two equivalent clusters of three SH groups and a further two SH groups in separate but equivalent positions.

My results agree with those of Murayama obtained at 0°, but not with those of Huisman and his collaborators, except that they also were unable to find a difference between the two haemoglobins. Their figures for the silver and mercury uptake of native haemoglobin A and S are very close indeed to those obtained in the present, and previous, experiments with samples fully denatured by sodium dodecyl sulphate. Their method of preparing the haemoglobin is a different and perhaps a harsher one than the method used in this work; this might explain the high uptake of heavy metal without previous denaturation. With respect to the first of the two breaks in the titration plot obtained with a mercury-coated electrode, it is possible that rather small changes in the titration conditions might make it almost invisible.

SUMMARY

1. The numbers of sulphydryl groups in native and sodium dodecyl sulphate-denatured sickle-cell haemoglobin are the same as in the normal haemoglobin. Their configuration is probably also the same.

2. Two kinds of sulphydryl groups, which have different affinities for silver, can be estimated by amperometric titration with silver nitrate at the mercury-treated platinum electrode estimate. The groups correspond apparently to the sulphydryl groups available to silver at the untreated electode in native and in denatured human haemoglobin.

3. Storage at 4° leads to a loss in sulphydryl groups available in the native protein.

The author is very grateful to Dr M. F. Perutz for encouraging this investigation. The experiments reported were made possible through the courtesy of Dr A. C. Allison, Oxford, and Dr G. Seaman, Cambridge, who supplied samples of haemoglobin S and of homozygous sickle-cell blood.

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The Oxidation of Tryptamine to 3-Indolylacetaldehyde by Plant Amine Oxidase

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(*Received* 13 *August* 1956)

3-Indolylacetic acid (IAA) is believed to be a principal auxin of higher plants. There is much evidence that it is formed in plants from tryptophan but the metabolic route is not yet established. It has been suggested that the route is by way of tryptamine and 3-indolylacetaldehyde (IAc) or 3-indolylpyruvic acid and IAc. Two oxidases, or groups of oxidases, catalysing the oxidation of amines have been found in higher plants. The presence of a monoamine oxidase was shown by Werle & Roewer (1950, 1952), and that of a histaminase or diamine oxidase by Werle & Zabel (1948) and Werle & Pechmann (1949). Kenten & Mann (1952*a*) found that the diamine oxidase catalysed the oxidation of both mono- and diamines and preferred the name plant amine oxidase. It was shown that the oxidation of tryptamine is catalysed by the enzyme and this reaction, followed by the action of an aldehyde oxidase on the product, was suggested as a possible mechanism of formation of IAA in higher plants. Subsequent attempts to prove that IAc is the product of the oxidation of tryptamine catalysed by plant amine oxidase, with the relatively crude preparations of the enzyme described by Kenten & Mann (1952*a*), were unsuccessful. In these experiments the observed oxygen uptake was much greater than that required for oxidation of tryptamine to the corresponding

aldehyde, which suggested the presence in the crude enzyme preparations of factors catalysing the oxidation of IAc. Mann (1955) has since purified plant amine oxidase several hundredfold, and the results of an investigation of the oxidation of tryptamine catalysed by such purified preparations of the enzyme are here recorded.

EXPERIMENTAL

Amine oxidase. Purified preparations of amine oxidase were obtained by the method of Mann (1955) with some slight modifications. Pea seedlings (*Pisum sativum* L.) were grown for 8–12 days in sand. The washed cotyledons alone were used as starting material, since Kenten & Mann (1952*a*) have shown that most of the enzyme is present in the cotyledons at this stage of growth. The cotyledons were macerated for 2 min. in an Ato-Mix blender (Measuring and Scientific Equipment Ltd.) with 0·067 M phosphate buffer, pH 7, previously cooled to 0–5° (100 ml./100 g. of cotyledons) and the macerate was squeezed through strong cotton cloth. The residual pulp was mixed with more buffer (50 ml./100 g. of cotyledons) and again squeezed through cloth. The combined extracts were cooled in an ice–salt freezing mixture to between 0 and 5°, and a mixture of ethanol–chloroform (20 ml. of ethanol and 10 ml. of chloroform/100 ml. of extract), previously cooled to −10°, was added slowly during vigorous mechanical stirring. The stirring was continued for a further 90 min. after the addition of the ethanol–chloroform mixture. The subsequent procedure was that of Mann (1955). The volume of the final enzyme solutions was such that 1 ml. was equivalent to 50 g. of cotyledons. About thirty such preparations were used in the work and the N contents and specific activities (Mann, 1955) of seven were estimated. The N contents varied from 425 to 690 μg./ml. with an average of 590 μg./ml. The specific activities varied from 64000 to 114000 with an average of 92000.

Relatively crude dry preparations of amine oxidase were prepared from pea-seedling extracts by fractional precipitation with ammonium sulphate followed by precipitation with acetone, as described by Kenten & Mann (1952*a*). These are referred to in the text as the dry preparations of amine oxidase.

Peroxidase. This was prepared from horse-radish roots by the method of Kenten & Mann (1954), except that the final fractional precipitation with ethanol was omitted. The Purpurogallinzahl (P.Z.) (Willstätter & Stoll, 1918) of the preparation, i.e. mg. of purpurogallin formed by 1 mg. of enzyme preparation in 5 min. from pyrogallol and H_2O_2 under fixed conditions, was estimated by the method of Keilin & Hartree (1951) with an EEL (Evans Electroselenium Ltd.) colorimeter with Ilford Bright Spectrum Blue filter 622 (maximum transmission at λ465 mμ.). The preparation of P.Z. 900 used was about 80% peroxidase.

The peroxidase activities of some of the amine oxidase preparations were also estimated by the same method.

Catalase. This was prepared from ox liver by the method of Sumner & Dounce (1937). The activity of the preparations was estimated as described previously (Kenten & Mann, 1952*b*). The Katalasefähigkeit (Kat. f.) values of the two preparations used were 11 000 and 27 000, suggesting purities of about 20 and 50% respectively. From the equation of Chance & Herbert (1950) that Kat. f. = 520 k_1'/M, where M is the molecular weight of catalase (230000), the velocity constants of the preparations are:

$$k_1' = 4{\cdot}9 \times 10^6 \text{ M}^{-1} \text{ sec.}^{-1} \quad \text{and} \quad 11{\cdot}9 \times 10^6 \text{ M}^{-1} \text{ sec.}^{-1}.$$

In experiments with added catalase, 100 μg. of the preparation of Kat. f. 11000 or 50 μg. of that of Kat. f. 27000 were used/3 ml. of reaction mixture.

Notatin. This was a preparation given by the Boots Pure Drug Co. in 1952.

Buffers. Phosphate buffers (0·2 M) were prepared from solutions of KH_2PO_4 and KOH. Pyrophosphate buffers (0·2 M) were prepared from solutions of $Na_2H_2P_2O_7$ and $K_4P_2O_7$. Phosphate–borate buffers were made by mixing equal volumes of 0·4 M-KH_2PO_4 and 0·4 M-H_3BO_3 and adjusting to the required pH with KOH, finally diluting to twice the original volume.

