palmitostearo-olein 28·3 %) and 58·9 % of monosaturated–diunsaturated glycerides (hexadecenooleopalmitin 25·0 %, hexadeceno-oleostearin 10·7 % and palmitodiolein 23·2 %). No triunsaturated glyceride was found.

The experimental values for the distribution of acyl groups in the glycerides of wild-boar fat, together with the computed values of the 'even' and 'random' types, are given in Table 8, and it is clear that wild-boar fat follows the rule of 'even' distribution, according to which the component fatty acids tend to be distributed as widely as possible among all the triglyceride molecules. In this respect wild-boar fat does not fall into line with the general land-animal type of fat. The 'random' distribution would require an appreciable amount of fully saturated triglycerides, lower proportions of disaturated–mono-unsaturated, and monosaturated–diunsaturated glycerides, and an appreciable quantity of triunsaturated glycerides. As the wild-boar fat follows the 'even-distribution' pattern, Kartha's (1953) theory of restricted random distribution need not be discussed.

SUMMARY

1. The composition of the depot fat from a wild-boar (*Sus cristatus indicus*) has been studied. The mixed acids were separated by applying the lead salt–ethanol method and their composition was studied by the ester-fractionation procedure.

2. The proportions of individual fatty acids in the fat were, in general, similar to those in the fats of other herbivorous animals and were very close to those of pig fat.

3. The glyceride structure of the wild-boar fat has been investigated by low-temperature crystallization of neutral fat with acetone and ether as crystallizing medium. Three main fractions thus obtained were studied separately and possible glyceride composition was computed from the observed data.

4. The observed glyceride pattern in wild-boar fat follows Hilditch's rule of 'even distribution' and so it does not correspond with general land-animal types.

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Plant Enzyme Reactions Leading to the Formation of Heterocyclic Compounds

3. PLANT AMINE OXIDASE AND THE FORMATION OF PYRROLIDINE AND PIPERIDINE ALKALOIDS*

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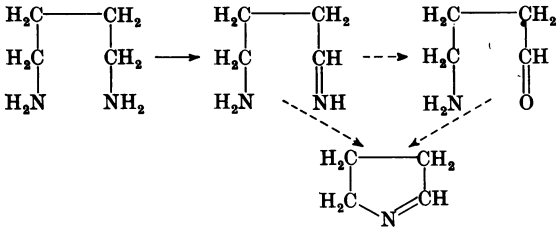
The products of the oxidation of 1:4-diaminobutane and 1:5-diaminopentane, catalysed by plant amine oxidase in the presence of catalase, accumulate in the reaction mixtures as compounds of Δ'-pyrroline and of 2:3:4:5-tetrahydropyridine respectively (Mann & Smithies, 1955*a*; Hasse & Maisack, 1955). Mann & Smithies (1955*a*) suggested that these compounds arise by spontaneous cyclization of the aldimines, which are probably the primary products of the enzyme reactions, or of the amine aldehydes formed from the aldimines by spontaneous reaction with water. Thus with 1:4-diaminobutane as substrate, the suggested reactions were those given in Scheme 1. With 1:4-diaminobutane and 1:5-diaminopentane as enzyme substrates the amine aldehydes postulated as possible intermediates in the reactions would be γ-aminobutyraldehyde and δ-aminovaleraldehyde.

* Part 2: Mann & Smithies (1955*b*).

These aldehydes and their *N*-methyl derivatives, or the cyclic forms of these compounds, have been used as starting materials in model synthesis of alkaloids of the pyrrolidine and piperidine series. Such syntheses depend on the condensation of the



Scheme 1

aldehydes, or their cyclic forms, with β-oxo acids, which undergo decarboxylation during the reactions which result in the formation of β-(2-pyrrolidyl)- or β-(2-piperidyl)-ketones (Anet, Hughes & Ritchie, 1949*a*, *b*; Galinovsky, Wagner & Weiser, 1951; Robinson, 1936; Schöpf, 1949, 1955). The fact that in some cases these condensations occur in aqueous media at pH 7·0 supports the suggestion of Robinson (1917*a*) that such reactions may be involved in the biosynthesis of pyrrolidine and piperidine alkaloids.

The present work records the results of a study of the reactions occurring when the oxidation of a series of aliphatic diamines of varying hydrocarbon chain length, catalysed by plant amine oxidase, was carried out in the presence of certain β-oxo acids. Evidence was obtained that, in some cases, alkaloids were formed as reaction products.

EXPERIMENTAL

Enzyme preparations. Plant amine oxidase preparations were made from the cotyledons of pea seedlings by the method of Clarke & Mann (1957). Fourteen preparations were used during the work. The contents of N varied from 420 to 840 μg./ml., with an average of 590 μg./ml. The specific activities (oxidase units/mg. of N) varied from 60 000 to 156 000, with an average of 94 000. A unit of amine oxidase is defined as the amount which at 28° gives an uptake of 1 μl. of O_2/hr. (calculated from initial rates) in the presence of 0·01 M-1:4-diaminobutane, catalase and 67 mM-phosphate buffer, pH 7·0, in a total volume of 3 ml. (Mann, 1955). Catalase was prepared from ox liver by the method of Sumner & Dounce (1937). The activity of the preparation was estimated by the method of Sumner & Somers (1943). The rate of decomposition of 0·01 N-H_2O_2 in 6·7 mM-phosphate buffer, pH 7·0, at 0°, by a suitable amount of the catalase, was followed by iodine titration of samples taken at 0, 3, 6, 9 and 12 min. From the equation

$$K = \frac{1}{t} \log \frac{a}{a-x},$$

where a is the initial H_2O_2 concentration and $a-x$ is the concentration at time t (min.), K values for the time intervals were calculated and the K value at zero time was obtained by extrapolation. With this value for K the Katalasefähigkeit (Euler & Josephson, 1927; Kat.f. = K/g. of enzyme) was found to be 42 000, suggesting a purity of about 80%. From the equation of Chance & Herbert (1950) that Kat.f. = 520 k_1'/M, where M is the molecular weight of catalase (230 000), the velocity constant of the preparation $k_1' = 1{\cdot}9 \times 10^7\ M^{-1}$ sec.$^{-1}$.

Chemicals. 1:4-Diaminobutane dihydrochloride, 1:5-diaminopentane dihydrochloride, 1:6-diaminohexane and 1:10-diaminodecane were obtained from L. Light and Co. Ltd. Solutions of the free bases were neutralized with 0·2 N-HCl. Acetoacetic acid was prepared by the method of Krueger (1952). Benzoylacetic acid was obtained by hydrolysis of ethyl benzoylacetate, prepared by the method given by Hauser & Hudson (1942). Acetonedicarboxylic acid was prepared by the procedure described by Adams, Chiles & Rassweiler (1941). Pyruvic acid was obtained from British Drug Houses Ltd. and was redistilled immediately before use. Oxaloacetic acid, α-oxoglutaric acid and sodium mesoxalate were obtained from L. Light and Co. Ltd. Solutions of the keto acids were prepared immediately before use and brought to the required pH with 0·2 N-NaOH. Solutions of sodium mesoxalate were brought to the required pH with 0·2 M-KH_2PO_4. *o*-Aminobenzaldehyde was prepared by the method of Bamberger & Demuth (1901, 1927). α-Tripiperideine (perhydro-4a:8a:12a-triazatriphenylene) and *iso*-tripiperideine (perhydro-1:8a:12a-triazatriphenylene) were prepared by the method of Schöpf, Komzak, Braun & Jacobi (1948). Solutions of these compounds were prepared immediately before use and brought to the required pH with 0·2 N-HCl.

Buffers. Phosphate buffers (0·2 M) were prepared from solutions of KH_2PO_4 and KOH.

