Plant Enzyme Reactions Leading to the Formation of Heterocyclic Compounds

2. THE FORMATION OF INDOLE

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Mann & Smithies (1955) showed that the oxidation of 1:4-diaminobutane and 1:5-diaminopentane, catalysed by plant amine oxidase, results in the formation of Δ^1-pyrroline and 2:3:4:5-tetrahydropyridine compounds respectively. The ring compounds are presumably formed by the spontaneous cyclization of the aldimines which are the probable products of the enzyme-catalysed reactions or of the amine aldehydes subsequently formed by the reaction of the aldimines with water. Plant amine oxidase, unlike animal diamine oxidase, attacks phenylalkylamines as well as aliphatic diamines (Kenten & Mann, 1952; Mann, 1955). Thus the oxidation of 2-phenylethylamine is catalysed with the formation of phenylacetaldehyde (Kenten & Mann, 1952). In view of the ring-compound formation resulting from the oxidation of 1:4-diaminobutane and 1:5-diaminopentane it appeared possible that, by using as substrates phenylalkylamines containing a second $-NH_2$ group not attacked by the enzyme, cyclization of the oxidation products might occur. In the present work this was tested with 2-(2-aminophenyl)ethylamine as substrate which was found to be quantitatively converted into indole. The result is not unexpected, since the work of Stephen (1925) suggests 2-(2-aminophenyl)-acetaldehyde, the probable product of the enzyme-catalysed reaction, readily forms indole *in vitro*. Krebs, Hafez & Eggleston (1942) showed that *Escherichia coli* formed indole from 2-(2-aminophenyl)ethanol and suggested that the primary step was probably the formation of 2-(2-aminophenyl)acetaldehyde.

MATERIALS AND METHODS

Enzyme preparations. These were as described in the preceding paper (Mann & Smithies, 1955).

Preparation of 2-(2-aminophenyl)ethylamine. This was prepared from *o*-nitrocinnamic acid by the series of reactions described by Ruggli, Steiger & Schobel (1945). The condensation of the acid chloride with activated sodium azide could not, however, be carried out in dry benzene solution as described by these authors. The reaction, however, went smoothly in acetone solution. The acid chloride (prepared from 9·7 g. of acid) was taken up in acetone (150 ml.), sodium azide (3·9 g.) in 9 ml. water added and the mixture shaken gently for 20 min. at room temp. The cinnamoyl azide was precipitated with water as pale-yellow crystals, m.p. 86°. The yield was 8·5 g. The dry product was refluxed in 50 ml. benzene for 3 hr. then, after the addition of methanol (20 ml.), for a further 45 min. The yield was 8·5 g. On recrystallization from ethanol, 7·8 g. of the yellow crystals were obtained, m.p. 147°. Ruggli *et al.* (1945) give m.p. 146–147·5° for methyl *o*-nitrostyrylcarbamate. The further reactions were carried out as described by Ruggli *et al.* (1945). The 2-(2-aminophenyl)-ethylamine dihydrochloride obtained (5·4 g.) softened at 177–179° (Jaenisch, 1923, reported 184°). It did not melt at 205–210° as reported by Ruggli *et al.* (1945) though bubbles appeared at the higher temp. The dibenzoyl derivative was obtained in quantitative yield and after one recrystallization from ethanol melted at 136–137°. Jaenisch (1923) reported m.p. 139–140°.

Melting points. These are uncorrected.

Analytical methods. Indole was estimated by the Ehrlich reagent with the solution used by Morgan & Elson (1934) for the estimation of glucosamine (2 g. *p*-dimethylaminobenzaldehyde dissolved in a mixture of 100 ml. acetic acid and 5 ml. of conc. HCl). Tracey (personal communication) has shown that this solution is suitable for the estimation of indole. Samples of the reaction mixtures containing 2–20 μg. indole were diluted to 2 ml. with water, 8 ml. of acetic acid was added followed by 1 ml. of the reagent. The mixtures were thoroughly stirred and, after standing for 40 min. at room temp., the colour intensities were read in an EEL (Evans Electroselenium Ltd.) colorimeter using Ilford Bright Spectrum Yellow–Green Filter 625 (max. transmission, 540 mμ.). The readings were compared with those obtained when using known amounts of indole over the range of 2–20 μg. Ammonia was estimated by diffusion in Conway dishes at room temp.

