers were prepared from KH₂PO₄ and KOH.

Manometric methods. O₂ uptake was measured on 3 ml. of reaction mixture at 25° in air, with 0.2 ml. of 5 N-KOH in the centre well, in a Warburg apparatus (Braun model V) from the Shandon Scientific Co. Ltd. (London, W. 10).

Determination of ammonia. Ammonia formed in the reaction mixtures was usually determined by the micro-diffusion method of Conway (1950). When 1,4-diaminobutane was the substrate the oxidation products were volatile and interfered with this determination; ammonia was therefore measured enzymically with the system glutamate dehydrogenase, α -oxoglutarate and NADH (Kirsten, Gerez & Kirsten, 1963). With the large amount of α -oxoglutarate used, ammonia was converted quantitatively into L-glutamate. The reaction was followed by measuring the decrease in *E*₃₆₆. When the reaction mixture contained 1,5-diaminopentane results obtained by the two methods agreed closely.

Determination of amines. The amines remaining in the

reaction mixtures at the end of the incubation period were estimated spectrophotometrically with 2,4,6-trinitrobenzenesulphonic acid. This reagent combines with the :CH·NH₂ group of amines, amino acids and peptides to give coloured derivatives (Okuyama & Satake, 1960; Satake, Okuyama, Ohashi & Shinoda, 1960). Ammonia does not interfere with this reaction. A total volume of 3 ml. containing 1.0 ml. of reagent (0.1% trinitrobenzenesulphonic acid tetrahydrate in 0.01 N-HCl), 1.0 ml. of 0.2 M-phosphate buffer, pH 8.0, 0.1–0.5 ml. of the Warburg reaction mixture and water was incubated at 40° for 90 min. The *E*₄₃₀ value was measured against a control from which the Warburg reaction mixture had been omitted. Under these conditions, with silica cuvettes of 1 cm. light-path, 0.1 µmole of 1,4-diaminobutane or 1,5-diaminopentane gave *E*₄₃₀ 0.365; *E*₄₃₀ was proportional to the concentration in the range 0.01–0.3 µmole of amine.

Spectrophotometry. Extinctions were measured in a Unicam SP.500 spectrophotometer or an Optica CF4 DR recording spectrophotometer with silica cells of 1 cm. light-path.

Determination of Δ^1 -pyrroline and Δ^1 -piperideine. The amount of Δ^1 -pyrroline present in reaction mixtures initially containing 1,4-diaminobutane was determined by the method of Holmstedt, Larsson & Tham (1961). Samples (0.5 ml.) of the reaction mixtures were mixed with 1 ml. of 0.01 M-*o*-aminobenzaldehyde (in 0.02 M-phosphate buffer, pH 7.0), 1 ml. of 0.2 M-phosphate buffer, pH 7.0, and water to give a total volume of 3 ml. The mixtures were left at room temperature for 30 min. and the Δ^1 -pyrroline content was calculated from the *E*₄₃₀, assuming ϵ 1860 cm.⁻¹.

The amount of Δ^1 -piperideine present in the reaction mixtures initially containing 1,5-diaminopentane was determined similarly, except that *E*₄₆₆ was measured, assuming ϵ 2058 cm.⁻¹.

RESULTS

Effect of pyridoxal on the oxidation of 1,4-diaminobutane and 1,5-diaminopentane by pea-seedling extracts. The reaction catalysed by diamine oxidase is represented by the equation:

which in the presence of catalase becomes:

Fig. 1 shows that extracts of 9-day-old pea seedlings rapidly oxidize 1,4-diaminobutane, and, although after 2 hr. the total oxygen absorbed slightly exceeded that expected from eqn. (2), the ammonia formed was close to the expected amount (Table 1). 1,5-Diaminopentane was also rapidly oxidized for the first 20 min., but the reaction then slowed considerably and Table 1 shows that at the end of 2 hr. both the oxygen uptake and amount of ammonia formed were much less than predicted. Pyridoxal had little effect on either the oxygen absorbed or the ammonia produced with 1,4-diaminobutane. In the presence of pyridoxal both the absorption of