Paper chromatography. A descending one-dimensional technique was used with Whatman no. 1 paper. The solvent was the upper phase of butan-1-ol–acetic acid–water (4:1:5, by vol.). The detecting agents were 0·1% (w/v) solution of ninhydrin or of isatin or 0·2% (w/v) solution of *o*-aminobenzaldehyde in actone or the modified Dragendorff reagent of Munier & Macheboeuf (1951). With *o*-aminobenzaldehyde and Munier & Macheboeuf's reagent, the colours were developed at room temperature and with ninhydrin and isatin by heating at 100° for 5–10 min.

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. Carbon dioxide was measured by Warburg's direct method (Dixon, 1943). Acetoacetic acid was estimated manometrically by measuring the CO_2 formed in its decarboxylation, catalysed by aniline citrate, according to the method of Edson (1935), except that the reaction was carried out at 28° and the CO_2 evolution was measured over 1 hr.

Melting points. These are uncorrected.

RESULTS

Decarboxylation of β-oxo acids by plant amine oxidase systems

Decarboxylation of acetoacetate. The reactions catalysed by amine oxidases have generally been represented by the equation

$$R{\cdot}CH_2{\cdot}NH_2 + H_2O + O_2 = R{\cdot}CHO + H_2O_2 + NH_3.$$

In the presence of catalase the net reaction is expressed by:

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

Thus in the oxidation of 1:4-diaminobutane, catalysed by plant amine oxidase in the presence of catalase, the total O_2 uptake was 0·5 mole/mole of amine and no CO_2 was formed. If, however, the oxidation of 1:4-diaminobutane was carried out in the presence of acetoacetate an output of gas took place (Fig. 1). This output was at first comparatively slow, but after a short lag period it reached a rate similar to that of the O_2 uptake. It continued after the oxidation reaction was completed and finally exceeded the O_2 uptake. If either the amine or the amine oxidase was omitted from the reaction mixtures, or if heat-treated enzyme (10 min. at 100°) was used, very little gas output was observed. When the rate of gas output in the complete reaction mixture had fallen to a low level, the amount of acetoacetate present was estimated by decarboxylation with aniline citrate. The CO_2 evolved in this estimation was less than that required for the acetoacetate originally present, by an amount approximately equal to the gas output during the oxidation reaction. This gas was CO_2, produced by decarboxylation of the acetoacetate. Only traces of carbonyl compounds, steam-volatile at pH 5·2, were found in the complete reaction mixture. The reaction therefore differs from the known decarboxylation of β-oxo acids by primary amines, which yields acetone from acetoacetic acid. The fact that such CO_2 evolution was dependent on the presence of both the amine and the amine oxidase suggested that it was due to a reaction between an oxidation product of the amine and the acetoacetate and not to the amine acting as a decarboxylating agent.

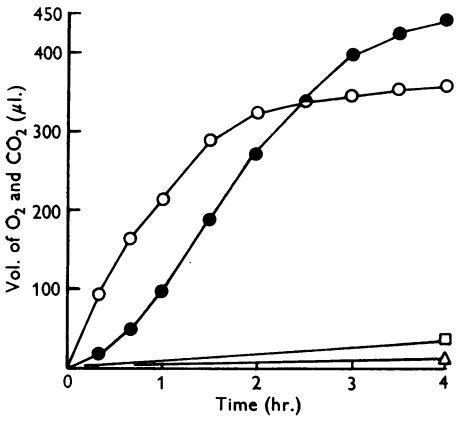


Fig. 1. Uptake of O_2 and output of CO_2 during the amine oxidase-catalysed oxidation of 1:4-diaminobutane in the presence of acetoacetate. Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase (0·02 ml.), catalase (50 μg.) and acetoacetate (30 μmoles) in 33 mM-phosphate, pH 5·2. The substrate (30 μmoles of 1:4-diaminobutane) was added from the side arm. The amine and the amine oxidase respectively were omitted from control-reaction mixtures. Gas phase, air; temp., 28°. Uptake of O_2: ○, complete reaction mixture. Output of CO_2: ●, complete reaction mixture; □, amine oxidase omitted; △, 1:4-diaminobutane omitted.

Effect of variation of diamine substrate and of pH *on the decarboxylation of acetoacetate by plant amine oxidase systems.* The oxidation of a number of diamines, catalysed by plant amine oxidase in the presence of catalase, was carried out in phosphate buffers, pH 5·2, 8·0 and 8·5, in reaction mixtures containing acetoacetate at the same concentration as that of the diamines (0·01 M). The reaction mixtures were incubated for 5 hr., by which time the O_2 uptakes had reached, or exceeded, the theoretical values, and the evolution of CO_2 (which lagged behind the O_2 uptakes, as in the experiment of Fig. 1) had fallen to a very low rate. Samples (1 ml.) of the reaction mixtures were then transferred to Warburg vessels with two side arms, and bound CO_2 was measured over a 10 min. period after the addition of 0·3 ml. of 50% (w/v) citric acid. Residual acetoacetate was then estimated in the same samples by measuring the CO_2 output in 1 hr., after the addition of aniline citrate (0·4 ml. of aniline–50% citric acid, 1:1) from the second side arm. The values given in Table 1 for total CO_2 and decrease in acetoacetate are corrected for the spontaneous decomposition of acetoacetate in control-reaction mixtures from which the amine oxidase was omitted; the CO_2 formed did not exceed 2 μmoles. The O_2 uptakes in the reaction mixtures at pH 7 and 8·5 were generally higher than the theoretical values. The causes of such increased O_2 uptakes, with 1:5-diaminopentane and 1:10-diaminodecane as substrates, have been investigated by Mann & Smithies (1955*a*). With 1:5-diaminopentane it was attributed to the further oxidation, catalysed by Mn^{2+} ions and peroxidase, of the ring compounds formed by the cyclization of the primary reaction product. With 1:10-diaminodecane, the increased O_2 uptakes have been shown to be due to the slow oxidation of the amine aldehyde, which is the product of the primary reaction.

The total CO_2 (free and bound) varied with the amine used as substrate for the oxidase. It was greatest with 1:4-diaminobutane, where it exceeded the O_2 uptake, and was least with 1:10-diaminodecane. Changes in pH, from 5·2 to 8·5, did not alter the amount of CO_2 formed. The CO_2 outputs were accompanied by corresponding decreases in the concentration of acetoacetate.

Effect of variation of keto acid. The oxidation of 1:4-diaminobutane, catalysed by the amine oxidase in the presence of a number of different keto acids,

and the CO_2 outputs, were measured (Table 2). The reaction mixtures were buffered with phosphate, pH 5·5, and no correction was made for bound CO_2. The experiments were designed to test the relative rates of decarboxylation of the different keto acids by the system. The evolution of CO_2 was measured over a period of 1 hr., for the amount of enzyme preparation used was such that the O_2 uptakes approached the theoretical value in this time. The results show that, of the acids tested, only the β-oxo acids were decarboxylated by the amine oxidase system. The rates of decarboxylation of benzoylacetic acid and of acetonedicarboxylic acid were much greater than that of acetoacetic acid. The effect of the amine oxidase system on the decarboxylation of oxaloacetic acid was not clearly shown under the conditions of Table 2, owing to the comparatively rapid spontaneous decarboxylation of this acid and the marked effect of 1:4-diaminobutane alone on the rate of decarboxylation. The fact that the amine oxidase system is much more active in the reaction than the amine alone is shown more clearly by the experiments of Fig. 2, in which lower concentrations of oxaloacetate and 1:4-diaminobutane were used to reduce the rates of CO_2 output in the control-reaction mixtures.

