activity defined as the quantity of enzyme which at 20° reduces 0·01 μmole triphosphopyridine nucleotide per minute.

3. The mean values of glucose 6-phosphate dehydrogenase activity, determined at pH 7·6, and of 6-phosphogluconate dehydrogenase activity, determined at pH's 7·6 and 9·0, were respectively 46 ± 3, 59 ± 8 and 147 ± 10 units/g. liver in male rats. All these levels were consistently higher in females.

4. Both dehydrogenases are activated by magnesium, calcium and manganese and inhibited by mercury. 6-Phosphogluconate dehydrogenase is also inhibited by cupric and zinc ions.

5. Evidence is presented that the activity of 6-phosphogluconate dehydrogenase is dependent on the presence of active sulphydryl groups.

6. Adenosine triphosphate acts as a competitive inhibitor of 6-phosphogluconate dehydrogenase but not of glucose 6-phosphate dehydrogenase.

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The Oxidation of Tryptophan in Pea-seedling Tissues and Extracts

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Tryptophan is known to be a source of nicotinic acid in animals (literature reviewed by Dalgliesh, 1951). There is evidence (Gustafson, 1949; Galston, 1949; Nason, 1950) that this conversion occurs also in some higher plants, but few of the intermediate compounds found in animals have been detected. One intermediate, anthranilic acid, accumulates in the anthers and seedling leaves of a maize mutant (Teas & Anderson, 1951).

Tryptophan may also be a precursor of indolylacetic acid in spinach leaves (Wildman, Ferri & Bonner, 1947), in tobacco ovaries (Wildman & Muir, 1949), in pineapple leaves (Gordon & Nieva, 1949) and in crown-gall tissue of sunflower (Henderson & Bonner, 1952). Conversion to indole, as by *Escherichia coli* (Happold, 1950) has not been reported in higher plants.

In the present work with pea seedlings, no conversion to indolylacetic acid was observed, but some experiments on oxidation of indolylacetic acid are reported. Certain similarities were found to the early stages of tryptophan breakdown in animal tissue.

Most studies of the mechanism of tryptophan oxidation have been made with cells of bacteria or animals previously exposed to an unusually high concentration of tryptophan (Stanier & Tsuchida, 1949; Knox & Mehler, 1950). In such cells adaptive enzymes were produced and tryptophan was

oxidized much faster than in normal cells. Of several plant tissues examined, pea seedlings contained the fastest tryptophan-oxidizing system. Seedlings of maize and dwarf bean, potato tuber, and leaves of tobacco, dwarf bean, horseradish, sugar beet and broccoli had lower activities.

Knox & Mehler (1950) found that the oxidation of tryptophan in liver extracts of several animals was accelerated by addition of enzymes and substrates producing hydrogen peroxide. This has now been found to be the case with pea-seedling tissues and extracts. Oxidation depended on the supply of enzymically generated peroxide which was used in a 'coupled oxidation'. The oxidation of aliphatic amines by amine oxidase (Kenten & Mann, 1952) provided a convenient source of peroxide.

$$R.CH_2NH_2 + O_2 + H_2O \xrightarrow{\text{amine oxidase}} R.CHO + NH_3 + H_2O_2. \quad (1)$$

The peroxide formed in reaction (1) was usually decomposed by catalase so that 0·5 mole oxygen was used per mole amine oxidized. When tryptophan was added, even in the presence of catalase, it combined with peroxide from reaction (1) according to reaction (2) thus increasing the oxygen consumption

$$\text{Tryptophan} + 2H_2O_2 \xrightarrow{\text{peroxidase}} \text{Products}. \quad (2)$$

The oxidation of tryptophan by plant peroxidase, which has been reported briefly by Sizer (1947), is the subject of this paper.

METHODS AND MATERIALS

Plant material. Pea seedlings were grown for 7–10 days either (*a*) in dishes of moist sand kept in the dark or (*b*) in seedboxes of John Innes potting compost in a greenhouse with normal lighting. Method (*a*) gave better growth in winter. The seedlings were washed at harvest to remove sand or soil and any diseased parts were discarded. Where indicated in the Experimental section they were dissected with scissors into root, cotyledon and epicotyl portions.

Tissue slices. Tissues were cut freehand with a razor blade moistened with 0·067 M-potassium phosphate buffer at pH 7. Growing tips of roots and epicotyls were discarded. Slices from a number of plants were pooled and washed thrice with buffer, then suspended in the same buffer saturated with toluene.

Tissue extracts. Fresh tissue was weighed and minced and the juice pressed through cloth. The residue was extracted by grinding for 2 min. in a Townson and Mercer (Croydon) macerator with 0·067 M buffer equal to the fresh weight; the extract was pressed through cloth and combined with the juice. Large particles were removed by centrifugation at 1000 rev./min. for 5 min.

