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The Oxidation of Schiff Bases of Pyridoxal and Pyridoxal Phosphate with Amino Acids by Manganous Ions and Peroxidase

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1. Oxygen was taken up rapidly when pyridoxal or pyridoxal phosphate was added to mixtures of pea-seedling extracts and Mn^{2+} ions. 2. The increases in total oxygen uptake were proportional to the pyridoxal or pyridoxal phosphate added and were accompanied by the disappearance of these compounds. 3. In addition to Mn^{2+} ions, the reactions depended on two factors in the extracts, a thermolabile one in the non-diffusible material and a thermostable one in the diffusate; these factors could be replaced in the reactions by horse-radish peroxidase (donor–hydrogen peroxide oxidoreductase, EC 1.11.1.7) and amino acids respectively. 4. When pyridoxal phosphate was added to mixtures of amino acids and Mn^{2+} ions oxygen uptake was rapid after a lag period of 30–90min.; the lag period was shortened to a few minutes by peroxidase, particularly in the presence of traces of *p*-cresol, or by light. 5. When pyridoxal replaced pyridoxal phosphate relatively high concentrations were required and peroxidase had only a small activating effect. 6. Pyridoxal or pyridoxal phosphate disappeared during the reactions and carbon dioxide and ammonia were formed. 7. With phenylalanine as the amino acid present, benzaldehyde was identified as a reaction product. 8. It is suggested that the reactions are oxidations of the Schiff bases formed between pyridoxal or pyridoxal phosphate and amino acids, mediated by a manganese oxidation–reduction cycle, and resulting in oxidative decarboxylation and deamination of the amino acids.

The present work started with observations that pyridoxal and pyridoxal phosphate slightly accelerated the uptake of oxygen by pea-seedling extracts; when Mn^{2+} ions were also added, oxygen was taken up rapidly. The increases in total oxygen uptake were proportional to the amounts of pyridoxal or pyridoxal phosphate added and were accompanied by the disappearance of these compounds.

Kenten (1953) showed that sap from many plants, including pea seedlings, oxidizes phenylacetaldehyde. He found that the oxidizing system in pea-seedling sap consisted of a thermolabile factor, apparently a peroxidase, and a thermostable factor that could be partially replaced by Mn^{2+} ions. A system with similar properties to that in pea-seedling sap was constructed with horse-radish peroxidase and Mn^{2+} ions. With either system, benzaldehyde and formic acid were formed. The only other aldehydes oxidized by the system were 1-naphthylacetaldehyde and isobutyraldehyde; formaldehyde, acetaldehyde, glyoxal, *n*-butyraldehyde, isovaleraldehyde, cinnamaldehyde and phenylpropionaldehyde were not oxidized. Pyridoxal and pyridoxal phosphate were not tested.

Mazelis (1959, 1960) reported the oxidative decarboxylation of methionine by an enzyme preparation from particulate fractions of cabbage leaves in the presence of Mn^{2+} ions and pyridoxal phosphate, and then showed that horse-radish peroxidase, in the presence of Mn^{2+} ions and pyridoxal phosphate, catalyses the oxidative decarboxylation of methionine and many other amino acids (Mazelis, 1962). 3-Methylpropionamide and indol-3-ylacetamide were identified as products of the reactions with methionine and tryptophan respectively (Mazelis & Ingraham, 1962; Riddle & Mazelis, 1964).

MATERIALS AND METHODS

Plant extracts. The plant material, usually roots from 8-day-old pea seedlings, was frozen at −20° and then ground to a powder in a mortar. The powder was ground with water and the paste was squeezed in cotton cloth. The extract was diluted with water so that 1ml. corresponded to 1g. of tissue. The diffusate and non-diffusible material were made by dialysing the extract for 48hr. at 0–5° against 1 and 40 vol. of water respectively.

Preparation of the thermostable factor. A 100g. portion of frozen pea-seedling roots was powdered in a mortar and ground with 200ml. of 80% (v/v) ethanol in an MSE Ato-Mix homogenizer. The mixture was squeezed in cloth and the extract filtered, concentrated by distillation under

reduced pressure and evaporated to dryness in a vacuum desiccator to give 2·6g. of product. Solutions of the thermostable factor were made by grinding 1g. of the product with 10ml. of water and removing insoluble material by centrifugation. Inhibitors were removed with partially inactivated charcoal prepared by the method of Schramm & Primosigh (1943). Decolorizing charcoal was washed with 5% (v/v) acetic acid and then washed free from acid. The solution (10ml.) of the thermostable factor was mixed with a suspension of 1g. of the washed charcoal in 10ml. of water and the mixture shaken for 5min. at room temperature, or kept at 0–5° for 24hr. with intermittent shaking, and then filtered on a Buchner funnel through a layer of kieselguhr.

Enzymes. Horse-radish peroxidase was made by the method of Kenten & Mann (1954) as far as the second fractional precipitation with ethanol. The activity of the preparation was estimated with pyrogallol as hydrogen donor by the method of Keilin & Hartree (1951), except that the purpurogallin formed was estimated by measuring the $E_{430\,m\mu}$ and taking ϵ to be 2470 (Chance & Maehly, 1955). A unit of peroxidase is defined as the amount catalysing the use of 1μmole of H_2O_2/min. in the reaction at 20°; 3μmoles of H_2O_2 are used in the formation of 1μmole of purpurogallin. The specific activity (units/mg.) of the preparation was 2200 and the haemin content, estimated from $E_{557\,m\mu}$ after conversion into pyridine haemochromogen (Paul, Theorell & Åkeson, 1953), was 0·89%. Crystalline ox-liver catalase (EC 1.11.1.6) was obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.) and from the Sigma (London) Chemical Co. Ltd. (London, S.W. 6). Glutamate dehydrogenase (EC 1.4.1.2) was obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany).

Chemicals. Pyridoxal hydrochloride, pyridoxal 5-phosphate, pyridoxine hydrochloride, pyridoxamine dihydrochloride and 4-pyridoxic acid were obtained from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.), and pyridoxamine 5-phosphate was from the Sigma (London) Chemical Co. Ltd. L-Ornithine monohydrochloride and DL-homoserine were obtained from L. Light and Co. Ltd., and other amino acids were from British Drug Houses Ltd. (Poole, Dorset). NADH was obtained from C. F. Boehringer und Soehne G.m.b.H.

Buffers. Phosphate buffers were prepared from KH_2PO_4 and KOH. Borate buffers were prepared from $Na_2B_4O_7$ and NaOH.

Manometric methods. Oxygen uptake was measured on 3ml. of reaction mixture in the Warburg apparatus at 25° in air, with 0·2ml. of 5N-KOH in the centre well. Carbon dioxide was measured by Warburg's direct method (Dixon, 1943). Two models of Warburg apparatus were used; both were obtained from the Shandon Scientific Co. Ltd. (London, W.10). Unless otherwise stated the experiments were made in the Braun model V apparatus. This has a steel water bath and light therefore only reaches the reaction mixtures from above. In some experiments (subdued light) the light intensity in the room was decreased from 150–200 ft.-candles to about 5ft.-candles; in other experiments light was almost completely excluded from the reaction mixtures by covering the Warburg vessels with bags of dark cloth. Experiments in bright light were made in the Braun photochemical Warburg apparatus model VL, which has a water bath of transparent plastic. The light sources consisted of 40w tungsten-filament lamps mounted directly below the reaction flasks.

Estimation of ammonia. Unless otherwise stated NH_3 was estimated by distilling samples (usually 1ml.) of the reaction mixtures with 2ml. of 0·1M-borate buffer, pH10, in the Markham (1942) still. The distillate was collected in 2ml. of 0·1% boric acid and titrated against 0·07N-HCl. In some experiments the method of Pugh & Quastel (1937), for estimating NH_3 in presence of aliphatic amines, was used. The distillate was collected in 2ml. of 0·2M-phosphate buffer, pH7, and the NH_3 was absorbed on to yellow HgO. The washed HgO,NH_3 complex was transferred to the still and decomposed with 2ml. of 5N-NaOH; the distillate was collected in boric acid and titrated with HCl. In other experiments NH_3 was estimated enzymically with the system glutamate dehydrogenase, α-oxoglutarate and NADH by the method of Kirsten, Gerez & Kirsten (1963). At the high concentration of α-oxoglutarate used added NH_3 was converted quantitatively into L-glutamate. The reaction was followed by measuring the fall in $E_{366\,m\mu}$.

Estimation of pyridoxal and pyridoxal phosphate. Pyridoxal and pyridoxal phosphate were estimated by the procedure of Wada & Snell (1961), which depends on the extinction of the phenylhydrazones at 410mμ.

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

Chromatography. One-dimensional chromatograms were prepared by the descending technique on Whatman no. 1 paper with butan-1-ol saturated with N-HCl as developing agent.