Ammonia. This was estimated by diffusion in Conway dishes at room temperature.

IAA. 3-Indolylacetic acid was estimated by means of the Salkowski reaction. Houff, Hinsvark, Weller, Wittwer & Sell (1954) have shown that this reaction, the formation of a red colour when IAA reacts with Fe^{3+} ions in acid solution, depends on the oxidation of IAA to *N*-hydroxy-IAA. The estimation procedure used in the present work was one of those suggested by Gordon & Weber (1951). Samples of the reaction mixtures, preferably containing 20–70 μg. of IAA, were diluted to 10 ml. with water and 15 ml. of modified Salkowski reagent (a mixture of 5 ml. of 0·5 M-$FeCl_3$, 250 ml. of water and 150 ml. of conc. H_2SO_4) was added. The solutions were thoroughly mixed and allowed to stand at least 1 hr., and not more than 4 hr., at room temperature. The optical densities at 530 mμ. were then measured in matched tubes in a Unicam model SP. 400 spectrophotometer. Samples of reaction mixtures without added tryptamine were treated with the reagent in the same way and used in the blank tube. The calibration curve obtained with IAA, a mixture of 10 ml. of water and 15 ml. of Salkowski reagent being used in the blank tube, was almost linear with amounts of IAA up to 70 μg. The optical density at 530 mμ. reached a maximum in about 45 min. and little difference was found in the calibration curve whether the time allowed for colour development was 1 or 4 hr. These results agree with the results of Gordon & Weber (1951).

IAc. 3-Indolylacetaldehyde was oxidized with silver oxide to IAA, which was then estimated spectrophotometrically with Salkowski reagent. Oxidation with silver oxide forms the basis of several methods of estimating aldehydes but some difficulty was experienced in adapting the existing oxidation procedures. Separately prepared silver oxide, even when freshly precipitated, gave erratic results and the method finally evolved was derived from those of Pondorff (1931) and Siegel & Weiss (1954) in which the silver oxide was precipitated in the reaction mixtures. Samples of the reaction mixtures, not more than 0·5 ml. and preferably containing 100–300 μg. of IAc, were diluted with water to 1 ml.; 1 ml. of 0·1 M-$AgNO_3$ was then added, followed by 1 ml. of 0·1 M-NaOH and, lastly, 1 ml. of 0·2 M phosphate, pH 7. When a series of estimations were being done simultaneously the phosphate was added immediately after the NaOH. The solutions were thoroughly mixed after each addition and finally shaken mechanically for 5 min. in darkness. They were then filtered through Whatman no. 44 filter papers and 1 ml. samples of the filtrates were used for

the estimation of IAA. Control reaction mixtures without added tryptamine were treated with silver oxide in the same way and the filtrates with added Salkowski reagent were used in the blank tube in the IAA estimations.

Manganese. After wet digestion of enzyme samples the manganese present was oxidized with potassium periodate to permanganate, which was estimated colorimetrically.

Absorption curves. These were determined in a Unicam model SP. 500 spectrophotometer with 1 cm. cells.

Paper chromatography. A descending one-dimensional technique was used with Whatman no. 4 paper. The solvent was a mixture, *iso*propanol–NH_3 soln. (sp.gr. 0·880)–water (10:1:1, by vol.), as used by Bennet-Clark, Tambiah & Kefford (1952) and Stowe & Thimann (1954). The papers were dried at room temperature and sprayed with Ehrlich reagent (2 g. of *p*-dimethylaminobenzaldehyde in a mixture of 80 ml. of ethanol and 20 ml. of conc. HCl).

Manometric methods. Measurements of O_2 uptake were made, in air, in the Warburg apparatus at 28°. The volume of the reaction mixtures was usually 3 ml., and 0·2 ml. of 5N-KOH was present in the centre well unless otherwise stated. In a few experiments 9 ml. reaction mixtures were used in large Warburg vessels of total volume about 60 ml. Formation of CO_2 was measured by Warburg's direct method (Dixon, 1943).

Melting points. These are uncorrected.

RESULTS

Manometric studies of the oxidation

Effect of pH. The variation of activity with pH was tested in reaction mixtures containing amine oxidase, catalase and tryptamine (0·0067M) in phosphate–borate buffers, pH 6–10. Mann & Smithies (1955) showed that, at least over the pH range 7–8, borate does not inhibit plant amine oxidase. The pH–activity curve (Fig. 1) was obtained by plotting the O_2 uptakes in the first 10 min. against the final pH. The curve shows an optimum at about pH 8·1, but very little change in activity was found over the range of pH 7·9–8·4. The activity at pH 7 was about half that at the optimum.

Effect of substrate concentration. The effect of varied substrate concentration on the rate of oxidation was tested in the presence of catalase, in phosphate buffers at pH 7 and 8. At pH 8 the maximum initial rate of O_2 uptake was reached at about 0·004M tryptamine. Thereafter further increases in substrate concentration up to 0·02M produced little or no change in the rate. At pH 7 the initial rate of oxidation increased rapidly as the substrate concentration was raised to 0·004M, but thereafter the rate continued to increase, though relatively slowly, as the substrate concentration was raised to 0·02M.

Whereas at pH 8 the initial rate of oxidation is independent of change in substrate concentration from 0·004 to 0·02M, inhibition or inactivation of the enzyme is more rapid at the higher concentrations and, in consequence, the total O_2 uptake may decrease with increasing substrate. Thus the results of Fig. 2 show progressively decreasing total O_2 uptakes by reaction mixtures at pH 8 as the initial tryptamine concentration was raised from 0·0033 to 0·02M. Similar results were obtained at pH 7, though here the differences in the total O_2 uptakes

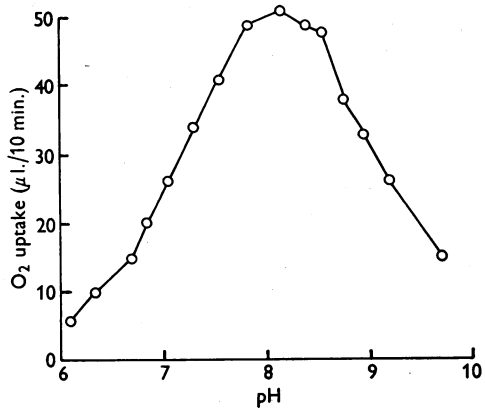

Fig. 1. Effect of pH on the rate of oxidation of tryptamine. Amine oxidase (0·1 ml.) and catalase in phosphate–borate buffers. The final concentration both of phosphate and borate was 0·033M. The substrate, 0·2 ml. of 0·1M tryptamine, was added from the side arms; KOH was omitted from the centre wells. The total volume of the reaction mixtures was 3 ml. Gas phase, air; temp., 28°. Control reaction mixtures without tryptamine showed no oxygen uptake in 10 min.

