slices, appear for both male and female animals. Glover *et al.* (1952) found that after administration of 7-dehydrocholesterol to guinea pigs, the substance had a 'transient existence' in the liver. Tuboi (1948), perfused rabbit liver with ergosterol and 7-dehydrocholesterol, when these substances disappeared, and gave rise to cholic and deoxycholic acids in each case. These acids do not show selective absorption in the region of 290 mμ., so that the maximum at 290 mμ. may be due to an intermediate in the conversion of ergosterol into the acids.

SUMMARY

1. The use of propylene glycol, Tween 80 and lecithin for producing aqueous solutions of ergosterol has been investigated.

2. The effect of the above substances on the anaerobic glycolysis rate of rat-liver slices has been studied: 0·01 % (v/v) Tween 80 and 1 % propylene glycol show appreciable inhibition of glycolysis, whereas lecithin up to 0·36 % has no effect.

3. Solutions of ergosterol and ergocalciferol in aqueous lecithin have been used to investigate the effect of these sterols on the anaerobic glycolysis rate of rat-liver slices; there is increasing inhibition of glycolysis with concentration, for the range 10^{-3}–10^{2} μg./ml., ergosterol having a greater effect on male tissue, and ergocalciferol a greater effect on female tissue.

4. The spectra of aqueous ergosterol and ergocalciferol solutions have been investigated, after incubation with rat-liver slices.

Thanks are due to Professor J. Gillman for kindly providing laboratory facilities, to Mr O. H. Callaghan, for assistance during part of the experimental work, and to Dr J. Glover of Liverpool University for assistance with the manuscript.

Acknowledgement is also due to Dr T. F. Macrae and Dr J. C. Cain of Glaxo Laboratories for generous gifts of ergosterol and ergocalciferol.

REFERENCES

Burris, R. H., in Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, p. 44 *et seq.* 2nd ed. Burgess: Minneapolis.

Chiu, C. Y. (1950). *Biochem. J.* **46**, 120.

Dirscherl, W., Bergmeyer, H.-U. & Breuer, H. (1952). *Biochem. Z.* **322**, 245.

Dirscherl, W. & Hauptmann, K. H. (1950). *Biochem. Z.* **320**, 199.

Dirscherl, W. & Knüchel, W. (1950). *Biochem. Z.* **320**, 228.

Festenstein, G. N. (1951). Ph.D. Thesis, University of Liverpool.

Feyel-Cabanes, T. (1949). *C.R. Soc. Biol., Paris*, **143**, 1454.

Glover, M. (1949). Ph.D. Thesis, University of Liverpool.

Glover, M., Glover, J. & Morton, R. A. (1952). *Biochem. J.* **51**, 1.

Hanahan, D. J. & Wakil, S. J. (1953). *Arch. Biochem. Biophys.* **44**, 150.

Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.

Levrat, M. & Brette, R. (1951). *Pr. méd.* **59**, 1454.

Morton, R. A. & Rosen, D. G. (1949). *Biochem. J.* **45**, 612.

Nicolaysen, R. & Eeg-Larsen, N. (1953). *Vitam. & Horm.* **11**, 29.

Pangborn, M. C. (1951). *J. biol. Chem.* **188**, 471.

Paschkis, K. E. & Rakoff, A. E. (1950). *Recent Progr. Hormone Res.* **5**, 118.

Pincussen, L. (1941). *J. Chem.* **140**, ci.

Simonsen, D. H. & van Wagtendonk, W. J. (1947). *J. biol. Chem.* **170**, 239.

Stadie, W. C. & Riggs, B. C. (1944). *J. biol. Chem.* **16**, 187.

Szego, C. M. (1952). *Endocrinology*, **50**, 429.

Tuboi, S. (1948). *Biochem. J., Tokyo*, **20**, 139.

Zetterström, R. (1951). *Acta chem. scand.* **5**, 343.

Zetterström, R. & Ljunggren, M. (1951). *Acta chem. scand.* **5**, 283.

Purification and Properties of the Amine Oxidase of Pea Seedlings

By P. J. G. MANN

Biochemistry Department, Rothamsted Experimental Station, Harpenden Hertfordshire

(*Received* 6 *September* 1954)