Fig. 1. Rate of oxidation of 1,4-diaminobutane and 1,5-diaminopentane by pea-seedling extract in the presence of pyridoxal. Reaction mixtures and conditions were similar to those used in Table 1. ○, 10 μ moles of 1,4-diaminobutane; △, 10 μ moles of 1,4-diaminobutane + 10 μ moles of pyridoxal; ●, 10 μ moles of 1,5-diaminopentane; ▲, 10 μ moles of 1,5-diaminopentane + 10 μ moles of pyridoxal.

Table 1. Effect of pyridoxal on the oxidation of 1,4-diaminobutane and 1,5-diaminopentane by pea-seedling extracts

A total volume of 3 ml. contained 0.1 ml. of an extract of 9-day-old pea seedlings in 67 mM-phosphate buffer, pH 7.0. Additions (10 μ moles of amine and 10 μ moles of pyridoxal) were made as indicated, the amine being added from the side arm of the Warburg vessel.

Addition	NH ₃ formed (μ moles/2 hr.)	O ₂ uptake (μ g.atoms/2 hr.)
1,4-Diaminobutane	10.7	13.0
1,4-Diaminobutane + pyridoxal	10.8	14.0
1,5-Diaminopentane	8.1	8.2
1,5-Diaminopentane + pyridoxal	10.1	12.6

oxygen and the formation of ammonia with 1,5-diaminopentane were greatly increased and were similar to those with 1,4-diaminobutane.

Effect of boiled pea-seedling extract on the oxidation of 1,5-diaminopentane by purified pea-seedling diamine oxidase. When 1,5-diaminopentane was oxidized by diamine oxidase in the presence of pea-seedling extract heated at 100° for 30 min., there was no inactivation of the reaction and both the uptake of oxygen and the formation of ammonia

were close to the theoretical values. This suggested that the inactivation in crude extracts (Fig. 1) depends on a thermolabile factor, other than diamine oxidase, present in the extract.

Oxidation of various amines by purified pea-seedling diamine oxidase in the presence of horseradish peroxidase. Peroxidase is abundant in pea seedlings, and since hydrogen peroxide, a product of the diamine oxidase reaction, is one of its substrates, the effect of peroxidase on the oxidation of various amines by diamine oxidase was studied. The results (Table 2) show that peroxidase had a negligible effect on the absorption of oxygen and the formation of ammonia when 1,4-diaminobutane, 1,6-diaminohexane, 1,10-diaminodecane, lysine, lysine methyl ester or spermidine was the diamine oxidase substrate. However, with 1,5-diaminopentane, peroxidase depressed both the absorption of oxygen and the formation of ammonia; this effect was not found with any of the other amines tested.

When tyramine, phenethylamine, or histamine was the diamine oxidase substrate, peroxidase had little effect on the production of ammonia, but appreciably increased the absorption of oxygen. This increase was attributed to the peroxidase-catalysed oxidation of the *p*-hydroxyphenylacetaldehyde, phenylacetaldehyde and imidazolylacetaldehyde formed by the diamine oxidase reaction (Kenton, 1953).

Effect of pH on the oxidation of 1,5-diaminopentane by purified diamine oxidase in the presence of peroxidase. Fig. 2 shows that peroxidase had only a slight effect on the oxidation of 1,5-diaminopentane by diamine oxidase at pH 6, but that at pH 7 and pH 8 there was a marked time-dependent inhibition. For the first 15 min. oxygen was taken up at much the same rate with or without peroxidase, but thereafter the uptake was slower in those reaction mixtures containing peroxidase. Table 3 shows that peroxidase inhibited the removal of 1,5-diaminopentane and the formation of ammonia more than the uptake of oxygen. The differences between the theoretical values (based on the oxygen absorbed) for 1,5-diaminopentane oxidation and ammonia production, and those observed, increased as the pH was raised and were always twice as much for 1,5-diaminopentane removal as for ammonia formation. The ammonia formed may have reacted further forming an amino compound that interfered with the determination of the remaining 1,5-diaminopentane.

