Plant Enzyme Reactions Leading to the Formation of Heterocyclic Compounds

1. THE FORMATION OF UNSATURATED PYRROLIDINE AND PIPERIDINE COMPOUNDS

BY P. J. G. MANN AND W. R. SMITHIES

Biochemistry Department, Rothamsted Experimental Station, Harpenden, Hertfordshire

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Zeller (1938) suggested that the reactions catalysed by animal diamine oxidase can be represented by the equation

The reaction is an oxidative deamination in which only one of the —NH_2 groups of the substrate is attacked with the formation of an amine aldehyde. Tabor (1951) pointed out that the evidence of the formation of such aldehydes in reactions catalysed by animal diamine oxidase consists mainly in the demonstration of the capacity of the reaction products to fix bisulphite or cyanide. With histamine as substrate Tabor (1951) obtained further evidence of the aldehyde nature of the reaction product by showing its oxidation was catalysed by liver aldehyde oxidase, and by xanthine oxidase. With 1:4-diaminobutane (putrescine) and 1:5-diaminopentane (cadaverine) as substrates, however, the oxidation of the reaction products was not catalysed by these enzymes. In attempts to demonstrate the presence of carbonyl compounds by the method of Clift & Cook (1932), in which fixed bisulphite is estimated by iodine titration, it was found that, particularly with 1:5-diaminopentane as substrate, the oxidation products reacted directly with iodine in absence of added bisulphite. The oxidation products gave the colour tests with *o*-aminobenzaldehyde described by Schöpf, Komzak, Braun & Jacobi (1948) for Δ^1 -pyrroline and 2:3:4:5-tetrahydropyridine compounds. It was, therefore, suggested that while the oxidation of 1:4-diaminobutane and 1:5-diaminopentane catalysed by animal diamine oxidase results in the formation of amine aldehydes, the latter undergo rapid cyclization to form Δ^1 -pyrroline and 2:3:4:5-tetrahydropyridine respectively. The direct reaction with iodine was attributed to the reactive double bonds of these compounds.

Mann (1955) has shown that preparations of plant amine oxidase catalyse the oxidation not only of diamines but also, though less readily, of L- and D-lysine. In subsequent preliminary experiments on the course of oxidation of L-lysine,

evidence was obtained of ring compound formation. The present work was therefore undertaken to investigate ring compound formation resulting from oxidations catalysed by plant amine oxidase, using as substrates L- and D-lysine and aliphatic diamines of the general formula $\text{NH}_2(\text{CH}_2)_n\text{NH}_2$, where $n = 4, 5$ and 10 .

MATERIALS AND METHODS

Enzyme preparations. Plant amine oxidase was prepared from pea seedlings by the method of Mann (1955). Some of the preparations used were those described by Mann (1955), the average N content of which was about $600 \mu\text{g./ml.}$ Many other preparations were used, but the N content of only a few of these was determined. The catalase preparations were those described by Mann (1955). The amount of catalase added to each reaction mixture was $50 \mu\text{g.}$ of the crystalline preparation (Katalasefähigkeit = 19 000) or an equivalent amount of less active preparations/3 ml. of reaction mixture. The peroxidase preparation used was obtained from horseradish by following the earlier stages of the method of Kenten & Mann (1954). The Purpurogallinzahl (P.Z.) (Willstätter & Stoll, 1918) of the preparation estimated by the method of Keilin & Hartree (1951), using an EEL (Evans Electro Selenium Ltd.) colorimeter and Ilford Bright Spectrum Blue Filter 622 (max. transmission $445\text{--}500 \text{ m}\mu$), was 760.

Compounds. *o*-Aminobenzaldehyde was prepared by the method of Bamberger & Demuth (1901, 1927). α -Tri-piperidine (perhydro-4a:8a:12a-triazatriphenylene, Scheme 1, formula II) and isotripiperidine (perhydro-1:8a:12a-triazatriphenylene, Scheme 1, formula III) were prepared by the method of Schöpf *et al.* (1948). DL-Pipecolinic acid hydrochloride was prepared by hydrogenation of picolinic acid hydrochloride with platinum oxide as catalyst by the method of Stevens & Ellman (1950), except that the H_2 was at atmospheric pressure and the reaction was continued until the uptake of H_2 stopped. Platinic oxide catalyst was prepared by the method of Adams, Vorhees & Shriner (1948).

Melting points. These are uncorrected.

Analytical methods. Total steam-volatile bases in the reaction mixtures were estimated by distilling samples in the Markham (1942) still with 2 ml. borate-carbonate (25 g. $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O} + 10 \text{ g. Na}_2\text{CO}_3/\text{l.}$) and titrating the distillate, collected in boric acid, against $\text{N}/140 \text{ HCl}$. Ammonia was estimated by treating the distillates collected in phosphate buffer, pH 7, with yellow HgO according to the procedure of Pugh & Quastel (1937). The washed

HgO.NH_3 complex was transferred to the still and decomposed with 2 ml. of 5*N*-NaOH. The distillate was collected in boric acid and titrated against $\pi/140$ HCl. Ammonia was also estimated by diffusion in Conway dishes at room temp. Carbonyl compounds were estimated by the method of Clift & Cook (1932).

Paper chromatography. Both the ascending and descending one-dimensional techniques were used with Whatman no. 4 paper. The descending technique was preferred when the solutions under investigation contained inorganic salts. The solvents used were the upper phases of mixtures of *n*-butanol, acetic acid and water (4:1:5 or 6:1:3 by vol.) as described by Partridge (1948), Campbell, Work & Mellanby (1951) and Morrison (1953). The latter more economical mixture was used in particular in the fractionations on cellulose columns. A 3:1:1 mixture was also found useful in separating the products of oxidation of 1:4-diaminobutane. The papers were dried at room temp. and sprayed with, or dipped in, the appropriate reagent. The reagents used were 0.1% (w/v) solutions of ninhydrin or of isatin or 0.2% (w/v) *o*-aminobenzaldehyde in acetone. With ninhydrin and isatin the colours were developed by heating the paper at 100° for 5–10 min. With *o*-aminobenzaldehyde the colours were developed at room temp. No special precautions were taken to ensure that all samples of the solvents were equally esterified. No great accuracy is, therefore, claimed for the R_F values recorded.

Cellulose columns. These were prepared with Whatman standard-grade cellulose powder suspended in acetone according to the procedure of Campbell *et al.* (1951). The acetone was washed from the columns with the 6:1:3 or 4:1:5 mixture of *n*-butanol, acetic acid and water. The rate of solvent flow through the column was estimated by running methyl orange several inches in advance of the mixture under investigation.

Resin columns. Columns of Zeo-Karb 215 (50–100 mesh/in.) were prepared by a procedure based on that of Partridge & Westall (1949).

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 $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ and $\text{K}_4\text{P}_2\text{O}_7$.

