The Oxidation of Schiff Bases of Pyridoxamine with α -Oxo Acids by Manganous Ions and Peroxidase

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1. Oxygen was taken up rapidly when pyruvate was added to mixtures of pyridoxamine and Mn^{2+} ions after lag periods that were shortened by peroxidase (donor–hydrogen peroxide oxidoreductase, EC 1.11.1.7). 2. The total oxygen uptake was proportional to the pyridoxamine added and was accompanied by the disappearance of pyridoxamine; the pyruvate acted catalytically and hydrogen peroxide was not formed. 3. At pH 6 more than half the pyridoxamine that disappeared was accounted for as pyridoxal and ammonia; it is suggested that the primary reaction is the oxidative deamination of the pyridoxamine. 4. Results were similar when α -oxobutyrate or glyoxylate were substituted for pyruvate, except that the reactions were slower and the yield of pyridoxal less. 5. The oxidative decarboxylations of α -oxoglutarate and phenylpyruvate are catalysed by Mn^{2+} ions and these reactions are activated by peroxidase; pyridoxamine increased both the rates and total oxygen uptakes in these reactions, and ammonia was produced. 6. The lag periods in the oxidation of mixtures of pyridoxamine and α -oxo acids, catalysed by Mn^{2+} ions, were also shortened by traces of colloidal manganese dioxide. 7. It is suggested that the activating effect of peroxidase depends on its catalysis of manganese oxidation.

Many of the reactions of amino acids catalysed by enzymes containing pyridoxal phosphate are also catalysed in model systems by pyridoxal and metal ions. Metzler *et al.* (1954) suggested that the mechanism of these reactions depends on the properties of the metal chelates of the Schiff bases of the amino acids and pyridoxal, and that in the enzymic reactions the enzymes themselves act like the metals in the model reactions. The significance of these model reactions has increased as evidence has accumulated that some of the amine oxidases contain both copper and pyridoxal phosphate (Yamada & Yasunobu, 1962, 1963; Buffoni & Blaschko, 1964; Blaschko & Buffoni, 1965; Mondovi *et al.*, 1967). Mazelis (1962) showed that peroxidase initiated the oxidation of Schiff bases of amino acids and pyridoxal or pyridoxal phosphate with Mn^{2+} ions. However, for such reactions to be models of those catalysed by enzymes containing pyridoxal phosphate, the pyridoxal and pyridoxal phosphate must act catalytically. Hill & Mann (1966) reported that not only were the amino acids oxidatively decarboxylated and deaminated but that the pyridoxal and pyridoxal phosphate were also changed during the reactions, probably by oxidation, and had little or no catalytic activity.

The present work shows that Schiff bases of pyridoxamine with α -oxo acids are also oxidized by Mn^{2+} ions. Evidence is presented that in reactions with pyruvate as the α -oxo acid, it acts catalytically, and

that the primary reaction is an oxidative deamination of pyridoxamine to pyridoxal.

Experimental

Materials

Enzymes. The horseradish peroxidase preparation was that described by Hill & Mann (1966). Crystalline rabbit muscle lactate dehydrogenase (L-lactate–NAD oxidoreductase, EC 1.1.1.27) was bought from BDH Chemicals Ltd. (Poole, Dorset, U.K.).

Chemicals. Pyridoxamine dihydrochloride, pyridoxal hydrochloride, 2-oxoglutaric acid and glyoxylic acid were bought from Koch–Light Laboratories (Colnbrook, Bucks., U.K.); sodium pyruvate was from Hopkin and Williams (Chadwell Heath, Essex, U.K.); sodium 2-oxobutyrate was from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.); NADH was from Boehringer Corp. (London) Ltd. (London W.5, U.K.); and 2,4,6-trinitrobenzenesulphonic acid and 2,6-dichloro-*p*-benzoquinone-4-chloroimine were from BDH. Hydrated manganese dioxide was prepared by the method of Heintze & Mann (1949). The preparation was centrifuged at 3000g for 20 min to remove the larger particles, and the supernatant colloidal solution was used.

Buffers. Phosphate buffers were prepared from KH_2PO_4 and KOH, borate buffers from $Na_2B_4O_7$

and NaOH and veronal buffers from sodium 5,5-diethylbarbiturate and HCl.

Methods

Manometric methods. Oxygen uptake was measured on 3 ml of reaction mixture in a Warburg apparatus at 25°C in air, with 0.2 ml of 5M-KOH in the centre well. Carbon dioxide was measured by Warburg's direct method (Dixon, 1951).