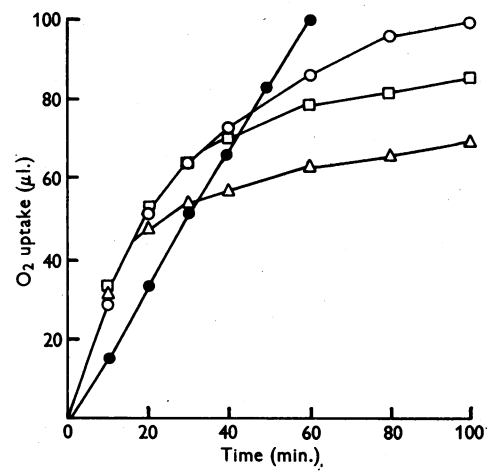

Fig. 2. Enzyme inactivation or inhibition produced by high tryptamine concentrations. Amine oxidase (0·08 ml.) and catalase in 0·033M phosphate buffer, pH 8, with varying concentrations of tryptamine; KOH was present in the centre wells. Other conditions were similar to those in Fig. 1. ○, 0·0033M Tryptamine; □, 0·0067M tryptamine; △, 0·02M tryptamine; ●, 0·02M 2-phenylethylamine (0·02 ml. of amine oxidase).

were smaller. This was due, at least in part, to the fact that the initial reaction rates at this pH were greater at the higher substrate concentrations.

Werle & Pechmann (1949) showed that plant amine oxidase is inactivated during the course of the reactions it catalyses, particularly at high substrate concentrations, and attributed the inactivation to the hydrogen peroxide formed as a reaction product. Mann (1955) showed that the enzyme is readily inactivated by hydrogen peroxide only in presence of its substrates, and that with 1:4-diaminobutane or 1:5-diaminopentane (0·01 M) as substrate the inactivation is prevented by catalase. The amounts of catalase used in the present work were sufficient to prevent inactivation of the enzyme when 2-phenylethylamine (0·02 M) was used as substrate (Fig. 2). The decreasing O_2 uptakes with increasing tryptamine concentrations were unaffected by a tenfold increase in catalase. These results show that the decreasing O_2 uptake is dependent on the fact that tryptamine was used as substrate. In experiments with animal diamine oxidase Zeller, Schär & Staehlin (1939) and Zeller (1941) showed that substrates such as histamine and agmatine, which contain two basic groups with very different affinities for the enzyme, act as inhibitors at high concentrations. Such inhibition, like that observed by Mann & Smithies (1955) using plant amine oxidase with 2-(2-aminophenyl)ethylamine as substrate, and unlike that with tryptamine, is apparent from the start of the reaction. Further work is necessary to establish whether inactivation by hydrogen peroxide or by excess of substrate is the cause of the decreasing O_2 uptakes. A third possibility is that combination takes place between tryptamine and its oxidation product to form an inhibiting compound; this would be favoured by high tryptamine concentrations.

Oxidation of 5-hydroxytryptamine. The oxidation of 5-hydroxytryptamine is also catalysed by the enzyme. In phosphate buffer, pH 7, the initial rate of O_2 uptake with 5-hydroxytryptamine creatinine sulphate (10 μmoles) as substrate was about one-half of that with the same concentration of tryptamine. No O_2 uptake was observed in control experiments in which creatinine sulphate was tested as substrate.

Total oxygen uptake. The reactions catalysed by amine oxidases have generally been represented by the equation

$$R.CH_2.NH_2 + H_2O + O_2 = R.CHO + H_2O_2 + NH_3.$$

In presence of catalase the net reaction is expressed

$$2R.CH_2.NH_2 + O_2 = 2R.CHO + 2NH_3.$$

If the reaction studied consists only of the oxidation of tryptamine to IAc, the total O_2 uptake in the presence of catalase should be 0·5 mole of O_2/mole of tryptamine. The total O_2 uptake was measured, in the presence of catalase, in phosphate buffers, pH 7 and 8. Since the amine oxidase is inhibited or inactivated during the reaction sufficient enzyme was used to give a rapid initial rate of O_2 uptake, and low concentrations of tryptamine were used. The results obtained at pH 7 are shown in Fig. 3; those at pH 8 were similar. With the purified enzyme as catalyst the O_2 uptake rapidly reached a value slightly greater than 0·5 mole/mole of tryptamine, but thereafter further uptake took place though this was relatively slow. The total O_2 uptake reached in the experimental time was about 0·6 mole. No CO_2 was formed in the reaction. The results suggest that the oxidation of tryptamine to IAc is the main reaction with the purified enzyme as catalyst. The excess of O_2 uptake over the theoretical with the purified enzyme was not inhibited by pyrophosphate and was not therefore the same as that observed by Mann (1955), using lysine as substrate.

When the dry preparations of amine oxidase were used to catalyse the reaction the O_2 uptake reached

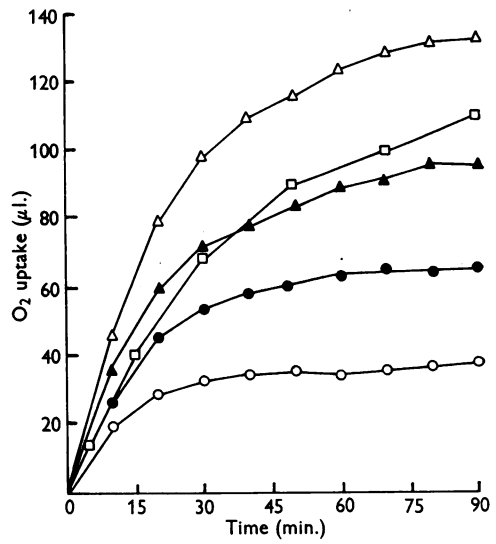

Fig. 3. Course of the oxygen uptake during the catalysis of the oxidation of tryptamine by the purified and dry preparations of amine oxidase. Amine oxidase (0·2 ml. of the purified preparation or 100 mg. of the dry preparation) and catalase in 0·033 M phosphate buffer, pH 7; KOH was present in the centre wells. Other conditions were similar to those in Fig. 2. The oxygen uptakes of control reaction mixtures without tryptamine, 0–5 μl. with the purified amine oxidase and 52 μl. with the dry preparation, were subtracted. ○, 2·5 μmoles of tryptamine; ●, 5 μmoles of tryptamine; ▲, 7·5 μmoles of tryptamine; △, 10 μmoles of tryptamine with the purified amine oxidase; □, 5 μmoles of tryptamine with the dry preparation of amine oxidase. Oxygen uptakes of 0·5 mole equivalents would be 28, 56, 84 and 112 μl. with 2·5, 5, 7·5 and 10 μmoles of tryptamine.

1 mole/mole of tryptamine (Fig. 3) and exceeded this value when the experimental time was increased. This higher O_2 uptake with the dry preparation may have been due to the oxidation of IAc, possibly to IAA. When the purified enzyme was used to catalyse the oxidation of tryptamine the final reaction mixtures gave a golden-yellow colour with Salkowski reagent, but when the dry preparation was used the reaction mixtures, like IAA, gave a red colour with the reagent. The yield of IAA, assuming the optical density at 530 mμ. to be entirely due to this compound, was very small. Thus in the experiment of Fig. 3 where 5 μmoles of tryptamine were oxidized with the dry preparation the IAA estimated to be present was less than 0·04 μmole.