Effect of peroxidase concentration on oxidation of 1,5-diaminopentane by diamine oxidase. Fig. 3 shows the effect of different amounts of peroxidase on the oxygen used in the oxidation of 1,5-diaminopentane by diamine oxidase. Oxygen uptake always started at about the same rate but decreased

Table 2. *Oxidation of various amines by purified pea-seedling diamine oxidase in the presence and absence of horseradish peroxidase*

A total volume of 3 ml. contained catalase (25 μ g.) and diamine oxidase in 67 mM-phosphate buffer, pH 7.0. Where indicated (+), 10 μ g. of horseradish peroxidase was also present. Amine (10 μ moles) was added from the side arm after equilibration. The reaction time was 2 hr.

Diamine (10 μ moles)	Peroxidase	Diamine oxidase (i.u.)	O ₂ uptake (μ g.atoms)	NH ₃ formed (μ moles)
1,4-Diaminobutane	—	0.2	10.2	10.9
1,4-Diaminobutane	+	0.2	10.6	11.1
1,5-Diaminopentane	—	0.2	9.4	9.7
1,5-Diaminopentane	+	0.2	6.4	6.0
1,6-Diaminohexane	—	0.8	9.4	10.6
1,6-Diaminohexane	+	0.8	9.2	10.3
1,10-Diaminodecane	—	1.0	9.6	8.8
1,10-Diaminodecane	+	1.0	9.6	8.8
Tyramine	—	5.0	8.2	8.4
Tyramine	+	5.0	11.8	8.2
Phenethylamine	—	5.0	9.8	9.6
Phenethylamine	+	5.0	13.2	9.5
Histamine	—	6.0	10.0	9.7
Histamine	+	6.0	16.6	9.7
Lysine	—	10.0	4.0	4.1
Lysine	+	10.0	4.6	4.1
Lysine methyl ester	—	0.4	6.2	5.2
Lysine methyl ester	+	0.4	6.2	5.2
Spermidine	—	5.0	6.0	6.2
Spermidine	+	5.0	6.4	6.4

Fig. 2. Effect of change of pH on the rate of oxidation of 1,5-diaminopentane by purified pea-seedling diamine oxidase in the presence and absence of peroxidase. Reaction mixtures and conditions were similar to those used in Table 3. ○, pH 8 without peroxidase; ●, pH 8 plus peroxidase; △, pH 7 without peroxidase; ▲, pH 7 plus peroxidase; □, pH 6 without peroxidase; ■, pH 6 plus peroxidase.

progressively earlier with increasing concentration of peroxidase. Table 4 shows the effect of peroxidase concentration on the oxygen uptake, ammonia formation and the 1,5-diaminopentane oxidation

Table 3. *Effect of pH on the oxidation of 1,5-diaminopentane by diamine oxidase in the presence and absence of peroxidase*

The reaction mixture contained purified pea-seedling diamine oxidase (0.2 i.u.) and catalase (25 μ g.) in 3 ml. of 67 mM-phosphate buffer, pH 6.0, 7.0 or 8.0. Where indicated (+), 10 μ g. of peroxidase was also present. After equilibration, 10 μ moles of 1,5-diaminopentane were added from the side arm of the vessel. The reaction time was 2 hr.

pH	Peroxi- dase	O ₂ uptake (μ g.atoms)	NH ₃ formed (μ moles)	1,5-Diamino- pentane oxidized (μ moles)
6.0	—	9.2	9.2	8.8
6.0	+	8.2	7.9	7.5
7.0	—	10.0	10.1	9.6
7.0	+	6.4	6.0	5.5
8.0	—	5.8	5.6	5.4
8.0	+	4.6	3.4	2.1

in 2 hr. Peroxidase always inhibited the oxidation of 1,5-diaminopentane by diamine oxidase, and the inhibition began sooner with increase of peroxidase.

