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THE OXIDATION OF PYRIDOXAL AND RELATED COMPOUNDS BY PEA-SEEDLING EXTRACTS OR SYSTEMS CONTAINING PEROXIDASE

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Abstract—Pyridoxal, pyridoxal phosphate, pyridoxine and pyridoxamine increase the oxygen uptake of pea-seedling extracts in the presence of 1,4-diaminobutane. These increases are greatest with root extracts and are accompanied by a disappearance of pyridoxal or related compounds. Two thermolabile factors in pea-seedling extracts, probably a diamine oxidase and a peroxidase, are involved in this reaction. The diamine oxidase and 1,4-diaminobutane can be replaced by hydrogen peroxide or another system that produces hydrogen peroxide. Pyridoxal and related compounds containing the 3-hydroxypyridine structure are substrates for peroxidase; when pyridoxal or pyridoxine are incubated with pea-seedling root extracts and 1,4-diaminobutane or with peroxidase and a hydrogen peroxide producing system, the 3-hydroxypyridine structure is destroyed. The increase in oxygen uptake when pyridoxal is incubated with legume-seedling extracts catalysing the oxidation of diamines is therefore not, as has been suggested, evidence for a pyridoxal moiety in the prosthetic group of plant diamine oxidase but is due at least in part to a coupled oxidation of pyridoxal by peroxidase.

INTRODUCTION

PYRIDOXAL phosphate has been suggested to be the active carbonyl component of some amine oxidases of animal and microbial origin.[1-4] Legume seedling diamine oxidase also contains an active carbonyl component[5,6] and because the oxidative deamination of 1,5-diaminopentane by dialysed clover-seedling extracts was increased by adding pyridoxal it has been suggested that this enzyme might also contain a pyridoxal moiety.[5] However neither pyridoxal nor pyridoxal phosphate stimulates the activity of purified pea-seedling diamine oxidase[7,8] and all attempts to demonstrate the presence of pyridoxal or a derivative in this enzyme have failed.[7-9]

The increase in oxidative deamination of 1,5-diaminopentane by pea-seedling extracts which occurs on adding pyridoxal is due not to an activation of diamine oxidase but to the prevention of formation of an inhibitor of this enzyme.[10] The formation of this inhibitor is catalysed by peroxidase and in experiments in which legume-seedling extracts or purified systems containing peroxidase, diamine oxidase and a diamine oxidase substrate, the oxygen

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uptake was often increased more than the ammonia formation by the addition of pyridoxal. The present paper shows that some of this increase in oxygen uptake may come from a peroxidase-catalysed oxidation of pyridoxal or related compounds having the 3-hydroxypyridine structure, using hydrogen peroxide produced in the diamine oxidase reaction.

RESULTS

The effect of pyridoxal and related compounds on the oxygen uptake of roots, epicotyls, cotyledons or whole plants of 13-day-old pea seedlings was tested in the presence of 1,4-diaminobutane. This compound was used as the diamine oxidase substrate because, in contrast to 1,5-diaminopentane, no inhibitor of diamine oxidase is formed when peroxidase is present.[10] Slight increases in the oxygen uptake when pyridoxal or pyridoxal phosphate were

TABLE 1. EFFECT OF PYRIDOXAL AND RELATED COMPOUNDS ON THE OXIDATION OF 1,4-DIAMINOBUTANE BY PEA-SEEDLING ROOT EXTRACTS

Expt.	Vol. of extract (ml)	Addition	O_2 uptake/2 hr (μmole)
1	1·4	None	2·95
	1·4	Pyridoxal (2·5 μmoles)	3·44
	1·4	Pyridoxal (10 μmoles)	5·09
	1·4	Pyridoxine (2·5 μmoles)	3·34
	1·4	Pyridoxine (10 μmoles)	4·69
2	1·4	None	3·40
	1·4	Pyridoxal phosphate (2·5 μmoles)	4·15
	1·4	Pyridoxal phosphate (10 μmoles)	4·25
	1·4	Pyridoxamine (2·5 μmoles)	3·83
	1·4	Pyridoxamine (10 μmoles)	4·69