Effect of variation of diamine substrate on the decarboxylation of benzoylacetate. The decarboxylation of benzoylacetate by amine oxidase systems was tested in reaction mixtures, with different diamine substrates, in phosphate buffer, pH 5·2. The O_2 uptakes and CO_2 outputs of the complete reaction mixtures and of control-reaction mixtures, from which either the amine oxidase or the amine was omitted, were measured over 4 hr. The results (Table 3) show that the CO_2 formed was much higher than in similar experiments with acetoacetate (Table 1) and, with 1:4-diaminobutane as

Table 1. *Effect of variation of* pH *and of diamine substrate on the decarboxylation of acetoacetate by amine oxidase systems*

Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase, catalase (50 μg.) and acetoacetate (30 μmoles) in 33 mM-phosphate. Diamine substrates (30 μmoles) were added from the side arms. Amine oxidase was omitted from the control vessels. The O_2 uptakes and CO_2 outputs were measured for 5 hr. at 28° in air. Bound CO_2 and residual acetoacetate were then estimated on samples of the reaction mixtures. Values for CO_2 formed and decrease in acetoacetate are corrected for the spontaneous decomposition of acetoacetate in the control-reaction mixtures.

Amine	Amine oxidase (ml.)	Phosphate (pH)	Uptake of O_2 (μmoles)	Output of CO_2 (μmoles)	Decrease in acetoacetate (μmoles)
1:4-Diaminobutane	0·02	5·2	14·6	15·6	17·6
1:4-Diaminobutane	0·01	7·0	15·0	17·6	19·0
1:4-Diaminobutane	0·02	8·5	15·6	15·8	12·7
1:5-Diaminopentane	0·02	5·2	14·5	6·6	8·1
1:5-Diaminopentane	0·01	7·0	16·2	5·2	5·4
1:5-Diaminopentane	0·02	8·5	15·8	8·9	8·6
1:6-Diaminohexane	0·1	5·2	15·5	14·5	13·7
1:6-Diaminohexane	0·01	7·0	18·9	13·1	15·3
1:6-Diaminohexane	0·005	8·5	18·4	13·7	14·0
1:10-Diaminodecane	0·1	5·2	14·1	1·1	1·1
1:10-Diaminodecane	0·02	7·0	21·2	1·7	2·4
1:10-Diaminodecane	0·02	8·5	16·5	0·6	1·0

Table 2. *Decarboxylation of keto acids by amine oxidase systems*

Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase (0·02 ml.), catalase (50 μg.) and the sodium salts of the keto acids (30 μmoles) in 33 mM-phosphate, pH 5·5. Substrate (30 μmoles of 1:4-diaminobutane) was added from the side arm. Amine oxidase and 1:4-diaminobutane respectively were omitted from control-reaction mixtures. The O_2 uptakes and CO_2 outputs were measured over 1 hr. Other conditions were similar to those described in Fig. 1.

Keto acid (as sodium salt)	Uptake of O_2 (μl./hr.) by complete reaction mixture	Output of CO_2 (μl./hr.)		
		1:4-Diaminobutane omitted	Amine oxidase omitted	Complete reaction mixture
Pyruvic acid	301	2	4	2
Acetoacetic acid	308	8	13	114
Benzoylacetic acid	291	7	4	307
Mesoxalic acid	310	7	0	2
Oxaloacetic acid	314	73	275	474
α-Oxoglutaric acid	323	2	2	21
Acetonedicarboxylic acid	282	5	27	466

substrate, approached 1 mole of CO_2/mole of amine. With 1:10-diaminodecane as substrate, a slight white turbidity was noted at the end of the experiment. This was not observed in the control-reaction mixtures, nor in the complete reaction mixtures with the other diamines as substrates.

Decarboxylation of β-oxo acids by the products of the oxidation of the diamines

Evidence that the observed decarboxylation of acetoacetate is brought about by the products of the oxidation of the diamines was obtained in experiments in which the enzymic oxidation of the diamines was taken to completion (0·5 mole of O_2/mole of amine) before the addition of acetoacetate. Fig. 3 shows the subsequent CO_2 outputs in one such experiment, with 10, 20 and 30 μmoles of 1:4-diaminobutane as substrate in phosphate buffer, pH 5·2. Similar CO_2 outputs were observed

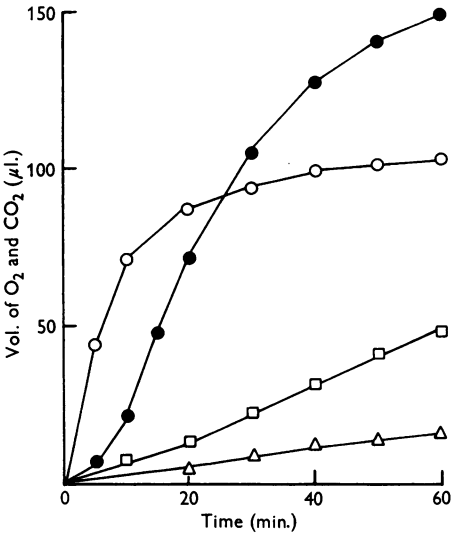


Fig. 2. Decarboxylation of oxaloacetate by amine oxidase systems. Reaction mixtures consisted of amine oxidase (0·02 ml.), catalase (50 μg.) and oxaloacetate (10 μmoles) in 33 mM-phosphate, pH 5·5. The substrate (10 μmoles of 1:4-diaminobutane) was added from the side arm. The amine and the amine oxidase respectively were omitted from control-reaction mixtures. Other conditions were similar to those in Fig. 1. Uptake of O_2: ○, complete reaction mixture. Output of CO_2: ●, complete reaction mixture; □, amine oxidase omitted, △, 1:4-diaminobutane omitted.

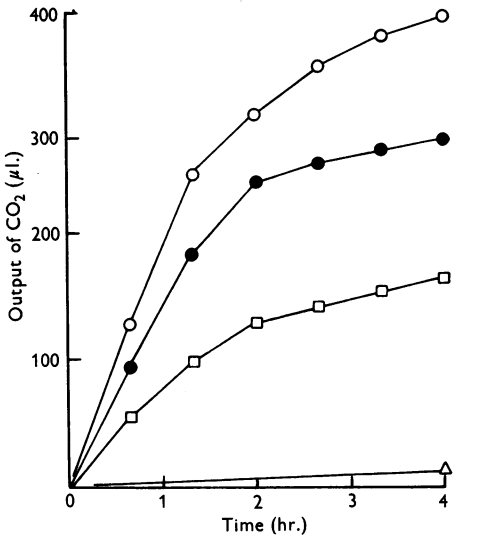


Fig. 3. Decarboxylation of acetoacetate by the products of the amine oxidase-catalysed oxidation of 1:4-diaminobutane. Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase (0·05 ml.) and catalase (50 μg.) in 33 mM-phosphate, pH 5·2. The substrate (10, 20 and 30 μmoles of 1:4-diaminobutane) was added from the side arm. In a control-reaction mixture the amine was omitted. Incubation was continued until the O_2 uptake stopped (0·5 mole of O_2/mole of amine). Acetoacetate (30 μmoles) was then added to the complete and control-reaction mixtures and the subsequent outputs of CO_2 were measured. Other conditions were similar to those of Fig. 1. Output of CO_2: ○, 30 μmoles of 1:4-diaminobutane; ●, 20 μmoles of 1:4-diaminobutane; □, 10 μmoles of 1:4-diaminobutane; △, control-reaction mixture without 1:4-diaminobutane.

Table 3. *Decarboxylation of benzoylacetate by amine oxidase systems*

Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase, catalase (50 μg.) and benzoylacetate (30 μmoles) in 33 mM-phosphate, pH 5·2. Diamine substrates (30 μmoles) were added from the side arms. Amine and the amine oxidase respectively were omitted from control-reaction mixtures. The O_2 uptakes and CO_2 outputs were measured over 4 hr. Other conditions were similar to those of Fig. 1.