Buffers. 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. Phosphate buffers were made from KH_2PO_4 and KOH. Except where otherwise stated, the final concentration of phosphate (and of borate where present) in the reaction mixtures was 0·033 M.

Manometric measurements. Measurements of O_2 uptake were made in air in the Warburg apparatus at 28°. Except where otherwise stated, the volume of the reaction mixtures was 3 ml. and 0·2 ml. 5 N-KOH was present in the centre cups.

RESULTS

Manometric studies on the oxidation of 2-(2-aminophenyl)ethylamine

Effect of pH. The variation of activity with pH was tested, using 0·01 M 2-(2-aminophenyl)ethylamine with amine oxidase, in presence of catalase, in phosphate–borate buffers over the range of pH 7–9·5. The pH curve (Fig. 1) was obtained by plotting the O_2 uptake in 20 min. against the final pH. The curve shows an optimum at pH 8·8–8·9

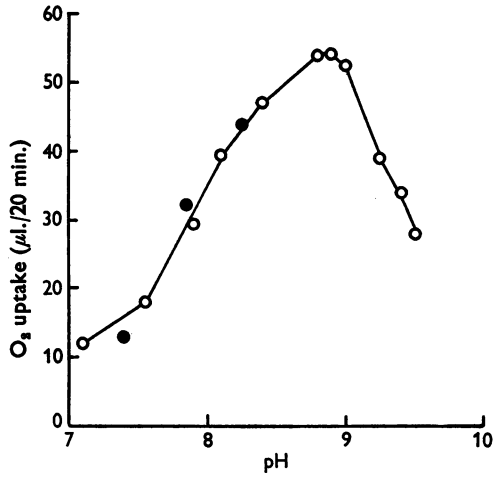

Fig. 1. Effect of pH on the rate of oxidation of 2-(2-aminophenyl)ethylamine. Amine oxidase (0·05 ml.), catalase and 0·01 M 2-(2-aminophenyl)ethylamine in phosphate buffers or phosphate–borate buffers. ●, Phosphate buffers; ○, phosphate–borate buffers. The final concentration of phosphate and borate (where present) was 0·033 M. The total volume of each reaction mixture was 3 ml. The substrate was added from the side arms. Gas phase, air; temp., 28°.

and closely resembles that obtained by Kenten & Mann (1952) with 2-phenylethylamine as substrate. Over the range of pH 7–8 a few points were also included, using phosphate buffer alone. The results suggest that, over this pH range at least, borate has no inhibiting effect.

Effect of substrate concentration. The effect of substrate concentration on initial reaction velocity was tested in phosphate–borate buffer, pH 8·5, in presence of catalase. Maximum velocity was reached with 0·002 M 2-(2-aminophenyl)ethylamine. High substrate concentrations caused some decrease in the initial reaction velocity (Fig. 2). The curve differed markedly from that obtained by Kenten & Mann (1952) with 2-phenylethylamine as substrate and the pea-seedling extract as catalyst where it was found that increase in substrate concentration was accompanied by increased initial reaction velocity up to a substrate concentration of at least 0·01 M. In view of this difference the effect of 2-phenylethylamine concentration on the initial reaction velocity was tested with the purified amine oxidase preparation in phosphate–borate buffer, pH 8·5. The results obtained were very similar to those obtained by Kenten & Mann (1952). The results suggest that 2-(2-aminophenyl)-ethylamine has a high affinity for the enzyme as have the diamines such as 1:4-diaminobutane (Kenten & Mann, 1952). It is possible that this high affinity is due to the combination of both of the