Extracts of roots of 13-day-old pea-seedlings were prepared as described in the Experimental section. The reaction mixtures contained pea-seedling root extract and additions as indicated in the total volume of 3 ml potassium phosphate buffer, pH 7. After an equilibration period of 30 min the reaction was started by addition of the 1,4-diaminobutane (10 μmoles) and the oxygen uptake was measured over 2 hr.

added in the absence of 1,4-diaminobutane were attributed to the formation of Schiff's bases with amino acids and their subsequent oxidation catalysed by Mn^{2+} ions;[11] the increases were always greater in the presence of 1,4-diaminobutane. Table 1 shows the oxygen uptake of pea-seedling root extracts catalysing the oxidation of 1,4-diaminobutane with and without pyridoxal, pyridoxine, pyridoxamine and pyridoxal phosphate; the results are corrected for endogenous oxygen uptakes of 1·5 μmoles in experiment 1 and 1·9 μmoles in experiment 2, when 1,4-diaminobutane was absent from the reaction mixtures. Pyridoxal and related compounds increased the oxygen uptake of these reaction mixtures containing root extracts and 1,4-diaminobutane. Similar results were obtained with extracts of epicotyls, cotyledons and whole plants but the increases in oxygen uptake were less than those with root extracts.

Spectrophotometry showed that the increases in oxygen uptake were accompanied by a decrease in concentration of pyridoxal and the other related compounds. Samples of the reaction mixtures from Table 1 containing 10 μmole pyridoxine, were diluted 40-fold with

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either 0·1 M phosphate buffer, pH 7, or with 0·1 M HCl and spectra were plotted from 210 nm to 400 nm using reference solutions prepared from the appropriate reaction mixtures containing no pyridoxine. The absorption spectra of reaction mixtures containing pyridoxine incubated with root extract or 1,4-diaminobutane alone were almost identical with that of the pyridoxine originally added. When 1,4-diaminobutane and root extract were both present during the incubation with pyridoxine, changes occurred in the spectra; at pH 7 there was a decrease of 27 per cent in the absorption of the 324 nm band whilst the spectrum in 0·1 N HCl showed a decrease of 32 per cent in the 291 nm absorption band. As the absorption by pyridoxine in acid solution at this wavelength has been attributed to a 3-hydroxypyridine structure[12] these results suggest that the oxidation of pyridoxine involves a change of the 3-hydroxyl group.

Results were similar with pyridoxal; although its concentration decreased slightly when it was incubated with root extract alone the decrease was much greater in the presence of 1,4-diaminobutane.

Effect of Heating Root Extracts

Heating a root extract of 13-day-old pea seedlings to 100° for 30 sec decreased its ability to catalyse the oxidation of 1,4-diaminobutane, pyridoxal or pyridoxine (Table 2). When heating was prolonged to 2 min these compounds were not oxidized at all. Addition of purified diamine oxidase restored only the ability to oxidize 1,4-diaminobutane. These results suggest that two thermolabile factors are involved in the oxidations catalysed by pea-seedling extracts; the first, probably diamine oxidase, catalysing the oxidation of 1,4-diaminobutane and the other coupling this oxidation with that of pyridoxal or pyridoxine.

TABLE 2. EFFECT OF HEATING PEA-SEEDLING ROOT EXTRACTS ON THE OXYGEN UPTAKE IN THE PRESENCE OF 1,4-DIAMINOBUTANE, PYRIDOXINE, OR PYRIDOXAL WITH AND WITHOUT ADDED DIAMINE OXIDASE

Addition	O_2 uptake (μmole/2 hr) Time of heating (sec)		
	0	30	120
None	3·9	2·9	0·1
Pyridoxine (10 μmoles)	8·6	4·3	0·0
Pyridoxal (10 μmoles)	8·5	4·4	0·3
Diamine oxidase (0·2 I.U.)	6·5	5·1	3·1
Diamine oxidase (0·2 I.U.) + pyridoxine (10 μmoles)	9·1	6·6	2·9
Diamine oxidase (0·2 I.U.) + pyridoxal (10 μmoles)	10·3	7·5	3·0

Root extracts of 13-day-old pea-seedling were heated to 100° for 30 sec and 120 sec and then rapidly cooled in ice. Reaction mixtures contained heated or unheated root extracts (1 ml) catalase (25 μg) and additions as given, total volume of 3 ml in a 67 mM phosphate buffer, pH 7. The reaction was started by the addition of 10 μmoles 1,4-diaminobutane.