Oxidation of phenylacetaldehyde and IAA by the dry preparation. No significant increase in O_2 uptake was found when acetaldehyde, benzaldehyde, salicylaldehyde and hypoxanthine were tested as substrates for the dry preparation. The dry preparation, but not the purified preparation, catalysed the oxidation of phenylacetaldehyde and IAA (Table 1). Kenten (1953) has shown that the oxidation of phenylacetaldehyde is catalysed by many plant saps and that in pea-seedling sap the oxidizing system consists of a peroxidase together with a thermostable factor which can be partially replaced by Mn^{2+} ions. The oxidation of IAA, catalysed by horse-radish peroxidase, is also stimulated by Mn^{2+} ions (Kenten, 1955*a*). The dry preparation of amine oxidase had P.Z. 1 and contained 12 μg. of manganese/100 mg. The peroxidase activity of the amounts of purified amine oxidase used (0·2 ml.) was only about 1–2 % of that of 100 mg. of the dry preparation. Amounts of peroxidase and Mn^{2+} of the same order as those present in 100 mg. of the dry preparation catalysed the oxidation of phenylacetaldehyde and IAA (Table 1).

Oxidation of the product of the amine oxidase reaction by the dry preparation and by peroxidase. When the oxidation of tryptamine, catalysed by the purified amine oxidase, was allowed to proceed until the O_2 uptake reached 0·5 mole equivalent little increase was produced in the subsequent slow rate of O_2 uptake by adding more of the purified amine oxidase. If the dry preparation was added at this point, instead of the purified preparation, the rate of oxidation was markedly increased and the total O_2 uptake approached 1 mole equivalent (Fig. 4). This further oxidation was also catalysed by peroxidase, particularly in presence of Mn^{2+} ions. This was shown in similar experiments in which, after the initial oxidation was complete, peroxidase, Mn^{2+} ions or peroxidase together with Mn^{2+} ions were added from the second side arm (Fig. 5). Peroxidase caused an increased rate of O_2 uptake which differed little whether 0·1 or 0·3 mg. of peroxidase was used. The Mn^{2+} ions (11 μg.) alone had no effect on O_2 uptake but added together with peroxidase produced a larger effect than that given by peroxidase alone. Heat-treated peroxidase (30 min. at 100°), with or without Mn^{2+} ions, only slightly increased the rate of O_2 uptake. The results suggest that the greater O_2 uptake observed when the dry preparation of amine oxidase was used instead of the purified preparation, to catalyse the oxidation of tryptamine, was due at least in part to the oxidation of the product of the amine oxidase reaction by a peroxidase system. Results reported below show that the product of the amine oxidase reaction is IAc. Tests with Salkowski reagent showed that, in the experiments of Fig. 5, only traces of IAA accumulated in the reaction mixtures whether peroxidase or peroxidase together with Mn^{2+} ions was used to catalyse the secondary oxidation.

Table 1. *Oxidation of phenylacetaldehyde and IAA by the dry preparation of amine oxidase and by peroxidase* ± Mn^{2+} *ions*

Purified or dry preparations of amine oxidase, or peroxidase, with and without $MnSO_4$, were tested as catalysts for the oxidation of phenylacetaldehyde or IAA in reaction mixtures containing catalase in 0·033 M phosphate buffer, pH 7. The substrates, 10 μmoles of phenylacetaldehyde or IAA, were added from the side arms. In the experiments with phenylacetaldehyde KOH was omitted from the centre wells and measurements of the O_2 uptake were not started until 5 min. after the addition of the substrate, which was dissolved in ethanol (0·1 ml.). The O_2 uptakes of control reaction mixtures of the dry preparation of amine oxidase without substrate were subtracted. These were 10 μl. (KOH absent) and 28 μl. (KOH present). Other conditions were similar to those in Fig. 2.

Catalyst	Substrate	O_2 uptake (μl./1 hr.)
Amine oxidase (100 mg. of dry preparation)	Phenylacetaldehyde	57
Amine oxidase (100 mg. of dry preparation)	IAA	53
Purified amine oxidase (0·2 ml.)	Phenylacetaldehyde	0
Purified amine oxidase (0·2 ml.)	IAA	5
Peroxidase (0·1 mg.)	Phenylacetaldehyde	22
Mn^{2+} ions (11 μg.)	Phenylacetaldehyde	8
Peroxidase (0·1 mg.) + Mn^{2+} ions (11 μg.)	Phenylacetaldehyde	44
Peroxidase (0·1 mg.)	IAA	48
Mn^{2+} ions (11 μg.)	IAA	0
Peroxidase (0·1 mg.) + Mn^{2+} ions (11 μg.)	IAA	63

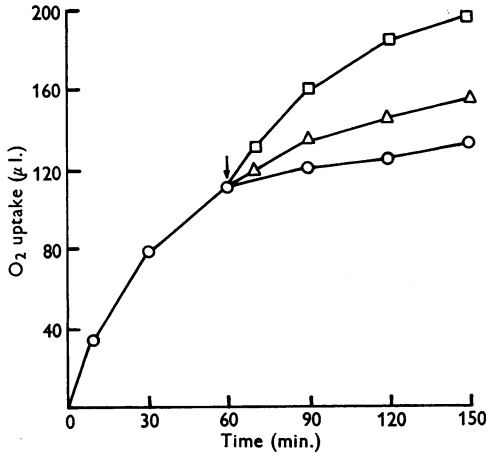

Fig. 4. Oxidation of the product of the amine oxidase-catalysed reaction by the dry crude preparation of amine oxidase. Amine oxidase (0·2 ml. of the purified preparation) and catalase in 0·033 M phosphate buffer, pH 7, in reaction vessels with two side arms. Tryptamine (10 μ-moles) was added from the side arm. Other conditions were similar to those in Fig. 1. ○, No further addition; △, an additional 0·2 ml. of purified amine oxidase was added from the second side arm at ↓; □, 60 mg. of the dry preparation of amine oxidase suspended in 0·3 ml. of water was added at ↓. The oxygen uptakes of control reaction mixtures without added substrate were subtracted. These were 4 μl. with 0·2 ml. of amine oxidase, increasing to 6 μl. where a further 0·2 ml. was added and to 46 μl. where the second addition was that of the dry preparation.

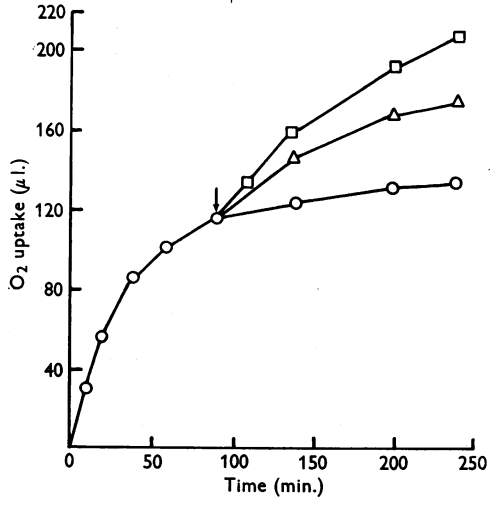

Fig. 5. Oxidation of the product of the amine oxidase-catalysed reaction by peroxidase ± Mn^{2+} ions. Conditions were as in Fig. 4 but the additions from the second side arms at ↓ were: ○, no addition or 0·2 ml. of mM-$MnSO_4$ (11 μg. of Mn^{2+} ions); △, 0·1 mg. of peroxidase; □, 0·1 mg. of peroxidase + 11 μg. of Mn^{2+} ions.