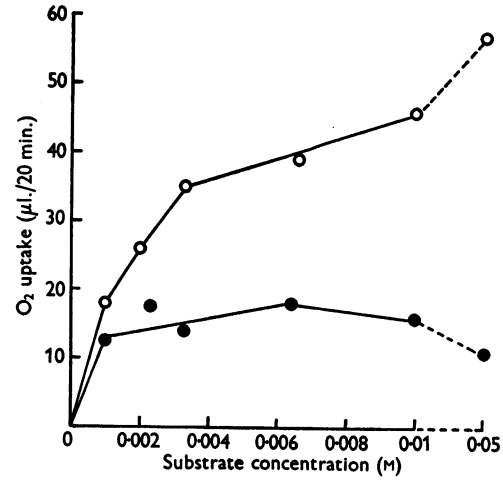

Fig. 2. Effect of substrate concentration on the rates of oxidation of 2-phenylethylamine and 2-(2-aminophenyl)-ethylamine. Amine oxidase (0·03 ml.) and catalase in phosphate–borate buffer pH 8·5. ○, 2-Phenylethylamine; ●, 2-(2-aminophenyl)ethylamine. Conditions similar to those in Fig. 1.

amino groups of the substrate with the enzyme in the formation of the enzyme–substrate compound. It is known that with animal diamine oxidase substrates like histamine and agmatine, which contain two basic groups with very different affinities for the enzyme, act as inhibitors at high concentrations (Zeller, Schär & Staehlin, 1939; Zeller, 1941).

Total oxygen uptake, ammonia and indole formation. The total O_2 uptake and the amount of ammonia and indole formed were measured, using a range of concentrations of 2-(2-aminophenyl)-ethylamine in phosphate–borate buffer, pH 8·5, with amine oxidase, in presence of catalase. The results (Table 1) show that the total O_2 uptake approached 0·5 mol. O_2/mol. substrate. The ammonia formed corresponded closely to 1 mol.

NH_3/mol. amine suggesting that only one of the amine groups of the substrate was attacked. The results of indole estimation suggested that the oxidation product accumulated quantitatively as indole. This was confirmed by the isolation of indole, in almost quantitative yield, from the reaction mixtures (p. 104). The results of the indole estimations were sometimes erratic with the reaction mixtures containing the highest substrate concentration. This was apparently due to precipitation of indole and consequent inaccurate sampling. When the vessels were rinsed with a little ethanol and the reaction mixtures together with the washings made up to a known volume samples gave consistent values for indole. The results with the highest substrate concentration in Table 1 were obtained in this way. Control experiments showed that the amount of ethanol used did not affect the indole estimation.

Table 1. *Oxygen uptake and ammonia and indole formation during the oxidation of* 2-(2-*aminophenyl)ethylamine by plant amine oxidase*

Reaction mixtures, incubated for 85 min. at 28°, consisted of 0·3 ml. amine oxidase, 50 μg. catalase, 2-(2-aminophenyl)ethylamine and phosphate–borate buffer, pH 8·5. The substrate was added from the side arm. The total volume of each reaction mixture was 3 ml. Gas phase, air.

2-(2-Aminophenyl)-ethylamine added (μmoles)	Total O_2 uptake (μmoles)	Ammonia formed (μmoles)	Indole formed (μmoles)
10	4·8	10·2	10·1
20	9·6	19·2	19·7
30	13·9	29·2	32·4

No carbonyl compounds were found in the reaction mixtures by the method of Clift & Cook (1932) and no precipitate or colour reaction was given with 2:4-dinitrophenylhydrazine. This suggests that if the reaction takes place by way of 2-(2-aminophenyl)acetaldehyde subsequent ring closure to indole must be very rapid.