Enzymes. Catalase was prepared from human erythrocytes by Bonnichsen's method (1947). Purified liver catalase, notatin and horseradish peroxidase were presented by Dr E. F. Hartree. Peroxidase activity was measured by Keilin & Hartree's method (1951) except that an EEL colorimeter (Evans Electroselenium Ltd.) and filter 622 were used instead of a Spekker absorptiometer. The number of enzyme units (E.U.) in a preparation $= Vw/1000v$, where V is the total volume of solution, w = mg. purpurogallin formed by v ml. of solution.

Dry weight. Tissue was dried to constant weight at 110°.

Deproteinization. Samples were precipitated either (*a*) by addition of trichloroacetic acid solution to a final concentration of 5% (w/v) or (*b*) by addition of an equal volume of 5% $(CH_3COO)_2Zn$, $2H_2O$ and half the volume of 0·4 N-NaOH, and filtered or centrifuged.

Analytical methods. Tryptophan was estimated colorimetrically (Horn & Jones, 1945) by reaction with *p*-dimethylaminobenzaldehyde (Ehrlich reagent) in acid solution. This reaction is not specific for tryptophan. Tryptamine gives an equivalent amount of the same blue colour, and colours are given by all pyrroles with at least one free methene hydrogen, by indoles and by furans (Reichstein, 1932). Since it was found that the blue colour was readily bleached by H_2O_2, catalase was added to decompose peroxide before addition of Ehrlich reagent. In some experiments duplicate estimations of tryptophan were made by Eckert's method (1943).

Ammonia was estimated (*a*) by steam-distillation in the Markham apparatus, or (*b*) by diffusion in Conway dishes at room temperature. Method (*b*) was used when putrescine was included in the reaction mixture because the product of its oxidation is a steam-volatile base (Kenten & Mann, 1952).

Total nitrogen was estimated by the Kjeldahl method.

Manometric measurements. O_2 uptake and CO_2 production were measured in the Warburg apparatus in air at 28°. The total volume of each reaction mixture was 3 ml., and KOH was present in the centre wells, except when CO_2 was measured (by addition of 0·2 ml. of 2 N-HCl at the end of the incubation).

RESULTS

Tryptophan oxidation by tissue slices

The respiration of pea-seedling slices was slow (Q_{O_2} 1 to 4, Table 1). It was increased by addition of substrates for amine oxidase, such as putrescine or ethanolamine, but not by tryptophan. In the presence of amines, addition of tryptophan increased the oxygen consumption, and part of the tryptophan was oxidized. The rate of oxidation averaged 0·5 μmole/g. dry wt./hr. without amine, and 0·8 μmole/hr. with amine, when the tryptophan concentration was 0·00025 M. At 0·004 M-tryptophan, the averages were 4·6 and 9·1 μmoles/hr. Tryptophan oxidation was followed in deproteinized filtrates by colorimetry and by the decrease in absorption at 279 mμ. No product was detected, and no colour was formed.

Tryptophan oxidation by tissue extracts

Tryptophan was oxidized at about the same rate by whole tissue extracts as by slices (Table 2) and the oxidation was more definitely accelerated by ethanolamine. This amine rather than putrescine was used for most of the experiments with extracts

because the product of oxidation of putrescine reacts with Ehrlich reagent to give a red compound which interfered with the tryptophan assays. Ethanolamine was oxidized comparatively slowly so that a long incubation was necessary. Toluene was added to control bacterial infection.

Table 1. *Oxidation of tryptophan by pea-seedling tissue slices*

(Slices shaken at room temperature in 25 ml. flasks containing 5 ml. 0·067 M-phosphate buffer, pH 7, saturated with toluene. Expt. 1: 0·5 g. slices, 1·25 μmoles tryptophan, 50 μmoles amine, incubated 16 hr. Expt. 2: 1·0 g. slices, 20 μmoles tryptophan, 1 m-mole amine, incubated 12 hr. Q_{O_2} = μl. O_2/mg. dry wt./hr.)

Tissue	Q_{O_2}	Additions	Tryptophan oxidized (μmoles) Expt. 1	Expt. 2
Root	4·0	None	0·22	4·44
		Putrescine	0·37	.
		Ethanolamine	0·54	6·52
Cotyledon	1·0	None	0·22	2·35
		Putrescine	0·29	.
		Ethanolamine	0·35	7·00
Epicotyl	2·6	None	0·25	3·09
		Putrescine	0·30	.
		Ethanolamine	0·54	5·80

Table 2. *Oxidation of tryptophan by pea-seedling extracts*

(Extract equivalent to 15 g. fresh wt. of tissue shaken at room temperature in 100 ml. flasks containing 20 μmoles tryptophan, 1·5 m-moles ethanolamine, 0·067 M-phosphate buffer, pH 7, and water to 30 ml.)