Amine	Amine oxidase (ml.)	Uptake of O_2 (μmoles) by complete reaction mixture	Output of CO_2 (μmoles)		
			Amine omitted	Amine oxidase omitted	Complete reaction mixture
1:4-Diaminobutane	0·02	15·9	2·0	1·8	25·7
1:5-Diaminopentane	0·02	14·6	1·8	1·9	13·3
1:6-Diaminohexane	0·1	17·6	1·8	1·8	21·7
1:10-Diaminodecane	0·1	14·1	1·7	2·1	7·0

when the amine oxidase-reaction mixtures were filtered, in an ultrafiltration apparatus of the type described by Paterson, Pirie & Stableforth (1947), before addition of the acetoacetate, showing that once the oxidation of the diamine was complete the subsequent decarboxylation reaction was independent of the presence of the enzyme. In contrast with the results of Fig. 1, where the acetoacetate was present during the oxidation, there was little or no lag period before the CO_2 output started. Although the evolution of CO_2 was not complete in the 4 hr. experimental period, particularly with the higher concentrations of 1:4-diaminobutane, the results suggest a stoicheiometric relation between the amount of amine oxidized and the amount of acetoacetate decarboxylated. The CO_2 evolution was most nearly complete with the lowest amount of amine, where it reached about 160 μl. (theoretical amount for mole/mole reaction 224 μl. of CO_2). The stoicheiometric relation between the amount of 1:4-diaminobutane oxidized and the CO_2 output was shown more clearly in similar experiments in which acetonedicarboxylate was used instead of acetoacetate (Fig. 4). In these experiments, the amounts of 1:4-diaminobutane used were 5, 10 and 15 μmoles. The CO_2 outputs were complete within the experimental period and were only slightly less than 1 mole of CO_2/mole of 1:4-diaminobutane oxidized. The results also show that acetonedicarboxylate is rapidly decarboxylated by low concentrations of the oxidation product of 1:4-diaminobutane.

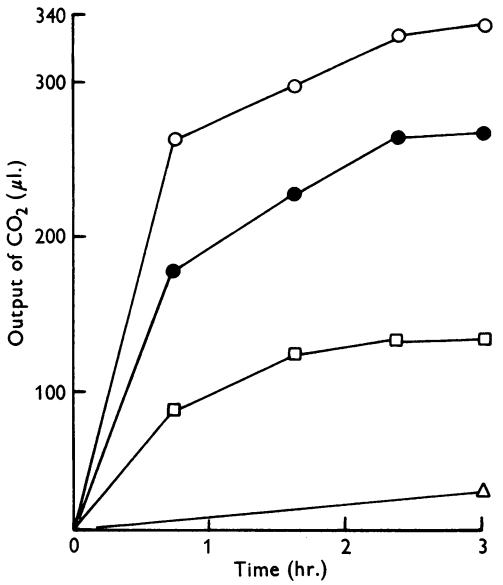


Fig. 4. Decarboxylation of acetonedicarboxylate by the products of the amine oxidase-catalysed oxidation of 1:4-diaminobutane. Conditions were as in Fig. 3, except that acetonedicarboxylate (30 μmoles) was substituted for acetoacetate and the amounts of 1:4-diaminobutane used were lower. Outputs of CO_2: ○, 15 μmoles of 1:4-diaminobutane; ●, 10 μmoles of 1:4-diaminobutane; □, 5 μmoles of 1:4-diaminobutane; △, control-reaction mixture without 1:4-diaminobutane.

Effect of variation of pH *and of diamine substrate on the decarboxylation of acetoacetate by the products of amine oxidase reactions.* The effects of variation of pH and of diamine substrate on the decarboxylation of acetoacetate by the products of the amine oxidase reactions were also studied (Table 4). The oxidations were carried out in phosphate buffers pH 5·2, 7·0 and 8·5, with comparatively large

Table 4. *Decarboxylation of acetoacetate by the products of the amine oxidase-catalysed oxidation of various diamines*

Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase and catalase (50 μg.) in 33 mM-phosphate. Diamine substrates (30 μmoles) were added from the side arms. Reaction mixtures were incubated, in air, until the O_2 uptakes reached about 0·5 mole/mole of substrate. The vessels were then filled with N_2 and acetoacetate (30 μmoles) was added from the side arms. Subsequent CO_2 outputs were measured over 4 hr. Bound CO_2 was then measured over 10 min., after the addition of 0·3 ml. of 50% (w/v) citric acid from the side arm. The values given for total CO_2 are corrected for those obtained in control-reaction mixtures with acetoacetate but without the diamines.

Amine	Amine oxidase (ml.)	Phosphate (pH)	Oxidation time (min.)	Uptake of O_2 (μmoles)	Total output of CO_2 (μmoles)
1:4-Diaminobutane	0·04	5·2	60	14·3	20·2
1:4-Diaminobutane	0·02	7·0	35	15·6	12·7
1:4-Diaminobutane	0·04	8·5	45	14·6	13·3
1:5-Diaminopentane	0·04	5·2	60	13·7	8·7
1:5-Diaminopentane	0·03	7·0	50	14·1	2·4
1:5-Diaminopentane	0·04	8·5	40	14·5	1·1
1:6-Diaminohexane	0·3	5·2	66	14·1	13·2
1:6-Diaminohexane	0·03	7·0	66	13·9	11·2
1:6-Diaminohexane	0·02	8·5	55	15·0	9·1
1:10-Diaminodecane	0·3	5·2	40	13·9	0·7
1:10-Diaminodecane	0·04	7·0	50	15·4	0·3
1:10-Diaminodecane	0·03	8·5	45	15·2	0·6

amounts of the enzyme preparation, so that the theoretical O_2 uptakes were reached within 1 hr. Under these conditions, the secondary oxidations already noted in connexion with the experiments of Table 1 became more marked, and for this reason the subsequent reactions with acetoacetate were carried out in an atmosphere of N_2. The CO_2 outputs were measured over a period of 4 hr. after addition of the acetoacetate. The results showed that, with 1:4-diaminobutane and 1:6-diaminohexane as substrates, the CO_2 formed was not much less than that when the acetoacetate was present during the oxidation of the amines, and decreased with rise in pH (Table 1). However, with 1:5-diaminopentane at pH 7·0 and 8·5 the CO_2 outputs were much smaller than those of Table 1 and with 1:10-diaminodecane no significant amounts of CO_2 were formed. This connexion between CO_2 output and pH suggested that the oxidation products causing the decarboxylation were most stable at pH 5·2, and that the oxidation product formed from 1:5-diaminopentane was less stable than those from 1:4-diaminobutane and 1:6-diaminohexane.

Decarboxylation of acetoacetate and acetonedicarboxylate by α- and iso*-tripiperideine.* The unsaturated ring compound resulting from the amine oxidase-catalysed oxidation of 1:5-diaminopentane is 2:3:4:5-tetrahydropyridine. The work of Schöpf and his collaborators (e.g. Schöpf *et al.* 1948; Schöpf, Braun & Komzak, 1956) has shown that this compound is only stable in strongly acid solutions. In weakly acid, neutral or alkaline solutions it polymerizes to the dimer, tetrahydroanabasine or to the two trimers, α- and *iso*-tripiperideine. The effect of these two trimers on the decarboxylation of acetoacetate and acetonedicarboxylate was tested in phosphate buffers, pH 5·2, 7·0 and 8·5. The reaction mixtures were incubated in N_2 and the total CO_2 (free and bound) formed in 4 hr. was measured. The results (Table 5) show the rates of decarboxylation of acetoacetate and acetonedicarboxylate by both trimers was slow. Similar results were obtained when solutions of α-tripiperideine buffered at pH 5·2, 7·0 and 8·5 were incubated for 5 hr. at 28°, before addition of the keto acid solutions. This preliminary treatment converts the greater part of the α-tripiperideine into the dimer, tetrahydroanabasine (Schöpf *et al.* 1956). The volumes of CO_2 evolved from acetoacetate were less than those in the corresponding experiments of Table 1, where acetoacetate was incubated with amine oxidase-reaction mixtures containing 1:5-diaminopentane as substrate, but of the same order as those of Table 4, where acetoacetate was incubated with the oxidation product of 1:5-diaminopentane. The results suggest that, at least in part, the comparatively low activity in the decarboxylation of β-oxo acids shown by amine oxidase-reaction mixtures containing 1:5-diaminopentane as substrate may be due to the tendency of the reactive cyclic form of the oxidation product to polymerize. Another factor which may be responsible for the comparatively low activity in the decarboxylation of β-oxo acids by amine oxidase systems with 1:5-diaminopentane as substrate, particularly under the conditions of Table 4, where the diamine was oxidized before addition of the acetoacetate, is the secondary oxidation which takes place so readily with this amine as the substrate (Mann & Smithies, 1955*a*).