RESULTS

Experiments with plant extracts

Effect of change in concentration of Mn^{2+} *ions.* Fig. 1 shows that the oxygen uptake of a pea-seedling root extract was increased by adding either pyridoxal or Mn^{2+} ions but that the increase with both was much greater than the sum of the separate effects. There was a lag period of several minutes before the maximum rate of oxygen uptake was observed. The rate increased with additions of Mn^{2+} ions up to 1μmole; greater additions caused little further increase. Control reaction mixtures, without the root extract, took up little or no oxygen. Oxygen uptake was also rapid when pyridoxal was added to extracts of whole pea seedlings or of the cotyledons or shoots in the presence, but not in the absence, of Mn^{2+} ions. With bean-seedling extracts (*Phaseolus vulgaris* and *P. multiflorus*) there was a lag period of an hour or more after pyridoxal was added before oxygen uptake became rapid.

Similar results were obtained with pyridoxal phosphate but not with pyridoxine, pyridoxamine, 4-pyridoxic acid, acetaldehyde, benzaldehyde or salicylaldehyde. Mn^{2+} ions could not be replaced by Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, vanadate, molybdate or tungstate ions.

Effect of variations in the concentrations of pyridoxal and pyridoxal phosphate. Fig. 2 shows the effect

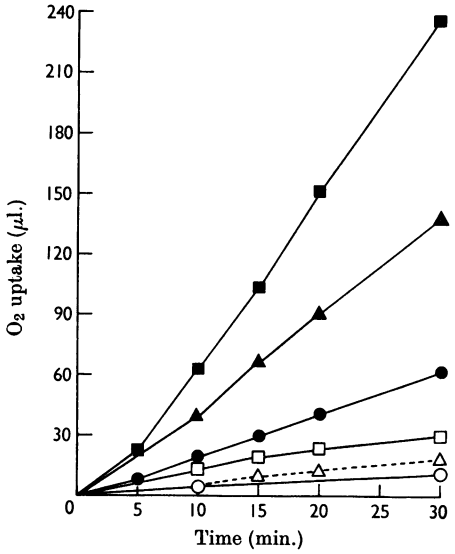

Fig. 1. Effect of Mn^{2+} ions on the rate of O_2 uptake of mixtures of pea-seedling root extract and pyridoxal. The control reaction mixture, of 3 ml. total volume, contained 1 ml. of root extract in 67 mM-phosphate buffer, pH 7. Other reaction mixtures also contained either or both pyridoxal and $MnSO_4$. Pyridoxal was added from the side arm after equilibration. The gas phase was air and the temperature 25°. KOH was present in the centre cups. The additions were: ○, none (control reaction mixture); △, 10 μmoles of pyridoxal; □, 1 μmole of $MnSO_4$; ●, 10 μmoles of pyridoxal and 0·1 μmole of $MnSO_4$; ▲, 10 μmoles of pyridoxal and 0·3 μmole of $MnSO_4$; ■, 10 μmoles of pyridoxal and 1 μmole of $MnSO_4$.

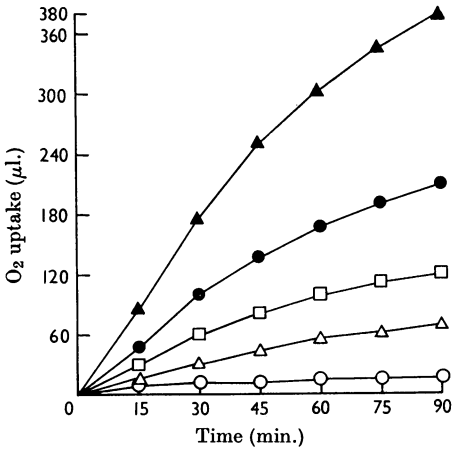

Fig. 2. Effect of variation in pyridoxal concentration. Reaction mixtures, of 3 ml. total volume, contained 1 ml. of root extract and 1 μmole of $MnSO_4$ in 67 mM-phosphate buffer, pH 7. Pyridoxal was added from the side arm after equilibration. Other conditions were as given in Fig. 1. Pyridoxal additions: ○, none (control reaction mixture); △, 1 μmole; □, 2 μmoles; ●, 4 μmoles; ▲, 8 μmoles.

of 1–8 μmoles of pyridoxal on the oxygen uptake of a mixture of pea-seedling root extract and Mn^{2+} ions. The increases in the rates of the reactions and in the total oxygen uptakes during the experimental time were proportional to the pyridoxal added. Fig. 3 shows the results of similar experiments in which pyridoxal was replaced by pyridoxal phosphate. The lag period increased with the amount of pyridoxal phosphate added but the maximum rates of the subsequent reactions were almost the same whether 1 or 8 μmoles of pyridoxal phosphate were added. The increases in total oxygen uptake were proportional to the pyridoxal phosphate added and were about 2 moles of oxygen/mole of pyridoxal phosphate.

Effect of the concentration of root extract. Fig. 4 shows that with small concentrations of root extract the reactions were much faster with pyridoxal phosphate than with pyridoxal. The duration of the reactions increased with the amount of extract and with excess of pyridoxal phosphate the total oxygen uptakes were proportional to the amount of extract. These results, in conjunction with those of Fig. 3, suggested that both pyridoxal phosphate and a factor in the extract were oxidized in the reactions.

Evidence of the disappearance of pyridoxal and pyridoxal phosphate during the reactions. When pyridoxal was added to mixtures of 1 ml. of root extract and phosphate buffer, pH 7, with or without added Mn^{2+} ions, a yellow colour developed within a few minutes. Similarly the yellow colour of neutral pyridoxal phosphate solutions was intensified by the root extract. These colour changes, like those

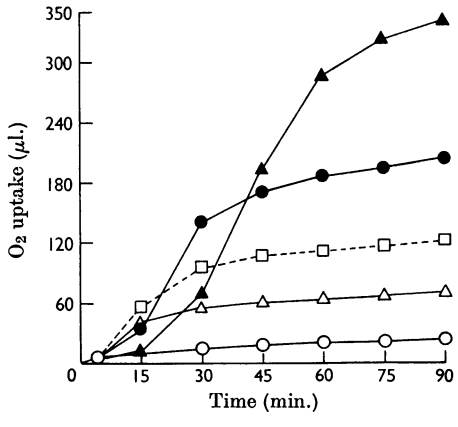

Fig. 3. Effect of variation in pyridoxal phosphate concentration. Reaction mixtures were similar to those given in Fig. 2 except that pyridoxal phosphate replaced pyridoxal. Pyridoxal phosphate additions: ○, none (control reaction mixture); △, 1 μmole; □, 2 μmoles; ●, 4 μmoles; ▲, 8 μmoles.

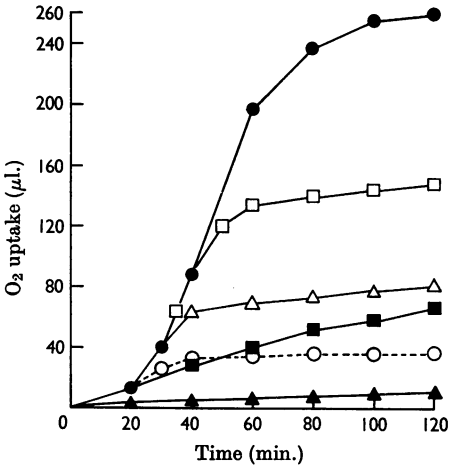

Fig. 4. Effect of variation in the concentration of root extract. Reaction mixtures, of 3 ml. total volume, contained different amounts of pea-seedling root extract and 1 μmole of $MnSO_4$ in 67 mM-phosphate buffer, pH 7. Pyridoxal or pyridoxal phosphate (10 μmoles) was added from the side arm after equilibration. Other conditions were as in Fig. 1. ○, △, □ and ●, Pyridoxal phosphate with 0·01, 0·025, 0·05 and 0·1 ml. of extract respectively; ▲ and ■, pyridoxal with 0·01 and 0·1 ml. of extract respectively.