Oxidation of tryptamine by peroxidase systems. Another secondary reaction which may occur when the dry preparation is used as catalyst is the oxidation of tryptamine by the hydrogen peroxide formed in the primary reaction. Colorimetric evidence suggesting that peroxidase catalyses this oxidation was obtained in test-tube experiments at room temperature. Reaction mixtures of a total volume of 3 ml. contained 1 mg. of peroxidase and 20 μmoles of tryptamine in 0·067 M phosphate buffer, pH 7. Additions of 0·05 ml. of 0·02 M hydrogen peroxide were made at intervals of 10 min. over a period of 200 min. The reaction mixtures turned yellow after the first addition of hydrogen peroxide. This colour slowly deepened with subsequent additions of hydrogen peroxide and after 200 min. an orange-brown solution with slight turbidity resulted. After standing at room temperature for a further period of several hours a brown precipitate was deposited. No colour or precipitate formation was observed in reaction mixtures from which peroxidase was omitted or in which heat-treated peroxidase (30 min. at 100°) was used. As in the peroxidase-catalysed oxidation of tryptophan (Wiltshire, 1953) the reaction apparently depends on the maintenance of a low concentration of hydrogen peroxide. When the hydrogen peroxide (1 ml. of 0·02 M) was all added at the start of the reaction the subsequent visual signs of tryptamine oxidation were comparatively slight.

The oxidation of tryptamine by hydrogen peroxide in the presence of peroxidase was also demonstrated in manometric experiments in which the notatin-catalysed oxidation of glucose was used as the source of the hydrogen peroxide. The basal reaction mixture consisted of notatin, catalase and glucose (10 μmoles) in phosphate buffer, pH 7. Tryptamine (10 μmoles) or peroxidase (0·1 or 1 mg.) added separately to such reaction mixtures had no effect on the O_2 uptake, but where both were added together the O_2 uptake was increased and was doubled with the larger amount of peroxidase (Table 2). These increases in O_2 uptake were accompanied by the formation of an orange-brown colour in the reaction mixtures where 0·1 mg. of peroxidase was used and a brown precipitate with 1 mg. of peroxidase. Heat treatment of the peroxidase (30 min. at 100°) decreased, but did not entirely eliminate, the peroxidase effect. The effect of peroxidase (0·1 mg.) was not increased by the addition of Mn^{2+} ions (1·1 or 11 μg.).

Identification of IAc as the oxidation product of tryptamine

Spectrophotometric evidence. When the oxidation of tryptamine, catalysed by amine oxidase in the presence of catalase, was allowed to proceed until the O_2 uptake reached 0·5 mole equivalent, samples of the reaction mixture gave a golden-yellow

Table 2. *Oxidation of tryptamine by peroxidase systems*

The basal reaction mixture consisted of tryptamine (10 μmoles), notatin (0·15 mg.) and catalase in 3 ml. of 0·033 M phosphate buffer, pH 7. Glucose (10 μmoles) was added from the side arms. Other reactions contained, in addition, peroxidase, $MnSO_4$ or heat-treated peroxidase (30 min. at 100°); KOH was present in the centre wells. Other conditions were as in Fig. 2.

Catalyst	O_2 uptake (μl./4 hr.)
Notatin	108
Notatin + 11 μg. of Mn^{2+} ions	106
Notatin + 0·2 mg. of peroxidase	168
Notatin + 0·2 mg. of peroxidase + 1·1 μg. of Mn^{2+} ions	165
Notatin + 0·2 mg. of peroxidase + 11 μg. of Mn^{2+} ions	158
Notatin + 1 mg. of peroxidase	228
Notatin + 0·2 mg. of peroxidase (heat-treated)	115
Notatin + 1 mg. of peroxidase (heat-treated)	134

Table 3. *Spectrophotometric evidence of the formation of IAc as the product of the oxidation of tryptamine catalysed by amine oxidase*

Reaction mixtures consisted of amine oxidase (0·2 ml.), catalase and tryptamine (10 μmoles) in 3 ml. of 0·033 M phosphate buffer, pH 7. Other conditions were as in Fig. 2. The reaction mixtures were incubated for 1 hr., at which time the O_2 uptake had reached 0·5 mole equivalent. Control reaction mixtures, without tryptamine, were incubated for the same time. After addition of Salkowski reagent to samples of the reaction mixture, before and after various treatments, the optical density at 530 mμ. was measured. In each case 0·25 ml. of the reaction mixture, or equivalent amounts of the treated reaction mixtures, was diluted to 10 ml. with water and 15 ml. of Salkowski reagent was added. The blank cells contained Salkowski reagent and control reaction mixtures without tryptamine treated in the same way as the experimental mixture.

Sample	Treatment	Colour with Salkowski reagent	Optical density at 530 mμ.
Reaction mixture (0·25 ml.)	None	Golden-yellow	0·053
Reaction mixture (0·25 ml.)	0·125 ml. of a sat. soln. of 2:4-dinitrophenylhydrazine in 2 N-HCl added. Bulky orange-brown ppt. removed by centrifuging	Yellow*	0·006
Reaction mixture (0·25 ml.)	0·125 ml. of 2 N-HCl added. Slight ppt. removed by centrifuging	Golden-yellow	0·047
Reaction mixture (0·25 ml.)	Oxidized with silver oxide	Red	0·258
Reaction mixture (0·25 ml.)	Oxidized with silver oxide, then treated with 2:4-dinitrophenyhydrazine reagent. No ppt. formed	Red	0·260
Tryptamine (0·83 μmole)	None	None	0·007
Tryptamine (0·83 μmole)	Oxidized with silver oxide	None	0·008
IAA (0·83 μmole)	None	Red	0·362

* This colour was due, at least in part, to the presence of 2:4-dinitrophenylhydrazine.