Determinations. Ammonia was determined by steam-distilling samples (usually 1 ml) of the reaction mixtures with 2 ml of 0.1M-borate buffer, pH 10. The distillate was collected in 2 ml of 1% boric acid and titrated against 3.5M-H₂SO₄. Pyridoxamine was determined by a modification of the procedure described by Okuyama & Satake (1960) for the determination of amino acids and amines. Pyridoxamine (0.025–0.25 μmol) or samples (0.1 ml) of the reaction mixtures were incubated for 2 h at 40°C in stoppered tubes with 1 ml of 0.2M-phosphate buffer, pH 8, and 1 ml of the trinitrobenzenesulphonic acid reagent (0.1%, w/v, in 0.01M-HCl) in a total volume of 3 ml. The mixtures were cooled to room temperature and the E_{437} was measured and found to be proportional to the amount of pyridoxamine used ($\epsilon = 9600 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). Pyridoxal was determined by the method of Metzler & Snell (1952); pyridoxal (0.05–0.2 μmol) or samples of the reaction mixtures (0.1 ml) in a total volume of 2.25 ml with water were mixed with 0.75 ml of ethanolamine and the E_{365} was measured. Pyridoxal was also determined by the method of Wada & Snell (1961). The reaction mixtures were diluted 20-fold with 10mM-phosphate buffer, pH 7. Samples (1 ml) of this dilution were mixed with 2.8 ml of water and 0.2 ml of phenylhydrazine reagent (2%, w/v, phenylhydrazine hydrochloride in 5M-H₂SO₄), heated to 60°C for 20 min and then kept at room temperature for 10 min before measurement of the E_{410} . Pyruvate was determined enzymically with lactate dehydrogenase and NADH as described by von Korff (1969) and also as the 2,4-dinitrophenylhydrazine as described in the section on chromatography.

The method for measuring the 3-hydroxypyridine compounds was based on the procedures described by Scudi (1941) and Hochberg *et al.* (1944). The reaction mixtures were diluted 20-fold and samples (0.2 ml) were mixed with 0.8 ml of water, 1 ml of 0.05M-veronal buffer, pH 7.6, 2.5 ml of propan-2-ol and 0.5 ml of 2,6-dichloro-*p*-benzoquinone 4-chloroimine (0.04%, w/v, in propan-2-ol) reagent. The blue colour given by pyridoxamine and related 3-hydroxypyridine derivatives reached a maximum intensity in 10 min and was stable for a further 5 min. The E_{650} was measured 10 min after adding the chloroimine reagent and was used to calculate the amount of 3-hydroxypyridine components in the reaction mixtures.

Chromatography. Residual pyridoxamine and its reaction products were separated by one-dimensional chromatography by using the descending technique on Whatman no. 1 paper with the solvent system 2-methylpropan-2-ol–90% (w/w) formic acid–water (14:3:3, by vol.) as described by Peterson & Sober (1954) for the separation of pyridoxine and related compounds. The preparation, chromatography and determination of pyruvic acid 2,4-dinitrophenylhydrazone were based on the methods of Isherwood & Cruickshank (1954) and Hulme *et al.* (1964). Sufficient 0.01M-2,4-dinitrophenylhydrazine reagent in 0.2M-HCl was added to the reaction mixtures to ensure that the amount of 2,4-dinitrophenylhydrazine was at least twice that of the pyruvate initially present. After at least 1 h at room temperature, 50 μl of the solution was applied as a streak 2.5 cm long to Whatman no. 3 paper that had previously been dipped in 0.02M-phosphate buffer, pH 6.2, and air-dried. The chromatogram was developed by the descending technique by using the upper phase of a mixture of 2-methylbutan-2-ol–ethanol–water (50:10:40, by vol.). After the paper had been dried in air, the positions of the hydrazones were marked under u.v. light. The pyruvic acid 2,4-dinitrophenylhydrazone gave two spots (R_F 0.37 and 0.56) caused by the *cis*- and *trans*-isomers (Isherwood & Cruickshank, 1954). The two isomers were eluted each with a total of 5 ml of 0.2M-NaHCO₃, and the eluates were combined and the concentration of 2,4-dinitrophenylhydrazone determined by measuring the E_{370} (Hulme *et al.*, 1964).

Spectrophotometry. Extinctions were measured with a Unicam SP.500, an Optica CF4DR or an Optica CF4R spectrophotometer with Spectrosil cells of 1 cm light-path.