Table 5. *Decarboxylation of acetoacetate and acetonedicarboxylate by α- and* iso*-tripiperideine*

Reaction mixtures, of 3 ml. total volume, consisted of 10 μmoles of α- or *iso*-tripiperideine in 33 mM-phosphate, pH 5·2, 7·0 and 8·5. Acetoacetate or acetonedicarboxylate (30 μmoles) was added from the side arm. The CO_2 outputs were measured over 4 hr. Bound CO_2 was then measured during 10 min., corrected for values obtained with control-reaction mixtures without α- or *iso*-tripiperideine. Gas phase, N_2; temp. 28°.

Amine	Phosphate (pH)	Total CO_2 (μmoles) Aceto-acetate	Acetone-dicarboxylate
α-Tripiperideine	5·2	4·3	14·1
α-Tripiperideine	7·0	2·2	7·7
α-Tripiperideine	8·5	1·6	9·9
iso-Tripiperideine	5·2	2·5	10·7
iso-Tripiperideine	7·0	1·9	8·6
iso-Tripiperideine	8·5	2·4	11·3

Chromatographic examination of the reaction products

The oxidation of each of the four amines, catalysed by plant amine oxidase in the presence of catalase, was carried out in the presence and in the absence of acetoacetate or benzoylacetate, respectively, under conditions similar to those of Table 2 except that the buffer used was 0·02M-phosphate, pH 5·2. After incubation for 5 hr. the reaction mixtures were evaporated to dryness *in vacuo*. The residues were each suspended in 0·2 ml. of ethanol, and 0·005 ml. samples of the supernatants were chromatographed on Whatman no. 1 paper with the butan-1-ol–acetic acid–water (4:1:5, by vol.) solvent mixture. The results obtained with the various detecting agents are summarized in Table 6. Only the most clearly defined spots are recorded. It was shown by Schöpf & Oechler (1936) and Schöpf *et al.* (1948) that compounds of Δ′-pyrroline and of 2:3:4:5-tetrahydropyridine give yellow and orange colours respectively with *o*-aminobenzaldehyde, owing to the formation of dihydroquinazolinium compounds. With this detecting agent on the paper

Table 6. *Paper chromatography of the products of the oxidation of various diamines in the presence and the absence of acetoacetate or benzoylacetate*

Reaction mixtures, of 3 ml. total volume, consisted of amine oxidase, catalase (50 μg.) and diamine (30 μmoles) with and without acetoacetate or benzoylacetate (30 μmoles) in 0·02 M-phosphate, pH 5·2. Amounts of amine oxidase used varied from 0·02 to 0·10 ml., with the different diamines, as in Table 1. KOH was omitted from the centre wells. Other conditions were similar to those described in Table 1. Incubations were continued for 5 hr. and reaction products were then investigated by paper chromatography as described in the text.

Detecting agents		*o*-Aminobenzaldehyde		Ninhydrin		Isatin		Munier & Macheboeuf's (1951) reagent	
Amine	Keto acid	Colour	Mean R_F	Colour	Mean R_F	Colour	Mean R_F	Colour	Mean R_F
1:4-Diaminobutane	None	Yellow	0·18	Yellow–brown	0·17	None	—	Pink	0·19
1:4-Diaminobutane	Acetoacetic	None	—	Yellow	0·47	Dark blue	0·47	Pink	0·48
1:4-Diaminobutane	Benzoylacetic	None	—	Yellow	0·72	Dark blue	0·72	Pink	0·72
1:5-Diaminopentane	None	Orange	0·27	Purple–brown	0·27	None	—	Pink	0·27
1:5-Diaminopentane	Acetoacetic	None	—	Purple with yellow centre	0·56	Pale blue	0·57	Pink	0·56
1:5-Diaminopentane	Benzoylacetic	None	—	Purple with yellow centre	0·80	Pale blue	0·78	Pink	0·80
1:6-Diaminohexane	None	Weak yellow	0·34	Purple–slate	0·34	None	—	None	—
1:6-Diaminohexane	Acetoacetic	None	—	Weak purple	0·63	Pale blue	0·65	Pink	0·64
1:6-Diaminohexane	Benzoylacetic	None	—	Purple with yellow centre	0·79	Pale blue	0·79	Pink	0·81
1:10-Diaminodecane	None	Weak yellow	0·82	Purple Purple	0·66 0·80	None —	— —	Pink —	0·65 —
1:10-Diaminodecane	Acetoacetic	None	—	Purple	0·66	None	—	Pink	0·70
1:10-Diaminodecane	Benzoylacetic	None	—	Purple Purple	0·65 0·87	None —	— —	Pink Pink	0·63 0·86

chromatograms, strong yellow or orange spots respectively were given by the reaction mixtures containing 1:4-diaminobutane and 1:5-diaminopentane as substrates, in the absence of the keto acids. With 1:6-diaminohexane and 1:10-diaminodecane as substrates, in the absence of keto acids, yellow spots were obtained with the reagent, but these were weak, particularly where 1:10-diaminodecane was the substrate. The compounds detected with *o*-aminobenzaldehyde gave weak or negative colour reactions with the other detecting agents. In the complete reaction mixtures containing acetoacetate or benzoylacetate in addition to the amine substrates, the compounds reacting with *o*-aminobenzaldehyde were not detected, suggesting that the reactions with these keto acids involved the disappearance of the unsaturated ring compounds. In such reaction mixtures, however, different products appeared which were detected with ninhydrin and Munier & Macheboeuf's reagent and, except in the 1:10:diaminodecane reaction mixtures, with isatin. In such reaction mixtures with 1:4-diaminobutane and 1:5-diaminopentane as substrates, the colours obtained with ninhydrin and isatin resembled those given by pyrrolidine and piperidine respectively, suggesting that the reactions with the β-oxo acids resulted in the formation of saturated ring compounds.

Isolation of reaction products

General procedure. Reaction mixtures, in 250 ml. Erlenmeyer flasks, each consisted of 1 ml. of amine oxidase, 0·5 mg. of catalase, 20 ml. of 0·1 M-diamine substrate (1:4-diaminobutane or 1:5-diaminopentane), 24 ml. of 0·1 M-acetoacetate and 6 ml. of 0·2 M-phosphate, pH 6·0, in a total volume of 60 ml. Four such reaction mixtures were used with each substrate. The flasks were shaken in the bath at 28° for 5 hr. To remove residual acetoacetate, the combined reaction mixtures and washings were passed through a column (50 cm. × 1·4 cm.) of De-Acidite FF (−16+50 mesh; The Permutit Co. Ltd., London, W. 4) in the hydroxide form, and washed through with 100 ml. of water. As the solution emerged from the column it was brought to pH 6·0 by dropwise addition of 2 N-H_2SO_4. The solution was cooled in ice, made strongly alkaline by adding 2·5 g. of KOH in 20 ml. of water and continuously extracted with ether for 48 hr. in an apparatus swept with N_2. Evaporation of the ether *in vacuo* left the free base as a colourless or pale-yellow liquid which rapidly darkened and resinified on exposure to air. In general it was more convenient to isolate the bases in the form of their hydrochlorides. A slight excess of an ethereal solution of hydrogen chloride was added immediately to the ether extract. After removal of the ether under reduced pressure and drying in a vacuum desiccator, a resinous product was obtained which slowly crystallized on standing. The product was extracted with successive portions of hot, dry ethyl acetate. On concentration and cooling of the combined ethyl acetate extracts, colourless crystals of the base hydrochloride were obtained.