Manometric methods. Measurements of O_2 uptake were made, in air, in the Warburg apparatus at 28°. The volume of the reaction mixtures was 3 ml., except where otherwise stated, and 0.2 ml. 5*N*-KOH was present in the centre cups. Hydrogenation of the products of the amine oxidase-catalysed reactions was measured in the Warburg apparatus using platinum black as catalyst. The platinum black was freshly prepared for each experiment by suspending 10 mg. platonic oxide in 1 ml. water in a Warburg vessel and shaking the suspension in an atmosphere of H_2 until the uptake stopped. The platinum black tended to coagulate and could not be pipetted accurately. In some experiments this difficulty was partially overcome by making the suspensions in 0.2% gum arabic. The hydrogenation of unsaturated compounds has previously been followed in the Warburg apparatus by Harrison (1939) using colloidal palladium as catalyst. On a larger scale hydrogenations were carried out at atmospheric temperature and pressure in conventional apparatus. The solutions were shaken with 20 mg. platonic oxide catalyst until the H_2 uptake stopped. Repeated additions of catalyst (10 mg.) were then made until such additions produced no further H_2 uptake other than that required for the formation of platinum black.

RESULTS

Total oxygen uptake and total steam-volatile bases, ammonia and carbonyl compounds formed

Oxygen uptake. With the diamines as substrates the O_2 uptake stopped, or nearly stopped, once a value of 0.5 mol. O_2 /mol. substrate had been reached, provided catalase was present and the amounts of amine oxidase used were such as to give a suitable initial rate of O_2 uptake. The oxidation of *L*- and *D*-lysine was catalysed much less readily than that of the diamines and required comparatively large amounts of the enzyme preparations. The total O_2 uptake with these substrates, in phosphate buffer at pH 8.5, approached 1 mol./mol. substrate. This increased O_2 uptake has been attributed by Mann (1955) to the catalysis of the oxidation of the products of the primary reactions by traces of heavy metals and of peroxidase in the amine oxidase preparations. It was shown by Mann (1955) that such secondary oxidation could be partially prevented by substituting pyrophosphate for phosphate in the reaction mixtures. With *L*- and *D*-lysine as substrates, in pyrophosphate buffer at pH 8.5, the rate of oxidation was slow after the O_2 uptake had reached 0.5 mol./mol. substrate. When large amounts of the amine oxidase preparations, such as those used with *L*- and *D*-lysine, were used to catalyse the oxidation of the diamines, the total O_2 uptake remained at little more than 0.5 mol./mol. substrate with 1:4-diaminobutane, but with the other diamines secondary oxidations occurred giving a final O_2 uptake of 1 mol. O_2 /mol. substrate.

Total steam-volatile bases and ammonia. Table 1 shows the results of analyses of the reaction mixtures for NH_3 and total steam-volatile bases. Except with 1:4-diaminobutane as substrate the reactions were studied at both levels of O_2 uptake by suitably varying the amount of enzyme and by the use of both phosphate and pyrophosphate buffers. The NH_3 values given in Table 1 are those obtained with the mercuric oxide method. Similar results were obtained by the Conway method. In all cases where the O_2 uptake was 0.5 mol./mol. substrate the NH_3 formed was about 1 mol./mol. substrate. This is in accordance with equation 1. Where oxidation was continued until O_2 uptake reached 1 mol./mol. substrate marked increases in the NH_3 produced were found only with 1:10-diaminodecane as substrate. Under these conditions oxidative deamination of both amino groups of this substrate took place with the formation of about 2 mol. NH_3 /mol. substrate. With 1:5-diaminopentane and with *L*- and *D*-lysine the further oxidation was of a different nature, since it was not accompanied by any appreciable increase in NH_3 formation. The fact that with *L*- and *D*-lysine as

Table 1. *Oxygen uptake and total steam-volatile bases and ammonia formed during the oxidation of a series of diamines and of L- and D-lysine by plant amine oxidase*

Reaction mixtures consisted of amine oxidase, catalase and substrate (30 μ moles) in 0.067M phosphate or pyrophosphate buffers. The results were corrected for those obtained with control reaction mixtures without substrate. The total volume of each reaction mixture was 3 ml. The substrates were added from the side arms. Gas phase, air; temp., 28°.

Substrate	Amine oxidase (ml.)	Buffer	Time of incubation (min.)	Total O ₂ uptake (μ moles)	Total steam-volatile bases (μ moles, calc. as NH ₃)	Ammonia (μ moles)
1:4-Diaminobutane	0.02	Phosphate, pH 7	120	14.1	39.4	27.4
1:5-Diaminopentane	0.03	Phosphate, pH 7	40	14.6	54.8	27.8
	0.20	Phosphate, pH 7	355	25.4	39.0	29.6
1:10-Diaminodecane	0.03	Phosphate, pH 7	175	14.9	35.6	29.2
	0.20	Phosphate, pH 7	365	27.1	56.4	49.7
L-Lysine	0.10	Pyrophosphate, pH 8.5	210	17.4	26.0	22.3
	0.30	Phosphate, pH 8.5	355	27.7	28.6	25.5
D-Lysine	0.10	Pyrophosphate, pH 8.5	240	14.9	23.0	23.6
	0.30	Phosphate, pH 8.5	405	27.2	27.0	25.3

Table 2. *Estimation of carbonyl compounds by the bisulphite method*

Reaction mixtures were those of Table 1. When the total O₂ uptake had reached the value shown, 1 ml. samples of the reaction mixtures were used for the estimation of carbonyl compounds by the bisulphite method. Samples (1 ml.) of the reaction mixtures were also titrated directly against iodine in absence of bisulphite but otherwise under the conditions of the method. The titrations were continued over a 10 min. period. The values given are corrected for those obtained with control reaction mixtures without substrates.

Substrate	Total O ₂ uptake (μ moles)	Titration values (ml. 0.01N iodine)					
		2 min.		4 min.		10 min.	
		Direct	Bisulphite	Direct	Bisulphite	Direct	Bisulphite
1:4-Diaminobutane	14.1	0.12	1.70	0.19	1.77	0.31	1.87
1:5-Diaminopentane	14.6	1.56	1.94	2.25	2.78	2.57	3.92
	25.4	0.44	0.78	0.60	0.94	0.74	1.22
1:10-Diaminodecane	14.9	0.0	2.15	0.0	2.15	0.03	2.19
	27.1	0.0	3.76	0.0	3.76	0.03	3.79
L-Lysine	17.4	0.55	0.73	0.60	0.77	0.74	0.89
	27.7	0.06	0.16	0.10	0.16	0.20	0.20
D-Lysine	14.9	0.72	0.79	0.75	0.88	0.84	0.98
	27.2	0.05	0.20	0.09	0.23	0.13	0.25

substrates, the amount of NH₃ formed is nearly as great in reaction mixtures buffered with pyrophosphate as in those buffered with phosphate, despite the disparity in O₂ uptake, is evidence that pyrophosphate eliminates the secondary oxidation. When the mercuric oxide procedure used in the NH₃ estimations was omitted and the distillates from the reaction mixtures were titrated directly against acid it was found that steam-volatile bases other than NH₃ were frequently present. The amount of such bases was dependent on the substrate and the stage of the oxidation. With L- and D-lysine as substrates no volatile bases other than NH₃ were found. The differences between the direct titrations and those after the mercuric oxide treatment were no greater than could be attributed to losses during this analytical procedure. The result is to be expected if the carboxyl group of the sub-

strate remains intact in the reaction product. With 1:5-diaminopentane as substrate, at the lower level of O₂ uptake, the value found for steam-volatile bases (calculated as NH₃) was about twice that for NH₃. A substantial difference was also found with 1:4-diaminobutane as substrate while with 1:10-diaminodecane the difference was smaller. The further oxidation was accompanied by a partial or complete disappearance of the steam-volatile bases other than NH₃.