Tissue	Additions	Tryptophan oxidized (μmoles) 6 hr.	16 hr.
Root	None	0·2	3·6
	Ethanolamine	5·3	10·2
Cotyledon	None	0·6	3·3
	Ethanolamine	8·5	16·2
Epicotyl	None	0	3·3
	Ethanolamine	7·5	15·4

The rate of oxidation by extracts was decreased by ageing or by dialysis, and was restored by addition of ethanolamine. The extract remained active for 1 week if saturated with toluene and stored at +2°.

Absorption spectra of deproteinized and diluted reaction mixtures of cotyledon extract are plotted in Fig. 1. The usual spectrum of tryptophan persisted in the control without ethanolamine. The product in the mixture containing ethanolamine, in which most of the tryptophan had been oxidized, showed new absorption maxima at 270 and 370 mμ.

Purification of the tryptophan-oxidizing enzyme system

Centrifugation. Nearly all the activity in extracts was associated with soluble enzymes. In a typical experiment, extract was centrifuged for 30 min. at

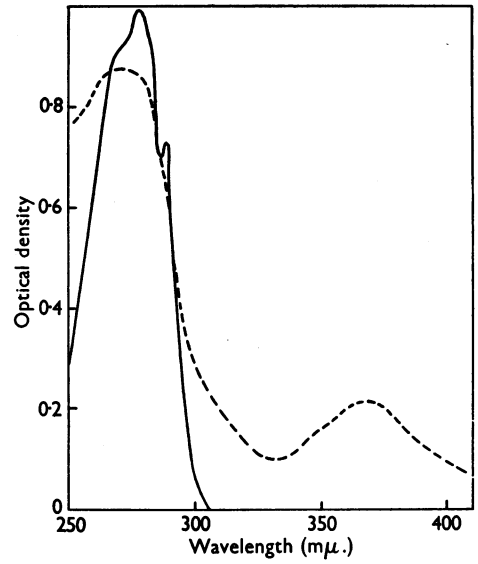

Fig. 1. Absorption spectra of tryptophan (continuous line) and of its oxidation product (interrupted line) in whole pea-seedling extract.

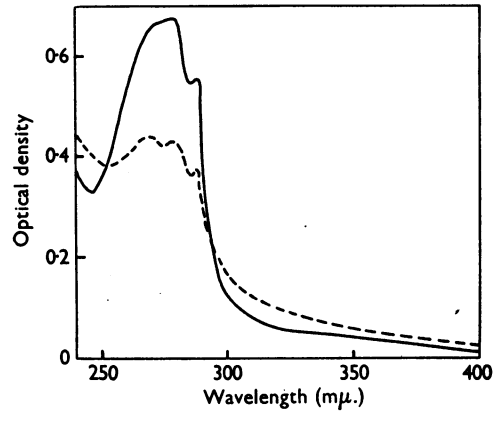

Fig. 2. Absorption spectra of tryptophan before (continuous line) and after (interrupted line) oxidation by enzyme *R*3.

10000 *g*. Tryptophan-oxidizing activity and peroxidase were assayed in samples of the clear supernatant (*S*1) and of the residue (*R*1) resuspended in one-tenth of the original volume of 0·067 M-phosphate buffer. *S*1 contained 90% of the peroxidase and of the tryptophan-oxidizing activity.

There was no increase of activity of *S*1 when *R*1 was added back to it. Subsequent steps in purification started with *S*1.

Precipitation with ammonium sulphate. *S*1 was fractionated by addition of ammonium sulphate to one-third and two-thirds saturation. The two precipitates and the supernatant at two-thirds saturation were examined for activity and protein nitrogen after dialysis to remove ammonium sulphate. The recoveries, based on activity and protein nitrogen of *S*1 were: 17% of activity and 36% of protein precipitated at one-third saturation; 34% of activity and 32% of protein precipitated between one-third and two-thirds saturation; 37% of activity and 13% of the protein in the supernatant at two-thirds saturation.

Precipitation with immiscible solvents. A great deal of inactive protein could be removed before ammonium sulphate precipitation by saturation of *S*1 with *n*-butanol (Morton, 1950) or better by addition of chloroform and ethanol. 0·25 Vol. of *n*-butanol, or 0·1 vol. of chloroform and 0·2 vol. of ethanol, were added to *S*1 with stirring. The organic layer and the bulky protein precipitate which floated at the interface on centrifugation were discarded. About half of the activity was recovered in the aqueous layer (*S*2) after butanol or chloroform-ethanol treatment, but the latter method was more often used because it removes more inactive protein.