(±)-2-*Pyrrolidylacetone* (*norhygrine*). The norhygrine hydrochloride (0·44 g.; 34 % yield based on 80 ml. of 0·1 M-1:4-diaminobutane) crystallized in needles from dry ethyl acetate. The deliquescent crystals had m.p. 114–115° (Found: N, 8·3. Calc. for $C_7H_{13}ON$,HCl: N, 8·6 %). Chromatographed on Whatman no. 1 paper with the solvent system butan-1-ol–acetic acid–water (4:1:5, by vol.), and isatin as detecting agent, the compound gave one dark-blue spot, R_F 0·47 (see Table 5). Both the free base and the hydrochloride gave positive iodoform tests. Attempts to prepare the picrate of the base gave an orange–red gum which could not be crystallized.

The hydrochloride, or the free base, readily yielded a semicarbazone. A solution of the base (62 mg.) in pyridine (1 ml.) was treated with a slight excess of semicarbazide hydrochloride in aqueous pyridine (0·5 ml.; 1:1, v/v). The mixture was then heated on a water bath for 1 hr. On cooling the reaction mixture a fine white powder separated which was filtered off and dried (53 mg.; m.p. 193–194°). Recrystallization from a small volume of methanol gave a colourless crystalline product, m.p. 195–196°, which after drying *in vacuo* over phosphorus pentoxide had m.p. 204–205° (Found: C, 40·6; H, 8·3; N, 23·1. Calc. for $C_8H_{16}ON_4,H_2O$,HCl: C, 40·3; H, 8·0; N, 23·5 %).

A solution of platinic chloride in 2 N-HCl was added to an aqueous solution of the base hydrochloride and the mixture allowed to stand for 30 min. The precipitate which formed was filtered off and washed thoroughly with water and then with ethanol. The buff-coloured precipitate decomposed in hot water and was insoluble in organic solvents. Without further purification the product was dried *in vacuo*. The product had m.p. 228° (decomp.) [Found: C, 25·6; H, 4·5; N, 3·9. Calc. for $(C_7H_{13}ON)_2,H_2PtCl_4$: C, 25·4; H, 4·2; N, 4·2 %]. The residue after ignition was 28·8 % of the initial weight, giving a molecular weight of 135 for a mono-acid base. The molecular weight expected for norhygrine is 127.

Norhygrine and its derivatives are not recorded in the literature, though C. Schöpf (personal communication, 1956) has prepared norhygrine by condensation of Δ′-pyrroline with acetoacetate.

(±)-2-*Piperidylacetone* (*isopelletierine*). The base was isolated in the manner previously described, and on treatment with an ethereal solution of hydrogen chloride yielded the crude *iso*pelletierine

hydrochloride. The base hydrochloride was extracted with portions of hot, dry ethyl acetate and the extracts were concentrated. On cooling the resulting solution, white flocculent crystals of the hydrochloride were obtained, m.p. 137–140°, raised to 141–143° on recrystallization from ethyl acetate (0·14 g.; 12% yield) (Found: C, 54·05; H, 8·3; N, 8·1; Cl, 20·2. Calc. for $C_8H_{16}ONCl$: C, 54·1; H, 8·4; N, 7·9; Cl, 20·0%). Mortimer & Wilkinson (1957) give m.p. 144–145°. Chromatographed on Whatman no. 1 paper with the solvent system butan-1-ol–acetic acid–water (4:1:5, by vol.), and isatin as detecting agent, the compound gave one pale-blue spot R_F 0·57.

A sample of the base hydrochloride was used to prepare the semicarbazone derivative in a manner similar to that given for norhygrine hydrochloride. The semicarbazone was obtained as a white powder, m.p. 158–160°, after recrystallization from methanol, m.p. 167–168° (Found: C, 42·9; H, 8·1; N, 21·9. Calc. for $C_9H_{18}O_4N,H_2O,HCl$: C, 42·8; H, 8·4; N, 22·2%). Mortimer & Wilkinson (1957) give m.p. 169–171°. An authentic sample kindly supplied by Dr Wilkinson had m.p. 169–170° unchanged by admixture with the semicarbazone described above.

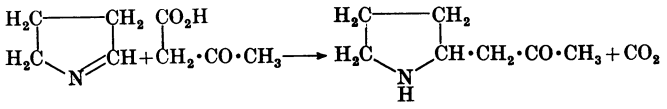
The picrate was obtained by treating a solution of the free base in ethanol with an ethanolic solution containing the calculated amount of picric acid. The picrate crystallized in feathery clusters of needles and, after recrystallization from ethanol, had m.p. 147–148°. Mortimer & Wilkinson (1957) give m.p. 149–150°. An authentic sample kindly supplied by Dr Wilkinson had m.p. 147–148° unchanged by admixture with the sample described above (Found: C, 45·55; H, 5·0; N, 15·4. Calc. for $C_8H_{15}ON,C_6H_3O_7N_3$: C, 45·4; H, 5·0; N, 15·1%). Determinations of the molecular weight of the picrate by the method of Cunningham, Dawson & Spring (1951) gave values of 361 and 358. The value expected for the picrate of *iso*pelletierine is 370.

DISCUSSION

The results of the present work suggest that the observed decarboxylation of β-oxo acids by the amine oxidase systems used is brought about by stoicheiometric reactions between the products of the amine oxidase reactions and the β-oxo acids. Of the diamines used as substrates 1:4-diaminobutane, 1:5-diaminopentane and 1:6-diaminohexane were active in the system, whereas 1:10-diaminodecane was comparatively inactive. It is already known (Hasse & Maisack, 1955; Mann & Smithies, 1955*a*) that the amine aldehydes which are the probable products of the amine oxidase-catalysed oxidation of 1:4-diaminobutane and 1:5-diaminopentane spontaneously cyclize to give unsaturated pyrrolidine or piperidine compounds. Preliminary investigations of the oxidation product of 1:6-diaminohexane suggest that it exists under the experimental conditions as an equilibrium mixture of the open chain and cyclic forms. Evidence that the oxidation product of 1:10-diaminodecane reacts primarily as an amine aldehyde was given by Mann & Smithies (1955*a*). The present results therefore support the suggestion of van Tamelen & Knapp (1955), for such reactions with δ-aminovaleraldehyde, that the unsaturated cyclic forms of the amine aldehydes are the reactive species. Thus the reaction between the oxidation products of 1:4-diaminobutane and acetoacetic acid to form norhygrine may be represented as in Scheme 2.

van Tamelen & Knapp (1955) suggested that the reaction involves a cyclic intermediate from which carbon dioxide is eliminated simultaneously with the condensation. An intermediate of this type provides a pathway of decarboxylation differing from that suggested by Pederson (1934) and Westheimer & Jones (1941) for the primary amine-catalysed decarboxylation of β-oxo acids. The slight decarboxylating activity of amine oxidase systems with 1:10-diaminodecane as substrate may be due to the presence of traces of the cyclic form of the oxidation product of this amine. This is suggested by the weakly positive colour test with *o*-aminobenzaldehyde given by reaction mixtures containing this substrate in the absence, but not in the presence, of β-oxo acids. Preliminary results suggest that, contrary to the results of Mann (1955), the oxidation of ornithine is slowly catalysed by the amine oxidase with the formation of a pyrroline carboxylic acid, and that this compound and the tetrahydropicolinic acid formed by the oxidation of lysine also condense with β-oxo acids.