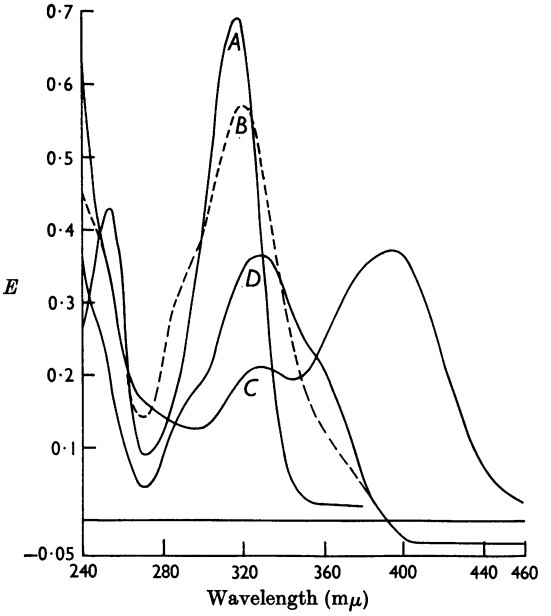

Fig. 5. Absorption spectra of reaction mixtures of root extract, Mn^{2+} ions and pyridoxal or pyridoxal phosphate before and after incubation. Reaction mixtures, of 3 ml. total volume, contained 1 ml. of root extract, 10 μmoles of pyridoxal or pyridoxal phosphate and 1 μmole of $MnSO_4$ in 67 mM-phosphate buffer, pH 7. Before and after incubation for 4 hr. samples of the reaction mixtures were diluted 40-fold with 10 mM-phosphate buffer, pH 7. The absorption spectra of the diluted reaction mixtures were determined with similarly diluted control reaction mixtures without pyridoxal or pyridoxal phosphate in the blank cell. Absorption spectra: *A* and *B*, pyridoxal-containing reaction mixtures before (*A*) and after (*B*) incubation; *C* and *D*, pyridoxal phosphate-containing reaction mixtures before (*C*) and after (*D*) incubation.

occurring when pyridoxal and pyridoxal phosphate are mixed with neutral solutions of amino acids (Metzler, 1957; Matsuo, 1957), may be attributed to the formation of Schiff bases between the amino acids of the extract and the pyridoxal or pyridoxal phosphate. After incubation until the oxygen uptake had nearly stopped, the control reaction mixtures, without added Mn^{2+} ions, remained yellow, whereas with added Mn^{2+} ions the yellow colour was almost completely discharged. Adding more pyridoxal or pyridoxal phosphate at this stage restored the yellow colours and caused a renewed rapid oxygen uptake. The disappearance of the colour was correlated with the appearance in the reaction mixtures of compounds with an intense blue fluorescence in ultraviolet light, a property of 4-pyridoxic acid and its lactone (Huff & Perlzweig, 1944).

Fig. 5 shows the difference spectra of reaction mixtures of root extract and Mn^{2+} ions, with and without pyridoxal or pyridoxal phosphate, before and after incubation for 2 hr. The reaction mixtures were similar to those used for Figs. 2 and 3 except that 10 μmoles of pyridoxal or pyridoxal phosphate were added. The difference spectrum of the pyridoxal reaction mixture before incubation showed the two bands at about 250 and 320 mμ characteristic of neutral solutions of pyridoxal. The difference spectrum of the incubated reaction mixture showed a diminution and slight shift of the 320 mμ band and the 250 mμ band now appeared only as an inflexion; an inflexion also appeared at about 290 mμ. The initial difference spectrum of the reaction mixtures containing pyridoxal phosphate showed the characteristic bands at about 390 and 330 mμ. In the difference spectrum of the incubated reaction mixtures these bands were replaced by a single maximum at about 327 mμ and inflexions at about 355 and 290 mμ. In acid solution (0·1 N-hydrochloric acid) the maximum shifted to 297 mμ and intensified slightly; in alkaline solution (0·1 N-sodium hydroxide) the reaction mixture turned yellow and showed general absorption over the range 280–420 mμ with inflexions at about 305 and 390 mμ. The spectra in neutral and acid solution resemble those of pyridoxamine phosphate but in alkaline solution this compound gives an intense band with a maximum at 305 mμ (Peterson & Sober,

1954). Neutral solutions of pyridoxic acid phosphate absorb maximally at 318mμ (Morrison & Long, 1958). The absorption spectra determined before and after incubation of control reaction mixtures of root extract and pyridoxal or pyridoxal phosphate, without added Mn^{2+} ions, showed relatively small changes. The results suggest that the reactions were accompanied by changes in pyridoxal and pyridoxal phosphate that did not involve rupture of the pyridine ring and that the increased oxygen consumption might be due, at least in part, to oxidation of these compounds.

Further evidence of the disappearance of pyridoxal and pyridoxal phosphate during the reactions was obtained by converting these compounds into their phenylhydrazones. Reaction mixtures were similar to those used for Fig. 5. Before and after incubation for 4hr. samples of the reaction mixtures were mixed with equal volumes of 30% (w/v) trichloroacetic acid and centrifuged. Samples (0·05ml.) of the supernatant solutions were mixed with 3·75ml. of water and 0·2ml. of the phenylhydrazine reagent. The mixtures were kept at room temperature for 10min. (pyridoxal phosphate-containing reaction mixtures) or heated at 60° for 20min. (pyridoxal-containing reaction mixtures) before the absorption spectra were determined. Similarly treated control reaction mixtures, without pyridoxal or pyridoxal phosphate were used in the blank cell. The spectra of the initial reaction mixtures after this treatment resembled those of the phenylhydrazones of pyridoxal and pyridoxal phosphate with an intense band at 410mμ (E 0·44) and a shoulder at about 330mμ (E 0·10). In the spectra of the incubated reaction mixtures initially containing pyridoxal or pyridoxal phosphate, $E_{410\,m\mu}$ decreased to 0·14 and 0·07 respectively and $E_{330\,m\mu}$ increased to 0·36 and 0·17 respectively with the formation of definite bands in this region. The decreases in $E_{410\,m\mu}$ showed that most of the pyridoxal and almost all the pyridoxal phosphate had disappeared; the increases in $E_{330\,m\mu}$ suggested that carbonyl compounds were among the reaction products.

Incubated reaction mixtures of root extract, Mn^{2+} ions and pyridoxal or pyridoxal phosphate and control reaction mixtures of root extract incubated alone, with Mn^{2+} ions, or with pyridoxal or pyridoxal phosphate, were examined by paper chromatography. When dry, the chromatograms were examined in ultraviolet light before and after exposure to ammonia. Pyridoxal and related compounds appeared as blue-fluorescent spots (Peterson & Sober, 1954; Rodwell, Volcani, Ikawa & Snell, 1959). Pyridoxal and pyridoxal phosphate were also detected as green-fluorescent spots in ultraviolet light after spraying the chromatograms with the semicarbazide reagent of Håkanson (1964). The marker compounds, and their mean R_F values, were: pyridoxal (0·39, 0·68), pyridoxamine (0·03), pyridoxic acid (0·52), pyridoxic acid lactone (0·23), pyridoxal phosphate (0·60) and pyridoxamine phosphate (0·02). A solution of pyridoxic acid lactone was prepared by heating a solution of the acid in 0·5N-hydrochloric acid for 30min. in a boiling-water bath (Huff & Perlzweig, 1944). As reported by Håkanson (1964) pyridoxal gave two spots on the chromatograms. In our experiments the second spot (R_F 0·68) was detected only with the sensitive semicarbazide reagent. The chromatograms of the complete reaction mixtures examined in ultraviolet light each showed one spot, with strong blue fluorescence, at R_F 0·46 (pyridoxal reaction mixture) and R_F 0·36 (pyridoxal phosphate reaction mixture). These spots did not appear on the chromatograms of the control reaction mixtures. The pyridoxal and pyridoxal phosphate markers were not detected until after exposure of the chromatograms to ammonia, when they appeared as pale (pyridoxal) or intense (pyridoxal phosphate) yellow spots in daylight with blue fluorescence in ultraviolet light. Ammonia also intensified the spots already detected on the chromatograms of the complete reaction mixtures and further blue-fluorescent spots appeared at R_F 0·23 (pyridoxal reaction mixture) and R_F 0·46 (pyridoxal phosphate reaction mixture). At this stage pyridoxal and pyridoxal phosphate were detected on the chromatograms of the appropriate control reaction mixtures but not on those of the complete reaction mixtures. With the semicarbazide reagent traces of pyridoxal and pyridoxal phosphate were also detected on the chromatograms of the complete reaction mixtures. This reagent also gave evidence of the presence of pyridoxal, possibly formed by phosphatase action, in the control reaction mixture of root extract and pyridoxal phosphate, without added Mn^{2+} ions. The results confirmed the disappearance of pyridoxal and pyridoxal phosphate and suggested that, except possibly for pyridoxic acid lactone (R_F 0·23), the products detected were not identical with any of the marker compounds.

Oxygen consumption and the formation of carbon dioxide and ammonia. Table 1 shows that more oxygen was consumed and more carbon dioxide and ammonia were formed with pyridoxal than with pyridoxal phosphate present. The formation of carbon dioxide suggested that among the reactions involved was the oxidative decarboxylation of amino acids by the peroxidase system described by Mazelis (1962). In such reactions oxidative deamination of amino acids has not hitherto been reported, but in Table 1 the ammonia equalled or exceeded the carbon dioxide formed. In two experiments values for ammonia only slightly less than those in Table 1 were obtained by the more specific

Table 1. *Oxygen uptake and the formation of carbon dioxide and ammonia in the reaction between pea-seedling root extract, Mn^{2+} ions and pyridoxal or pyridoxal phosphate*

Reaction mixtures, of 3 ml. total volume, contained 1 ml. of root extract and 1 μmole of $MnSO_4$ in 67 mM-phosphate buffer, pH 7. Pyridoxal or pyridoxal phosphate was added from the side arm after equilibration. The results were corrected for those obtained with control reaction mixtures without pyridoxal or pyridoxal phosphate. The reactions were stopped before estimation of NH_3 by adding 30 μmoles of EDTA from the second side arm.