solution on treatment with Salkowski reagent. The optical density of these coloured solutions at 530 mμ., where IAA treated with Salkowski reagent shows an absorption maximum, was low in relation to that required for quantitative conversion of the tryptamine into IAA (Table 3). When the reaction mixture was treated with 2:4-dinitrophenylhydrazine in 2 N-HCl a bulky orange-brown precipitate was formed. If this precipitate was removed at once by centrifuging, the supernatant treated with Salkowski reagent showed no absorption at 530 mμ. Treatment of the reaction mixture in the same way with 2 N-HCl alone produced little or no precipitate and the optical density at 530 mμ. remained almost unchanged. These results suggested that the observed absorption was due to the presence of a carbonyl compound. When the reaction mixture was oxidized with silver oxide, under the conditions described for estimation of IAc, the resultant solution, like IAA, gave a red colour with Salkowski reagent; the optical density at 530 mμ. suggested that about 70% of the tryptamine was recovered as IAA. Tryptamine gave little or no colour with Salkowski reagent before or after silver oxide treatment. The reaction mixture oxidized with silver oxide gave no precipitate with 2:4-dinitrophenylhydrazine, nor was the optical density at 530 mμ. affected by this reagent. The results suggested that IAA was formed by the oxidation of the carbonyl compound in the reaction

mixture. This is supported by the absorption curve of the silver oxide-treated mixture after the addition of the Salkowski reagent and 2:4-dinitrophenylhydrazine. Fig. 6 shows that the absorption curve was almost identical with that of IAA. A red colour and a very similar absorption curve were obtained with the reaction mixture oxidized by silver oxide with the more specific Salkowski reagent described by Gordon & Weber (1951), in which perchloric acid is substituted for sulphuric acid. Stowe & Thimann (1954) have shown that the coloured products this modified reagent forms with compounds related to IAA have different absorption maxima. Since pretreatment with 2:4-dinitrophenylhydrazine abolishes the observed absorption of the reaction mixture at 530 mμ. but does not affect it after oxidation with silver oxide the absorption after the latter treatment may be taken as a measure of the IAA present, and from this the amount of IAc in the original reaction mixture can be calculated.

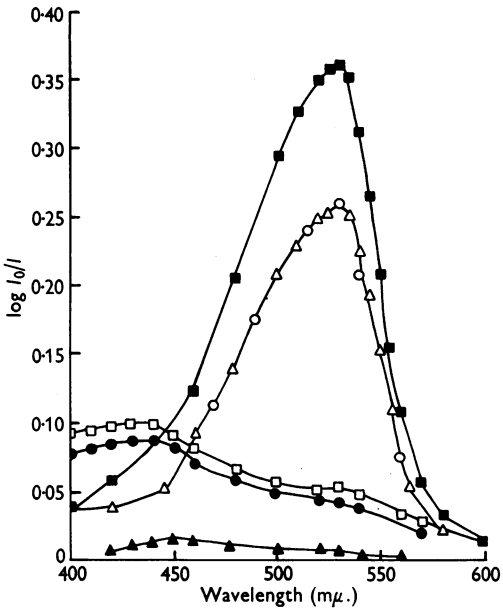

Fig. 6. Absorption spectra of the solutions obtained by mixing samples of the reaction mixture, before and after various treatments, with Salkowski reagent. Reaction mixtures containing 0·2 ml. of amine oxidase, catalase and tryptamine (10 μmoles) were incubated in 0·067 M pyrophosphate buffer, pH 7, until the oxygen uptake reached 0·5 mole equivalent. Control reaction mixtures without tryptamine were incubated for the same time. Other conditions were as in Fig. 2. In the Salkowski reaction 0·25 ml. of the untreated reaction mixture, or an equivalent amount of the treated reaction mixtures, was used. The blank cell contained appropriate controls made by mixing Salkowski reagent with samples of the control reaction mixture similarly treated. □, Untreated reaction mixture; ▲, supernatant after removal of 2:4-dinitrophenylhydrazone; ●, supernatant after treatment with 2 N-HCl; △, reaction mixture treated with silver oxide; ○, supernatant after oxidation with silver oxide and treatment with 2:4-dinitrophenylhydrazine; ■, 0·83 μ-mole of IAA.

Paper chromatography. Reaction mixtures, of a total volume of 3 ml., contained 0·3 ml. of amine oxidase and catalase in 0·002 M phosphate buffer, pH 7; KOH was omitted from the centre wells. Tryptamine (10 μmoles) was added from the side arms. The reaction mixtures were incubated until O_2 uptake was about 0·5 mole equivalent. One of the reaction mixtures was then shaken in the bath for a further 10 min. after the addition of 0·5 ml. of a suspension of silver oxide. The silver oxide was prepared immediately before use by mixing 10 ml. of 0·1 M-$AgNO_3$ with 10 ml. of 0·1 M-NaOH. The precipitated silver oxide was collected and washed by centrifuging and suspended in 2 ml. of water. Finally both reaction mixtures were filtered and the filtrates were concentrated in a vacuum desiccator until the volume of each was about 0·3 ml. Samples (0·005 ml.) were then chromatographed with the *iso*propanol solvent, with Ehrlich reagent as detecting agent. Tryptamine and IAA were used as reference compounds. The reaction mixture not treated with silver oxide gave a strong yellow-brown streak (R_F 0·90) and a weak purple spot identical with that given by tryptamine (R_F 0·73). Neither of these spots was given by control reaction mixtures without tryptamine nor by the reaction mixtures treated with silver oxide, but a new purple spot appeared with the latter identical with that given by IAA (R_F 0·44). Control reaction mixtures of tryptamine and heat-treated enzyme (10 min. at 100°) both before and after treatment with silver oxide showed only the one spot corresponding to tryptamine. The results suggest that the yellow-brown streak (R_F 0·90) was due to IAc, or to an artifact formed during the chromatography, and that the IAc was oxidized by the silver oxide to IAA. Weller, Wittwer & Sell (1954) record that IAc gives a yellow-brown colour with Ehrlich reagent on paper chromatograms.

Conditions affecting the yield of IAc. The effects of pH, reaction time and tryptamine concentration on the yield of IAc were investigated. The reaction mixture, in phosphate buffers at pH 7 and 8, were incubated until the O_2 uptakes reached 0·5 mole equivalent and the ammonia and IAc present were then estimated. The results (Table 4) show that the yields of IAc were of the order of 90 % of theoretical with the lowest concentration of tryptamine used but decreased progressively as the tryptamine concentration was increased. The decrease was due, at least in part, to the longer reaction times. Thus in the experiments with 30 μmoles of tryptamine the

Table 4. *Effect of tryptamine concentration,* pH *and oxidation time on the yield of IAc*

Reaction mixtures of 9 ml. total volume in 60 ml. Warburg vessels consisted of amine oxidase and catalase in 0·033 M phosphate buffers, pH 7 and 8. The substrate was added from the side arms; KOH was omitted from the centre wells. When the O_2 uptakes reached 0·5 mole equivalent the ammonia and IAc contents of the reaction mixtures were estimated. Values obtained with control reaction mixtures without tryptamine were subtracted.

Tryptamine (μmoles)	pH	Amine oxidase (ml.)	Time of incubation (min.)	Total O_2 uptake (μmoles)	Ammonia (μmoles)	IAc (μmoles)
15	7	0·6	15	7·5	15·3	13·0
15	8	0·3	10	7·8	15·2	13·9
30	7	0·6	25	14·8	27·3	24·3
30	8	0·3	15	15·0	27·6	23·2
30	7	0·3	70	15·2	25·2	19·8
30	8	0·15	60	15·2	27·3	17·7
60	7	0·6	80	30·6	48·0	40·7
60	8	0·3	140	29·4	45·6	29·1

yields of IAc fell when the reaction times were increased by using less of the enzyme preparation. The decrease in yield at the higher tryptamine concentrations was accompanied by the formation of an insoluble reaction product. The amount of this was greater at pH 8 than at pH 7 and was probably due, in part, to polymerization of IAc. Kenten (1953) found that aqueous solutions of phenylacetaldehyde rapidly became turbid in alkaline solution, presumably owing to polymerization. But the fact that, in the tryptamine reaction, the yield of ammonia also decreased with increase in tryptamine concentration, shows that reactions other than polymerization were involved. Some pigment formation occurred during the reaction, ranging from a slight yellow at low tryptamine concentrations to yellow-brown at high concentrations. It was more marked at pH 7 than at pH 8, and was apparently due to a highly dispersed insoluble product, since the supernatant solution obtained after centrifuging was almost colourless.