Mann & Smithies (1955*a*) suggested that the oxidation of 1:4-diaminobutane, 1:5-diaminopentane and lysine, catalysed by plant amine oxidase, might be a stage in the biosynthesis of pyrrolidine and piperidine compounds in higher plants. The reactions studied in the present work represent one possible mechanism of such biosynthesis and are of particular interest since the products are alkaloids.



Scheme 2

The results lend additional support to the theory of the biogenesis of such alkaloids advanced by Robinson (1917*a*). In connexion with the present work the following postulate put forward by Robinson (1949) in discussing the synthesis of alkaloids by condensation of amine aldehydes and β-oxo acids is of particular interest: 'Now suppose an aldehyde is produced by an oxidizing enzyme system, is it not possible that acetoacetic, or some other activated acetone derivative, is present in any case and that the reaction will go to completion.'

Unsaturated pyrrolidine and piperidine compounds are formed as a result of reactions catalysed by enzymes other than amine oxidases. Glutamic semialdehyde, which is presumably the primary product of the oxidation of ornithine by the amine oxidase, is an intermediate in the interconversion of glutamic acid, proline and ornithine in animal tissues (Stetten, 1951) and in *Neurospora* (Vogel & Bonner, 1954), and is a precursor of proline in *Escherichia coli* (Vogel & Davis, 1952). It may arise by oxidation of proline (Taggart & Krakaur, 1949), by reduction of glutamic acid (Vogel & Bonner, 1954), by the action of ornithine transaminase (Fincham, 1953; Meister, 1954*a*) or by oxidation of α-amino-δ-hydroxy-valeric acid (Yura & Vogel, 1957). The position in intermediary metabolism of the corresponding aldehyde derived from lysine which spontaneously cyclizes, probably to 2:3:4:5-tetrahydropicolinic acid, is not yet clear. It is a postulated precursor of lysine (Schweet, Holden & Lowy, 1954; Yura & Vogel, 1957) and Mann & Smithies (1955*a*) suggest that it may be a precursor of pipecolinic acid in higher plants. In animal tissues pipecolinic acid is formed from lysine by way of the corresponding α-oxo acid (Rothstein & Miller, 1954). The only enzyme, other than plant amine oxidase, which has so far been shown to catalyse the formation of lysine semialdehyde appears to be the ω-hydroxy-α-amino acid dehydrogenase of Yura & Vogel (1957). The α-oxo acids formed by the action of amino acid oxidases on ornithine and lysine cyclize spontaneously to Δ′-pyrroline-2-carboxylic acid and 3:4:5:6-tetrahydropicolinic acid (Boulanger & Osteux, 1952; Meister, 1954*b*; Boulanger, Coursaget, Bertrand & Osteux, 1957). It is probable that these unsaturated ring compounds, like those derived from the amine aldehydes, would undergo spontaneous condensation reactions with β-oxo acids. It is clear from these considerations that if biosynthesis of pyrrolidine and piperidine alkaloids in higher plants occurs by the type of reaction studied in the present work it is not necessarily dependent on the presence of plant amine oxidase.

With the diamines as substrates the products of the condensation reactions would be racemic mixtures and acetonedicarboxylic, benzoylacetic and propionylacetic acids would, as with acetoacetic acid, result in the formation of naturally occurring α-substituted pyrrolidine and piperidine alkaloids, from which other alkaloids might be formed subsequently by *N*-methylation or by reduction of the carbonyl group. Since one metabolic pathway of proline metabolism involves its oxidation to glutamic semialdehyde, which is in equilibrium with Δ′-pyrroline-5-carboxylic acid, it is possible that $\alpha\alpha_1$-disubstituted pyrrolidine and piperidine alkaloids might be formed by oxidation of the α-substituted alkaloids followed by condensation reactions. Such a possibility has been mentioned in connexion with alkaloids of *Lobelia inflata* (Schöpf & Kauffman, 1957). Where acetonedicarboxylic acid, or a half-ester of this acid, has taken part in the initial condensation, further condensation subsequent to oxidation of the ring of the monosubstituted pyrrolidine or piperidine compound could give rise to alkaloids having a tropane or ψ-pelletierine structure. This suggests a biosynthetic pathway alternative to the succinic dialdehyde synthesis of tropinone (Robinson, 1917*b*; Schöpf & Lehmann, 1935) and the glutaric dialdehyde synthesis of ψ-pelletierine (Menzies & Robinson, 1924; Schöpf & Lehmann, 1935) and lobelanine (Schöpf & Lehmann, 1935).

[$^{14}C_2$]Ornithine has been found (Leete, Marion & Spenser, 1954; Haga, 1956) to act as a precursor of hyoscyamine, a tropane alkaloid, and the bridgehead carbon atoms have been found equally labelled (Haga, 1956). A compound of Δ′-pyrroline derived from ornithine can be postulated as an intermediate in the biosynthesis of tropane alkaloids such as hyoscyamine, equal labelling of the bridgehead carbon atoms of the tropane skeleton arising by double-bond migration in the Δ′-pyrroline compound. The fact that ornithine, but not 1:4-diaminobutane, serves as a precursor of hyoscyamine in *Datura stramonium* (Leete *et al.* 1954) could be due to the presence of an enzyme system forming the Δ′-pyrroline compound from ornithine but not from 1:4-diaminobutane.

The biosynthesis of nicotine can be postulated as taking place by the condensation of a vinylogue of a β-oxo acid, 5-carboxy-5-hydro-2-pyridone and Δ′-pyrroline. Such a route meets the requirements suggested by feeding experiments with labelled compounds (Dawson, Christman, D'Adamo, Solt & Wolf, 1958; Grimshaw & Marion, 1958). As with hyoscyamine the α- and α′-carbon atoms of the pyrrolidine ring of nicotine have been found equally labelled (Leete, 1955; Dewey, Byerrum & Ball, 1955) and tautomerism of the postulated intermediate Δ′-pyrroline could be the cause rather than symmetrical intermediates such as pyrrolidine (Dewey *et al.* 1955) or 1:4-diaminobutane (Leete, 1955). Anabasine might be formed

in a similar manner by condensation of 5-carboxy-5-hydro-2-pyridone with 2:3:4:5-tetrahydropyridine or 2:3:4:5-tetrahydropicolinic acid. [$^{14}C_2$]-Lysine was found, in feeding experiments with *Nicotiana glauca* (Leete, 1956), to act as a precursor of the piperidine ring of anabasine, although experiments with excized leaves showed only very slight incorporation of [$^{14}C_2$]lysine into anabasine (Aronoff, 1956). In the experiments of Leete (1956) all the ^{14}C activity of the anabasine was found at the single carbon atom of the piperidine ring which is attached to the pyridine. This was interpreted as being due to an asymmetrical precursor, possibly 3:4:5:6-tetrahydropicolinic acid, in a preceding reaction. More recently, Leete (1958) has shown that 1:5-diamino[1:5-$^{14}C_2$]pentane is a more efficient precursor (of the piperidine ring of anabasine) than [$^{14}C_2$]lysine. The 1:5-diaminopentane was considered to be converted into 2:3:4:5-tetrahydropyridine before incorporation into the anabasine molecule, which appears to be equally labelled at the $C_{(2)}$ and $C_{(6)}$ atoms of the piperidine ring. Nicotine, anabasine and *iso*pelletierine have been found to occur together in *Duboisia myoporoides* (Mortimer & Wilkinson, 1957), which suggests the possibility of similar biosynthetic routes.