Addition (μmoles)		Time of incubation (hr.)	O_2 uptake (μmoles)	CO_2 formed (μmoles)	NH_3 formed (μmoles)
Pyridoxal	Pyridoxal phosphate				
4	—	2	12·2	—	8·2
4	—	4	12·8	8·2	9·2
8	—	2	20·0	—	14·1
8	—	4	24·8	15·7	17·4
8	—	6	25·1	—	17·8
—	4	2	8·3	—	4·7
—	4	4	9·5	5·2	5·1
—	8	2	15·6	—	10·1
—	8	4	18·0	9·9	11·2
—	8	5	18·4	—	11·5

method of Pugh & Quastel (1937). The possibility remained that the ammonia was formed during the estimations by breakdown of unstable products. Attempts to estimate the ammonia enzymically by the method of Kirsten *et al.* (1963) were unsuccessful.

Effect of heating the root extract. Previous heat treatment of the root extract prolonged the lag period between adding pyridoxal to the reaction mixtures and the increase in rate of oxygen uptake. The lag period increased with the duration of heating and was about 2 hr. after heating for 30 min. at 100°, but the reaction ultimately proceeded almost as fast as with unheated extracts, which suggests that a thermolabile factor is involved in initiating the reaction.

Effect of dialysis of root extracts. Fig. 6 shows that reaction mixtures containing pyridoxal, Mn^{2+} ions and either the diffusible or the non-diffusible fraction of the root extract consumed little or no oxygen; when both diffusible and non-diffusible material were present oxygen was taken up rapidly after lag periods that decreased with increase in non-diffusible material. The reaction was prevented by heat treatment (5 min. at 100°) of the non-diffusible material but not of the diffusate. Thus the root extract contained two factors active in the system, one thermolabile and non-diffusible and the other thermostable and diffusible. The reaction mixtures of Fig. 6 were made up in subdued daylight and light was maintained at low intensity throughout the experiments. If this precaution was not taken the control reaction mixtures, without non-diffusible material, frequently consumed oxygen rapidly after lag periods of several hours, suggesting that the reaction between pyridoxal, Mn^{2+} ions and the

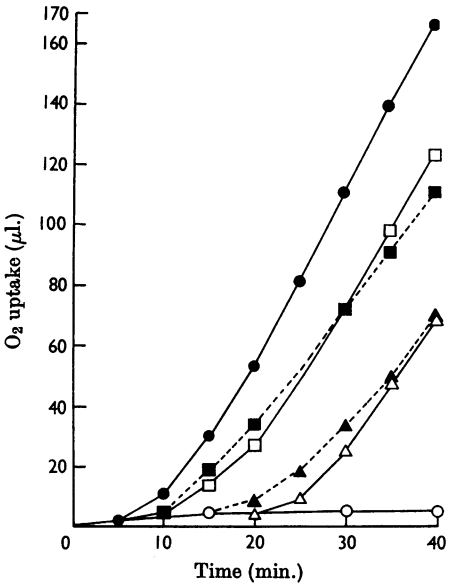

Fig. 6. Separation of two components of the system by dialysis of the root extract, and replacement of the non-diffusible material by horse-radish peroxidase. The dialysis procedure is described in the Materials and Methods section. Reaction mixtures, of 3 ml. total volume, contained 1 ml. of diffusate and 1 μmole of $MnSO_4$, with and without non-diffusible material or peroxidase, in 67 mM-phosphate buffer, pH 7. Pyridoxal (10 μmoles) was added from the side arm after equilibration. Additions: ○, none; △, 0·01 ml. of non-diffusible material; □, 0·03 ml. of non-diffusible material; ●, 0·1 ml. of non-diffusible material; ▲, 1 μg. of peroxidase; ■, 10 μg. of peroxidase. ○, Control reaction mixture containing non-diffusible material (0·1 ml.), $MnSO_4$ and pyridoxal.

diffusate might be initiated both by the non-diffusible material and by light. Unless otherwise stated all subsequent experiments were made in subdued light.

Replacement of non-diffusible fraction by peroxidase. The non-diffusible material could be replaced in the reaction by the horse-radish peroxidase preparation (Fig. 6). Heating the peroxidase for 5 min. at 100° prevented the initiation of the reaction by small amounts (1 μg.) and greatly extended the lag period with larger amounts (10 μg.). Assays showed that 0·1 ml. of the non-diffusible material had a peroxidase activity equivalent to 8 μg. of the horse-radish peroxidase preparation.

Experiments with the system of peroxidase and preparations of the thermostable factor

Effect of light. When the preparation of the thermostable factor was substituted for the diffusate in reaction mixtures containing pyridoxal and Mn^{2+} ions, a rapid oxygen uptake was initiated by peroxidase after lag periods ranging from 10 to 90 min. in different experiments. In some experiments control reaction mixtures without peroxidase consumed little or no oxygen, but in others a rapid oxygen uptake occurred after prolonged lag periods. When pyridoxal phosphate replaced pyridoxal the lag periods also varied but were shorter; control reaction mixtures without peroxidase always consumed oxygen rapidly though only after lag periods much longer than with peroxidase. Evidence was obtained that the wide variations in the lag period were related to differences in light-intensity. Fig. 7 shows the results of two experiments in subdued and bright light. The reaction is clearly initiated by light without peroxidase and light shortens the lag period with peroxidase.

Effects of catalase and hydrogen peroxide. Fig. 8 shows that 1 and 100 μg. of peroxidase were almost equally effective in initiating the reaction between pyridoxal phosphate, thermostable factor and Mn^{2+} ions. Catalase (25 μg.) inhibited the oxidation strongly when the peroxidase concentration was low. Similar results were obtained when pyridoxal replaced pyridoxal phosphate. With peroxidase, but without catalase, trace amounts of hydrogen peroxide almost completely eliminated the lag

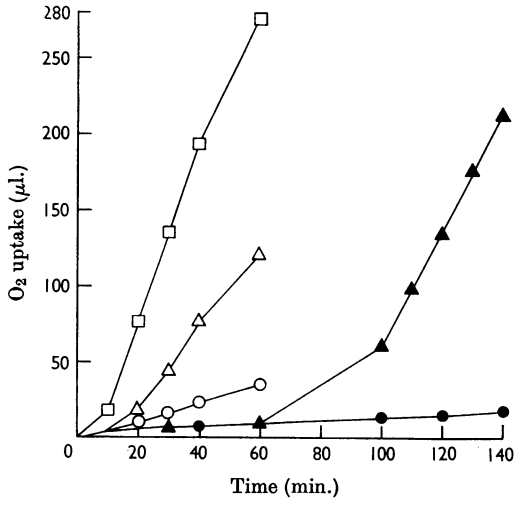

Fig. 7. Effect of light on the initiation of the reaction. Reaction mixtures each contained 0·2 ml. of the solution of the preparation of the thermostable factor in 67 mM-phosphate buffer, pH 7. The preparation of this solution is described in the Materials and Methods section. Other reaction mixtures contained, in addition, 1 μmole of $MnSO_4$ or 10 μg. of peroxidase or both. Pyridoxal (10 μmoles) was added from the side arm after equilibration. Two sets of reaction mixtures were made up. One set was incubated in bright light and the other set in subdued light. Additions to reaction mixtures in bright light: ○, no addition or 10 μg. of peroxidase; △, 1 μmole of $MnSO_4$; □, 1 μmole of $MnSO_4$ and 10 μg. of peroxidase. Additions to reaction mixtures in subdued light: ●, no addition or either 1 μmole of $MnSO_4$ or 10 μg. of peroxidase; ▲, 1 μmole of $MnSO_4$ and 10 μg. of peroxidase.

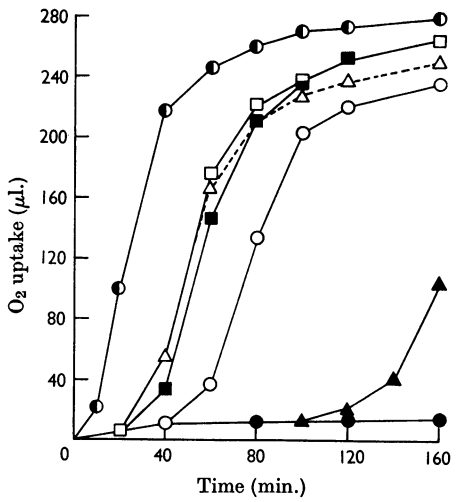

Fig. 8. Effects of catalase and H_2O_2. Reaction mixtures, of 3 ml. total volume, contained 0·2 ml. of the solution of the preparation of the thermostable factor and 1 μmole of $MnSO_4$ in 67 mM-phosphate buffer, pH 7. Other reaction mixtures contained, in addition, H_2O_2, peroxidase or catalase or both peroxidase and catalase. Pyridoxal phosphate (6 μmoles) was added from the side arm after equilibration. Additions: ○, none; △, 1 μg. of peroxidase; □, 100 μg. of peroxidase; ●, 25 μg. of catalase; ▲, 25 μg. of catalase + 1 μg. of peroxidase; ■, 25 μg. of catalase + 100 μg. of peroxidase. ◐, 0·1 μmole of H_2O_2 + 100 μg. of peroxidase.

period. The results suggest that hydrogen peroxide is formed in the reaction mixtures.