The effect of peroxidase. Kenten (1953) has shown that the oxidation of phenylacetaldehyde, which is the product of the amine oxidase-catalysed oxidation of 2-phenylethylamine (Kenten & Mann, 1952), is catalysed by peroxidase, particularly in presence of Mn^{2+}. Such oxidation was independent of H_2O_2 formed during the oxidation of the amine and of added H_2O_2. Since 2-(2-aminophenyl)acetaldehyde may be formed as a result of the action of amine oxidase on 2-(2-aminophenyl)-ethylamine the effect of peroxidase on the course of the oxidation of this amine was tested (Fig. 3). It was found that peroxidase, present from the start of the oxidation, caused an increased O_2 uptake. The effect was diminished but not completely eliminated by heat treatment of the peroxidase (30 min. at 100°). The effect was not increased by Mn^{2+} at the concentration used. Peroxidase added when the amine oxidase-catalysed reaction was completed did not cause any significant O_2 uptake. Further investigation showed that the increased O_2 uptake produced when peroxidase was present from the start of the oxidation was due to the oxidation of indole and was dependent on the H_2O_2 formed in the primary reaction.

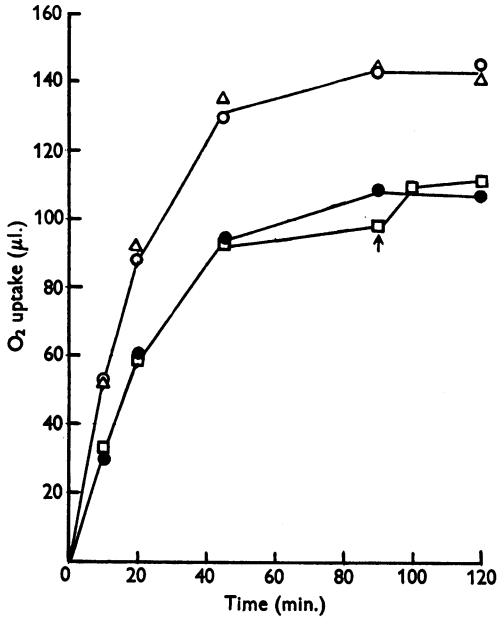

Fig. 3. The catalysis of the oxidation of the products of the amine oxidase-catalysed oxidation of 2-(2-aminophenyl)-ethylamine by peroxidase. The basal reaction mixtures consisted of amine oxidase (0·1 ml.), catalase and 2-(2-aminophenyl)ethylamine (0·1 ml. of 0·1 M) in phosphate–borate buffer, pH 8·5. To these were added at the start of the reaction peroxidase (0·1 mg.) alone, or peroxidase +0·3 ml. of 0·001 M-$MnSO_4$ or heat-treated peroxidase (30 min. at 100°). Peroxidase was also added to the basal reaction mixture at the point marked by the arrow when the amine oxidase-catalysed oxidation of the 2-(2-aminophenyl)ethylamine was complete. An O_2 uptake of 0·5 mole equivalents = 112 μl. ○, Basal reaction mixture + peroxidase; △, basal reaction mixture + peroxidase + Mn^{2+}; ●, basal reaction mixture + heat-treated peroxidase; □, basal reaction mixture. Conditions similar to those in Fig. 1.

Oxidation of indole by peroxidase systems

The fact that peroxidase catalyses indole oxidation was first demonstrated in test tube experiments in which the basal reaction mixture consisted of 2 ml. of 0·01 M indole and 1 ml. of

0·1 M phosphate buffer, pH 7. Other reaction mixtures contained, in addition, 0·2 mg. peroxidase, 0·2 ml. of 0·05 M-H_2O_2, both peroxidase and H_2O_2 and also heat-treated peroxidase with H_2O_2. At room temperature within a few minutes the reaction mixture containing both H_2O_2 and peroxidase developed a yellow colour which gradually became purple and at the end of 15 min. showed turbidity. After about an hour a purplish brown precipitate was deposited. A similar reaction occurred much more slowly in the reaction mixture containing heat-treated peroxidase and H_2O_2. All the other reaction mixtures remained colourless.