Effect of various substances on the oxidative deamination of 1,4-diaminobutane, 1,5-diaminopentane and histamine by diamine oxidase and peroxidase. Werle & Hartung (1956) showed that not only pyridoxal, but also some unrelated compounds, including catechol and α -oxoglutarate,

increased the oxidative deamination of 1,5-diaminopentane by clover-seedling extracts. Table 5 shows the effect of these compounds on the oxidative deamination of 1,4-diaminobutane, 1,5-diaminopentane and histamine by pea-seedling extracts. Only with 1,5-diaminopentane did pyridoxal, α -oxoglutarate and catechol consistently increase both the uptake of oxygen and the formation of ammonia. Table 6 shows that many compounds prevent the inhibition by peroxidase of the diamine oxidase-catalysed deamination of 1,5-diaminopentane, although complications arise because some of the compounds

Fig. 3. Effect of peroxidase concentration on the rate of oxygen uptake of the 1,5-diaminopentane-diamine oxidase system. Reaction mixtures and conditions were similar to those used for Table 4. 0, 2, 5, 10, 50 and 100 μ g. of horseradish peroxidase were present as indicated.

Table 4. Effect of peroxidase concentration on the oxidation of 1,5-diaminopentane by purified pea-seedling diamine oxidase

The basal reaction mixture contained purified pea-seedling diamine oxidase (0.2 i.u.), catalase (25 μ g.) and horseradish peroxidase in 3 ml. of 67 mM-phosphate buffer, pH 7.0. After equilibration, 10 μ moles of 1,5-diaminopentane were added from the side arm of the reaction vessel. The reaction time was 2 hr.

Peroxidase added (μ g.)	O ₂ uptake (μ g.atoms)	NH ₃ formed (μ moles)	1,5-Diaminopentane oxidized (μ moles)
0	9.0	9.0	9.5
2	8.6	8.4	7.8
5	7.6	7.4	6.9
10	6.8	6.5	6.2
50	5.6	5.1	4.6
100	5.4	4.6	4.0

used also inhibit diamine oxidase. Oxygen uptake sometimes increased; where this could not be correlated with increases in the formation of ammonia and the disappearance of 1,5-diaminopentane it was assumed to result from a coupled oxidation of the added substance by peroxidase. The inhibition by peroxidase of the oxidative deamination of 1,5-diaminopentane was prevented by pyridoxal, pyridoxamine, pyridoxal phosphate, catechol, α -oxoglutarate, manganese sulphate, EDTA and *o*-aminobenzaldehyde, but not, or only partially, by pyridoxine, histamine and α -tripiperidine. The concentration of α -tripiperidine used was equivalent to that which would be formed by oxidation of 10 μ moles of 1,5-diaminopentane. *o*-Aminobenzaldehyde was used because it will condense with Δ^1 -piperidine, a product of the oxidative deamination of 1,5-diaminopentane, to give the corresponding quinazolinium compound (Schöpf *et al.* 1948), which can be determined spectrophotometrically at 466 m μ . With 10 μ moles of *o*-aminobenzaldehyde at pH 7, peroxidase had no significant effect on the Δ^1 -piperidine formed from 1,5-diaminopentane: 7.2 μ moles and 7.5 μ moles of Δ^1 -piperidine respectively were found in the presence and absence of peroxidase. In the absence of *o*-aminobenzaldehyde, peroxidase considerably diminished the formation of Δ^1 -piperidine; in the presence of peroxidase 4.4 μ moles were formed and in its absence 7.4 μ moles. The differences in the Δ^1 -piperidine found between reaction mixtures not containing peroxidase, with and without *o*-aminobenzaldehyde, were attributed, in part, to polymerization of the Δ^1 -piperidine.