The unsaturated ring compounds may also condense with nucleophilic species other than those derived from β-oxo acids. Thus van Tamelen & Knapp (1955) prepared β-(2-piperidyl)indole by condensing 2:3:4:5-tetrahydropyridine with indole and, in work on the synthesis of oxygenated tetracyclic lupin alkaloids, van Tamelen & Baran (1956) obtained 3-α-pyridylquinolizidine-1-carboxylic acid as the product of the reaction between 2:3:4:5-tetrahydropyridine and 2-(α-pyridyl)allylmalonic acid. Preliminary experiments suggest β-(2-piperidyl)indole is formed when the oxidation of 1:5-diaminopentane, catalysed by plant amine oxidase, is carried out in the presence of indole.

If alkaloids are waste products of metabolism it seems likely that they might be formed by non-specific mechanisms such as that described in the present work. The lag period in the production of carbon dioxide, which occurs with the amine oxidase reaction mixtures containing β-oxo acids, suggests that the condensation reactions are unlikely to occur to any great extent where rapid specific pathways exist for the metabolism of the unsaturated ring compounds. Recent work suggests that specific enzymes may exist which catalyse the direct addition of hydrogen to unsaturated compounds of this type to give pyrrolidine and piperidine compounds, and oxidation to aromatic ring compounds is a possible alternative metabolic pathway. Thus Δ′-pyrroline-5-carboxylic acid is reduced to proline by extracts of *Neurospora* (Yura & Vogel, 1955) and by rat-liver preparations (Smith & Greenberg, 1956, 1957). Meister & Buckley (1957) have shown that 3:4:5:6-tetrahydropicolinic acid is reduced to pipecolinic acid by enzyme preparations from rat or rabbit liver. The same preparations also catalyse the reduction of Δ′-pyrroline-2-carboxylic acid and Δ′-pyrroline-5-carboxylic acid to proline. The results of further investigations (Meister, Radhakrishnan & Buckley, 1957) suggest that different enzymes catalyse the reduction of each of the two last-named compounds and show that extracts of *Pisum sativum* and *Phaseolus radiatus* reduce 3:4:5:6-tetrahydropicolinic acid and Δ′-pyrroline-2-carboxylic acid to pipecolinic acid and proline respectively. Preparations of D-amino acid oxidase catalyse the formation of pyrrole-2-carboxylic acid from hydroxy-D- and *allo*hydroxy-D-proline (Radhakrishnan & Meister, 1956, 1957; Letellier & Bouthillier, 1956). Radhakrishnan & Meister (1957) also observed enzymic conversion of hydroxy-L-proline into pyrrole-2-carboxylic acid by intact- and broken-cell suspensions of a soil bacterium and obtained evidence that Δ′-pyrroline-4-hydroxy-2-carboxylic acid, which spontaneously forms pyrrole-2-carboxylic acid, is an intermediate in all three reactions. In a preliminary note Hasse & Berg (1957) report the oxidation of 1:5-diaminopentane to anabasine by pea-seedling extracts. The tetrahydroanabasine formed as a result of the action of the amine oxidase present is oxidized by a system of two components in the extracts, one of which can be replaced by Mn^{2+} ions. These considerations suggest the possibility that oxidations catalysed by amine oxidase may be stages in the biosynthesis not only of pyrrolidine and piperidine but also of pyrrole and pyridine compounds.

Lastly it should be pointed out that Krueger (1955) observed decarboxylation of β-oxo acids by *o*-quinones formed by the action of tyrosinase. The postulated mechanism of reaction involved condensation of the β-oxo acid with the *o*-quinone.

SUMMARY

1. The oxidation of 1:4-diaminobutane, 1:5-diaminopentane, 1:6-diaminohexane and 1:10-diaminodecane, catalysed by purified plant amine oxidase preparations in the presence of catalase, took place without formation of carbon dioxide. When these oxidations were carried out in the presence of β-oxo acids carbon dioxide was formed. The outputs of carbon dioxide sometimes exceeded the uptakes of oxygen and arose by decarboxylation of the β-oxo acids.

2. The rates of decarboxylation in such systems depended on the amine used as substrate. It was greatest with 1:4-diaminobutane and least with 1:10-diaminodecane.

3. Benzoylacetic acid, oxaloacetic acid and acetonedicarboxylic acid were decarboxylated more readily than was acetoacetic acid. The α-oxo acids tested were not decarboxylated.

4. In manometric experiments evidence was obtained that the decarboxylations were due to stoicheiometric reactions between the β-oxo acids and the oxidation products of the amines.

5. The results of paper-chromatographic investigations suggested that the decarboxylations were dependent on condensation reactions between the unsaturated ring compounds, formed by the spontaneous cyclization of the products of oxidation of the amines, and the β-oxo acids, which resulted in the formation of saturated ring compounds. This was established by the isolation of norhygrine and *iso*pelletierine from large-scale reaction mixtures in which 1:4-diaminobutane and 1:5-diaminopentane respectively were oxidized in the presence of acetoacetate.

6. The possible significance of the results in relation to alkaloid biosynthesis is discussed.

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Calcium and Magnesium Metabolism in Calves

4. BONE COMPOSITION IN MAGNESIUM DEFICIENCY AND THE CONTROL OF PLASMA MAGNESIUM*

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Orent, Kruse & McCollum (1934) first showed that bone magnesium is depleted in rats kept on magnesium-deficient diets. This was subsequently also shown to be true for calves (Knoop, Krauss & Hayden, 1939; Blaxter, Rook & MacDonald, 1954; Blaxter & Sharman, 1955; Parr, 1957). It has also been shown that in neither of these animals are the soft tissues usually appreciably depleted even under the most severe conditions of magnesium deficiency (Cunningham, 1936; Blaxter *et al.* 1954; Parr, 1957; and our own unpublished observations) although MacIntyre & Davidsson (1958) have recently reported a small fall in skeletal-muscle magnesium in magnesium-deficient rats.

It appears therefore that bone magnesium represents a store of this element which can be called upon under conditions of deficiency to supply the needs of the soft tissues. It has been shown by killing rats at different times after introducing a magnesium-deficient diet that their bone magnesium can rapidly be mobilized (Duckworth, Godden & Warnock, 1940; Duckworth & Godden, 1941). Similar experiments with calves have not been carried out but Blaxter (1956) has related bone magnesium to plasma magnesium in calves slaughtered with differing degrees of hypomagnesaemia developed as a result of protracted milk feeding.

Our experiments on the development of hypomagnesaemia in milk-fed calves (Smith, 1957, 1958, 1959) made it desirable to follow associated changes in bone composition in such animals and other magnesium-deficient calves. The use of vertebrae taken from the tail of the living animal for this purpose will be described.

* Part 3: Smith (1959).

EXPERIMENTAL

Sample preparation and analysis

Bones examined were the caudal vertebrae, rib shaft, femur head and shaft and the first phalanges. They were removed either after slaughter or, with some of the caudal vertebrae, from sections of the tail taken from the living animal, a local anaesthetic (Nupercaine) being used. In this way at least ten successive samples could be obtained from one calf if sections containing only one vertebra were removed at a time. The bones were thoroughly cleaned of adhering soft tissue, cartilage and fat. Samples of the vertebrae and phalanges consisted of whole bones which were split longitudinally. Samples taken from the femur and rib shafts (which were cut transversely from about the middle of the bone) and from the femur head each weighed about 1–2 g. The samples were extracted in a Soxhlet extractor first for 12 hr. with ethanol and then for 6 hr. with ether. They were dried in an oven at 100° to constant weight and then ashed at 600° for about 18 hr. The ash was dissolved in 2N-HCl, the solution made up to a suitable volume and a 5 ml. sample containing about 0·05 mg. of magnesium added to a 15 ml. conical centrifuge tube. With methyl orange as an indicator saturated sodium acetate was added dropwise to give a pH of about 4–5. Calcium was then precipitated as the oxalate and titrated with permanganate; magnesium was precipitated as magnesium ammonium phosphate and determined colorimetrically. The method was that of Green & Allcroft as described by Godden (1937). The accurate determination, by chemical means, of magnesium in the presence of a large excess of calcium is difficult. We found that with the above method