About 40 g./100 ml. of ammonium sulphate was required to saturate *S*2 as compared with 70 g./100 ml. for *S*1. The precipitate (*R*2) so obtained contained most of the amine oxidase, but did not oxidize tryptophan. Peroxidase was not precipitated in *R*2 (Table 3). If the organic solvent was first removed from *S*2 by dialysis, more ammonium sulphate could be dissolved, and the precipitate between one-third and two-thirds saturation contained amine oxidase, together with peroxidase and most of the tryptophan-oxidizing activity. This enzyme (*R*3), after dialysis, was a yellow solution which darkened on standing. It was dried over sodium hydroxide by evaporation in a desiccator under reduced pressure. An attempt to prepare a dry powder by addition of 2 vol. of acetone resulted in loss of activity on ethanolamine and on tryptophan.

Separation of components of the enzyme system

Several attempts were made to separate pea-seedling peroxidase from amine oxidase. A solution of *R*3 was carefully adjusted to pH 5·5. The precipitate (*R*4) was dissolved in phosphate buffer at pH 7 and the supernatant (*S*3) was readjusted to pH 7. The peroxidase and tryptophan-oxidizing activities of *S*1, *S*2, *S*3, *R*2, *R*3 and *R*4 are compared in Table 3. Most of the amine oxidase was precipitated at pH 5·5, while the peroxidase and tryptophan-oxidizing activity were not. Rather more efficient separation was achieved by treatment of *R*3 with calcium phosphate gel (prepared according to Singer & Kearney, 1950) at pH 5·5. Peroxidase (*P*) was not adsorbed, while amine oxidase (*A*) could be recovered by elution from the gel with phosphate buffer pH 8·5. Neither enzyme alone

Table 3. *Tryptophan oxidation and peroxidase activity in pea-seedling extracts purified in several ways*

(Enzymes prepared as described in the text, shaken at room temperature in flasks containing 20 μmoles tryptophan, 1·5 m-moles ethanolamine and 0·067 M-phosphate buffer at pH 7. Activity expressed as μmoles tryptophan oxidized/ml./hr. and peroxidase as E.U./100 ml.)

	Tryptophan-oxidizing activity		Peroxidase	
Enzyme	Expt. 1	Expt. 2	Expt. 1	Expt. 2
*S*1	0·9	0·7	4·56	5·3
*S*2	0·4	.	1·99	.
*S*3	.	0·8	.	6·1
*R*2	0·04	.	0·07	.
*R*3	2·2	.	4·3	.
*R*4	.	0·1	.	0·8

Table 4. *Tryptophan oxidation by amine oxidase and peroxidase from pea seedlings*

(Enzymes *A* (amine oxidase) and *P* (peroxidase) prepared as described in the text. Warburg vessels contained enzyme as indicated, 10 μg. catalase, 20 μmoles ethanolamine, 0·067 M-phosphate buffer, pH 7.)

Enzyme (ml.)			Tryptophan	Oxygen uptake (μmoles)		
A	*P*	Boiled *P*	(μmoles)	Expt. 1	Expt. 2	Expt. 3
0·2	.	.	.	2·6	4·6	.
0·2	.	.	25	2·8	5·6	.
.	1·5	.	.	0·4	0·8	.
.	1·5	.	25	0·5	1·2	.
0·2	1·5	.	.	2·9	5·3	.
0·2	1·5	.	25	4·7	8·3	.
.	.	1·5	.	.	.	0·3
.	.	1·5	25	.	.	0·4
0·2	.	1·5	.	.	.	3·0
0·2	.	1·5	25	.	.	3·1

oxidized tryptophan. Oxidation by the reconstituted enzyme was followed by the oxygen uptake in Warburg manometers (Table 4). Boiled peroxidase preparation was inactive.

Course of the reaction

It was found impossible to measure accurately the gas exchange during tryptophan oxidation by *S*1 because of the rapid respiration of the enzyme itself. Dried enzyme *R*3 retained reasonable activity with substrates added, while its blank respiration was about 1% of that of *S*1. Enzyme *R*3 diluted to contain about 1 mg. protein nitrogen/ml. was used in the following experiments.

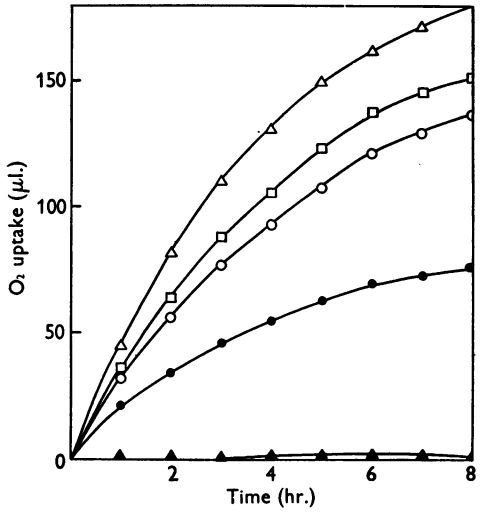

Fig. 3. Coupled oxidation of tryptophan during oxidation of ethanolamine by enzyme *R*3. Warburg vessels contained 1 ml. *R*3, 10 µg. catalase, 10 µmoles ethanolamine and 0·067 M-phosphate buffer, pH 7. The oxygen uptake of the enzyme has been subtracted. No tryptophan, ●—●; 5 µmoles tryptophan, ○—○; 10 µmoles tryptophan, □—□; 37·5 µmoles tryptophan, △—△; 37·5 µmoles tryptophan without ethanolamine, ▲—▲.