Researches extending over the past twenty-six years have established the presence in animal tissues of two oxidases, or groups of oxidases, which catalyse the oxidation of amines. Amine oxidase, or monoamine oxidase, catalyses the oxidation of aliphatic monoamines, of phenylalkylamines and of diamines of the general formula $NH_2(CH_2)_nNH_2$ with a long hydrocarbon chain. Thus Blaschko & Hawkins (1950) reported that such diamines were oxidized by amine oxidase when $n > 6$. The relative rates of oxidation of different substrates by monoamine oxidase vary with the source of the enzyme. It is not yet clear how far this is due to variations in the specificity of the enzyme from different sources or to the presence of a mixture of enzymes. Werle & Roewer (1952) have advanced evidence for the existence in both animal and plant tissues of distinct enzymes catalysing the oxidation of aliphatic monoamines and phenylalkylamines respectively. Diamine oxidase catalyses the oxidation of diamines

of the general formula $NH_2(CH_2)_nNH_2$, where $n = 2$–8 (Zeller, 1938; Blaschko & Duthie, 1945), and of certain substituted diamines such as histamine and agmatine. As with monoamine oxidase the relative rates of oxidation of different substrates by diamine oxidase vary with the source of the enzyme. The two groups of oxidases have hitherto been differentiated not only by their differences in substrate specificity but also by the effects of inhibitors. Diamine oxidase, in contrast to monoamine oxidase, is inhibited by cyanide and by carbonyl reagents. Hirsch (1953) described an amine oxidase of sheep and ox serum, spermine oxidase, which does not fall into either of these groups. It catalyses the oxidation of spermine and spermidine but not that of 1:4-diaminobutane or of histamine, typical substrates of diamine oxidase. Like diamine oxidase it is inhibited by cyanide and carbonyl reagents. The results of Tabor, Tabor & Rosenthal (1945), with a purified preparation of the enzyme from ox plasma, suggest that, apart from the oxidation of spermine and spermidine, the enzyme is more closely related to monoamine oxidase than diamine oxidase in substrate specificity. Thus the oxidation of both aliphatic monoamines and phenylalkylamines is catalysed by the enzyme though the rate of oxidation of tyramine, a typical substrate of monoamine oxidase, is slow. Also, like the monoamine oxidase, it catalyses the oxidation of the long-chain diamine, 1:10-diaminodecane.

The enzymes of higher plants which catalyse the oxidation of amines have, until recent years, received little attention. Cromwell (1943) showed the presence in *Atropa belladonna* of an enzyme catalysing the oxidation of 1:4-diaminobutane (putrescine), and Werle & Raub (1948) found a histaminase in a number of plant families, especially in leguminosae. Werle & Pechmann (1949) found that this enzyme also attacks 1:4-diaminobutane and 1:5-diaminopentane and claimed that it corresponds to the diamine oxidase of animal tissues. Like the latter enzyme it is inhibited by cyanide and semicarbazide. A plant enzyme differing in distribution from the plant diamine oxidase and catalysing the oxidation of aliphatic monoamines and phenylalkylamines was described by Werle & Roewer (1950). Like animal monoamine oxidase this enzyme is not inhibited by cyanide or semicarbazide.

Kenten & Mann (1952) found that extracts of pea seedlings (*Pisum sativum* L.), and partially purified enzyme preparations made therefrom, catalysed the oxidation not only of aliphatic diamines but also of phenylalkylamines. The oxidation of the phenylalkylamines was apparently not due to the presence of the monoamine oxidase of Werle & Roewer (1950) since, like that of the diamines, it was inhibited by cyanide and semicarbazide. The aliphatic monoamines tested were not readily attacked but some increase in oxygen uptake was obtained with ethanolamine, ethylamine, allylamine, and *n*-heptylamine. Of the amino acids tested, L-lysine caused a small but definite increase in the oxygen uptake of the extracts. The evidence, though not conclusive, suggested that the oxidation of the mono- and diamines is catalysed by the same enzyme plant amine oxidase.

The present work was undertaken when it was found that under conditions of pH different from those used by Kenten & Mann (1952), L-lysine was more readily oxidized by the pea-seedling extracts and enzyme preparations. A method is described by which a 300-fold purification of the amine oxidase can be achieved. The purified preparations catalysed the oxidation of diamines and phenylalkylamines and also, though much less readily, that of L- and of D-lysine and of aliphatic monoamines.

MATERIALS AND METHODS

Amine oxidase. The amine oxidase preparations were obtained from pea seedlings grown as described by Kenten & Mann (1952). There were three main steps in the method. The first consisted in the removal of much inactive material by treatment of the seedling extracts with the reagent of Tsuchihashi (1923). This was followed by fractional precipitation with $(NH_4)_2SO_4$. In this stage much of the peroxidase present was separated from the amine oxidase and a pea seedling peroxidase fraction was obtained. Lastly, after dialysis, the amine oxidase was repeatedly precipitated at pH 5, a method used by Tabor (1951) in the preparation of animal diamine oxidase.