Carbonyl compounds. In attempts to estimate carbonyl compounds in the reaction mixtures by the bisulphite method of Clift & Cook (1932) it was found, in confirmation of the results of Tabor (1951) with animal diamine oxidase, that the reaction products, in some cases, reacted directly with iodine. In control experiments therefore, samples of the reaction mixtures were titrated

directly against iodine under the conditions of the bisulphite method but without adding bisulphite and, in view of the nature of the results obtained, the titrations were continued over a 10 min. period. Considered as an attempt to estimate carbonyl compounds the experiments were successful only with 1:10-diaminodecane as substrate. The oxidation products of this diamine did not react directly with iodine. At the level of O_2 uptake of 0.5 mol./mol. substrate the titration value obtained was in agreement with the formation of the amine aldehyde according to equation 1. The titration required by theory for the sample taken would be 2 ml. of 0.01N iodine. The titration reached about double this value with reaction mixtures where the O_2 uptake approached 1 mol./mol. substrate, and since it was also accompanied by the formation of 2 mol. NH_3 (Table 1) the increased titration was presumably due to the further oxidation of the amine aldehyde to the dialdehyde. Since the oxidation of aliphatic monoamines is catalysed by plant amine oxidase (Kenten & Mann, 1952; Mann, 1955), though slowly in comparison with that of the diamines, it is probable that the slow secondary oxidation with 1:10-diaminodecane as substrate is catalysed by the amine oxidase itself. The oxidation products of all the other substrates reacted directly with iodine. This direct reaction was slow where 1:4-diaminobutane was substrate and the rapid reaction in the presence of bisulphite gave a titration value approaching that required for the amine aldehyde in agreement with the results of Kenten & Mann (1952). The direct reaction was particularly rapid with 1:5-diaminopentane as substrate, as found by Tabor (1951) with animal diamine oxidase as catalyst. With this substrate at the lower level of O_2 uptake the bisulphite titration was higher than that required for the formation of the aldehyde, while with the L- and D-lysine reaction mixtures it was lower. In all cases where the reaction was studied at both levels of O_2 uptake, except with 1:10-diaminodecane as substrate, the iodine titration was greater at the lower level of O_2 uptake, both in presence and absence of bisulphite. The

results suggest that only with 1:10-diaminodecane, of the diamine substrates used, does the primary reaction product accumulate as the amine aldehyde and that with L- and D-lysine as substrates the corresponding aldehydes do not accumulate. Tabor (1951) has attributed the direct reaction with iodine of the products of the oxidation of 1:4-diaminobutane and 1:5-diaminopentane, catalysed by animal diamine oxidase, to the presence of Δ^1 -pyrroline and 2:3:4:5-tetrahydropyridine formed by the spontaneous cyclization of the amine aldehydes which are the probable products of the enzyme-catalysed reaction. 2:3:4:5-Tetrahydropyridine (I) itself is unknown but a number of its polymers have been described. Experiments were made to test the direct reaction of two of these polymers (α -tripiperideine (II), and isotripiperideine (III)) with iodine and their capacity to fix bisulphite. The

(I)

(II)

(III)

results (Table 3) resemble both qualitatively and quantitatively those obtained with the oxidation products of 1:5-diaminopentane at the level of O_2 uptake of 0.5 mol./mol. substrate.

Reaction mixtures similar to those of Tables 1 and 2 were tested for the presence of carbonyl compounds with a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl. At a level of O_2 uptake of 0.5 mol./mol. substrate a positive reaction, consisting of a yellow precipitate, was

Table 3. *The fixation of bisulphite by α -tripiperideine and isotripiperideine and the direct reaction of these compounds with iodine*

Bisulphite fixation was tested with 1 ml. samples of 0.0033M α -tripiperideine and isotripiperideine under the conditions of the bisulphite method for estimation of carbonyl compounds. Samples (1 ml.) were also titrated directly against 0.01N iodine under the conditions of this method but in absence of bisulphite. The titrations were continued over a period of 10 min.

Substrate	Titration values (ml. 0.01N iodine)					
	2 min.		4 min.		10 min.	
	Direct	Bisulphite	Direct	Bisulphite	Direct	Bisulphite
α -Tripiperideine	1.42	2.88	1.76	3.34	2.10	3.60
Isotripiperideine	1.46	2.84	1.64	3.20	2.03	3.46

obtained only with the oxidation product of 1:10-diaminodecane. The other reaction mixtures showed no significant precipitate or colour change either on adding the reagent or on subsequent addition of alkali. Steam distillates of the reaction mixtures, at the lower level of O_2 uptake, gave a clear positive test only where 1:10-diaminodecane was the substrate. The results suggest that the difference between the value for steam-volatile bases and that for ammonia (Table 1) with 1:10-diaminodecane as substrate, but not with the other diamine substrates, was due to the distillation of part of the amine aldehyde formed as the oxidation product.

The reaction with o-aminobenzaldehyde

According to Schöpf & Oechler (1936) and Schöpf *et al.* (1948), compounds of Δ^1 -pyrroline and 2:3:4:5-tetrahydropyridine give yellow and orange colours respectively with *o*-aminobenzaldehyde at room temperature owing to the formation of dihydroquinazolinium compounds. This reagent was used in the present work to test for the presence of Δ^1 -pyrroline and 2:3:4:5-tetrahydropyridine compounds in reaction mixtures similar to those of Table 1. The reaction mixtures were adjusted to pH 4–5 with *N*-HCl and an equal volume of a 2% (w/v) solution of *o*-aminobenzaldehyde in 0.1M citrate buffer, pH 4.7, was added. At a level of O_2 uptake of 0.5 mol./mol. substrate the reaction mixtures with 1:4-diaminobutane as substrate gave a strong yellow colour with the reagent, while those with 1:5-diaminopentane and *L*- or *D*-lysine gave a strong orange colour. The colours increased in intensity on standing at room temperature. Tabor (1951) reported similar colour reactions with the products of the oxidation of 1:4-diaminobutane and 1:5-diaminopentane, catalysed by animal diamine oxidase. No colour reaction with *o*-aminobenzaldehyde was given by the oxidation products of 1:10-diaminodecane. Yellow or orange colour reactions were also given by the reaction mixtures other than those of 1:10-diaminodecane at a level of O_2 uptake of 1 mol./mol. substrate but the colours were much less intense. The results suggest that the oxidation products of 1:4-diaminobutane accumulate in the reaction mixtures as Δ^1 -pyrroline compounds and those of 1:5-diaminopentane and of *L*- and *D*-lysine as 2:3:4:5-tetrahydropyridine compounds. The differences found between the values for steam-volatile bases and ammonia (Table 1) with 1:4-diaminobutane and 1:5-diaminopentane as substrates was apparently due to the steam volatility of the ring compounds. The steam distillates of these reaction mixtures at a level of O_2 uptake of 0.5 mol./mol. substrate gave the colour reaction with *o*-aminobenzaldehyde but, unlike those of