Effect of the α -tripiperidine and isotripiperidine, and of the products of their oxidation by peroxidase, on the oxidation of 1,4-diaminobutane by diamine oxidase. The Δ^1 -piperidine formed by the oxidative deamination of 1,5-diaminopentane rapidly condenses to give α -tripiperidine and isotripiperidine (Schöpf *et al.* 1948); Mann & Smithies (1955) have shown that these compounds are substrates for peroxidase. The possibility was considered that an oxidation product of one of these tripiperidines inactivated diamine oxidase. Both α -tripiperidine and isotripiperidine were oxidized by peroxidase at pH 7 until the oxygen uptake ceased. Table 7 shows that there was little difference between the effects of the unoxidized and oxidized forms on the oxidation of 1,4-diaminobutane by diamine oxidase. 1,4-Diaminobutane was used as the substrate for diamine oxidase in these experiments because its oxidation would be unaffected by any peroxidase present.

DISCUSSION

My results suggest that oxygen uptake is increased when pyridoxal is added to a legume-seedling

Table 5. *Oxidation of 1,4-diaminobutane, 1,5-diaminopentane and histamine by pea-seedling extracts in the presence of α -oxoglutarate, catechol and pyridoxal*

The reaction mixture of 3 ml. total volume contained 0.1 ml. of pea-seedling extract and additions as indicated in 67 mM-phosphate buffer, pH 7.0. The diamine oxidase substrate (10 μ moles) was added from the side arm after equilibration. As controls, reaction mixtures were used from which the diamine oxidase substrate was omitted. The reaction time was 2 hr.

Substrate ...	1,4-Diaminobutane		1,5-Diaminopentane		Histamine	
	O ₂ uptake (μ g.atoms)	NH ₃ formed (μ moles)	O ₂ uptake (μ g.atoms)	NH ₃ formed (μ moles)	O ₂ uptake (μ g.atoms)	NH ₃ formed (μ moles)
None	13.0	10.7	8.2	8.1	11.2	7.5
α -Oxoglutarate (10 μ moles)	11.6	10.2	13.2	10.7	9.4	7.6
Catechol (10 μ moles)	19.0	9.7	19.6	10.0	12.8	7.7
Pyridoxal (10 μ moles)	14.0	10.8	12.6	10.1	9.8	8.0

Table 6. *Effect of various substances on the oxidation of 1,5-diaminopentane by the pea-seedling diamine oxidase-peroxidase system at pH 7*

The basal reaction mixture of 3 ml. total volume contained purified pea-seedling diamine oxidase (0.2 i.u.), catalase (25 μ g.), additions as indicated and horseradish peroxidase (10 μ g.) where shown (+), in 67 mM-phosphate buffer, pH 7.0. The reaction was started by the addition of 10 μ moles of 1,5-diaminopentane from the side arm of the reaction vessel.

Addition	Amount of addition (μ moles)	Peroxidase	O ₂ uptake (μ g.atoms/2 hr.)	NH ₃ formed (μ moles/2 hr.)	1,5-Diaminopentane used (μ moles/2 hr.)
None	0	—	9.4	9.4	9.4
None	0	+	6.4	6.1	5.8
Pyridoxal	10	—	8.2	8.6	8.3
Pyridoxal	10	+	10.0	9.8	9.7
Pyridoxine	10	—	7.8	8.5	7.7
Pyridoxine	10	+	5.4	4.7	5.1
Pyridoxamine	10	—	7.0	7.9	7.5
Pyridoxamine	10	+	7.4	7.5	7.2
Pyridoxal phosphate	10	—	9.0	9.8	8.7
Pyridoxal phosphate	10	+	10.0	10.1	9.1
Catechol	10	—	9.8	9.8	9.5
Catechol	10	+	12.4	10.5	9.6
α -Oxoglutarate	10	—	9.0	8.8	8.7
α -Oxoglutarate	10	+	9.2	9.6	9.2
Histamine	10	—	8.6	9.8	9.9
Histamine	10	+	7.6	8.8	8.7
EDTA	10	—	9.0	10.5	9.7
EDTA	10	+	10.0	10.9	9.8
<i>o</i> -Aminobenzaldehyde	10	—	6.4	7.0	6.4
<i>o</i> -Aminobenzaldehyde	10	+	7.0	7.7	7.2
α -Triperideine	3.3	—	4.0	3.7	4.0
α -Triperideine	3.3	+	6.0	2.6	2.5
MnSO ₄	3.0	—	10.8	12.8	10.0
MnSO ₄	3.0	+	10.4	12.4	9.9