The reaction catalysed by diamine oxidase produces hydrogen peroxide; hydrogen peroxide is a substrate for peroxidase and as this enzyme is more abundant in roots than in other parts of pea seedlings[13] the possibility that it was involved in the oxidation of pyridoxine or related compounds was considered.

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Oxidation of Pyridoxine and Pyridoxal by Diamine Oxidase and Peroxidase

Purified pea-seedling diamine oxidase has little or no peroxidase activity. Adding horse-radish peroxidase to reaction mixtures containing diamine oxidase, catalase and 1,4-diaminobutane slightly increased the oxygen uptake, larger increases occurred when pyridoxal and pyridoxine were also present (Table 3); pyridoxal and pyridoxine had little effect without peroxidase. Catalase was used in the reaction mixtures both to prevent the accumulation of amounts of hydrogen peroxide which inhibit the diamine oxidase and to allow the amount of hydrogen peroxide used in the peroxidase-catalysed reaction to be measured manometrically. Although catalase decomposes hydrogen peroxide its affinity ($K_m = 2{\cdot}5 \times 10^{-2}$ M) for this substrate is much lower than that of peroxidase ($K_m = 6 \times 10^{-6}$ M). The differences in affinity and the relative amounts of catalase and peroxidase used in the reaction mixtures make it unlikely that hydrogen peroxide would be decomposed by catalase unless hydrogen peroxide was

TABLE 3. OXIDATION OF PYRIDOXAL AND PYRIDOXINE BY HORSE-RADISH PEROXIDASE IN THE PRESENCE OF DIAMINE OXIDASE AND 1,4-DIAMINOBUTANE

Expt.	Addition	O_2 uptake/2 hr (μmole)
1	None	4·75
	Horseradish peroxidase (1 mg)	5·23
	Pyridoxine (10 μmoles)	4·78
	Pyridoxine (10 μmoles) + horse-radish peroxidase (1 mg)	7·36
2	None	4·20
	Horse-radish peroxidase (1 mg)	4·5
	Pyridoxal (10 μmoles)	4·4
	Pyridoxal (10 μmoles) + horse-radish peroxidase (1 mg)	8·12

Reaction mixtures contained 0·2 I.U. purified pea-seedling diamine oxidase, 25 μg catalase plus additions as given, in 3 ml 67 mM phosphate buffer, pH 7. The reaction was started by the addition of 1,4-diaminobutane (10 μmoles) from the side-arm of the reaction vessel.

formed at a greater rate than it could be used in the peroxidase-catalysed reaction. Peroxidase and Mn^{2+} ions catalyse the further oxidation of the product of diamine oxidase-catalysed oxidation of 1,4-diaminobutane[14] and probably the increases in oxygen uptake in the absence of pyridoxal or pyridoxine were from this reaction. The much larger increases when both peroxidase and either pyridoxal or pyridoxine were present indicate peroxidase catalysed the oxidation of pyridoxal or pyridoxine by hydrogen peroxide. Results were similar when a partially purified preparation of pea-seedling peroxidase, prepared by the method described by Mann,[15] was substituted for horse-radish peroxidase in these reactions.

Spectrophotometry showed that the amounts of both pyridoxal and pyridoxine diminished in reaction mixtures containing horse-radish peroxidase, diamine oxidase and 1,4-diaminobutane together, but not when only one or two of these components were present. The changes were similar to those with root extracts and support the suggestion that peroxidase catalyses the coupled oxidation of pyridoxal or pyridoxine.