Isolation of the 2:4-*dinitrophenylhydrazone of IAc.* Reaction mixtures in 250 ml. Erlenmeyer flasks each consisted of 1·6 ml. of amine oxidase, 0·6 mg. of catalase, 1·2 ml. of 0·1 M tryptamine and 6 ml. of 0·2 M phosphate buffer, pH 7, in a total volume of 36 ml. Five such reaction mixtures were used. The flasks were shaken in the bath at 28° for 75 min. A previous control experiment had shown that the O_2 uptake reached 0·5 mole equivalent under these conditions. The combined reaction mixtures and washings were brought to pH 4–5 with conc. HCl and centrifuged. The supernatant was added to 50 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2 N-HCl. After the addition of 50 ml. of 2 N-HCl the mixture was stored at 4° overnight. The orange-yellow precipitate (165 mg., 82% of theoretical yield), m.p. 184–188°, which formed, was separated by filtration and dried *in vacuo*. Part of the product (49 mg.) was gently warmed with benzene (5 ml.), filtered free from insoluble material (7 mg.) and the solution chromatographed on a kieselguhr–bentonite (5 g.:5 g.) column (Linstead, Elvidge & Whalley, 1955) by elution with benzene. The eluate was collected in 8 ml. portions. Evaporation of the combined fractions 3–6 and crystallization from a supersaturated benzene solution gave small orange-red prisms, m.p. 196–198°. (Found: C, 57·0; H, 3·9; N, 20·6. Calc. for $C_{16}H_{13}O_4N_5$: C, 56·6; H, 3·85; N, 20·65%.) Light-absorption max., 2690 and 3590 Å (ε 14 700 and 22 000 respectively). Brown, Henbest & Jones (1952) give m.p. 196–202°, light-absorption max., 2680 and 3580 Å (ε 16 800 and 23 600 respectively).

Isolation of the dimedone derivative of IAc. A total of 6 ml. of 0·1 M tryptamine was oxidized in reaction mixtures similar to those used for the preparation of the 2:4-dinitrophenylhydrazone. The combined reaction mixtures were brought to pH 5 with conc. HCl and extracted three times with ether, a total of 300 ml. of ether being used. The combined ether extracts were dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure left a viscous oil which was immediately dissolved in 10 ml. of aqueous methanol (1:1) and treated with a solution of dimedone (300 mg.) in the same solvent. The mixture after storage at 4° overnight had deposited 72 mg. of crystals. After storing the filtrate for 24 hr. at 4° a further 18 mg. was obtained. The total yield (90 mg.) was 36% of theoretical. Recrystallization from aqueous methanol (1:1) gave colourless crystals, m.p. 150–151°. Admixture with dimedone gave m.p. 122–127°. (Found: C, 73·7; H, 7·6. Calc. for $C_{26}H_{31}O_4N$: C, 74·1; H, 7·45%.) Brown *et al.* (1952) give m.p. 148–152°.

DISCUSSION

The results of the present work establish the fact that IAc is the product of the oxidation of tryptamine catalysed by plant amine oxidase. This is of interest in connexion with the metabolism of indolylalkylamines in both animals and plants.

In experiments with intact animals and perfused organs it has been shown that tryptamine is oxidized to IAA (Ewins & Laidlaw, 1913; Guggenheim & Loeffler, 1916) and that 5-hydroxytryptamine is oxidized to 5-hydroxy-IAA (Titus & Udenfriend, 1954; Erspamer, 1954, 1955). It is known that the oxidation of tryptamine (Blaschko, Richter & Schlossmann, 1937; Pugh & Quastel, 1937) and of 5-hydroxytryptamine (Blaschko & Philpot, 1953; Govier, Howes & Gibbons, 1953) is catalysed by the monoamine oxidase of animal tissues. This reaction is considered to be the first stage in the oxidation of these amines in animals, but no conclusive proof appears to have been obtained hitherto that, with these two amines as substrates, the corresponding aldehydes are the reaction products. The importance of these observations in animal metabolism depends on the pharmacological properties of certain naturally occurring indolylalkylamines, notably 5-hydroxytryptamine and its *N*-methyl derivatives. 5-Hydroxytryptamine occurs in animal tissues, and its oxidation product 5-hydroxy-IAA is a normal constituent of the urine (Erspamer, 1955; Udenfriend, Titus & Weissbach, 1955). 5-Hydroxytryptamine is formed from tryptophan by way of 5-hydroxytryptophan (Udenfriend, Titus, Weissbach & Peterson, 1956), which is then decarboxylated by the specific 5-hydroxytryptophan decarboxylase described by Udenfriend, Clark & Titus (1953) and Clark, Weissbach & Udenfriend (1954). The presence of IAA in urine was established by Herter (1908). Both IAA and indoleaceturic acid have since been found repeatedly as normal constituents of urine. But it is not yet clear whether such IAA is, in part, a product of metabolism of animal tissues or whether it is derived entirely from plant material in the diet and bacterial action on tryptophan in the intestine.

The literature on the mechanism of formation of IAA in plant tissues has been reviewed recently by Larsen (1951*a*) and Gordon (1954, 1956). Insufficient evidence is at present available to permit an assessment of the importance of the reaction studied in the present work in this connexion. The presence of tryptamine in certain *Acacia* species was established by White (1944) but there is no evidence, as yet, that it is widely distributed in plants. Isolated instances of the occurrence in plants of 5-hydroxytryptamine (Bowden, Brown & Batty, 1954), *NN*-dimethyltryptamine (Fish, Johnson & Horning, 1955) and 5-hydroxy-*NN*-dimethyltryptamine (Stromberg, 1954; Fish *et al.* 1955) have been reported. These findings suggest that the corresponding substituted indoleacetic acids may also be present in plants. Some plants are apparently unable to utilize tryptamine for the formation of IAA. Gordon (1956) reports that inhibitors of amine oxidase did not affect the formation of IAA from tryptophan by a plant-tissue preparation and suggests that tryptamine is not a normal intermediate in auxin formation. Evidence that IAc occurs in some higher plants and can act as a precursor of IAA has been obtained by Larsen (1944, 1949, 1951*b*), Gordon & Sanchez-Nieva (1949*a*, *b*) and by Yamaki & Nakamura (1952).