extract with 1,5-diaminopentane present, not because the apoenzyme of diamine oxidase is activated, as suggested by Werle & Pechmann (1949), but because the inactivation of this enzyme is prevented. Inactivation occurs only when 1,5-diaminopentane is the substrate, and depends on

the presence of a thermolabile factor in the extract. Purified systems containing diamine oxidase and peroxidase resemble crude pea-seedling extracts and the thermolabile factor is probably peroxidase. The inactivation of diamine oxidase in the presence of 1,5-diaminopentane and peroxidase was pre-

Table 7. *Oxidation of 1,4-diaminobutane by pea-seedling diamine oxidase in the presence of oxidized polymers of Δ^1 -piperidine*

The reaction mixtures of 3 ml. total volume contained purified pea-seedling diamine oxidase (0.2 i.u.), catalase (25 μ g.) and additions as listed, in 67 mm-phosphate buffer, pH 7.0. The reaction was started by the addition of 10 μ moles of 1,4-diaminobutane from the side arm of the reaction vessel. The oxidation products of both α -tripiperidine and isotripiperidine were prepared by oxidizing 3.3 μ moles of each of these compounds with 10 μ g. of horseradish peroxidase in 3 ml. of 67 mm-phosphate buffer, pH 7.0, at 25° until the oxygen uptake ceased. Samples (1 ml.) of these reaction mixtures were used in the experiments listed in this Table.

Addition	O ₂ uptake (μ g.atoms/2 hr.)	1,4-Diamino- butane used (μ moles/2 hr.)
None	10.0	9.9
Isotripiperidine (1.1 μ moles)	7.2	7.3
Oxidation product of isotripiperidine	6.6	7.5
α -Tripiperidine (1.1 μ moles)	7.8	7.9
Oxidation product of α -tripiperidine	7.8	8.4

vented by a wide range of apparently unrelated compounds, including pyridoxal, pyridoxal phosphate, dihydroxyphenols, α -oxoglutarate and *o*-aminobenzaldehyde. These results are similar to those of Werle & Hartung (1956) with clover-seedling extracts and support the suggestion that peroxidase in legume-seedling extracts inactivated the diamine oxidase in the presence of 1,5-diaminopentane.

The mechanism of the inactivation is not yet clear but oxidation of Δ^1 -piperidine by peroxidase may be involved. This suggestion is supported by the fact that *o*-aminobenzaldehyde, which combines with Δ^1 -piperidine to give a dihydroquinazolinium compound, prevents the inactivation. The oxidation products of the polymers of Δ^1 -piperidine, α -tripiperidine and isotripiperidine, do not inhibit the diamine oxidase reaction and it is therefore unlikely that these compounds are involved. The results indicate that, with peroxidase present, some of the ammonia formed may combine with an oxidation product of 1,5-diaminopentane and this compound may inactivate the diamine oxidase. Whatever the mechanism of the inactivation, the results diminish the relevance of the evidence of Werle & Pechmann (1949) that the plant diamine oxidase contains pyridoxal phosphate and they demonstrate the difficulties that can arise when crude extracts are used for such studies.