Results

Experiments with pyruvate

Effect of changes in concentration of reactants. Fig. 1 shows that when pyruvate was added to mixtures containing peroxidase, Mn²⁺ ions and pyridoxamine, oxygen was taken up after a lag period of about 5 min. The total oxygen uptake was proportional to the amount of pyridoxamine added and was about 0.8 mol of O₂/mol of pyridoxamine. Little or no oxygen was used when either pyridoxamine or pyruvate was omitted. The reaction rate was not further increased by adding more peroxidase or by *p*-cresol; *p*-cresol activates the oxidation by peroxidase and Mn²⁺ ions of the Schiff base formed between pyridoxal phosphate and methionine (Mazelis, 1962; Hill & Mann, 1966). The rate increased with additions of Mn²⁺ ions up to 1 μmol but decreased slowly with greater additions. Mn²⁺ ions catalysed the reaction in the absence of peroxidase but the lag period was longer.

Fig. 1. Oxidation of mixtures of pyridoxamine and pyruvate by Mn^{2+} ions and peroxidase: effect of variation of pyridoxamine concentration

Reaction mixtures (total volume 3 ml) contained 2.5 (●), 5 (□), 10 (Δ) or 15 (○) μ mol of pyridoxamine, 1 μ mol of $MnSO_4$ and 10 μ g of peroxidase in 67 mM-phosphate buffer, pH 7. Sodium pyruvate (30 μ mol) was added from the side-arm after equilibration. The gas phase was air and the temperature was 25°C. The centre cups contained 0.2 ml of 5 M-KOH.

Fig. 2. Effect of variation in pyruvate concentration

Reaction mixtures (total volume 3 ml) contained 10 μ mol of pyridoxamine and 1 μ mol of $MnSO_4$ and different amounts of pyruvate with and without 10 μ g of peroxidase in 67 mM-phosphate buffer, pH 7. Reaction mixtures with peroxidase: Δ, 2.5 μ mol of pyruvate; □, 10 μ mol of pyruvate; ○, 30 μ mol of pyruvate. Reaction mixtures without peroxidase: ▲, 2.5 μ mol of pyruvate; ■, 10 μ mol of pyruvate; ●, 30 μ mol of pyruvate.

Hill & Mann (1966) observed variations in the lag period in the oxidation by peroxidase and Mn^{2+} ions, of Schiff bases of pyridoxal phosphate and amino acids and attributed them to changes in light-intensity. The lag periods in the present experiments changed little with decrease in light intensity but increased with the age of the pyridoxamine solution used. In later experiments, fresh pyridoxamine solutions were prepared every few days and were stored in the dark at 0–5°C.

Fig. 2 shows that, with 10 μ mol of pyridoxamine present initially, the lag period increased as the addition of pyruvate was decreased from 30–2.5 μ mol. Peroxidase shortened the lag period; the subsequent reaction was very slow with 2.5 μ mol of pyruvate but increased with increase of concentration of pyruvate.

With 30 μ mol of pyridoxamine present initially, the reactions were rapid even with small additions of pyruvate. The total oxygen uptake was almost the same with 5 and 30 μ mol of pyruvate and suggests that pyruvate acts catalytically. The lag periods were usually short at the greater pyruvate concentrations, and here peroxidase had little effect; with 2 μ mol of pyruvate the long lag period was shortened by peroxidase.

Effect of the variation of pH. Fig. 3 shows that at

pH 5 and 5.5 the reaction was either not catalysed by Mn^{2+} ions or only after a very long lag period. With peroxidase also present, the lag periods were short and the subsequent reactions were almost as rapid as at pH 7. These results suggest that reactions under these acid conditions depend on the presence of peroxidase.

Evidence of the disappearance of pyridoxamine and the formation of pyridoxal. Fig. 4 shows the absorption spectra of reaction mixtures initially containing pyridoxamine, pyruvate, Mn^{2+} ions and peroxidase before and after incubation. Before incubation there were the two absorption bands at 254 and 327 nm characteristic of neutral solutions of pyridoxamine; after incubation the peak at 327 nm shifted to 318 nm where neutral solutions of pyridoxal absorb maximally. Fig. 4 also shows that when the reaction mixtures, after incubation, were treated with phenylhydrazine by the procedure of Wada & Snell (1961) the product, like pyridoxal phenylhydrazone, absorbed maximally at 410 nm; about half the pyridoxamine initially present could be accounted for as pyridoxal. In the presence of phenylhydrazine both the initial and final reaction mixtures absorbed strongly at 325 nm where pyruvic acid phenylhydrazone absorbs maximally; this E_{325} was affected by the absorption of the excess

Fig. 3. Effect of variation of pH

Reaction mixtures (total volume 3ml) contained pyridoxamine (10 μ mol), pyruvate (30 μ mol), MnSO_4 (1 μ mol), and peroxidase (10 μ g) in 67mM-phosphate buffer at pH 5 ($\square$), pH 5.5 ($\bullet$), and pH 6 ($\circ$). Results are shown for control reaction mixtures without peroxidase at pH 5 ($\blacktriangle$), pH 5.5 ($\blacktriangle$) and pH 6 ($\triangle$).

of phenylhydrazine reagent. The absorption maximum at 290nm shown by the initial reaction mixture was attributed to excess of phenylhydrazine. The E_{325} could not be used as an accurate measure of the pyruvate present, but the results suggest that the concentration of pyruvate changed little during the incubation period.