Effect of phenolic compounds. Oxidations catalysed by peroxidase without added hydrogen peroxide, the so-called peroxidase–oxidase reactions (Mason, 1957), are activated by monophenols and resorcinol and inhibited by other di- and tri-hydric phenols. Yamazaki & Piette (1963) classify these activators and inhibitors, which are all hydrogen donors for peroxidase, as 'redogenic' substrates (inhibiting) and 'oxidogenic' substrates (activating) according to the oxidation–reduction properties of the free radicals they form in peroxidase reactions. Mazelis (1962) reported similar activations and inhibitions, by phenolic compounds, of the oxidative decarboxylation of methionine by systems of peroxidase, Mn^{2+} ions and pyridoxal phosphate. This suggested that the lag periods in our reactions might be caused by the presence of inhibitory phenols in the preparation of the thermostable factor. Shaking the preparation with charcoal for 5min. to remove such inhibitors shortened from 50 to 10min. the lag period in the reaction between peroxidase, Mn^{2+} ions, pyridoxal and the thermostable factor. Adding 10 to 30mμmoles of catechol to systems containing the charcoal-treated preparation prolonged the lag periods to 50min. and 2hr. respectively. This inhibition by catechol could be prevented by adding 0·1μmole of hydrogen peroxide. In bright light in the photochemical Warburg apparatus, 10 and 20mμmoles of catechol increased the lag period only slightly whether or not peroxidase was present. Fig. 9 shows that, after treating the preparation of the thermostable factor with charcoal for 24hr., the reaction was initiated by Mn^{2+} ions alone in subdued light and the activating effect of peroxidase was much smaller than with the untreated preparation. Trace amounts of oxidogenic hydrogen donors, such as *p*-cresol, activated the reaction in the presence but not in the absence of peroxidase. This effect of *p*-cresol was not observed in reaction mixtures containing the untreated preparation. In similar experiments with pyridoxal phosphate the lag periods were shorter with the untreated preparation and were not affected so much by the charcoal treatment. However, as in the experiments with pyridoxal, *p*-cresol activated the reaction with the charcoal-treated preparation but not with the untreated preparation. The results suggest that peroxidase catalyses not only the removal of inhibitors but also the reactions between pyridoxal or pyridoxal phosphate, Mn^{2+} ions and the thermostable factor.

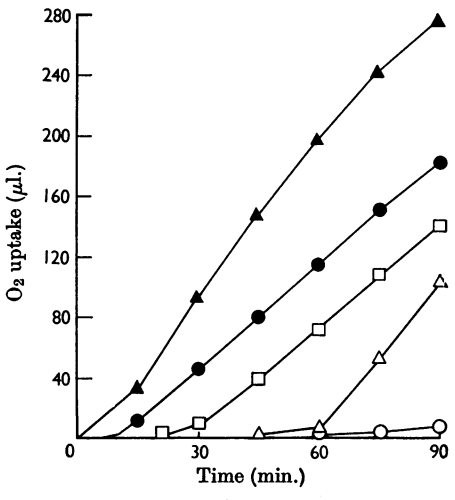

Fig. 9. Effect of treating the thermostable-factor preparation with charcoal on the lag period of the reaction. Reaction mixtures, of 3ml. total volume, contained 0·2ml. of a solution (100mg./ml.) of the preparation of the thermostable factor, or 0·4ml. of the solution obtained after charcoal treatment for 24hr., and 1μmole of $MnSO_4$ in 67mM-phosphate buffer, pH7. Other reaction mixtures contained, in addition, 10μg. of peroxidase or 30mμmoles of *p*-cresol or both peroxidase and *p*-cresol. Pyridoxal (10μmoles) was added from the side arm after equilibration. Additions with the untreated thermostable-factor preparation present: ○, none or *p*-cresol; △, peroxidase or peroxidase and *p*-cresol. Additions with the charcoal-treated thermostable preparation present: □, none or *p*-cresol; ●, peroxidase; ▲, peroxidase and *p*-cresol.

Experiments with systems of peroxidase and amino acids

Methionine. Mazelis (1962) demonstrated the oxidative decarboxylation of [*carboxy*-^{14}C]methionine by systems of peroxidase, Mn^{2+} ions and pyridoxal phosphate. A low concentration (0·25mM) of pyridoxal phosphate was used with which there was negligible decarboxylation of methionine without peroxidase; the non-enzymic decarboxylation increased with the pyridoxal phosphate concentration until, with 2·5mM-pyridoxal phosphate, the reaction was almost as rapid without as with peroxidase. At the concentrations of pyridoxal phosphate used by Mazelis (1962) the oxygen uptake of the reaction mixtures was too small to be measured accurately by our method. Fig. 10 shows that with 2mM-pyridoxal phosphate a catalytic effect of peroxidase was still apparent, particularly in the presence of trace amounts of *p*-cresol; without peroxidase, *p*-cresol had little or no effect on the rate of the reaction. Other experiments showed that the reaction was not accelerated by higher concentrations of peroxidase and *p*-cresol. Fig. 10 also shows that oxygen was taken up slowly when pyridoxal was added to mixtures of methionine and Mn^{2+} ions. Though peroxidase, in the presence of

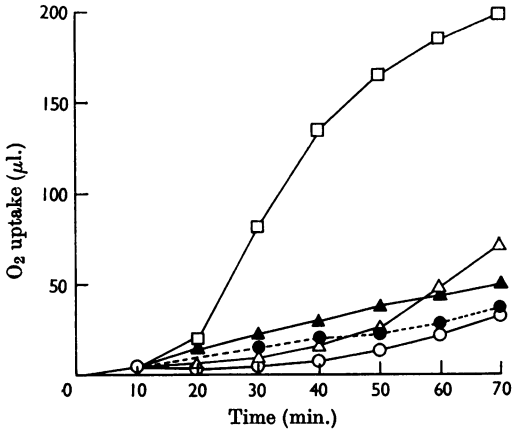

Fig. 10. Oxidation of mixtures of pyridoxal phosphate, or pyridoxal, and DL-methionine by Mn^{2+} ions in the presence and absence of peroxidase and *p*-cresol. Reaction mixtures, of 3 ml. total volume, contained 10 μmoles of DL-methionine and 1 μmole of $MnSO_4$ in 67 mM-phosphate buffer, pH 7. Pyridoxal phosphate or pyridoxal (6 μmoles) was added from the side arm after equilibration. Additions to reaction mixtures containing pyridoxal phosphate: ○, none, or 30 mμmoles of *p*-cresol; △, 10 μg. of peroxidase; □, 10 μg. of peroxidase and 30 mμmoles of *p*-cresol. Additions to reaction mixtures containing pyridoxal: ●, none; ▲, 10 μg. of peroxidase and 30 mμmoles of *p*-cresol.

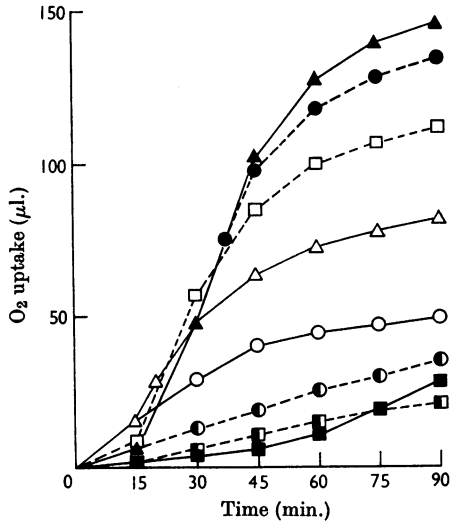

Fig. 11. Effect of variation in the concentration of DL-methionine. Reaction mixtures, of 3 ml. total volume, contained 1·5, 3, 6, 9 or 12 μmoles of DL-methionine, 1 μmole of $MnSO_4$, 30 mμmoles of *p*-cresol and 10 μg. of peroxidase in 67 mM-phosphate buffer, pH 7. Pyridoxal phosphate or pyridoxal (3 μmoles) was added from the side arm after equilibration. Peroxidase and *p*-cresol were omitted from control reaction mixtures. Methionine additions to reaction mixtures containing pyridoxal phosphate: ○, 1·5 μmoles; △, 3 μmoles; □, 6 μmoles; ●, 9 μmoles; ▲, 12 μmoles. ■, Control reaction mixture with 12 μmoles of methionine. Methionine additions to reaction mixtures containing pyridoxal: ◐, 12 μmoles. ◧, Control reaction mixture with 12 μmoles of methionine.

p-cresol, activated the reaction its effect was much smaller than with pyridoxal phosphate and the catalysed reaction remained relatively slow.