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Oxidation of Pyridoxine and Pyridoxal by Horse-radish Peroxidase and Hydrogen Peroxide

The peroxidase-catalysed oxidation of pyridoxal and pyridoxine was confirmed spectrophotometrically. Reaction mixtures containing 10 μmoles pyridoxine or pyridoxal, with and without peroxidase, were incubated at pH 7 with hydrogen peroxide, for 3 hr at 25°. Hydrogen peroxide was added as small samples at intervals throughout the course of the reaction because preliminary experiments showed that more pyridoxal and pyridoxine were oxidized under these conditions than when the same total amount of hydrogen peroxide was present from the start of the reaction. The fact that there were no changes in the spectra

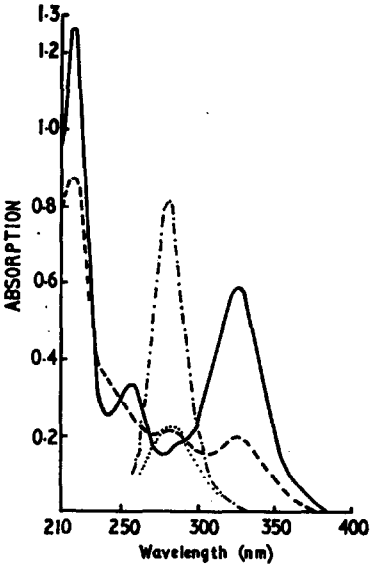

FIG. 1. ABSORPTION SPECTRA OF PYRIDOXINE BEFORE AND AFTER INCUBATION WITH PEROXIDASE AND HYDROGEN PEROXIDE.

A reaction mixture containing pyridoxine (10 μmole), peroxidase (2·7 mg), hydrogen peroxide (18 μmole, added as 1 μmole samples at 10 min intervals) and orthophosphate (200 μmole) in a total volume of 4 ml, pH 7, was incubated for 3 hr at 25°. After incubation, samples (0·1 ml) and catalase (25 μg) was diluted to 4 ml with either 0·01 M phosphate buffer, pH 7 (– – –) or 0·1 N HCl (·····) and their spectra plotted using similarly prepared solutions without pyridoxine as controls.

Similar reaction mixtures were prepared except that the hydrogen peroxide was added as one sample. The mixtures were diluted immediately as above and the spectra at pH 7 (———) and in 0·1 N HCl (— · — ·) were plotted against controls without pyridoxine.

before and after incubation of reaction mixtures treated with either hydrogen peroxide or peroxidase alone or with hydrogen peroxide and heat-treated peroxidase suggests that there was no loss of pyridoxal or pyridoxine. When both hydrogen peroxide and peroxidase were present during the incubation the spectrum at pH 7 showed considerable decreases in absorption at 220 and 324 nm whilst a new absorption band appeared at 284 nm; in 0·1 N HCl the spectrum showed a considerable decrease in the intensity of the 291 nm absorption band after incubation (Fig. 1). Similar results were obtained with pyridoxal except that new absorption bands were not formed.

Table 4 shows that the amount of the 3-hydroxypyridine component decreased in the reaction mixtures containing peroxidase, hydrogen peroxide and either pyridoxal or pyridoxine; this loss parallelled that found for pyridoxal and pyridoxine spectrophotometrically.

TABLE 4. DETERMINATION OF 3-HYDROXYPYRIDINE COMPONENT REMAINING AFTER OXIDATION OF PYRIDOXINE AND PYRIDOXAL WITH PEROXIDASE

Substrate	Peroxidase	3-Hydroxypyridine (μmole)
Pyridoxine	−	10·0
Pyridoxine	+	2·5
Pyridoxal	−	10·0
Pyridoxal	+	7·2

The 3-hydroxypyridine component remaining in the reaction mixtures used for Figs. 2 and 3 was determined. Samples of the reaction mixtures (0·1 ml) were treated with Gibb's chlorimide reagents as described in the Experimental and the 3-hydroxypyridine component was determined by measuring the absorption at 625 nm.