Paper chromatography confirmed that pyridoxamine disappeared and pyridoxal was formed during the reaction. Pyridoxamine and pyridoxal markers were detected on the chromatograms in u.v. light as blue fluorescent spots with mean R_F values of 0.52 and 0.71 respectively. Exposure of the chromatograms to ammonia intensified the spots and the one from pyridoxal was also yellow in daylight. Pyridoxamine was also detected as an orange spot after the chromatograms had been sprayed with ninhydrin (Peterson & Sober, 1954) and pyridoxal appeared as a green fluorescent spot after spraying with the semicarbazide reagent of Håkanson (1964). Little or no pyridoxamine was found in chromatograms of incubated reaction mixtures, but strong positive reactions were given for pyridoxal. In addition to the spot at R_F 0.71 attributed to pyridoxal, a

Fig. 4. Spectrophotometric evidence for the formation of pyridoxal as a reaction product

The reaction mixture (total volume 3ml) contained pyruvate (10 μ mol), pyridoxamine (10 μ mol), MnSO_4 (1 μ mol) and peroxidase (10 μ g) in 67mM-phosphate buffer, pH 7. Before and after incubation at 25°C for 4h, 0.1 ml samples were taken and diluted to 4ml with 10mM-phosphate buffer, pH 7, and the absorption spectrum was determined in 1 cm light-path cells. Absorption spectra were also determined after treatment of these diluted reaction mixtures with phenylhydrazine as described in the Experimental section. Absorption spectra: A, initial reaction mixture; B, incubated reaction mixture; C, initial reaction mixture treated with phenylhydrazine; D, incubated reaction mixture treated with phenylhydrazine; E, pyridoxal phenylhydrazone (20 μ M); F, pyruvic acid phenylhydrazone (40 μ M).

second blue fluorescent spot at R_F 0.62 was also detected after exposure of the chromatograms to ammonia.

Pyridoxal and ammonia as reaction products. Table 1 shows that, after incubation of the reaction mixtures for 4h, the total oxygen uptakes at pH 6, 7 and 8 were almost the same irrespective of whether the reactions were catalysed by Mn^{2+} ions alone or Mn^{2+} ions with peroxidase. Always less than 0.1 μ mol of the 10 μ mol of pyridoxamine originally present remained, but the greatest amount of pyridoxal found was only 6.2 μ mol at pH 6, and the yield decreased to 3.5 μ mol at pH 8. Pyridoxal was determined by measuring the

Table 1. *Oxygen uptake and the formation of pyridoxal and ammonia in the reaction between pyridoxamine, pyruvate and Mn^{2+} ions, with and without peroxidase*

Reaction mixtures (total volume 3ml) contained 10 μ mol of pyridoxamine and 1 μ mol of $MnSO_4$, with (+) and without (–) 10 μ g of peroxidase, in 67mM-phosphate buffer. Pyruvate (30 μ mol) was added from the side-arm after equilibration. The reaction mixtures were incubated for 4h at 25°C in the Warburg apparatus.

Peroxidase addition	pH of reaction mixture	O ₂ uptake (μ mol)	NH ₃ formed (μ mol)	Pyridoxal formed (μ mol)
–	6	8.4	6.9	6.0
+	6	8.8	7.3	7.2
–	7	8.1	6.4	5.5
+	7	8.4	6.6	5.7
–	8	8.5	5.1	3.3
+	8	8.7	5.6	3.5

E_{365} of the complex formed with ethanolamine (Metzler & Snell, 1952). This method was preferred to the determination as the phenylhydrazone by the method of Wada & Snell (1961) because there was a significant overlap at 410nm of the absorption bands in the spectra of the pyruvic acid and pyridoxal phenylhydrazones. Determinations with 2,6-dichloro-*p*-benzoquinone-4-chloroimine suggested that at pH7 there was little or no change in the concentration of the 3-hydroxypyridine compounds and only a small decrease at pH8. The results suggest that other 3-hydroxypyridine compounds besides pyridoxal were formed from pyridoxamine during the reaction.

Like the pyridoxal, the amount of ammonia formed decreased with increase in pH, but more ammonia than pyridoxal was always found, reaching 7.3 μ mol at pH6. In these experiments ammonia was measured by steam-distillation of samples of the reaction mixtures mixed with borate buffer at pH10. When 1M-NaOH was substituted for borate in the distillation, the amount of steam-volatile bases liberated, calculated as ammonia, increased to 10 μ mol. Little or no steam-volatile base was liberated from pyridoxamine under these conditions.