Under conditions otherwise similar to those of Fig. 10, the effect of changing the concentration of pyridoxal phosphate was tested. The rapid oxygen uptake, with peroxidase present, started after a lag period of 10–20 min.; the maximum rate was greater with 4 than with 2 μmoles of pyridoxal phosphate but was not much further increased with 6 μmoles. The reactions continued longer with the larger amounts of pyridoxal phosphate and the results resembled those of similar experiments with pea-seedling root extract (Fig. 3), suggesting a direct relation between total oxygen uptake and amount of pyridoxal phosphate added. When the reactions were catalysed by Mn^{2+} ions, the lag periods were much longer and ranged from 40–60 min. with 6 μmoles of pyridoxal phosphate to 80–100 min. with 2 μmoles. The rates of the subsequent reactions were little less than those when peroxidase was present. In similar experiments in which pyridoxal replaced pyridoxal phosphate the reactions proceeded slowly without peroxidase at rates proportional to the pyridoxal concentrations. Peroxidase, in the presence of *p*-cresol, had only a small activating effect.

Fig. 11 shows the effect of changing the methionine concentration with 3 μmoles of pyridoxal phosphate present initially. The results resemble those in Fig. 4 where the amount of pea-seedling root extract was varied. Fig. 11 also shows that the reactions were slower with pyridoxal than with pyridoxal phosphate. In further experiments the concentrations of both pyridoxal and methionine were increased. The reaction proceeded rapidly with reaction mixtures containing 10 μmoles of pyridoxal and 30 μmoles of methionine and the oxygen uptakes in 80 min. with and without peroxidase were 270 and 208 μl. respectively. The difference was greater in the early stages of the reaction because the main effect of peroxidase was to shorten the lag period.

Other amino acids. Glycine, DL-alanine, DL-homoserine, DL-β-phenylalanine, DL-aspartic acid, L-asparagine, L-glutamic acid, L-ornithine and L-lysine were tested in place of methionine. Homoserine was tested because it is the dominant amino acid in 8-day-old pea seedlings (Virtanen, Berg & Kari, 1953; Lawrence & Grant, 1963; Larson & Beevers, 1965). Fig. 12 shows that with DL-alanine, DL-homoserine, L-ornithine and L-lysine, as with the

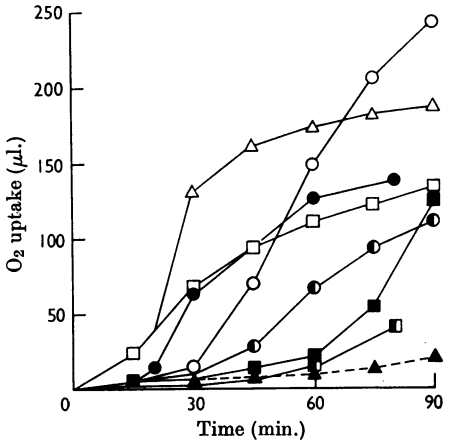

Fig. 12. Oxidation of mixtures of pyridoxal phosphate and L-lysine, L-ornithine, DL-alanine or DL-homoserine by Mn^{2+} ions in the presence and absence of peroxidase and *p*-cresol. Reaction mixtures, of 3ml. total volume, contained 10µmoles of amino acid, 1µmole of $MnSO_4$, 10µg. of peroxidase and 30mµmoles of *p*-cresol in 67mM-phosphate buffer, pH7. Pyridoxal phosphate (4µmoles) was added from the side arm after equilibration. Peroxidase and *p*-cresol were omitted from the control reaction mixtures. Complete reaction mixtures: ○, L-lysine; △, L-ornithine; □, DL-alanine; ●, DL-homoserine. Control reaction mixtures: ▲, L-lysine; ■, L-ornithine; ◐, DL-alanine; ◧, DL-homoserine.

other amino acids and with L-asparagine, the main effect of peroxidase was to shorten the lag period. The maximum rates of the reactions and the total oxygen uptakes were not much increased by peroxidase but maximum rates were reached much sooner when peroxidase was present. In all these experiments *p*-cresol was present; the question whether *p*-cresol always potentiates the peroxidase effect, as in the experiments with methionine, was not investigated. In similar experiments with DL-alanine and DL-β-phenylalanine, but with pyridoxal replacing pyridoxal phosphate, the reactions were very slow. As in the corresponding experiments with methionine, the rates of these reactions were increased by increasing the concentrations of amino acids and pyridoxal. The reactions were rapid, especially with phenylalanine, when the reaction mixtures contained 30µmoles of amino acid and 10µmoles of pyridoxal, but peroxidase had a much smaller effect than with pyridoxal phosphate.

Effect of catalase and hydrogen peroxide. Without catalase, 1 and 10µg. of peroxidase were almost equally effective in initiating the reaction between pyridoxal phosphate, Mn^{2+} ions and DL-alanine. The lag period was about 10min. with 10µg. of peroxidase and 15min. with 1µg.; thereafter the reactions proceeded at almost the same rate. Catalase (25µg.) had little effect in the presence of 10µg. of peroxidase but extended the lag period to 50min. with only 1µg. of peroxidase. Without peroxidase the lag period was increased from 40 to 70min. by 25µg. of catalase but was not further increased by 250µg. of catalase. Catalase (25 or 250µg.) added to the reaction mixtures from the second side arm after the lag period had little or no effect on the rate of the reaction. Without catalase 0·1µmole of hydrogen peroxide almost completely eliminated the lag period with peroxidase present and shortened it without peroxidase. Mazelis (1962) reported almost complete inhibition of the reaction by a freshly prepared solution of crystalline catalase though after storage for 5–9 months the solution inhibited less or even activated the reaction. He also reported inhibition of the reaction by hydrogen peroxide (0·3µmole). Since his assays were mostly made after a reaction time of 60min. extension of the lag period to 60min. or more would appear as complete inhibition.

Inhibition by EDTA. EDTA (1mM) present from the start, or added during the lag period, completely inhibited the reactions; when added after the rapid reaction had started it soon slowed the rate of oxygen uptake and almost stopped it in about 10min.

Evidence for the disappearance of pyridoxal and pyridoxal phosphate during the reactions. The amino acids used were DL-methionine and DL-alanine. The reaction mixtures resembled those of Fig. 12 and contained either 4µmoles of pyridoxal phosphate and 10µmoles of amino acid or 10µmoles of pyridoxal and 30µmoles of amino acid. The changes in the absorption spectra of the reaction mixtures on incubation resembled those when pyridoxal and pyridoxal phosphate were incubated with a mixture of pea-seedling root extract and Mn^{2+} ions (Fig. 5). Slightly smaller but otherwise similar changes in the spectra occurred when the control reaction mixtures were incubated with Mn^{2+} ions but without peroxidase.

Further evidence that pyridoxal phosphate disappears during the reactions was obtained by estimating this compound by the method of Wada & Snell (1961). After treatment with the phenylhydrazine reagent samples of the initial reaction mixtures had $E_{410\,m\mu}$ 0·56, where pyridoxal phosphate phenylhydrazone absorbs maximally; with the incubated reaction mixtures this fell to 0·05–0·06. $E_{330\,m\mu}$ increased from 0·12 with the initial reaction mixtures to 0·26 and 0·60 with the incubated reaction mixtures containing methionine and alanine respectively. The results resemble those of the corresponding experiments with root extract and suggest that carbonyl compounds are among the reaction products. The fact that these compounds

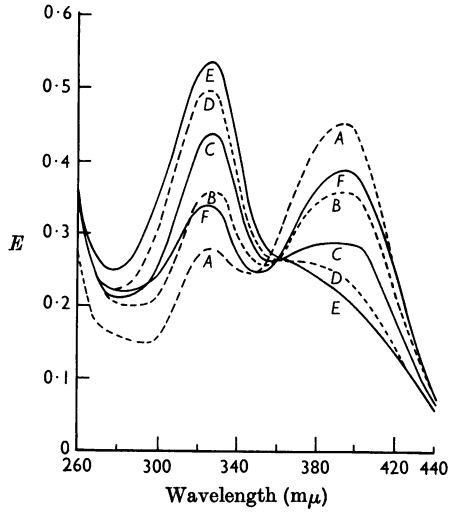

Fig. 13. Changes, during incubation, in the absorption spectrum of a reaction mixture containing 3 μmoles of DL-methionine, 0·1 μmole of $MnSO_4$, 30 mμmoles of *p*-cresol, 0·3 μmole of pyridoxal phosphate and 10 μg. of peroxidase. The reaction mixture, of 3 ml. total volume, was made up in 67 mM-phosphate buffer, pH 7, in a 1 cm. silica cell. The reaction was started by adding the pyridoxal phosphate and the absorption spectrum was determined immediately and after incubation at room temperatue for various times with a control reaction mixture without pyridoxal phosphate in the blank cell. Absorption spectra of complete reaction mixture: *A*, initially; *B*, *C*, *D* and *E*, after incubation for 15 (*B*), 30 (*C*), 60 (*D*) and 120 min. (*E*). *F*, Absorption spectrum of control reaction mixture without peroxidase incubated for 30 min.

differ in amount with the amino acid initially present suggests they may be oxidation products of the amino acids.