Oxidation of Pyridoxal and Related Compounds by Peroxidase in the Presence of Glucose and Glucose Oxidase

The oxidation of pyridoxal and pyridoxine by systems containing peroxidase, diamine oxidase and 1,4-diaminobutane could not be determined manometrically as the results were

TABLE 5. THE OXIDATION OF PYRIDOXAL AND RELATED COMPOUNDS BY PEROXIDASE-GLUCOSE OXIDASE SYSTEMS

Expt.	Additions (10 μmoles)	Peroxidase (mg)	O_2 uptake/3 hr (μmoles)
1	None	None	4·9
	None	1·0	5·1
	Pyridoxine	0·1	6·6
	Pyridoxine	0·3	7·8
	Pyridoxine	1·0	9·9
2	None	None	4·8
	Pyridoxal	None	5·1
	Pyridoxal	1·0	9·0
	Pyridoxamine	None	5·0
	Pyridoxamine	1·0	10·2
	Pyridoxal phosphate	None	4·9
	Pyridoxal phosphate	1·0	8·9
3	None	None	5·1
	2-Hydroxypyridine	None	5·1
	2-Hydroxypyridine	1·0	5·0
	3-Hydroxypyridine	None	5·0
	3-Hydroxypyridine	1·0	8·2
4	None	None	4·9
	2-Hydroxymethylpyridine	None	4·8
	2-Hydroxymethylpyridine	1·0	4·9
	3-Hydroxymethylpyridine	None	5·0
	3-Hydroxymethylpyridine	1.0	5·0

Reaction mixtures contained glucose oxidase (1·25 mg), catalase (25 μg), horse-radish peroxidase and additions as given in 3 ml 67 mM phosphate buffer, pH 7. The reaction was started by the addition of glucose (10 μmoles) from the side arm of the reaction vessel.

confused by further oxidation of the diamine-oxidation products. The glucose–glucose oxidase system was therefore used to supply hydrogen peroxide necessary for the manometric study of the oxidation of pyridoxal and related compounds because preliminary experiments showed that peroxidase did not catalyse a significant oxidation of the gluconic acid formed. In the presence of catalase 0·5 mole oxygen are taken up for each mole glucose oxidized; by using the hydrogen peroxide for secondary oxidations and thereby preventing its decomposition by catalase the addition of peroxidase can increase the oxygen taken up to a maximum of 1 mole per mole glucose oxidized. Table 5 shows that the oxygen uptake of reaction mixtures containing glucose, glucose oxidase, catalase and peroxidase, were increased by adding pyridoxine, pyridoxal, pyridoxal phosphate, pyridoxamine or 3-hydroxypyridine. All the

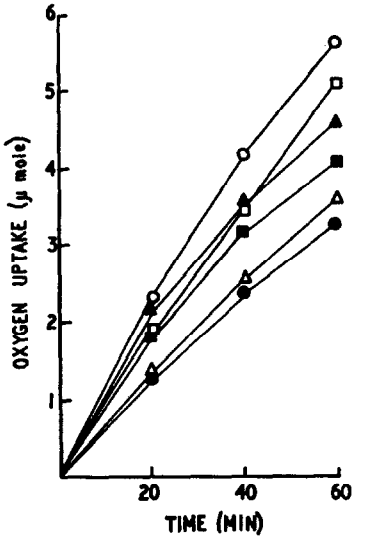

FIG. 2. RATE OF OXIDATION OF 3-HYDROXYPYRIDINE DERIVATIVES.

Reaction mixtures contained glucose oxidase (1·25 mg), catalase (25 μg) in 3 ml 67 mM orthophosphate buffer, pH 7, together with ●, no additions; △, horse-radish peroxidase (1 mg); ■, horse-radish peroxidase (1 mg) and pyridoxal phosphate (10 μmole); ▲, horse-radish peroxidase (1 mg) and 3-hydroxypyridine (10 μmole); □, horse-radish peroxidase (1 mg) and pyridoxal (10 μmole); ○, horse-radish peroxidase (1 mg) and pyridoxine (10 μmole). The reaction was started by the addition of glucose (10 μmole).

compounds effective in increasing the oxygen were unsubstituted at position 3 on the pyridine ring; 2-hydroxypyridine and 2- and 3-hydroxymethyl pyridines were ineffective. Little or no oxygen was taken up without glucose and there was no increase when pyridoxal or related compounds were absent. Figure 2 shows that the rate of oxygen uptake was greatest with pyridoxine and least with pyridoxal phosphate. The oxygen uptake of the glucose–glucose oxidase system was sometimes slightly increased by the presence of peroxidase but was always greater when the 3-hydroxypyridine derivatives were present.