Oxidative deamination of 10 μ mol of pyridoxamine to pyridoxal and ammonia requires an uptake of 5 μ mol of oxygen. The observed uptakes were 8.1–8.7 μ mol, suggesting that two or more oxidative reactions were involved; little or no CO_2 was formed. The small yield of pyridoxal suggested that the oxidation of pyridoxal might be one of the secondary reactions. In contrast with the oxidation of pyridoxal by peroxidase and H_2O_2 (Hill, 1970), little or no loss of the 3-hydroxypyridine compounds was detected in the present reaction mixtures. In further experiments with reaction mixtures at pH6, similar to those of Table 1, pyridoxal and pyridoxamine were determined after incubation times of 1, 2 and 4h. The ratio of pyridoxamine oxidized to pyridoxal formed changed little with time, which suggests at this pH

there was no continuing oxidation of the pyridoxal formed.

Effect of other metal ions. There was no oxygen uptake and no ammonia formed in reaction mixtures containing peroxidase, pyridoxamine and pyruvate at pH7 when Mn^{2+} ions were replaced by Mg^{2+} , Al^{3+} , Ca^{2+} , Fe^{2+} , Fe^{3+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , Ce^{2+} or Ce^{3+} . However, some pyridoxal was formed, and although the amount after incubation for 4h was greatest with Cu^{2+} and Fe^{3+} , it was less than in similar reaction mixtures containing Mn^{2+} ions. Both oxygen uptake and ammonia formation by mixtures of pyridoxamine and pyruvate, with or without peroxidase plus 1 μ mol of Mn^{2+} ions, were strongly inhibited by 1 μ mol of Cu^{2+} ions.

Catalytic effect of pyruvate in the reaction. The results in the reaction with 30 μ mol of pyridoxamine suggested that pyruvate had a catalytic effect on the reaction, and this was confirmed by measuring the pyruvate remaining after incubation. Reaction mixtures with or without Mn^{2+} ions and peroxidase, similar to those in Table 1 except that 10 μ mol of pyruvate were present initially, were incubated for 2h. Oxygen was not absorbed when peroxidase and Mn^{2+} ions were omitted, but 6.4 μ mol of O_2 was when these components were present. The pyruvate contents after incubation, determined by the lactate dehydrogenase method, were 9.1 and 9.6 μ mol respectively with and without peroxidase and Mn^{2+} ions. The results were confirmed by measuring the pyruvic acid 2,4-dinitrophenylhydrazone isolated by paper chromatography. In addition to the two spots (R_F 0.37 and 0.56) attributed to the *cis*- and *trans*-isomers of pyruvic acid 2,4-dinitrophenylhydrazone (Isherwood & Cruickshank, 1954), there was a third spot in the position of pyridoxal 2,4-dinitrophenylhydrazone (R_F 0.71). The E_{370} of the solutions of the pyruvic acid 2,4-dinitrophenylhydrazones obtained by eluting the spots with 0.2M- $NaHCO_3$ (Hulme *et al.*, 1964) from chromatograms of the

reaction mixtures with and without peroxidase and Mn^{2+} ions, were 0.178 and 0.181 respectively.

Experiments with other α -oxo acids

Table 2 shows that Mn^{2+} ions also catalysed the oxidation of mixtures of pyridoxamine with α -oxobutyrate or glyoxylate. The lag periods were longer than in corresponding experiments with pyruvate and ranged from 30 to 60 min in different experiments. Peroxidase shortened the lag periods to a few minutes, but the subsequent rates of reaction were not much faster with than without peroxidase and were slower than when pyruvate was used. Some pyridoxamine remained after incubation and the yields of pyridoxal and ammonia were smaller than with pyruvate.

When α -oxoglutarate and phenylpyruvate were used the reactions were more complex because Mn^{2+} ions and peroxidase catalyse the oxidation of these compounds. Kalnitsky (1953) showed that 30 mM-

Mn^{2+} ions at pH 8 catalysed the oxidative decarboxylation of α -oxoglutarate. In the present experiments at pH 7 the oxidation with 0.33 mM- Mn^{2+} ions was very slow but was clearly increased by peroxidase; when pyridoxamine was also present a much more rapid reaction occurred catalysed by Mn^{2+} ions and peroxidase with little or no lag period, and by Mn^{2+} ions alone after a lag period of 30–60 min. The oxidation of phenylpyruvate to benzaldehyde, CO_2 and other products is catalysed by peroxidase and Mn^{2+} ions (Conn & Seki, 1957). In the present experiments phenylpyruvate was rapidly oxidized by Mn^{2+} ions and peroxidase at pH 7, but the rate and the total oxygen uptake were both increased by adding pyridoxamine.