Fig. 13 shows that the reaction involving pyridoxal phosphate occurs at low concentrations of the reactants. In these experiments with DL-methionine the concentration of pyridoxal phosphate was 0·1 mM. This allowed direct determination of the absorption spectra without diluting the reaction mixtures, which were made up in silica cells. The spectra were determined initially and at intervals during incubation for 2 hr. at room temperature. The disappearance of the pyridoxal phosphate with formation of the compound absorbing maximally at 327 mμ was catalysed slowly by 0·03 mM-Mn^{2+} ions but much faster by the system of peroxidase, *p*-cresol and Mn^{2+} ions. In similar experiments with pyridoxal replacing pyridoxal phosphate the absorption spectrum of the reaction mixture, which resembled that of pyridoxal, remained almost unchanged after 2 hr. whether or not peroxidase was present. This was in agreement with the results of the manometric experiments showing that the reaction involving pyridoxal required relatively large concentrations of pyridoxal and amino acids.

The incubated complete reaction mixtures showed an intense blue fluorescence in ultraviolet light and evidence was obtained by paper chromatography that the fluorescent compounds present were identical with those formed in the corresponding experiments with root extract. The amino acids used were DL-methionine, DL-alanine and DL-β-phenylalanine. The chromatograms of the incubated reaction mixtures initially containing pyridoxal phosphate all showed a blue-fluorescent spot at R_F 0·34–0·36, which was intensified by exposure to ammonia, when a second blue-fluorescent spot at R_F 0·44 appeared on the chromatograms of the reaction mixtures containing alanine or phenylalanine. In the corresponding experiments with pyridoxal, blue-fluorescent spots at R_F 0·46 and 0·23–0·24 were detected, which also appeared on the chromatograms when peroxidase was omitted but not when Mn^{2+} ions were. It is suggested that the blue-fluorescent compounds are identical with those formed in the corresponding experiments with root extract and are oxidation products of pyridoxal and pyridoxal phosphate.

Oxygen consumption and the formation of carbon dioxide and ammonia. Table 2 shows the values obtained for oxygen consumed and carbon dioxide and ammonia formed in the oxidation of mixtures of 4 μmoles of pyridoxal phosphate with various amino acids (10 μmoles) or with L-asparagine. Usually about 8 μmoles of oxygen were taken up but the amounts varied between 5 and 12·5 μmoles. As in the experiments with root extract (Table 1) both carbon dioxide and ammonia were formed in amounts varying from 1·5 to 4·8 μmoles. When peroxidase was omitted and the reactions were catalysed by Mn^{2+} ions alone, the oxygen consumed and carbon dioxide and ammonia formed in 3 hr. were always slightly less than the values given in Table 2. The values for ammonia given in Table 2 were obtained by the distillation method. In some experiments the ammonia was checked by the glutamate-dehydrogenase method. Systems of peroxidase, Mn^{2+} ions and *p*-cresol catalyse the oxidation of NADH to NAD^+ (Akazawa & Conn, 1958) but the small samples (0·1 ml.) of the reaction mixtures required for the ammonia estimations usually caused little oxidation of NADH without glutamate dehydrogenase. The values for ammonia found by this method in the experiments with alanine, ornithine and lysine agreed with those given in Table 2. Attempts to check the ammonia in the reaction mixtures containing methionine by this method gave unsatisfactory results, as the reaction mixtures oxidized NADH slowly without the addition of glutamate dehydrogenase.

Table 2. *Oxygen uptake and the formation of carbon dioxide and ammonia in the reaction between pyridoxal phosphate and amino acids catalysed by systems of peroxidase, Mn^{2+} ions and* p-*cresol*

Reaction mixtures, of 3 ml. total volume, contained 10 μmoles of amino acid (or L-asparagine), 1 μmole of $MnSO_4$, 30 mμmoles of *p*-cresol and 10 μg. of peroxidase in 67 mM-phosphate buffer, pH 7. Pyridoxal phosphate (4 μmoles) was added from the side arm after equilibration. The reaction mixtures were incubated for 3 hr. The values are corrected for those obtained with control reaction mixtures without $MnSO_4$, *p*-cresol and peroxidase.

Amino acid or amide	O_2 uptake (μmoles)	CO_2 formed (μmoles)	NH_3 formed (μmoles)
Glycine	7·6	2·2	2·3
DL-Alanine	7·3	2·8	4·8
DL-Homoserine	7·3	2·4	3·4
DL-Methionine	8·5	4·1	3·4
DL-β-Phenylalanine	8·9	3·9	1·8
DL-Aspartic acid	5·0	2·8	1·5
L-Glutamic acid	7·4	2·7	3·0
L-Ornithine	8·6	4·3	4·3
L-Lysine	12·5	3·8	4·4
L-Asparagine	9·5	3·6	4·3

Formation of benzaldehyde from phenylalanine. When reaction mixtures of DL-β-phenylalanine, pyridoxal, Mn^{2+} ions, peroxidase and *p*-cresol were incubated the absorption spectrum, as in the experiments with DL-alanine and DL-methionine, showed a decrease in the intensity of the 320 mμ band but the intensity of the 250 mμ band was greatly increased. The reaction mixture smelt of almonds, suggesting that the increased absorption at 250 mμ might be due to benzaldehyde. Steam-distillation of the reaction mixture gave a solution that, like solutions of benzaldehyde in water, absorbed maximally at 250 mμ with a shoulder at about 290 mμ. Taking ϵ to be 12 600 (Kenten, 1953), it was calculated from the absorption at 250 mμ that about 3 μmoles of benzaldehyde were present in the reaction mixture, representing a yield of 30% based on the pyridoxal added. Similar reaction mixtures in which pyridoxal phosphate replaced pyridoxal also showed increased absorption at 250 mμ after incubation, because of a steam-volatile compound. The presence of benzaldehyde was established in the experiments with pyridoxal by its isolation from large-scale reaction mixtures as the 2,4-dinitrophenylhydrazone. Five reaction mixtures each containing 90 μmoles of DL-β-phenylalanine, 30 μmoles of pyridoxal, 0·3 μmole of manganous sulphate, 0·09 μmole of *p*-cresol and 30 μg. of peroxidase in a total volume of 9 ml. of 67 mM-phosphate buffer, pH 7, were incubated for 3 hr. in large Warburg vessels each of about 60 ml. total volume. The combined reaction mixtures were steam-distilled into 12·5 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid. About 50 ml. of distillate was collected. After standing overnight at room temperature the orange precipitate was filtered off, washed with water and dried in a vacuum desiccator. The product (12 mg.)

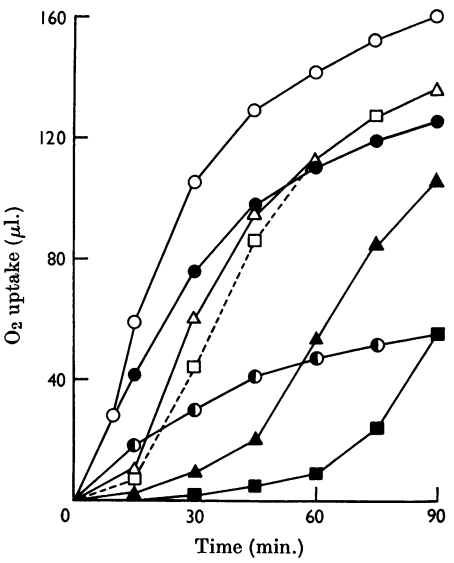

Fig. 14. Initiation of the reaction by light. Three pairs of reaction mixtures were made up, each of 3 ml. total volume, containing 10 μmoles of DL-alanine, 1 μmole of $MnSO_4$ and 30 mμmoles of *p*-cresol with and without 10 μg. of peroxidase in 67 mM-phosphate buffer, pH 7. Pyridoxal phosphate (4 μmoles) was added from the side arm after equilibration. One pair of reaction mixtures and a control reaction mixture containing pyridoxal phosphate in phosphate buffer, pH 7, were incubated in the photochemical Warburg apparatus. The lights were switched on immediately after the pyridoxal phosphate was added from the side arm. A second pair was incubated in subdued light and light was excluded from the third pair. Reaction mixtures containing peroxidase: ○, in bright light; △, in subdued light; □, in the dark. Reaction mixtures without peroxidase: ●, in bright light; ▲, in subdued light; ■, in the dark. ◐, Control reaction mixture containing pyridoxal phosphate in phosphate buffer in bright light.

had m.p. 233–235° (uncorr.), unchanged on mixture with an authentic specimen of benzaldehyde 2,4-dinitrophenylhydrazone, m.p. 233–235° (uncorr.). The yield was 28% based on the pyridoxal added. Conn & Seki (1957) reported that plant mitochondrial preparations, or mixtures of peroxidase and Mn^{2+} ions, catalyse the oxidation of phenylpyruvic acid to benzaldehyde, carbon dioxide and other products.