1:10-diaminodecane, gave little or no reaction with 2:4-dinitrophenylhydrazine.

Vogel & Davis (1952) and Fincham (1953) have used *o*-aminobenzaldehyde to trap Δ^1 -pyrroline-5-carboxylic acid formed in biological systems. It may be used for a similar purpose in reactions catalysed by plant amine oxidase. When the oxidations were carried out in presence of *o*-aminobenzaldehyde the characteristic colours developed in the reaction mixtures and the further oxidation past the stage of 0.5 mol. O_2 /mol. substrate, caused by using large amounts of the enzyme preparation, was very slow. The effect of *o*-aminobenzaldehyde on the course of the oxidation of *L*-lysine in reaction mixtures buffered with phosphate is illustrated in Fig. 1. Similar effects were observed on the course

Fig. 1. Effect of *o*-aminobenzaldehyde on the course of the oxidation of *L*-lysine in phosphate buffer. Reaction mixtures consisted of 0.02 ml. amine oxidase, catalase and substrate (0.2 ml. of 0.1M *L*-lysine) in 0.067M phosphate buffer, pH 8.5, with and without *o*-aminobenzaldehyde. An O_2 uptake of 0.5 mole equivalents = 224 μ l. o—o, No *o*-aminobenzaldehyde; x—x, 0.013M *o*-aminobenzaldehyde. The total volume of each reaction mixture was 3 ml. The substrate was added from the side arm. Gas phase, air; temp., 28°.

of the oxidation of all the other substrates including 1:10-diaminodecane. Only a slight yellow colour was produced during the oxidation of the latter compound in presence of *o*-aminobenzaldehyde, and the inhibition of the secondary oxidation was in this case presumably due to the combination with

the *o*-aminobenzaldehyde, of the amine aldehyde formed as the primary oxidation product to give a Schiff's base.

The effect of peroxidase and Mn²⁺

Mann (1955) showed that, as a result of the amine oxidase-catalysed oxidation of L- and D-lysine, compounds accumulated in the reaction mixtures which were readily oxidized by peroxidase. Further investigation, in the present work, showed that all the oxidations studied, except that of 1:10-diaminodecane, yielded compounds whose oxidation was catalysed by peroxidase. Such oxidations

using peroxidase and Mn²⁺ together to catalyse the secondary oxidations are shown in Fig. 2. In these experiments the amine oxidase-catalysed reactions were continued until the O₂ uptake reached 0.5 mol./mol. substrate. Peroxidase and manganese sulphate were then added from the side arm and caused an immediate resumption of the O₂ uptake except with the oxidation product of 1:10-diaminodecane. Since catalase was present in the reaction mixtures this peroxidase effect is not dependent on the hydrogen peroxide formed in the primary reactions. The oxidation of both α -tripiperidine and isotripiperidine is catalysed by

Fig. 2. Oxidation of the products of amine oxidase-catalysed reactions by peroxidase + Mn²⁺. Reaction mixtures consisted of 0.02–0.03 ml. amine oxidase, catalase and substrate (0.3 ml. of 0.1M) in 0.067M phosphate buffer, pH 7. When the O₂ uptakes reached 0.5 mol./mol. substrate (336 μl.), at the times shown by the arrows, a mixture of peroxidase and MnSO₄ (0.1 mg. peroxidase and 0.3 ml. of 0.001M MnSO₄) was added to each reaction mixture from the side arms. Δ—Δ, 1:4-Diaminobutane; ○—○, 1:5-diaminopentane; □—□, 1:10-diaminodecane. Conditions similar to those in Fig. 1.

were very rapid with the oxidation products of 1:5-diaminopentane and of L- and D-lysine but slow with that of 1:4-diaminobutane and were only partially prevented by heat treatment (30 min. at 100°) of the peroxidase. Traces of Mn²⁺ also catalysed the secondary oxidations and, with the oxidation product of 1:4-diaminobutane, the effect of peroxidase and Mn²⁺ together was greater than the sum of their separate effects. Results obtained

Fig. 3. Catalysis of the oxidation of isotripiperidine by peroxidase and Mn²⁺. Reaction mixtures consisted of 0.3 ml. of 0.033M isotripiperidine and catalase in 0.067M phosphate buffer, pH 7, with and without peroxidase (0.1 mg.) and Mn²⁺ (0.3 ml. of 0.001M-MnSO₄). ○—○, Peroxidase and Mn²⁺; ×—×, heat-treated peroxidase (30 min. at 100°) and Mn²⁺; Δ—Δ, Mn²⁺; □—□, peroxidase; ●—●, heat-treated peroxidase; ■—■, isotripiperidine alone. Conditions similar to those in Fig. 1.

peroxidase and by Mn²⁺. Results with isotripiperidine are shown in Fig. 3. In these experiments the amounts of peroxidase and Mn²⁺ used were the same as for those of Fig. 2. The results of further experiments suggest that, with these compounds, the effect produced by lower concentrations of peroxidase and Mn²⁺ together may be greater than the sum of their separate effects. The results suggest that the observed effect of per-

oxidase and Mn^{2+} on the products of the amine oxidase-catalysed reactions studied is dependent on the cyclization of these products.