Experiments with pyridoxal

When pyruvate was replaced by pyridoxal in reaction mixtures similar to those in Table 1, oxygen was rapidly taken up in daylight with little or no lag

Table 2. *Oxygen uptake and formation of pyridoxal and ammonia in the reaction between pyridoxamine, glyoxylate or α -oxobutyrate, and Mn^{2+} ions with and without peroxidase*

Reaction mixtures (total volume 3 ml) contained pyridoxamine (10 μ mol) and $MnSO_4$ (1 μ mol), with and without peroxidase, in 67 mM-phosphate buffer, pH 7. Glyoxylate or α -oxobutyrate (30 μ mol) was added from the side-arm after equilibration.

Additions		Time of incubation (h)	O_2 uptake (μ mol)	Pyridoxamine remaining (μ mol)	Pyridoxal formed (μ mol)	NH_3 formed (μ mol)
α -Oxo acid	Peroxidase (μ g)					
Glyoxylate	0	4	3.6	4.7	1.5	3.0
Glyoxylate	10	4	5.4	2.5	2.3	3.6
α -Oxobutyrate	0	6	5.4	5.2	1.7	3.0
α -Oxobutyrate	10	6	8.0	2.6	2.4	3.7

Table 3. *Oxygen uptake, increase in pyridoxal and formation of ammonia in the reaction between pyridoxamine, pyridoxal and Mn^{2+} ions with and without peroxidase at pH 7*

Reaction mixtures (total volume 3 ml) contained 1 μ mol of $MnSO_4$, with (+) and without (–) 10 μ mol of pyridoxamine and with (+) and without (–) 10 μ g of peroxidase in 67 mM-phosphate buffer, pH 7. Amounts of pyridoxal as indicated in the Table were added from the side-arm after equilibration and the reaction mixtures were then incubated for 4 h at 25°C in the Warburg apparatus.

Present in initial reaction mixtures			Found after incubation			
Peroxidase	Pyridoxamine	Pyridoxal (μ mol)	O_2 uptake (μ mol)	NH_3 formed (μ mol)	Pyridoxal (μ mol)	Pyridoxamine (μ mol)
+	–	10	0.58	0	9.7	0
+	+	0	0.40	0	0.23	9.45
–	+	5	7.90	5.6	9.3	0.22
+	+	5	8.16	5.6	8.3	0.23
–	+	10	7.45	6.8	13.7	0.06
+	+	10	8.25	6.9	13.6	0.23
–	+	30	7.68	7.2	31.9	0.09
+	+	30	8.36	7.4	31.5	0.22

where both peroxidase and Mn^{2+} ions were present and with a lag period of about 15min with only Mn^{2+} ions. The extent of the lag period was light-dependent; when reaction mixtures were kept in the dark, the lag period increased to 30min with peroxidase and Mn^{2+} ions together and to 60min with Mn^{2+} ions alone. After the lag period there was little or no difference in the rate of oxygen uptake between reaction mixtures in light or dark. The rate of oxygen uptake depended on and increased with the concentration of pyridoxal, but the total oxygen uptake was only slightly increased. Table 3 shows that ammonia formation increased slightly with increase in pyridoxal concentration and that the amount of pyridoxal after incubation always exceeded that originally added, but was never as much as predicted by assuming complete deamination of the pyridoxamine. The amount of 3-hydroxypyridine component found after incubation was also less than expected from the total amount of pyridoxal and pyridoxamine originally added, but was similar to the sum of the pyridoxal and pyridoxamine remaining. This suggests that in these reaction mixtures some of the 3-hydroxypyridine component of the pyridoxamine and pyridoxal originally present was changed by a secondary reaction.

Elimination of the lag periods by manganese dioxide

Fig. 5 shows that at pH 5.5 the oxidation of mixtures of pyridoxamine and pyruvate was initiated by Mn^{2+} ions only after a very long lag period. This lag period was eliminated not only by peroxidase but also by traces of colloidal manganese dioxide. Without Mn^{2+} ions, neither manganese dioxide nor peroxidase, at the concentrations used, catalysed the reaction. Similar results were obtained at pH 5, where the lag period of the reaction catalysed by Mn^{2+} ions alone exceeded 6h. Traces of manganese dioxide could also replace peroxidase in the initiation of the oxidation of mixtures of pyridoxamine and α -oxo-butyrate by Mn^{2+} ions at pH 7. The lag period of the reaction catalysed by Mn^{2+} ions alone was 90min and this was shortened to 5min by adding peroxidase or manganese dioxide.