Initiation of the reactions by light. The experiments with amino acids so far reported were made in subdued light. Fig. 14 shows that when light was excluded the lag period of the reaction was prolonged, more so when the reaction was catalysed by Mn^{2+} ions alone than when peroxidase was also present. The effect of peroxidase therefore appeared greater in darkness than in subdued light. In bright light the lag period was almost eliminated whether the reaction was catalysed by Mn^{2+} ions alone or by the peroxidase system though more oxygen was taken up in the presence of peroxidase. The results suggest that the oxidation of mixtures of pyridoxal phosphate, amino acids and Mn^{2+} ions can be initiated either by peroxidase or by light. The results in bright light were complicated by the photolysis of pyridoxal phosphate. Morrison & Long (1958) showed that this produces the pyridoin, which, in oxygen, is oxidized to the pyridyl and to pyridoxic acid phosphate.

DISCUSSION

The results suggest that the increases in oxygen uptake caused by adding pyridoxal or pyridoxal phosphate to mixtures of pea-seedling extracts and Mn^{2+} ions are due, at least in part, to oxidation of the amino acids and amino acid amides present by the peroxidase system described by Mazelis (1962). The possibility that amides are among the products of the reactions with other amino acids, as found by Mazelis & Ingraham (1962) and Riddle & Mazelis (1964) with methionine and tryptophan respectively, was not investigated. Under our conditions the system both deaminates and decarboxylates the amino acids but the variations in the relative amounts of ammonia and carbon dioxide formed with the amino acids used may reflect variations in amide yield. Pyridoxal and pyridoxal phosphate have little or no catalytic activity in the system but are themselves changed, possibly oxidized. The blue fluorescence of the reaction mixtures suggests the products may be related to 4-pyridoxic acid, which also has this property (Huff & Perlzweig, 1944).

The reactions are apparently related to peroxidase–oxidase reactions that are also independent of an external source of hydrogen peroxide and are activated by Mn^{2+} ions but require peroxidase for both the oxidase and peroxidase stages of the overall oxidation. The present reactions are catalysed by Mn^{2+} ions alone and peroxidase merely shortens the lag period. Subsequently the oxygen uptake is almost as rapid in the absence as in the presence of peroxidase; the greater total oxygen uptakes with peroxidase present probably result from secondary oxidations catalysed by this enzyme. The requirement for Mn^{2+} ions and the activation by peroxidase, particularly in the presence of *p*-cresol, suggests that manganese oxidation may be involved in the reaction mechanism. Kenten & Mann (1949, 1950) showed that peroxidase systems catalyse the oxidation of manganous to manganic manganese and suggested that the oxidation of some dicarboxylic acids by peroxidase in the presence of Mn^{2+} ions depends on a manganese oxidation–reduction cycle (Kenten & Mann, 1953). The activation of other peroxidase–oxidase reactions by Mn^{2+} ions was also attributed to this cycle by Maclachlan & Waygood (1956) and Yamazaki & Piette (1963), but Chance (1952) and Akazawa & Conn (1958) attributed it to activation of a peroxidase–peroxide complex by Mn^{2+} ions. Following Maclachlan & Waygood (1956), we suggest that the lag period in our reactions represents the time necessary for manganic manganese to reach a threshold concentration; the activating effect of peroxidase is attributed to its catalysis of this reaction. The manganic manganese oxidizes the Schiff base, formed between the pyridoxal or pyridoxal phosphate and the amino acid, and a product of this reaction (presumably a free radical) reacts directly with molecular oxygen, yielding a product able to oxidize manganous to manganic manganese without the intervention of peroxidase. A similar reaction was postulated by Drummond & Waters (1954) in the catalysis of the oxidation of malonate by manganic pyrophosphate.

Light can replace peroxidase both in initiating the reactions and in the removal of inhibitors. The latter effect of light is apparent in the experiments with the preparation of the thermostable factor where the reaction proceeds with Mn^{2+} ions alone in bright but not in subdued light. The long lag periods in the experiments with extracts of bean seedlings (*P. vulgaris* and *P. multiflorus*) also presumably reflect the presence of inhibitors. The activation of peroxidase–oxidase reactions by light has been the subject of much investigation since the observation of Galston & Baker (1949) that the oxidation of indol-3-ylacetate by a plant enzyme system was light-activated. Galston, Bonner & Baker (1953) postulated that the enzyme system comprised a light-sensitive flavoprotein and a peroxidase acting sequentially. Kenten (1955*a*) found that the oxidation of indol-3-ylacetate by peroxidase was activated by oxidogenic hydrogen donors and Mn^{2+} ions and that the rate of the oxida-

tion was increased by strong daylight. In experiments on the oxidation of β-indol-3-ylpropionate by systems of peroxidase and Mn^{2+} ions the lag period of the reaction depended on the light-intensity (Kenten, 1955*b*). Later work has shown that, in addition to the direct activating effect of light on indol-3-ylacetate oxidation, the light conditions under which a plant is grown can affect the activators and inhibitors of the oxidase present. Much of the recent work on the effect of light conditions on auxin concentration has been reviewed by Hare (1964). The activation of the present reactions by light, like that by peroxidase, may involve manganese oxidation. Andreae (1955) showed that Mn^{2+} ions are oxidized by light in the presence of a suitable light-sensitizer, e.g. riboflavine, and catalytic amounts of an oxidogenic hydrogen donor. Kenten & Mann (1955) showed that chloroplast preparations oxidize Mn^{2+} ions in light but not, or to a much less extent, in the dark. Homann (1965) reported the oxidation of 2,3-dioxogulonate by Mn^{2+} ions in light, or by systems of Mn^{2+} ions and peroxidase in the dark; the oxidation was attributed to a manganese oxidation–reduction cycle. Mazelis (1961) showed that light stimulated the formation of an oxidant in mixtures of pyridoxal phosphate, Mn^{2+} ions and amino acids. The nature of the oxidant was not established but it seems likely that it was manganic manganese. The reaction mixtures used in the present work gave strongly positive tests for manganic manganese with the benzidine reagent of Kenten & Mann (1952) whether the reactions were catalysed by Mn^{2+} ions alone or initiated by peroxidase or light.

Many of the reactions of amino acids catalysed by enzymes containing pyridoxal phosphate are also catalysed in model systems by pyridoxal and metal ions. The significance of these model reactions has increased as evidence has accumulated that some amine oxidases contain both copper and pyridoxal phosphate. This has been shown most recently with the histaminase (benzylamine oxidase) of pig plasma (Buffoni & Blaschko, 1964; Blaschko & Buffoni, 1965). In the model reactions Cu^{2+} ions have usually proved most effective and Mn^{2+} ions relatively ineffective (Longenecker & Snell, 1957). Metzler, Ikawa & Snell (1954) proposed a mechanism for the model reactions based on the formation of a chelate of the metal ion with the Schiff base of pyridoxal and amino acid. There is much evidence that the present reactions also depend on the formation of a Schiff base as suggested by Mazelis (1962). The yellow colour of the reaction mixtures, the facts that pyridoxine, pyridoxamine and 4-pyridoxic acid are inactive in the system and that pyridoxal phosphate is effective at much lower concentrations than pyridoxal all support this suggestion. In neutral solution pyridoxal is present mainly as the internal hemiacetal and therefore forms Schiff bases to a much smaller extent than pyridoxal phosphate, which cannot form the hemiacetal. The inhibition of the reactions by EDTA suggests that the reaction mechanism involves complexes of manganese, possibly with the Schiff bases. The fact that EDTA not only prevents the reactions when present initially but also stops them when added after the induction period is evidence that Mn^{2+} ions are essential both for the initiation and propagation of the reactions. Matsuo (1957) found that the relative effectiveness of metal ions tested in forming metal chelates with Schiff bases of pyridoxal phosphate and amino acids was in the order: $Co^{2+} \geqslant Al^{3+} > Cu^{2+} > Zn^{2+} > Ni^{2+} > Mn^{2+}$. The specific activity of Mn^{2+} ions in the present reactions cannot therefore be attributed to the stability of the complexes they form with the Schiff bases.

The results suggest that pyridoxal and pyridoxal phosphate have little or no catalytic activity in the reactions but are themselves changed. Unless the changes are reversible *in vivo* the reactions can have little quantitative significance in amino acid metabolism though they may be significant in the metabolism of pyridoxal and pyridoxal phosphate. Preliminary results of further work suggest that the oxidation of Schiff bases of pyridoxamine with α-oxo acids is also catalysed by Mn^{2+} ions and peroxidase systems; pyridoxal and ammonia are reaction products. It is known that in neutral or acid solutions of several pyridoxal phosphate-containing enzymes the pyridoxal phosphate is bound to the protein apoenzymes as Schiff bases. In the reactions catalysed by these enzymes Schiff bases formed between enzyme-bound pyridoxal phosphate and amino acid substrate are postulated intermediates. The present results suggest the possibility that such enzymes, in the presence or absence of their substrates, may be susceptible to attack by Mn^{2+} ions and peroxidase systems.

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