Requirement for hydrogen peroxide. It is apparent from the experiment presented in Fig. 3 that tryptophan oxidation is dependent on a supply of hydrogen peroxide. The oxygen consumption of enzyme with tryptophan was almost the same as that of enzyme alone. Oxygen uptake with ethanolamine was doubled in the presence of sufficient tryptophan. This implies that peroxidase competed successfully with catalase for the peroxide. The amount of peroxide so utilized depended on the concentrations of tryptophan (Fig. 3) and of enzyme (Fig. 4). The reaction mixture in both experiments contained 10 µg. added catalase besides that already present in the enzyme, which was by itself enough to destroy all the peroxide formed. Higher catalase concentrations had no effect on oxygen uptake with ethanolamine, but inhibited the extra oxygen uptake with tryptophan, supporting the inference that peroxide is essential. Oxidation was inhibited by 31% by 50 µg. catalase, 52% by 100 µg. and 76% by 500 µg. catalase.

Oxygen consumption. Results from several experiments using different preparations of enzyme (Table 5) indicate that 1 mole of tryptophan is oxidized with the uptake of 1 mole of oxygen. The oxygen uptake with tryptophan was never more than double that with ethanolamine alone, even when the tryptophan concentration was high compared with that of ethanolamine. The reaction is therefore between 1 molecule of tryptophan and

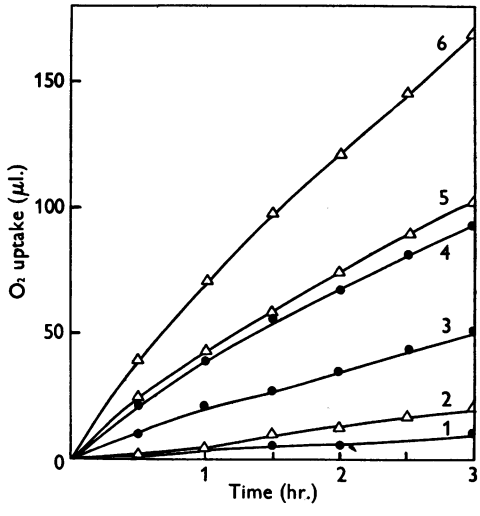

Fig. 4. Coupled oxidation of tryptophan. Warburg vessels contained 10 µg. catalase, 10 µmoles ethanolamine and 0·067 M-phosphate buffer, pH 7. Curve 1, 0·1 ml. *R*3; curve 2, 0·1 ml. *R*3 + 25 µmoles tryptophan; curve 3, 0·5 ml. *R*3; curve 4, 0·5 ml. *R*3 + 25 µmoles tryptophan; curve 5, 1·0 ml. *R*3; curve 6, 1·0 ml. *R*3 + 25 µmoles tryptophan.

2 molecules of hydrogen peroxide (reaction 2 above).

Production of ammonia and carbon dioxide. The amount of ammonia formed when tryptophan was oxidized with ethanolamine as the peroxide donor was the same as that due to oxidation of ethanolamine without tryptophan (Table 5). When the peroxide came from notatin and glucose (Table 6) no ammonia was formed. Less than 0·25 mole of carbon dioxide was formed per mole of tryptophan oxidized.

The oxidation product. The product of tryptophan oxidation by purified extracts is not that formed in whole extract. The most striking difference is that in the purified extract the product is coloured reddish brown, a feature which was first observed by

Elliott (1932*a*) for milk peroxidase. Elliott (1932*b*) was unable to oxidize tryptophan with horseradish peroxidase, and considered this an important difference between the two enzymes. The absorption spectrum of the product (Fig. 2) did not show maxima at 270 and 370 mμ. The changes in absorption were similar to those observed by Weil, Gordon & Buchert (1951) in the photochemical oxidation of tryptophan catalysed by methylene blue. The photochemical oxidation also involved hydrogen peroxide, which was formed by reversible oxido-reduction of the dye. The extent of decomposition of the indole nucleus was more extensive in that 4 moles of oxygen were consumed and 2 moles of carbon dioxide and 0·3 mole of ammonia produced per mole of tryptophan oxidized. The site of attack was considered to be the five-membered ring of the indole nucleus, because the absorption maximum at about 280 mμ., due to the ethylenic linkage in the pyrrole ring conjugated with the benzene nucleus, disappeared, while a new maximum appeared at 260 mμ. suggesting that the benzene chromophore persisted in the oxidation product. The indole nucleus of tryptophan is also destroyed by irradiation with cathode rays (Proctor & Bhatia, 1953), but in this case considerable amounts of ammonia are released.