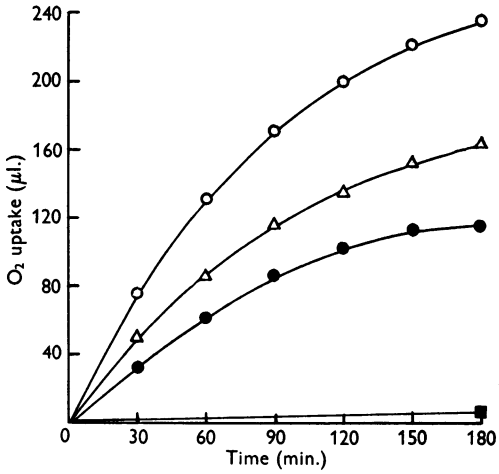

Fig. 4. Coupled oxidation of indole catalysed by peroxidase in presence of an amine oxidase system. The basal reaction mixture consisted of amine oxidase (0·3 ml.), catalase and ethanolamine (0·1 ml. of 0·1 M) in 0·067 M phosphate buffer, pH 7. An O_2 uptake of 0·5 mole equivalents = 112 µl. ●, Basal reaction mixture alone, or with peroxidase (0·1 mg.) or with indole (1·0 ml. of 0·01 M); ○, basic reaction mixture + indole + peroxidase; △, basal reaction mixture + indole + heat-treated peroxidase (30 min. at 100°); ■, indole + peroxidase. Conditions similar to those in Fig. 1.

The peroxidase-catalysed oxidation of indole by H_2O_2 was also demonstrated in manometric experiments in which the amine oxidase-catalysed oxidation of ethanolamine was used as the source of H_2O_2. The basic reaction mixture consisted of amine oxidase, catalase and ethanolamine in 0·067 M phosphate buffer, pH 7. Addition of indole (1 ml. of 0·01 M) or peroxidase (0·1 mg.) separately to the reaction mixtures caused no increase in the O_2 uptake, but added together they caused a doubling of the O_2 uptake (Fig. 4). This effect was only partially eliminated by heat treatment of the peroxidase. Peroxidase alone caused no oxidation of the indole. Peroxidase added when the amine oxidase-catalysed oxidation of ethanolamine was complete did not induce oxidation of the indole. The effect of peroxidase in catalysing the oxidation of the indole was, therefore, dependent on the H_2O_2 formed in the primary reaction. The colour reactions observed were similar to those in the test tube experiments. The increased O_2 uptake in presence of peroxidase was accompanied by a diminution in the amount of indole as estimated by the Ehrlich reagent. Wiltshire (1953) showed that peroxidase catalyses the oxidation of tryptophan by H_2O_2 and used the system of amine oxidase and ethanolamine to provide the H_2O_2 for this oxidation.

Isolation of indole following the amine oxidase-catalysed oxidation of 2-(2-aminophenyl)ethylamine

A reaction mixture was made up containing 10 ml. 0·1 M 2-(2-aminophenyl)ethylamine, 2 ml. amine oxidase, 1 mg. catalase, and 20 ml. phosphate-borate buffer, pH 8·5, in a total volume of 60 ml. A sample (3 ml.) of this reaction mixture was placed in a Warburg vessel and the O_2 uptake was followed at 28°. The rest of the reaction mixture was put in a 250 ml. Erlenmeyer flask and shaken at 28°. When the O_2 uptake stopped the reaction mixtures were combined and the solution was brought to pH 2 with conc. HCl. The solution was then extracted four times with 60 ml. of light petroleum (b.p. 60–80°). The combined extracts were concentrated to a small volume by distillation under reduced pressure and finally taken to dryness in a vacuum desiccator. The residue consisted of 0·106 g. of white shining leaflets, m.p. 50–51°, unchanged by admixture with an authentic sample of indole m.p. 51-52°. The yield (calculated as indole) was 91%. The derivative with 1:3:5-trinitrobenzene had m.p. 188–190° unchanged on admixture with an authentic sample, m.p. 187–189°.