The extent to which amine oxidase-catalysed oxidation of tryptamine occurs in plants may be restricted not only by the distribution of tryptamine but by that of the amine oxidases. Plant amine oxidase is known to be present in many leguminous plants. In the most complete survey of its distribution so far undertaken, based on its histaminase activity, Werle & Zabel (1948) obtained evidence of its presence in many other dicotyledonous plants but it was not found in the monocotyledons and gymnosperms examined. Little is known of the distribution of the plant monoamine oxidase which was shown to catalyse the oxidation of tryptamine by Werle & Roewer (1950).

It is now clear that the failure to identify IAc as the product of the oxidation of tryptamine in previous experiments with crude preparations of plant amine oxidase was due to the secondary reactions catalysed by such preparations. The results suggest that two of these reactions are peroxidase-catalysed oxidations of tryptamine and of IAc. Such oxidation of tryptamine was found to depend on an external source of hydrogen peroxide and was unaffected by Mn^{2+} ions at the concentrations used. The oxidation of IAc was independent of an external source of hydrogen peroxide and the rate of oxidation was increased by Mn^{2+} ions. Kenten (1953) identified benzaldehyde as the main product of the oxidation of phenylacetaldehyde by peroxidase systems and suggested that if IAc was oxidized by such systems 3-indolealdehyde would be a possible reaction product. In the present work tests with Salkowski reagent suggest that only traces of IAA accumulate during the reaction though this does not exclude the possibility that IAA is an intermediate since peroxidase systems also catalyse the oxidation of IAA (e.g. Galston, Bonner & Baker, 1953; Kenten, 1955*a*).

It is already known that the oxidation of indole (Mann & Smithies, 1955) and that of a number of its compounds is catalysed by peroxidase systems. Besides IAA these compounds include tryptophan (Wiltshire, 1953), β-(3-indolyl)propionic acid and γ-(3-indolyl)-*n*-butyric acid (Kenten, 1955*b*). An oxidation of tryptamine to give 5-hydroxytryptamine, a reaction already discussed by Erspamer & Vialli (1952), may also occur. In the present work preliminary colorimetric tests for 5-hydroxytryptamine as a product of the oxidation of tryptamine by peroxidase systems with 1-nitroso-2-naphthol (Udenfriend, Weissbach & Clark, 1955) have so far

given negative results. The pigment formation occurring during the oxidation suggests that the final products are complex. Pigment formation was also observed during the oxidation of tryptamine (Pugh & Quastel, 1937) and of 5-hydroxytryptamine (Blaschko, 1952) catalysed by preparations of the monoamine oxidase of animal tissues. This pigment formation has been further studied by Blaschko & Hellmann (1953), who attribute it to the products of the oxidation of the aldehydes formed in the oxidative deamination reactions.

Lastly it should be pointed out that the amine oxidase-catalysed oxidation of tryptamine provides a simple means of obtaining IAc. The possibility that this might prove to be the case was suggested by Gordon (1954). The work should therefore facilitate investigation designed to assess the importance of IAc as a precursor of IAA in plants. Much of the previous work on this subject has been done with preparations of IAc made by treating tryptophan with ninhydrin or isatin and containing only 2% of IAc, or by the use of plant extracts containing IAc. While a chemical method of synthesis is now available by which pure IAc can be obtained (Brown *et al.* 1952), because of the instability of the compound the enzymic method of preparation has obvious advantages for the proposed type of work. It may also be possible to prepare 5-hydroxy-IAc by the oxidation of 5-hydroxytryptamine, catalysed by the amine oxidase.

SUMMARY

1. Plant amine oxidase catalyses the oxidation of tryptamine to 3-indolylacetaldehyde (IAc). This was established by the isolation of the 2:4-dinitrophenylhydrazone and dimedone derivatives of IAc from the reaction mixtures.

2. The oxidation of 5-hydroxytryptamine is also catalysed by the enzyme.

3. A method has been worked out for the estimation of IAc based on its oxidation, with silver oxide, to 3-indolylacetic acid (IAA). The IAA so formed was estimated with Salkowski reagent.

4. With this method the effect of pH, substrate concentration and reaction time on the yield of IAc was investigated. The enzyme was rapidly inactivated at high tryptamine concentrations. With low tryptamine concentrations and short reaction times (15 min.) yields of IAc of up to 90% of theoretical were obtained at pH 7–8. The yield decreased with increase in reaction time, particularly with alkaline reaction mixtures. This was probably due, in part at least, to polymerization of the IAc.

5. It is suggested that the reaction forms a useful method of obtaining IAc.

6. Two of the secondary reactions which occur when crude preparations of the amine oxidase are used to catalyse the reaction were identified as peroxidase-catalysed oxidations of tryptamine and IAc.

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The Excretion of Bilirubin as a Diglucuronide Giving the Direct van den Bergh Reaction

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(*Received* 9 *July* 1956)

van den Bergh & Müller (1916) reported that there was a difference between bilirubin and the bilirubin-like pigment which is excreted in bile. They observed that bilirubin, and the serum pigment of patients with haemolytic jaundice, required ethanol for coupling with diazotized sulphanilic acid in acid solution. These materials were said to give an 'indirect' reaction. In contrast, the pigment of bile, and of sera from patients with obstructive jaundice and hepatitis, reacted 'directly', without the addition of ethanol. Cole & Lathe (1953) found that the direct and indirect types of pigment could be separated chromatographically, and Cole, Lathe & Billing (1954) showed that the direct-reacting pigment had two components (pigments I and II). Pigment II is the more polar of the two, and is also the chief pigment of human bile. It occurs, with pigment I, in the serum and urine of patients with obstructive jaundice and hepatitis.

Billing (1954) compared the products of diazotization of bilirubin and of pigment II and found that the azo pigment (pigment B) formed from pigment II was more polar than pigment A formed from bilirubin. The relation between these pigments is shown in Fig. 1. It was suggested that the difference between bilirubin and pigment II could

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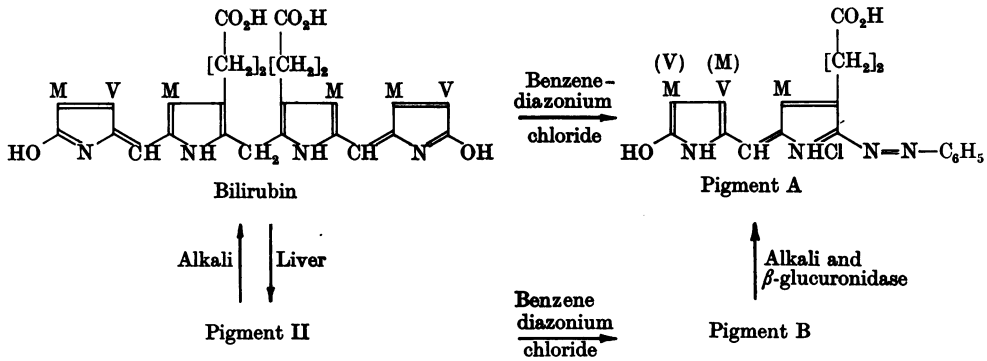

Fig. 1. Relation between bilirubin, pigment II and azo pigments A and B. M, Methyl; V, vinyl.