Effect of catalase. Catalase (25 μg) had little effect on the lag period when peroxidase (10 μg) initiated the oxidation of fresh pyridoxamine solutions (10 μmol) with pyruvate (30 μmol) and Mn^{2+} ions (1 μmol), and slightly increased the lag period when peroxidase was absent. With older solutions of pyridoxamine, catalase greatly extended the lag period, and with a solution of pyridoxamine kept for one week at $+2^\circ\text{C}$, the lag period with peroxidase was increased from about 10 to 25min by catalase. Once the reaction began catalase had no effect on the rate of total oxygen uptake, suggesting that hydrogen peroxide was not being produced.

Fig. 5. Initiation of the reaction by MnO_2

Reaction mixtures (total volume 3ml) contained pyridoxamine (10 μmol) and pyruvate (30 μmol) in 67mm-phosphate buffer, pH 5.5. Other additions: Δ , 10nmol of MnO_2 ; $\blacktriangle$, 1 μmol of MnSO_4 ; $\square$, 1 μmol of MnSO_4 + 10nmol of MnO_2 ; $\blacksquare$, 1 μmol of MnSO_4 + 10 μg of peroxidase.

Discussion

Mixtures of pyridoxamine, pyruvate and Mn^{2+} ions take up oxygen rapidly after an initial lag period. The lag period is shortened by peroxidase, but peroxidase has little or no effect on the subsequent rate or the total amount of oxygen uptake; light cannot replace peroxidase in initiating the reaction. Pyridoxal and ammonia are end-products and pyruvate seems to act catalytically. These results contrast with those for the oxidation of mixtures of pyridoxal and amino acids with Mn^{2+} ions (Hill & Mann, 1966), where either peroxidase or light are effective in shortening the lag period, where the amino acids are oxidatively deaminated and decarboxylated, and pyridoxal is oxidized to unidentified products, possibly 4-pyridoxic acid. Mn^{2+} ions are essential for both reactions, and both probably involve a

chelate of trivalent manganese with the Schiff base formed between either pyridoxal and an amino acid or pyridoxamine and an α -oxo acid. The differences in patterns of oxidation may come from the difference of position of the azomethine bond in the two types of Schiff base. In the pyridoxal-amino acid Schiff base, the π -electrons of the azomethine bond tend to be delocalized by conjugation with the π -electrons of the aromatic ring, but such delocalization cannot occur in the pyridoxamine α -oxo acid Schiff base without intramolecular rearrangement. My results and those of Ikawa & Snell (1954) also show that pyridoxal-pyridoxamine mixtures are oxidized by Mn^{2+} ions and, although the pyridoxamine is deaminated, some of the pyridoxal is also changed.

The oxidation of one molecule of pyridoxamine to pyridoxal requires a minimum uptake of one atom of oxygen and should produce one molecule of ammonia. In the present work always less than one molecule each of pyridoxal and ammonia were found in the final reaction mixtures and more than one and less than two atoms of oxygen were absorbed for each molecule of pyridoxamine oxidized. NaOH, unlike borate at pH 10, liberated 1 equivalent of steam-volatile base, although control experiments had shown that this borate buffer would liberate all the free ammonia from the reaction mixtures and that pyridoxamine was not steam-volatile. The results with 2,6-dichloro-*p*-benzoquinone-4-chloroimine suggest that, unlike in the oxidation of pyridoxamine by peroxidase (Hill, 1970), the 3-hydroxypyridine structure was not changed. Amides have been identified as oxidation products in the oxidative deamination of methionine and tryptophan by pyridoxal phosphate and Mn^{2+} ions (Mazelis & Ingraham, 1962; Riddle & Mazelis, 1964) and amides are readily hydrolysed by NaOH; pyridoxal may also be oxidized to 4-pyridoxic acid by this system (Hill & Mann, 1966). Therefore, as pyridoxal catalyses the oxidative deamination of pyridoxamine and is itself changed in the reaction, it is possible that in the oxidative deamination of pyridoxamine by α -oxo acids and Mn^{2+} ions an amide, possibly that of pyridoxic acid or a related compound, is produced by a secondary reaction and that this would account for the low yields of pyridoxal and ammonia found, and for the increase in oxygen uptake.

These reactions differ from most other Mn^{2+} ion-catalysed oxidations in that the results with catalase suggest that hydrogen peroxide is not a product and is not involved in the reaction; the stoichiometry precludes the reduction of oxygen to water. Like most other Mn^{2+} ion-catalysed oxidations the present reaction probably involves a change from bivalent to tervalent manganese. This is supported by the observation that the reaction is initiated both by peroxidase, which is known to catalyse the oxidation of Mn^{2+} to Mn^{3+} (Kenten & Mann, 1950, 1952), and

also by mixtures by MnO_2 and Mn^{2+} ions which, in contrast to Mn^{2+} ions or MnO_2 alone, can dismute to form Mn^{3+} ions.