Effect of pH

The variation of activity of enzyme *R*3 with pH was tested in 0·067 M potassium phosphate buffers from pH 6 to 8·5. Oxygen uptake was fastest at pH 7 and the rate fell off more sharply on the acid than on the alkaline side.

Effect of inhibitors

Tryptophan oxidation was completely inhibited by 10^{-3}M-potassium cyanide at pH 7. It was not inhibited by 10^{-6}M-sodium azide, but was inhibited to the extent of 8% at 10^{-4}M, 73% at 10^{-3}M and 95% at 10^{-2}M. Ethylenediaminetetraacetic acid (Sequestrene NA2, Alrose Chemical Co.,

Table 5. *Gas exchange in tryptophan oxidation by pea-seedling enzymes*

(Warburg vessels contained 1 ml. enzyme *R*3, 20 μg. catalase, 0·067 M-phosphate buffer, pH 7. Deductions have been made for oxygen uptake and ammonia and carbon dioxide formed with ethanolamine alone, and for oxygen uptake, ammonia and carbon dioxide formed and tryptophan oxidized with tryptophan alone.)

Additions		Oxygen uptake	Ammonia formed	Carbon dioxide formed	Tryptophan oxidized
Ethanolamine (μmoles)	Tryptophan (μmoles)	(μmoles)	(μmoles)	(μmoles)	(μmoles)
5	5	1·1	0	.	0·9
5	20	2·3	0	.	1·8
10	5	1·6	0	.	1·2
10	10	1·8	0	.	2·3
10	10	2·8	.	0·7	.
10	10	3·6	.	0·4	.
10	10	2·3	.	0·5	.
10	15	2·6	0	.	2·3
10	15	4·9	0	.	4·0
10	20	2·5	0	.	2·8
10	20	2·3	.	0·4	.
10	20	3·0	.	0·7	.
20	10	6·5	.	.	6·7

Table 6. *Tryptophan oxidation by purified peroxidases with notatin and glucose*

(Warburg vessels contained 20 μg. catalase and 0·067 M-buffer, pH 7, 20 μg. notatin, 22 μg. horseradish peroxidase, 1 ml. pea-seedling peroxidase, 20 μmoles glucose and 10 μmoles tryptophan where indicated.)

Additions					Oxygen uptake	Tryptophan oxidized
Notatin	Horseradish peroxidase	Pea-seedling peroxidase	Glucose	Tryptophan	(μmoles)	(μmoles)
−	+	−	+	−	1·6	.
−	+	−	+	+	2·4	2·5
+	−	−	+	−	10·9	.
+	−	−	+	+	11·5	1·2
+	+	−	+	−	12·0	.
+	+	−	+	+	18·0	4·2
+	−	+	−	−	0	.
+	−	+	−	+	1·1	1·6
+	−	−	+	−	8·4	.
+	−	−	+	+	9·1	0·2
+	−	+	+	−	11·2	.
+	−	+	+	+	15·6	3·6

Providence, R.I.) inhibited 21% at 10^{-3}M, 44% at 2×10^{-3}M and 100% at 5×10^{-3}M. The inhibition by potassium cyanide was in part attributable to inhibition of amine oxidase. Sodium azide and Sequestrene had very little effect on this enzyme; they inhibited peroxidase.

Specificity of reaction

Both optical isomers of tryptophan were oxidized at the same rate and neither was oxidized by *R3* without peroxide. Although indolylacetic acid was oxidized rapidly, its oxidation did not depend on peroxide; the oxygen uptake with ethanolamine and indolylacetic acid was the sum of that with the two substrates separately. Only the side chain was oxidized, as found by Tang & Bonner (1947), since the product retained the absorption spectrum typical of indoles and gave a blue colour with Ehrlich reagent, but did not give the red colour characteristic of indolylacetic acid with Salkowski reagent.