The spectrophotometric changes when pyridoxal and pyridoxine were oxidized by systems containing glucose oxidase and peroxidase resembled those when these compounds were oxidized by peroxidase and hydrogen peroxide. With pyridoxine the spectrophotometric changes at 324 nm were proportional to both the disappearance of 3-hydroxypyridine structure and the increase in oxygen uptake. When 10 μmoles of pyridoxine were oxidized 7·5

μmoles of oxygen were needed for the complete disappearance of the 324 nm absorption band, corresponding to 3 atoms of oxygen for every 2 molecules of pyridoxine oxidized. The results with pyridoxal were similar except that 5 atoms of oxygen were needed for the oxidation of 2 molecules of pyridoxal. It is probable that in this case 1 atom of oxygen per molecule of pyridoxal oxidized was used in the oxidation of the aldehyde group by a mechanism similar to that described for the peroxidase-catalysed oxidation of phenylacetaldehyde to benzoic acid.[16]

DISCUSSION

Pyridoxal phosphate is important as a component of the prosthetic group of many enzymes but little is known of the metabolism of this compound or the related compounds, pyridoxal, pyridoxine and pyridoxamine, in higher plants. The present results show that the oxidation of pyridoxal and related compounds can be catalysed by peroxidase present in extracts of higher plants. The results do not support the suggestion[5] that pyridoxal or a related compound is a co-factor for legume-seedling diamine oxidase. When pyridoxal is added to dialysed clover-seedling extracts catalysing the oxidation of 1,5-diaminopentane, both the oxygen uptake and ammonia formation are increased.[5] These increases were originally attributed to an activation of the diamine oxidase present by pyridoxal but it has since been shown that there are at least two alternative explanations for these increases which do not assume an activation of diamine oxidase.[10,11] The present results suggest a third explanation for part of the increase in oxygen uptake and show that the hydrogen peroxide produced in the diamine oxidase reaction can be used in the oxidation of pyridoxal catalysed by peroxidase.

Diamine oxidase is inhibited by hydrogen peroxide.[6] Werle and Hartung[17] reported the possibility that the diamine oxidase activity of clover-seedling extracts was increased because pyridoxal removed hydrogen peroxide, as the increase only occurred with 1,5-diaminopentane and not with histamine as the substrate. The oxidation product of histamine, imidazole acetaldehyde, is oxidized by peroxidase;[15-18] this oxidation in plant extracts would remove all the hydrogen peroxide produced by the diamine oxidase reaction and any oxidation of pyridoxal competing with this reaction for hydrogen peroxide would not increase the oxygen uptake. The oxidation products of 1,5-diaminopentane are only slowly oxidized in reactions catalysed by peroxidase and excess hydrogen peroxide would be decomposed by the catalase present. Adding pyridoxal to these reaction mixtures would increase the use of hydrogen peroxide by peroxidase and would thereby increase the oxygen uptake.