Catalytic hydrogenation of the oxidation products

The results so far obtained suggested that the oxidation products of 1:4-diaminobutane, 1:5-diaminopentane and of L- and D-lysine accumulated in the reaction mixtures as unsaturated ring compounds. It would be expected that in an atmosphere of H_2 and in presence of platinum black as

mixtures to pH 4-5. Freshly prepared platinum black suspension (0.2 ml.) was put in the second side arm of each vessel. The vessels were then filled with H_2 and, after equilibration, the platinum black was added to the reaction mixtures from the side arms. The oxidation products of 1:4-diaminobutane, 1:5-diaminopentane and of L- and D-lysine showed a rapid uptake of H_2 which approached 1 mol./mol. substrate. The H_2 uptake with the oxidation products of 1:10-diaminodecane was comparatively slow and, with this substrate, it was generally necessary to make further additions of platinum black to produce an uptake of H_2

Fig. 4. Catalytic hydrogenation of the products of amine oxidase-catalysed reactions. Reaction mixtures consisted of 0.01-0.04 ml. amine oxidase and catalase with 0.2 ml. of 0.1 M substrate, in 0.067 M phosphate buffer, pH 7, with 1:4-diaminobutane and 1:10-diaminodecane as substrates, and in 0.067 M pyrophosphate, pH 8.5, with L-lysine as substrate. Conditions similar to those in Fig. 1. When the O_2 uptake reached 0.5 mol./mol. substrate (224 $\mu\text{l.}$) sufficient 2 N-HCl was added from the side arms to give pH 4-5. The vessels were filled with H_2 and, after equilibration, platinum black suspension (0.2 ml.) was added from the side arms as shown by the arrows. The time taken to replace the air in the vessels with H_2 and to re-equilibrate the reaction mixtures is not shown. $\bigcirc$ — $\bigcirc$, 1:4-Diaminobutane; $\times$ — $\times$, 1:10-diaminodecane; $\square$ — $\square$, L-lysine.

catalyst, these compounds would take up H_2 and form the corresponding saturated ring compounds. This was tested in experiments in the Warburg apparatus. The amine oxidase-catalysed oxidations of the above substrates and that of 1:10-diaminodecane were allowed to proceed in vessels with two side arms until the O_2 uptake reached 0.5 mol./mol. substrate. Sufficient N-HCl was then added from the side arms of the vessels to bring the reaction

Fig. 5. Catalytic hydrogenation of α -tripiperidine and isotripiperidine. Reaction mixtures consisted of 0.2 ml. of 0.033 M α -tripiperidine or isotripiperidine in 0.067 M phosphate, pH 5. The vessels were filled with H_2 and after equilibration, platinum black suspension (0.2 ml.) was added from the side arms. The curves show the subsequent H_2 uptake. $\bigcirc$ — $\bigcirc$, α -Tripiperidine; $\square$ — $\square$, isotripiperidine. Conditions similar to those in Fig. 1.

approaching 1 mol./mol. substrate. Some of the results obtained are shown in Fig. 4. The curves shown are corrected for the small H_2 uptakes by control reaction mixtures with substrate. Under similar conditions both α -tripiperidine and isotripiperidine were rapidly hydrogenated, giving, respectively, H_2 uptakes of 70 and 55 % of those to be expected if these compounds reacted as 2:3:4:5-tetrahydropyridine (Fig. 5). Schöpf *et al.* (1948)

reported H_2 uptakes of 97 and 66.6–69.6% respectively, calculated on the same basis, when α -tripiperidine and isotripiperidine were catalytically hydrogenated in acid solution.

Paper chromatography of reaction products before and after hydrogenation

The oxidation products of the amine oxidase-catalysed reactions with 1:4-diaminobutane, 1:5-diaminopentane and L-lysine as substrates were investigated by paper chromatography, using *n*-butanol–acetic acid–water solvent mixtures and *o*-aminobenzaldehyde as detecting agent. Each reaction mixture gave two or more spots which were yellow with 1:4-diaminobutane as substrate and orange with 1:5-diaminopentane or L-lysine as substrate. These spots were not given by control reaction mixtures without substrate. α -Tripiperidine and isotripiperidine were used as reference compounds for the oxidation products of 1:5-diaminopentane. With the 6:1:3 solvent mixture these compounds both gave two orange spots with *o*-aminobenzaldehyde (R_F 0.45 and 0.66) which were identical in colour and position with the two leading spots given by the oxidation products of 1:5-diaminopentane. Examination of the hydrogenated reaction mixtures in the same way showed that the substances giving rise to the characteristic yellow and orange spots had partially or completely disappeared as a result of hydrogenation. Ninhydrin was also used as a detecting agent and pyrrolidine, piperidine, and DL-pipecolic acid as reference compounds. With ninhydrin the paper chromatogram of the hydrogenated 1:4-diaminobutane reaction mixture showed a yellow spot identical with that of pyrrolidine (R_F 0.63 with the 3:1:1 solvent mixture). The chromatogram of the hydrogenated 1:5-diaminopentane reaction mixture showed a purple spot identical with that given by piperidine (R_F 0.56 with the 6:1:3 solvent mixture) and that of the hydrogenated L-lysine reaction mixture a blue spot identical with that given by DL-pipecolic acid (R_F 0.42 with the 4:1:5 solvent mixture). These spots were not given by the reaction mixtures before hydrogenation or by control reaction mixtures without substrate. While the paper chromatograms showed other spots besides those suggesting the presence of pyrrolidine, piperidine and L-pipecolic acid, the results support the view that the products of the amine oxidase-catalysed oxidation of 1:4-diaminobutane, 1:5-diaminopentane and of L-lysine accumulate in the reaction mixtures as compounds of Δ^1 -pyrroline and 2:3:4:5-tetrahydropyridine. Attempts were made to establish this by isolation of derivatives of these compounds or of their hydrogenation products.

ISOLATION OF REACTION PRODUCTS

1:5-Diaminopentane reaction mixture

Isotripiperidine. Reaction mixtures, in Warburg vessels, consisted of 1 ml. of 0.1 M 1:5-diaminopentane dihydrochloride, 0.1 ml. amine oxidase and 50 μ g. catalase in 0.067 M phosphate buffer, pH 7.

A total of 24 such reaction mixtures were used (0.42 g. 1:5-diaminopentane dihydrochloride). Oxidation was continued until the O_2 uptake of each reaction mixture reached 1120 μ l. (0.5 mol. O_2 /mol. substrate). The contents of the vessels together with the washings were made alkaline and extracted with chloroform. The extract was dried with anhydrous Na_2SO_4 and concentrated by distillation under reduced pressure. The syrupy residue was dried over H_2SO_4 in a vacuum desiccator for several days. It gave a yellowish waxy solid containing some crystals. The weight was 0.21 g. (105% yield calculated as 2:3:4:5-tetrahydropyridine). It was dissolved in the minimum amount of hot acetone and was induced to crystallize by cooling in a freezing mixture of ice and salt. The colourless crystals (55 mg.) had m.p. 94–96° unchanged by admixture with an authentic sample of isotripiperidine (m.p. 95–96°) prepared according to Schöpf *et al.* (1948). By evaporating the mother liquor to dryness and repeating the crystallization from acetone a further 20 mg. of crystals were obtained of m.p. 91–94°.