Tryptophan oxidation in model systems containing purified peroxidases

Hydrogen peroxide decomposed slowly with either or both tryptophan and horseradish peroxidase (Table 7). The decomposition stopped after 2 hr. and the Warburg vessels were incubated for a further 4 hr. without change in manometer reading. Catalase (10 μg.) was then added from the side arms and the oxygen output measured. The total oxygen output before and after addition of catalase was in all cases nearly the same; it is concluded that there was no significant reaction between tryptophan and peroxide. All the reaction mixtures were colourless at the end of this experiment, but solutions of tryptophan in stronger (0·05M) peroxide became yellow after 12 or more hr. at room temperature, and in such solutions the absorption at 279 mμ. decreased. The reaction was not apparently accelerated by peroxidase. These observations are consistent with Elliott's conclusion that horseradish peroxidase does not oxidize tryptophan. However, when peroxide was added slowly to a solution of tryptophan and horseradish peroxidase, so that the peroxide concentration was low, tryptophan oxidation could be measured. In one experiment a mixture of 704 μg. horseradish peroxidase, 70 μg. catalase, 5 μmoles tryptophan, 0·067M-phosphate buffer at pH 7 and water to 20 ml. was stirred in a water bath at 28°, while 0·01M-hydrogen peroxide was run in from a horizontal burette with capillary delivery. The rate of addition of peroxide was 0·2 μmole/min. Disappearance of tryptophan was followed in successive samples from which peroxide was removed by addition of 7 μg. catalase. 27% of the tryptophan was oxidized in 6·5 hr. The red-brown colour of the product was noticed after 2 hr.

Table 7. *Reaction of tryptophan and peroxidase with hydrogen peroxide*

(All Warburg vessels contained 20 μmoles hydrogen peroxide and 0·067M-phosphate buffer, pH 7, 100 μg. horseradish peroxidase and 10 μmoles tryptophan where indicated. 10 μg. catalase added to all vessels after 6 hr.)

Additions		Oxygen output (μmoles)		
Peroxidase	Tryptophan	Before adding catalase	After adding catalase	Total
–	–	0·1	18·5	18·6
+	–	1·7	16·3	18·0
–	+	2·5	15·4	17·9
+	+	4·4	12·9	17·3

Tryptophan oxidation was also demonstrated in a coupled enzyme system in which peroxide was provided from the oxidation of glucose by glucose oxidase (notatin) and used by purified horseradish peroxidase (Table 6). The relation of oxygen used to tryptophan oxidized and the alteration in the absorption spectrum were the same as with the pea-seedling enzymes. Both D- and L-tryptophan were oxidized at the same rate, while indolylacetic acid, glutamic acid, alanine, tyrosine and histidine were not oxidized. Tryptophan was oxidized by pea-seedling peroxidase preparation *P* using notatin and glucose as peroxide source (Table 6).

Presence of peroxidase in pea seedlings

Pea seedlings contained about the same concentration of peroxidase as horseradish. The enzyme was present in all parts, but especially in the roots and cotyledons (Table 8). From the data of Table 6, 1 E.U. of purified horseradish peroxidase can oxidize

Table 8. *Distribution of peroxidase in 10-day-old etiolated pea seedlings*

Tissue	Fresh wt. (g.)	Dry wt. as % fresh wt.	Peroxidase content		
			E.U.*	E.U./g. fresh wt.	E.U./g. dry wt.
Root	100	5·8	2·94	0·0294	0·507
Cotyledon	124	28·2	7·24	0·0584	0·207
Epicotyl	164	5·9	1·72	0·0105	0·177

* E.U. = enzyme units.

52·2 μmoles of tryptophan/hr. The average content of peroxidase in pea-seedling tissue was 0·03 E.U./g. fresh wt., so that 1 g. fresh tissue might be expected to oxidize 1·6 μmoles tryptophan/hr. The rates observed (Table 1) were about one-third as fast.

DISCUSSION

It has been shown that tryptophan oxidation by tissues and extracts of pea seedlings is increased by addition of certain amines. This acceleration is brought about by hydrogen peroxide formed during oxidation of the amines by the amine oxidase, for amine and amine oxidase can be replaced by glucose and notatin or by a suitable concentration of hydrogen peroxide. Since the activity of extracts purified in several ways is parallel to their peroxidase content, and the same reaction is catalysed by purified horseradish or pea-seedling peroxidase, it is clear that tryptophan is oxidized by peroxidase in a coupled enzyme system using enzymically generated peroxide. The peroxidase reaction occurs in the presence of the normal concentration of catalase but is inhibited by higher concentrations of catalase.

Oxidation in tissues and whole extracts produced no coloured substance; a product with absorption maxima at 270 and 370 mμ. accumulated in whole extracts. It differed from tryptophan in that the red compound it formed with Ehrlich reagent did not change to blue on addition of sodium nitrite. This property is shared by 3-hydroxykynurenine (Makino, Satoh, Fujiki & Kawaguchi, 1952), which has absorption maxima at 268·5 and 370 mμ., and is a product of tryptophan oxidation in the rat (Dalgliesh, 1952).

In purified extracts and in model systems with horseradish peroxidase, a coloured substance possessing no definite absorption spectrum was formed. No ammonia and very little carbon dioxide was produced. The amount of tryptophan oxidized depended on the amount of peroxide supplied, and the oxygen uptake was equivalent to 2 moles hydrogen peroxide (1 mole oxygen)/mole of tryptophan oxidized.