DISCUSSION

The results of the present work show that ring-compound formation, as a result of amine oxidase activity, is not confined to those reactions in which aliphatic diamines are the substrates. Phenylalkylamines can also act as substrates leading to the formation of ring compounds. It is suggested that the indole formation like that of Δ^1-pyrroline and 2:3:4:5-tetrahydropyridine as a result of the enzyme-catalysed oxidation of 1:4-diaminobutane and 1:5-diaminopentane respectively (Mann & Smithies, 1955) may be due to the cyclization of the aldimine, which is the probable product of the enzyme-catalysed reaction, or to that of the aldehyde subsequently formed. The fact that the oxidation of 2-(2-aminophenyl)ethylamine leads to the formation of indole suggests that with other suitable phenylalkylamines as substrates quinolines and *iso*quinolines could be formed. The possibility of such ring-compound formation *in vivo*

depends, in the first place, on the availability of the substrates. The occurrence of 2-(2-aminophenyl)-ethylamine in plants has apparently not been recorded, and Baker, Happold & Walker (1946) have shown that 2-(2-aminophenyl)acetaldehyde is not an intermediate in the formation of indole from tryptophan by tryptophanase. Nevertheless, it is clear from the present results and from those of Mann & Smithies (1955) that the products of the reactions catalysed by plant amine oxidase frequently undergo spontaneous cyclization. Some of these cyclizations may be of importance *in vivo*. The possibility that the products formed by the action of plant amine oxidase on 1:4-diaminobutane and 1:5-diaminopentane may condense with plant metabolites to form alkaloids has already been referred to by Mann & Smithies (1955). It is possible that such condensations may also occur when phenylalkylamines are the substrates of the enzyme. In this connexion Blaschko (1952) has drawn attention to a particularly interesting model alkaloid synthesis by Späth & Berger (1930) in which 2-(3:4-dimethoxyphenyl)ethylamine condenses with the corresponding 2-(3:4-dimethoxyphenyl)acetaldehyde to give a compound which re-arranges itself to form tetrahydropapaverine. The amine is a substrate of animal amine oxidase (Bhagvat, Blaschko & Richter, 1939) and the aldehyde is presumably formed as a result of the enzyme-catalysed oxidation. Blaschko (1952) points out that the amine and aldehyde are, therefore, present simultaneously and that the presence of the amine oxidase creates the conditions under which alkaloid synthesis can take place.

Lastly it should be pointed out that the reaction described in the present work may be catalysed not only by plant amine oxidase but also by the plant monoamine oxidase described by Werle & Roewer (1950).

SUMMARY

1. The oxidation of 2-(2-aminophenyl)ethylamine is catalysed by plant amine oxidase. The oxidation product accumulates in the reaction mixture as indole.

2. The oxidation of indole by hydrogen peroxide is catalysed by peroxidase.

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Biosynthesis of Proteins

3. PRECURSORS IN THE SYNTHESIS OF CASEIN AND β-LACTOGLOBULIN*

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During recent years there has been much discussion of the role of peptides in protein synthesis. It has been suggested that one body protein can be converted into another without complete degradation to amino acids and that, for example, blood-plasma proteins may be used by the tissues without undergoing complete hydrolysis (Yuile, Lamson, Miller & Whipple, 1951). Evidence has been obtained also by Francis & Winnick (1953) that proteins present in embryo extracts can be used by tissue cultures without undergoing degradation and resynthesis. The idea of the conversion of one protein into another through peptide intermediates has also gained indirect support from the

* Part 2: Askonas, Campbell & Work (1954*a*).