In another experiment the dipicrate of 3-(3-amino-propyl)quinoline was prepared from the oxidation products of 1:5-diaminopentane under conditions similar to those described by Schöpf *et al.* (1948) for its preparation from α -tripiperidine or isotripiperidine. A total of 10 ml. of 0.1 M 1:5-diaminopentane dihydrochloride was oxidized in reaction mixtures as in the previous experiment. The reaction mixtures were combined, brought to pH 5 with *N*-HCl and heated in a boiling-water bath for 10 min. The suspension was cooled and centrifuged. The supernatant was heated to 100° on a boiling-water bath and added to a solution of 0.15 g. of *o*-aminobenzaldehyde in 50 ml. of 0.1 M citrate buffer, pH 4.7, also at 100°. The mixture was kept at 100° for 6 hr. and then filtered. The filtrate was again heated on the water bath while 1 g. picric acid was gradually added with stirring. The solution was left at room temp. overnight and the precipitate which had formed was filtered off and dried. The compound (0.36 g.) had m.p. 206–208° and represented a 56% yield (calculated as 3-(3-amino-propyl)quinoline dipicrate). After recrystallization from ethanol it had m.p. 208–210°. A sample of the dipicrate of 3-(3-amino-propyl)quinoline prepared from α -tripiperidine according to Schöpf *et al.* (1948) had m.p. 209–210°. Schöpf *et al.* (1948) reported m.p. 216–217°. The mixed m.p. was 208–210°.

The products of hydrogenation of α -tripiperidine and isotripiperidine

Before investigating the hydrogenated reaction mixtures of 1:5-diaminopentane the hydrogenation products of α -tripiperidine and isotripiperidine were studied. As found by Schöpf *et al.* (1948), α -tripiperidine, in dilute acid solution, took up the theoretical amount of H_2 expected for 2:3:4:5-tetrahydropyridine and yielded piperidine. Only one spot (purple) was detected on the paper chromatogram with the 6:1:3 solvent mixture and ninhydrin. This was

identical in colour and position with that given by piperidine (R_f 0.56). The benzenesulphonyl derivative prepared from the hydrogenated product had m.p. 88°. The mixed m.p. with the benzenesulphonyl derivative (m.p. 90°) prepared from piperidine was 88°.

According to Schöpf *et al.* (1948), isotripiperidine on hydrogenation yields piperidine, 2:3'-dipiperidine and *epi*-2:3'-dipiperidine. In the present work it was found that on hydrogenation in dil. acetic acid or HCl (pH 3-4) 70% of the theoretical amount of H_2 calculated for 2:3:4:5-tetrahydropyridine was taken up. The paper chromatogram with the 6:1:3 solvent mixture and ninhydrin showed two spots, one of which was purple and identical with that of piperidine and the second (R_f 0.34) had a purple leading edge and the remainder grey. In one experiment the product, after filtering and evaporating to dryness *in vacuo*, was transformed into the benzenesulphonyl derivative. From the crude mixture obtained the piperidine derivative was extracted with hot light petroleum (b.p. 40-60°). It had m.p. 88-89°, unchanged on admixture with an authentic specimen. In another experiment 0.1 g. isotripiperidine was hydrogenated. The product, after filtering and evaporating to dryness *in vacuo*, was fractionated on a cellulose column (2.2 × 40 cm.) with the 6:1:3 solvent mixture. The fractions were examined by paper chromatography and benzenesulphonyl derivatives prepared. The crude benzenesulphonyl derivative (7 mg.) from the piperidine fractions had m.p. 78-82° and on recrystallization from a mixture of light petroleum (b.p. 40-60°) and methanol had m.p. 88°. From the fractions giving the second spot (R_f 0.34) 0.22 g. benzenesulphonyl derivative was obtained of m.p. 60-65°. This consisted of two or more compounds. On fractional recrystallization from ethanol two crystalline fractions were obtained: *a*, m.p. 150-155°; *b*, m.p. 138-140°. Similar fractions were got from the residue left after the light petroleum extraction in the previous experiment and were combined with these. The part of fraction *a* relatively insoluble in methanol was filtered off. It had m.p. 158-160°. The mother liquor yielded rhombic crystals m.p. 161-162°.

Hydrogenated 1:5-diaminopentane reaction mixture

Piperidine. A total of 12 ml. of 0.1 M 1:5-diaminopentane dihydrochloride was oxidized in Warburg vessels as previously described. The combined reaction mixtures were brought to pH 3-4 and hydrogenated. About 80% of the amount of H_2 required for the reduction of a quantitative yield of 2:3:4:5-tetrahydropyridine was taken up. The filtrate was evaporated to dryness *in vacuo* and the benzenesulphonyl derivative prepared. The crude semi-solid product was repeatedly extracted with hot light petroleum (b.p. 40-60°). From the extracts were isolated crystals (40 mg.) of m.p. 82-83° which on recrystallization from a mixture of light petroleum and methanol gave a product of m.p. 89°. The mixed m.p. with the benzenesulphonyl derivative prepared from piperidine (m.p. 90°) was 89°.

In another experiment the gum (0.09 g.), prepared as previously described, by extracting the oxidation mixture with chloroform, was hydrogenated and took up 72% of the amount of H_2 required for hydrogenation of the theoretical yield of 2:3:4:5-tetrahydropyridine. The paper chromatogram, with the 6:1:3 solvent mixture and ninhydrin, gave three spots. The two faster-moving spots

were identical in colour and position with those obtained in the hydrogenation of isotripiperidine. The mixture was fractionated on a cellulose column in the same way as was the isotripiperidine mixture and benzenesulphonyl derivatives were prepared from the fractions. The yield of the crude piperidine derivative was 25 mg. The products giving the second spot were separated into faster and slower moving fractions. The faster moving fraction gave 44 mg. of a yellow powder from which no crystalline product could be obtained. The slower-moving fraction gave 60 mg. of derivative which on recrystallization from methanol gave two crystalline products. One, m.p. 158-160°, was relatively insoluble in methanol. The mixed m.p. with the fraction of the same m.p. from the hydrogenated isotripiperidine was 142-146°. The other, m.p. 148-150°, was readily soluble in methanol. After recrystallization it had m.p. 161-162°. The mixed m.p. with the rhombic crystals (m.p. 161-162°) from hydrogenated isotripiperidine was 160-161°.

The results suggest that the oxidation products of 1:5-diaminopentane accumulated in the reaction mixture as a mixture of polymers of 2:3:4:5-tetrahydropyridine. On hydrogenation other compounds besides piperidine were formed one of which was identified with a compound formed on hydrogenation of isotripiperidine. In view of the work of Schöpf *et al.* (1948) this compound is probably 2:3'-dipiperidine. The relative yield of piperidine on hydrogenation is higher than that given by isotripiperidine. This suggests that, in addition to isotripiperidine, α -tripiperidine, which gives only piperidine on hydrogenation, may be present in the reaction mixtures.