In some respects these results resemble those obtained by Knox & Mehler (1950) for tryptophan oxidation in liver extracts. Both oxidations depend on a peroxide-donating system. The amount of peroxide required is comparable with the tryptophan oxidized and its concentration must be low. Both are inhibited by catalase and in both the oxygen uptake is 1 mole/mole of tryptophan oxidized. Both are catalysed by enzymes which are not sedimented at 8000 rev./min. On the other hand, the pea-seedling enzyme is not specific to the L-isomer, and the animal enzyme does not oxidize typical peroxidase substrates, though it is inhibited by peroxidase inhibitors. The oxygen uptake of the liver tryptophan peroxidase, using D-alanine as source of hydrogen peroxide, was sometimes more than doubled by addition of tryptophan. This result led to the interpretation of the reaction as taking place in two steps, the second step providing peroxide which was used in the first, thus permitting 'cyclic oxidation'. Tryptophan never more than doubled the oxygen uptake with pea-seedling enzyme, even with several times more tryptophan than peroxide donor. The amount of tryptophan oxidized was strictly dependent on the peroxide available. There was no 'sparking' effect by peroxide, and a second reaction producing peroxide is improbable.

In spite of these differences, it is possible there may be some relation between the mechanism in the plant and animal. It may be suggested that in the plant tryptophan is oxidized by a series of enzymes of which peroxidase is the first, using two molecules of hydrogen peroxide. The product of this first reaction may be converted to 3-hydroxykynurenine by enzymes which are less stable or less active than peroxidase and which had been removed from the purified extracts used in this work. There is sufficient peroxidase in pea-seedling tissue to account for all the tryptophan oxidation observed. The rates averaged about 0·5 μmole/g. fresh wt./hr., which is comparable with the rate in normal rat liver (Knox, 1951) and more than 100 times as fast as the conversion of tryptophan to indolylacetic acid in tobacco ovaries (Wildman & Muir, 1949). Oxidation by peroxidase could therefore be the normal fate of tryptophan in this plant tissue.

SUMMARY

1. The oxidation of tryptophan in tissue slices and extracts from pea seedlings was accelerated by addition of certain amines.

2. The tryptophan-oxidizing activity of extracts was associated with peroxidase.

3. In the whole extract, the product of oxidation was a substance with absorption maxima at 270 and 370 mμ.

4. In purified extract two molecules of hydrogen peroxide oxidized one molecule of tryptophan to a coloured product.

5. Tryptophan was oxidized in model systems by purified horseradish peroxidase with free hydrogen peroxide or with peroxide-forming enzymes and substrates.

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The Colorimetric Estimation of Formaldehyde by Means of the Hantzsch Reaction

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Most of the colour reactions described for formaldehyde require severe conditions incompatible with the preservation of biological material, although consistent results can be obtained where complete destruction of this material can be tolerated (MacFadyen, 1945). Examples of such reactions are those employing chromotropic acid in hot strong sulphuric acid (Eegriwe, 1937), phenylhydrazine and ferricyanide in strong hydrochloric acid (Kersey, Maddocks & Johnson, 1940) and various tests quoted by Walker (1944), some using alkaloids and strong mineral acid and others using phenols and strong alkali. It would be convenient to have at least one colorimetric reaction that could be carried out under less violent conditions. It might also be desirable to preserve biological material on which an estimate of formaldehyde content was required, and from which the taking of samples was inconvenient or impossible.

In the course of work on the neutralization of formaldehyde in living bacterial suspensions, it was found that a yellow colour developed with acetylacetone in the presence of ammonium salts and, less rapidly, in the presence of glycine. This appeared to give a possible alternative to the reactions mentioned above and, as has already been briefly reported (Nash, 1952), was traced to the formation of 3:5-diacetyl-1:4-dihydrolutidine. Two-stage syntheses of this substance have been described using concentrated reagents and catalysts (Scholtz, 1897; Schneider & Sanger, 1903), but for the particularly simple molecules concerned the reaction can be quantitative for traces of formaldehyde under quite mild conditions.

General description of the reaction and its product

When traces of formaldehyde are added to approximately neutral solutions of acetylacetone and ammonium salt, a yellow colour gradually develops owing to the synthesis of diacetyldihydrolutidine (DDL). Under optimum conditions the molecular extinction in terms of formaldehyde has a smooth maximum of 8000 at 412 mμ., independent of dilution. The absorption curve is shown in Fig. 1, and was obtained using reagent *A* (see below); this has since been somewhat modified, but the effect on the absorption curve should be negligible. It can be seen that a good separation is obtained between the absorption maximum of DDL and the first absorption band of acetylacetone. The position of this curve coincided with that obtained from solutions of recrystallized DDL itself in