My results do not explain the observations of Goryachenkova (1956), that pyridoxal phosphate

and FAD sometimes increased the oxidative deamination of 1,6-diaminohexane by dialysed clover- and pea-seedling extracts. Zeller (1963) suggested that the apparent activation of diamine oxidase in animal tissue extracts by FAD results from an increase in activity of flavine enzymes that catalyse the further oxidation of the diamine-diamine oxidase reaction products. Suzuki (1966) reported that the copper-free apoenzyme of pea-seedling diamine oxidase could be reactivated after treatment with isonicotinoylhydrazide only by a mixture of pyridoxal phosphate and Cu²⁺ ions, and he suggested that this was further evidence for the presence of pyridoxal phosphate in plant diamine oxidase. These results recall the earlier work of Davison (1956), who found that the kinetics of the inhibition of pig-kidney diamine oxidase by isonicotinoylhydrazide paralleled those of the combination of this reagent with pyridoxal phosphate. He also found that the enzyme, after treatment with isonicotinoylhydrazide and subsequent dialysis, could be reactivated by pyridoxal phosphate. Goryachenkova & Ershova (1965) reported that, after pig-kidney diamine oxidase had been inhibited with chelating agents and dialysed, activity was restored only by adding Cu²⁺ ions and pyridoxal phosphate. In similar experiments with pea-seedling diamine oxidase, with sodium diethyldithiocarbamate as the inhibitor, Mann (1961) and Hill & Mann (1962) reported reactivation with Cu²⁺ ions alone. Much of the evidence for the presence of pyridoxal phosphate in the prosthetic group of the amine oxidases is unsatisfactory. Hill & Mann (1962) showed that Cu²⁺ ions at the concentrations used by Suzuki (1966) inactivate the pea enzyme, and that this inactivation could be prevented by chelating agents such as pyrophosphate. Isonicotinoylhydrazide is a chelating agent (Cymerman-Craig, Rubro, Willis & Edgar, 1955), and Cu²⁺ ions also form complexes with Schiff bases of pyridoxal phosphate (Matsuo, 1957). The reactivation of the pea enzyme reported by Suzuki (1966) possibly depended on the removal of the inhibiting isonicotinoylhydrazide as a copper complex of its Schiff base with pyridoxal phosphate.

Attempts in this Laboratory to demonstrate unequivocally the presence of pyridoxal phosphate in plant diamine oxidase have so far been unsuccessful. The methods used were similar to those used by Yamada & Yasunobu (1963) and Blaschko & Buffoni (1965). The negative results, coupled with differences between the properties of plant and plasma amine oxidases, suggest that the carbonyl component of the prosthetic group of the plant enzyme may not be pyridoxal phosphate. The most evident difference between the plant and plasma enzymes is their reaction with substrates under anaerobic conditions. Pea-seedling diamine oxidase

gives an intensely yellow product, attributed to an enzyme-substrate complex, with absorption maxima at 466, 437 and 350m μ (Mann, 1961; Hill & Mann, 1964), whereas the plasma amine oxidases give colourless products (Yamada & Yasunobu, 1962c; Buffoni & Blaschko, 1964). The copper-free pea-seedling diamine oxidase is orange-pink (Hill & Mann, 1964) with a maximum absorption at about 480m μ that is less intense than that of the holo-enzyme at 500m μ . The pink colour of plasma spermine oxidase disappears when copper is removed and the copper-free apoenzyme absorbs maximally at 380m μ . Yamada & Yasunobu (1962c, 1963) attributed this band at 380m μ to the pyridoxal phosphate in the enzyme; the band at 380m μ was not observed in the spectrum of copper-free pea-seedling diamine oxidase (Hill & Mann, 1964). These dissimilarities between the pea-seedling diamine oxidases and pyridoxal phosphate-containing plasma amine oxidases, in addition to the results presented in this paper, suggest that important differences exist between the prosthetic groups of these two types of enzyme.