Hydrogen peroxide in strongly alkaline solution oxidizes pyridoxal and pyridoxal phosphate to the corresponding *o*-dihydroxyphenols, 2-methyl-3,4-dihydroxy-5-hydroxymethylpyridine and 2-methyl-3,4-dihydroxy-5-pyridyl methyl phosphoric acid respectively.[19] The present results show that at pH 7 dilute hydrogen peroxide alone has little effect on pyridoxal, pyridoxal phosphate or related compounds having the 3-hydroxy pyridine structure; in the presence of peroxidase, hydrogen peroxide oxidizes these compounds if any contain the unsubstituted phenolic group but does not oxidize 2-hydroxypyridine derivatives or 3-hydroxymethylpyridine. Pyridoxine is oxidized more readily than the other compounds tested. The spectra of the oxidation products suggest that they are not *o*-dihydroxyphenols

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and that they do not retain the 3-hydroxypyridine structure. The results with chloroimide reagent support these observations. Chloroimide reagent reacts with phenols unsubstituted at the *para*-position to produce blue indophenols;[20] only pyridine derivatives with a phenolic group at position 3 form coloured complexes with this reagent.[21] Pyridoxal and pyridoxine, but not their oxidation products, react with chloroimide reagent, showing that on oxidation either the phenolic group or the group unsubstituted group *para* to this are changed. The final products of the peroxidase-catalysed oxidation of pyridoxal and pyridoxine were not chemically identified.

EXPERIMENTAL

Pea-Seedling Extracts

Pea seedlings (*Pisum sativum* L. var. Pilot) were grown in sand in the dark for 13 days at about 20°, then washed free from sand and either divided into roots, cotyledons and epicotyls, or left intact. The plant material was then ground with 0·2 M phosphate buffer, pH 7 (0·5 ml/g plant material) 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

Horse-radish peroxidase—Donor: hydrogen peroxide oxidoreductase—(EC 1.11.1.7) was made by following the method of Kenten and Mann[22] as far as the second precipitation with ethanol. The activity of the preparation was determined with pyrogallol as the hydrogen donor, by the method of Keilin and Hartree,[23] except that the amount of purpurogallin formed was found by measuring the absorption at 430 nm, the molecular extinction coefficient was taken to be 2470.[24] One unit of peroxidase is defined as the amount catalysing the use of 1 μmole hydrogen peroxide/min in the reaction at 20°; 3 μmoles hydrogen peroxide are used in the formation of 1 μmole purpurogallin. The specific activity (units/mg) of the preparation was 2200 and the haemin content, determined from the absorption at 557 nm after conversion into the pyridine haemochromogen,[25] was 0·89%. Pea-seedling diamine oxidase—Diamine: oxygen oxidoreductase (deaminating)—(EC 1.4.3.6) was prepared by the method of Hill and Mann.[26] The assay was based on the initial oxygen uptake by reaction mixtures containing diamine oxidase, catalase (25 μg), 10 mM 1,4-diaminobutane, 67 mM phosphate, pH 7, in a total volume of 3 ml. One unit of diamine oxidase activity is the amount that catalyses the oxidation of 1 μmole of 1,4-diaminobutane/min at 25° giving an initial rate of uptake of oxygen of 11·2 μl/min. Crystalline ox-liver catalase—Hydrogen peroxide:hydrogen peroxide oxidoreductase—(EC 1.11.1.6) and glucose oxidase—Glucose:oxygen oxidoreductase—(EC 1.1.3.4) were purchased from the Sigma (London) Chemical Co. Ltd.

Buffers

Phosphate buffers were prepared from KH_2PO_4 and KOH and veronal buffers from sodium barbitone and NaOH.

Manometric Methods

The oxygen uptakes were measured using 3-ml reaction mixtures at 25° in air, with 0·2 ml 5 N KOH in the centre well, in the Warburg apparatus.

Spectrophotometry

All measurements were made in silica cuvettes of 1 cm lightpath.

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Determination of 3-Hydroxypyridine Derivatives

The 3-hydroxypyridine component remaining after the oxidation of pyridoxal and pyridoxine was estimated using Gibb's chlorimide reagent.[27,28] The reaction mixture contained 1 ml 0·05 M veronal buffer, pH 7·6, 0·5 ml chloroimide reagent (10 mg 2,6-dichloro-*p*-benzoquinone-4-chloroimide in 25 ml isopropyl alcohol), 2·5 ml isopropyl alcohol, 0·1 ml of the Warburg reaction mixtures and water to give a total volume of 5 ml. After incubation at room temperature for 10 min, the absorption at 625 nm was measured.

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