Hydrogenated 1:4-diaminobutane reaction mixture

Pyrrolidine. A total of 24 ml. of 0.1 M 1:4-diaminobutane dihydrochloride was oxidized in 1 ml. portions in reaction mixtures as described for the oxidation of 1:5-diaminopentane. The product on hydrogenation at pH 4-5 took up 60% of the volume of H_2 expected if Δ^1 -pyrroline had been formed quantitatively as a result of the oxidation. The resulting solution was filtered, evaporated to dryness *in vacuo* and the residue was extracted several times, at room temp., with 90% (w/v) aqueous ethanol. The extract was evaporated to dryness *in vacuo* and examined by paper chromatography, using the 3:1:1 solvent mixture and ninhydrin. At least five spots appeared on development. The leading yellow spot (R_f 0.63) corresponded with that given by pyrrolidine. The second (yellow-purple) had R_f 0.46 and the other three spots, one yellow and two purple, had $R_f < 0.3$. The mixture was fractionated on a cellulose column (3.0 × 60 cm.) with the above solvent mixture. The fractions containing the substance giving the leading spot gave a crude toluene-*p*-sulphonyl derivative (30 mg.) of m.p. 114-118°. Recrystallized from a mixture of light petroleum (b.p. 40-60°) and methanol it had m.p. 117°. The mixed m.p. with the toluene-*p*-sulphonyl derivative prepared from pyrrolidine (m.p. 119°) was 118°. The fractions containing the compound giving the second spot yielded 67 mg. of toluene-*p*-sulphonyl derivative of m.p. 141-145°. Recrystallized from ethanol it had m.p. 163°. (Found: N, 6.53; mol.wt., 392. Calc. for $C_{22}H_{28}O_4N_2S_2$: N, 6.25%; mol.wt., 448.) $C_{22}H_{28}O_4N_2S_2$ represents a dimer of pyrrolidine as the di(toluene-*p*-sulphonyl) derivative. The products yielding the other three

spots were not well separated on the column. The combined fractions yielded a crude toluene-*p*-sulphonyl derivative (50 mg.) which softened at 190–200°. No crystalline products were obtained. It seems likely that these fractions included higher polymers of pyrrolidine.

Hydrogenated DL-lysine reaction mixture

DL-Pipecolic acid. Reaction mixtures in 250 ml. Erlenmeyer flasks, each consisted of 20 ml. 0.1M DL-lysine hydrochloride, 1.5 ml. amine oxidase, 2 mg. catalase and 20 ml. 0.2M pyrophosphate buffer, pH 8.5, in a total volume of 60 ml. A 3 ml. sample of one of the reaction mixtures was placed in a Warburg vessel. The Warburg vessel and Erlenmeyer flasks were shaken in the bath at 28° till the O₂ uptake as measured by the Warburg manometer reached 0.5 mol./mol. DL-lysine. The reaction mixtures were then brought to pH 4–5 with conc. HCl and placed in a boiling-water bath for 10 min. The suspensions were stored in the refrigerator overnight, centrifuged, and the supernatants hydrogenated. In 4 days a total of 120 ml. 0.1M DL-lysine was oxidized and the oxidation products hydrogenated. The platinum black was removed by centrifuging and the combined supernatants were concentrated by distillation under reduced pressure and finally taken to dryness *in vacuo* over H₂SO₄. The residue was extracted repeatedly with 90% (v/v) aqueous ethanol. The combined extracts were concentrated by distillation under reduced pressure and then evaporated to dryness *in vacuo* over H₂SO₄. The residue was taken up in about 40 ml. of water. The solution obtained was run on to a column of Zeo-Karb 215 (1.2 × 48 cm.) which was then washed with 250 ml. of water. The column was then treated with 0.15N-NH₃ and the effluent was collected in 5 ml. fractions which were examined by paper chromatography, using the 4:1:5 solvent mixture and ninhydrin. Those fractions giving a spot corresponding to that given by DL-pipecolic acid were evaporated to dryness *in vacuo* and the combined residues were extracted several times with the 4:1:5 solvent mixture. The combined extracts (about 50 ml.) were passed through a cellulose column (2.5 × 52 cm.), using the same solvent mixture. The effluent was collected in 5 ml. fractions which were examined by paper chromatography. Those fractions showing only one spot corresponding to that given by DL-pipecolic acid were taken to dryness *in vacuo*. The combined residues were dissolved in about 20 ml. of 0.1N-HCl and the solution was evaporated to dryness *in vacuo* over KOH. A slightly yellow crystalline product, m.p. 246–251° (127 mg.) was obtained. The yield calculated as DL-pipecolic acid hydrochloride was 6%. Attempts to purify the compound further by recrystallization were unsuccessful. An authentic sample of DL-pipecolic acid hydrochloride prepared according to Stevens & Ellman (1950) had m.p. 259–260°. These workers report m.p. 258–261° and Meister (1954) reports m.p. 264°. The mixed m.p. was 250–254°. The 2:4-dinitrophenyl derivative prepared according to the general method of Sanger (1945) and recrystallized from a mixture of ether and light petroleum (b.p. 60–80°) according to the procedure of Rao & Sober (1954) had m.p. 136–137°, unchanged on admixture with an authentic specimen (m.p. 136–137°) prepared from synthetic DL-pipecolic acid hydrochloride. Rao & Sober (1954) report m.p. 138–139°.

The results suggest that DL-pipecolic acid was one of the main products present in the hydrogenated reaction

mixture. The low yield obtained was in part due to incomplete fractionation on the cellulose column as a result of which many of the fractions containing DL-pipecolic acid were discarded. No attempt was made to isolate any of the other compounds present. On paper chromatograms two of these compounds, like the isolated compound, gave a blue colour with isatin and the blue spots with ninhydrin showed a magenta fluorescence, both of which reactions are given by pipecolic acid (Hulme & Arthington, 1952).

DISCUSSION

The results of the present work confirm and extend those of Tabor (1951) with animal diamine oxidase and suggest that the products of the oxidation of 1:4-diaminobutane, 1:5-diaminopentane and of lysine accumulate in the reaction mixtures as compounds of Δ¹-pyrroline and of 2:3:4:5-tetrahydropyridine. It may be, as suggested by Tabor (1951), that the ring-compound formation is due to the spontaneous cyclization of the aldehyde group and the second amine group with elimination of water. An alternative possibility seems to fit the facts equally well. It is generally considered that the reaction catalysed by amine oxidases is a dehydrogenation with the formation of an aldimine which undergoes a spontaneous reaction with water to form the aldehyde and ammonia. With 1:4-diaminobutane as substrate the enzyme-catalysed reaction may be formulated

It is known that the catalytic reduction of a nitrile leads to the formation of a primary and a secondary amine. The formation of the latter product has been held to arise from the interaction between the normal product of the reduction, the primary amine and the intermediate aldimine (Rodd, 1951).

The aldimine product of the enzyme action contains both the functional groups necessary for the reaction and therefore could undergo cyclization with elimination of ammonia. These alternative methods of cyclization are illustrated in the following scheme with 1:4-diaminobutane as substrate.

Ring-compound formation in these reactions depends, as would be expected, on the length of the hydrocarbon chain of the substrate. The oxidation product of 1:10-diaminodecane does not undergo ring closure.