REFERENCES

- Bergeret, B., Blaschko, H. & Hawes, R. (1957). *Nature, Lond.*, **180**, 1127.
- Blaschko, H. & Buffoni, F. (1965). *Proc. Roy. Soc. B*, **163**, 45.
- Buffoni, F. & Blaschko, H. (1964). *Proc. Roy. Soc. B*, **161**, 153.
- Conway, E. J. (1950). *Microdiffusion Analysis and Volumetric Error*, 3rd ed., p. 95. London: Crosby, Lockwood and Son Ltd.
- Cymerman-Craig, J., Rubro, S. D., Willis, D. & Edgar, J. (1955). *Nature, Lond.*, **176**, 34.
- Davison, A. N. (1956). *Biochem. J.* **64**, 546.
- Goryachenkova, E. V. (1956). *Biokhimiya*, **21**, 247.
- Goryachenkova, E. V. & Ershova, E. A. (1965). *Biokhimiya*, **30**, 165.
- Hill, J. M. & Mann, P. J. G. (1962). *Biochem. J.* **85**, 198.
- Hill, J. M. & Mann, P. J. G. (1964). *Biochem. J.* **91**, 171.
- Hill, J. M. & Mann, P. J. G. (1966). *Biochem. J.* **99**, 454.
- Hirsch, J. G. (1953). *J. exp. Med.* **97**, 345.
- Holmstedt, B., Larsson, L. & Tham, R. (1961). *Biochim. biophys. Acta*, **48**, 182.
- Kenten, R. H. (1953). *Biochem. J.* **55**, 350.
- Kenten, R. H. & Mann, P. J. G. (1952). *Biochem. J.* **50**, 360.
- Kenten, R. H. & Mann, P. J. G. (1954). *Biochem. J.* **57**, 347.
- Kirsten, E., Gerez, C. & Kirsten, R. (1963). *Biochem. Z.* **337**, 312.
- Mann, P. J. G. (1961). *Biochem. J.* **79**, 623.
- Mann, P. J. G. & Smithies, W. R. (1955). *Biochem. J.* **61**, 89.
- Matsuo, Y. (1957). *J. Amer. chem. Soc.* **79**, 2011.
- Mondovi, B., Rotilio, G., Finazzi, A. & Scioscia-Santoro, A. (1964). *Biochem. J.* **91**, 408.
- Okuyama, T. & Satake, K. (1960). *J. Biochem., Tokyo*, **47**, 454.
- Satake, K., Okuyama, T., Ohashi, M. & Shinoda, T. (1960). *J. Biochem., Tokyo*, **47**, 654.
- Schöpf, C., Komzak, A., Braun, F. & Jacobi, E. (1948). *Liebigs Ann.* **559**, 1.
- Smith, L. I. & Opie, J. W. (1955). *Org. Synth.* **3**, 56.
- Suzuki, Y. (1966). *Naturwissenschaften*, **53**, 504.
- Werle, E. & Hartung, G. (1956). *Biochem. Z.* **328**, 228.
- Werle, E. & Pechmann, E. (1949). *Liebigs Ann.* **562**, 44.
- Werle, E., Trautschold, I. & Aures, D. (1961). *Hoppe-Seyl. Z.* **326**, 200.
- Yamada, H., Odachi, O. & Ogata, K. (1965). *Agric. biol. Chem., Tokyo*, **29**, 912.
- Yamada, H. & Yasunobu, K. T. (1962a). *J. biol. Chem.* **237**, 1511.
- Yamada, H. & Yasunobu, K. T. (1962b). *I.U.B. Symp. Ser.* **30**, 453.
- Yamada, H. & Yasunobu, K. T. (1962c). *J. biol. Chem.* **237**, 3077.
- Yamada, H. & Yasunobu, K. T. (1963). *J. biol. Chem.* **238**, 2669.
- Zeller, E. A. (1938). *Helv. chim. Acta*, **21**, 880.
- Zeller, E. A. (1963). In *The Enzymes*, 2nd ed., vol. 8, part B, p. 322. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York and London: Academic Press Inc.