Pyridoxal and related 3-hydroxypyridines have properties characteristic of monohydric phenols. Kenten & Mann (1950) showed that peroxidase catalysed the aerobic oxidation of Mn^{2+} to Mn^{3+} in the presence of catalytic amounts of monohydric phenols. Peroxidase catalyses the oxidation of pyridoxal and pyridoxamine (Hill, 1970) and many metal ions catalyse the transamination of pyridoxamine to pyridoxal in the presence of a suitable oxo acid (Metzler & Snell, 1952). I suggest that in the present reaction peroxidase shortens the lag period by catalysing a slow oxidation of pyridoxamine (or pyridoxal formed by transamination) and Mn^{2+} ions to give a product, possibly Mn^{3+} , which then initiates the rapid peroxidase-independent oxidative deamination of the pyridoxamine- α -oxo acid complex. However, the mechanism of this peroxidase-independent deamination is unclear and no evidence was obtained to show where atmospheric oxygen was incorporated into the final products.

Hill & Mann (1966) showed that Mn^{2+} ions catalyse the oxidation of pyridoxal and amino acids. Amino acids will also be oxidatively deaminated by the mixtures of Mn^{2+} ions, pyridoxamine and pyruvate described above by using the pyridoxal formed. The biological significance of these reactions is unclear, but they indicate further ways in which vitamin B₆ analogues may be involved with Mn^{2+} ions in amino acid metabolism.

References

- Blaschko, H. & Buffoni, F. (1965) *Proc. Roy. Soc. Ser. B* **163**, 45-60
- Buffoni, F. & Blaschko, H. (1964) *Proc. Roy. Soc. Ser. B* **161**, 153-167
- Conn, E. E. & Seki, S. L. (1957) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **16**, 167
- Dixon, M. (1951) *Manometric Methods*, 3rd. edn., p. 51, Cambridge University Press, Cambridge
- Håkanson, R. (1964) *J. Chromatogr.* **13**, 263-265
- Heintze, S. S. & Mann, P. J. G. (1949) *J. Agr. Sci.* **39**, 80-94
- Hill, J. M. (1970) *Phytochemistry* **9**, 725-734
- Hill, J. M. & Mann, P. J. G. (1966) *Biochem. J.* **99**, 454-468
- Hochberg, M., Melnick, D. & Oser, B. L. (1944) *J. Biol. Chem.* **155**, 109-117
- Hulme, A. C., Smith, W. H. & Woollorton, L. S. C. (1964) *J. Sci. Food Agr.* **15**, 303-307
- Ikawa, M. & Snell, E. E. (1954) *J. Amer. Chem. Soc.* **76**, 4900-4902
- Isherwood, F. A. & Cruickshank, D. H. (1954) *Nature (London)* **173**, 121-122
- Kalnitsky, G. (1953) *J. Biol. Chem.* **201**, 817-823
- Kenten, R. H. & Mann, P. J. G. (1950) *Biochem. J.* **46**, 76-73

- Kenten, R. H. & Mann, P. J. G. (1952) *Biochem. J.* **52**, 125–130
- Mazelis, M. (1962) *J. Biol. Chem.* **237**, 104–108
- Mazelis, M. & Ingraham, L. L. (1962) *J. Biol. Chem.* **237**, 109–112
- Metzler, D. E. & Snell, E. E. (1952) *J. Amer. Chem. Soc.* **74**, 979–983
- Metzler, D. E., Ikawa, M. & Snell, E. E. (1954) *J. Amer. Chem. Soc.* **76**, 648–652
- Mondovi, B., Costa, M. T., Finazzi-Agro, A. & Rotilio, G. (1967) *Arch. Biochem. Biophys.* **119**, 373–381
- Okuyama, T. & Satake, K. (1960) *J. Biochem. (Tokyo)* **47**, 454–466
- Peterson, E. A. & Sober, H. A. (1954) *J. Amer. Chem. Soc.* **76**, 169–175
- Riddle, V. M. & Mazelis, M. (1964) *Nature (London)* **202**, 391–392
- Scudi, J. V. (1941) *J. Biol. Chem.* **139**, 707–720
- von Korff, R. W. (1969) *Methods Enzymol.* **13**, 519–523
- Wada, H. & Snell, E. E. (1961) *J. Biol. Chem.* **236**, 2089–2095
- Yamada, H. & Yasunobu, K. T. (1962) *J. Biol. Chem.* **237**, 3077–3082
- Yamada, H. & Yasunobu, K. T. (1963) *J. Biol. Chem.* **238**, 2669–2675