Weissman & Schoenheimer (1941) suggested that the α -keto acid corresponding to L-lysine would be expected to form a six-membered ring compound with great ease, yielding the ring homologue of proline. Meister (1954) has now shown that hydrogenation of the α -keto analogue of lysine results in the formation of pipecolic acid. Pipecolic acid is also formed by hydrogenation of the product formed by the action on L-lysine of the L-diamino acid dehydrogenase of turkey liver described by Boulanger & Osteux (1952*a, b*, 1954). Lowy, Holden & Schweet (1952) found that *Neurospora crassa* L-amino acid oxidase oxidizes L-lysine to a product which yields DL-pipecolic acid after catalytic hydrogenation. It is known that in plants L-pipecolic acid can be formed from L-lysine (Grobbelaar & Steward, 1953; Lowy, 1953). Meister (1954) suggested that a possible mechanism of formation of this amino acid is the oxidation or transamination of lysine followed by cyclization of the resulting α -oxo- ϵ -aminohexanoic acid to 3:4:5:6-tetrahydropicolinic acid, and optically specific reduction of the latter compound to the natural form of pipecolic acid. The scheme proposed for the formation of L-pipecolic acid from L-lysine by Weissman & Schoenheimer (1941) and Meister (1954) is based on deamination at the α -amino group. Rothstein & Miller (1954) have now shown that pipecolic acid containing ^{15}N is formed in the rat from DL-[ϵ -amino- ^{15}N]lysine. Their results provide conclusive evidence that the ϵ -amino group of lysine remains in large measure intact during the reaction. However, it was pointed out by Weissman & Schoenheimer (1941), that the biological degradation of L-lysine might start with deamination at the ϵ -amino group and this seems the more likely alternative in the amine oxidase-catalysed reaction, since both L- and D-lysine are attacked. This would lead to the formation of 2:3:4:5-tetrahydropicolinic acid whether the terminal aldimine or aldehyde group reacted with the residual amino group, whereas the cyclization of α -oxo- ϵ -aminohexanoic acid formed by α -deamination would yield 3:4:5:6-tetrahydropicolinic acid. In the former case the asymmetry of the α -carbon of the substrate would be retained in the oxidation product. It is, therefore, an attractive hypothesis that the amine oxidase-catalysed oxidation of L-lysine represents the first stage in the formation of L-pipecolic acid in plants. However, investigations so far reported suggest that the occurrence of L-pipecolic acid in plants is not limited to those plants containing the amine oxi-

dase, and it is possible, therefore, that one or more other enzymes are concerned in the primary attack on L-lysine. But it is probable that cyclization of the oxidation products to a tetrahydropicolinic acid leads, after reduction, to L-pipecolic acid. If so, the plant may also be capable of reducing tetrahydropyridine compounds and Δ^1 -pyrroline. The biosynthesis of proline from glutamic acid involves the reduction of Δ^1 -pyrroline-5-carboxylic acid (Vogel & Davis, 1952). The oxidation of 1:4-diaminobutane, 1:5-diaminopentane and of L-lysine studied in the present work may, therefore, be considered as initial stages in the synthesis of pyrrolidine and piperidine ring compounds.

The demonstration of the formation of heterocyclic compounds as a result of reactions catalysed by plant amine oxidase again raises the question whether the enzyme is connected with the synthesis of alkaloids. This was first suggested by Cromwell (1943), who discovered the enzyme during an investigation of the synthesis of hyoscyamine and related alkaloids in *Datura stramonium* and *Atropa belladonna*. The amine aldehydes which may be formed as the result of the enzyme-catalysed oxidation of 1:4-diaminobutane and 1:5-diaminopentane are key substances in model syntheses of alkaloids of the pyrrolidine and piperidine series. Thus Anet, Hughes & Ritchie (1949*a, b*; 1950), working along the general lines suggested by Robinson (1917), have recently synthesized hygrine, cuscohygrine, isopelletierine, methylisopelletierine and sparteine by reactions involving the condensation of these aldehydes or their methylated derivatives with such compounds as acetoacetic acid, acetonedicarboxylic acid and formaldehyde. In some cases the syntheses occurred in dilute aqueous solution at pH 7 and at room temperature. In the present studies with the purified amine oxidase some evidence has been obtained of the formation of complex ring compounds. In the 1:5-diaminopentane reaction mixtures one or more tripiperidine are present and in the hydrogenated reaction mixtures a dipiperidine. The presence of a dipyrrolidine is suggested in the hydrogenated 1:4-diaminobutane reaction mixtures. Schöpf *et al.* (1948) identified 2:3'-dipiperidine as the main product of the hydrogenation of isotripiperidine and pointed out that it is closely related to the alkaloid anabasin which is isometric with nicotine.

It appears probable that *in vivo* the metabolism of Δ^1 -pyrroline and 2:3:4:5-tetrahydropyridine and tetrahydropicolinic compounds will vary according to the enzyme systems with which the amine oxidase is associated. Direct reduction would produce the corresponding pyrrolidine and piperidine compounds. Condensation reactions would lead to more complex pyrrolidine and piperidine

compounds and possibly in some cases to alkaloids. Lastly the Δ^1 -pyrroline and tetrahydro-pyridine or -picolinic acid compounds may be oxidized in the plant. The results of the present work suggest that peroxidase may be a catalyst of such oxidations.

SUMMARY

1. The oxidation of 1:4-diaminobutane, 1:5-diaminopentane, 1:10:diaminodecane and of L- and D-lysine catalysed by plant amine oxidase has been studied.

2. Accumulation of carbonyl compounds as a result of the oxidations could be demonstrated only with 1:10-diaminodecane as substrate.

3. Evidence was obtained of the formation of Δ^1 -pyrroline compounds from 1:4-diaminobutane, of 2:3:4:5-tetrahydropyridine compounds from 1:5-diaminopentane and of 2:3:4:5-tetrahydro-picolinic acid from D- and L-lysine.

4. It is suggested that the formation of these ring compounds is due to the spontaneous cyclization of the aldehydes or of the aldimines which are the probable products of the enzyme-catalysed reactions.

5. The compounds formed as a result of the oxidation of 1:4-diaminobutane, 1:5-diaminopentane and of D- and L-lysine were readily hydrogenated in presence of platinum black as catalyst. Derivatives of pyrrolidine and piperidine were isolated from such hydrogenated reaction mixtures with 1:4-diaminobutane and 1:5-diaminopentane respectively as substrates. A derivative of DL-pipecolic acid was isolated from hydrogenated reaction mixtures with DL-lysine as substrate.

6. It is suggested that the amine oxidase-catalysed oxidation of 1:4-diaminobutane, 1:5-diaminopentane and L-lysine may represent the first stage in a biosynthesis of pyrrolidine and piperidine ring compounds. With L-lysine as substrate this would result in the formation of L-pipecolic acid.

7. The possibility that amine oxidase is concerned in the formation of alkaloids is discussed.

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