Pea seedlings (7–12 days old) were washed free from soil and any obviously diseased material was removed. The weighed seedlings (1000 g.) were minced in a chilled domestic meat mincer and squeezed through strong cotton cloth. The residual pulp was mixed with 500 ml. 0·067 M phosphate buffer, pH 7, macerated for 2 min. in a Townson & Mercer (Croydon) macerator, and again squeezed through cloth. The combined extracts were cooled in an ice–salt freezing mixture to 0–5°, and a mixture of ethanol (200 ml.) and chloroform (100 ml.), previously cooled to −10°, was added slowly during vigorous mechanical stirring. The stirring was continued for 30 min. and the mixture was then centrifuged. The almost clear yellow supernatant was poured off from the bulky white precipitate and from the bottom layer of chloroform and again cooled. It was then treated with $(NH_4)_2SO_4$ (45 g./100 ml.). Under these conditions the amine oxidase was precipitated while the bulk of the peroxidase remained in solution. On centrifuging, the precipitate formed a hard cake on the surface of the liquid. A broad spatula was inserted under the precipitate to support it while the liquid was poured off. The liquid (*A*) was retained for the preparation of the peroxidase fraction. The precipitate was ground to a smooth paste in a mortar and 500 ml. 0·02 M phosphate buffer, pH 7, was slowly added to give a smooth suspension which was stirred mechanically for 2 hr. at room temperature and stored overnight at 0–2°.

It was then centrifuged and the bulky precipitate was washed on the centrifuge with a further small amount of 0·02M phosphate buffer pH 7. The extracts were combined and cooled in ice. The small precipitate obtained by the addition of 18 g. $(NH_4)_2SO_4$/100 ml. extract was centrifuged off and discarded. To the cooled supernatant a further 18 g. $(NH_4)_2SO_4$/100 ml. was added. The precipitate, which contained the amine oxidase, was collected by centrifuging and suspended in 20 ml. of 0·2M phosphate buffer, pH 7. The suspension was dialysed for several hours against running tap water and then overnight at 0–2° against 2 l. of 0·005M phosphate buffer, pH 7. The dialysed suspension was centrifuged and the cooled supernatant brought to pH 5 by dropwise addition of 0·05N acetic acid. The resultant suspension was stored at 0–2° for several hours until the precipitate flocculated. The precipitate was collected by centrifuging, triturated with 20 ml. of water and brought into solution by adjusting to pH 7 with 0·05N-KOH. The precipitation at pH 5 was repeated twice. The final solution at pH 7 was centrifuged clear and stored at −10°. Preparations stored in this way showed little loss of activity over several months. Little or no loss of activity occurred during dialysis against distilled water or 0·005M phosphate buffer, pH 7 even when this was prolonged over several days. During prolonged dialysis against distilled water the enzyme tended to precipitate but the precipitate redissolved on addition of phosphate buffer.

A unit of amine oxidase is defined as the amount which at 28° gives an O_2 uptake of 1 μl./hr. (calculated from initial rates) in presence of 0·01M 1:4-diaminobutane, catalase, and 0·067M phosphate buffer at pH 7, in a total volume of 3 ml. The degree of purity of the enzyme is given by the specific activity which equals the number of oxidase units present per mg. N of the preparation.

Peroxidase. Peroxidase preparations of 40–60% purity were obtained from horseradish by following the earlier stages of the method of Kenten & Mann (1954). In addition, peroxidase preparations were made from pea seedlings using fraction (*A*) obtained during the preparation of amine oxidase. The purpurogallinzahl (P.Z.) (Willstätter & Stoll, 1918) of the horseradish preparations, i.e. mg. purpurogallin formed by 1 mg. of enzyme preparation in 5 min. from pyrogallol and H_2O_2 under fixed conditions was estimated by the method of Keilin & Hartree (1951) using an EEL (Evans Electroselenium Ltd.) colorimeter with Ilford Bright Spectrum Blue Filter 622. The preparations of horseradish peroxidase used had P.Z. = 450–750. A peroxidase unit (P.G.) is defined as the amount forming 1 mg. purpurogallin under the conditions of the estimation.

Pea-seedling peroxidase fraction. Experimental details of one preparation are given here. 700 g. of pea seedlings (7 days old) were used for the amine oxidase preparation. The initial extract contained 58000 P.G. units. Fraction *A* was concentrated by distillation under reduced pressure to about 75% of its original volume to remove the ethanol and chloroform. It was then saturated with $(NH_4)_2SO_4$. The precipitate was separated by centrifuging and suspended in 25 ml. of 0·2M phosphate buffer, pH 7. The suspension was dialysed for several hours against running tap water and then overnight in the refrigerator against 2 l. of 0·005M phosphate buffer, pH 7. The precipitate was removed by centrifuging. The clear supernatant liquid contained 26000 P.G. units.

Catalase. Catalase was prepared from human erythrocytes by the method of Bonnichsen (1947) and from ox liver by the method of Sumner & Dounce (1937). The activity of the preparations was estimated as described previously (Kenten & Mann, 1952). The Katalasefähigkeit (Kat.f.) values found were 8000–19 000. Two of the preparations (Kat.f. 19 000) were crystalline. Using the conversion factor of Chance & Herbert (1950) that Kat.f. $= 520\, k_1/M$, where M is the molecular weight of catalase (230 000), $k_1 = 3{\cdot}5 \times 10^6$ M^{-1} sec.$^{-1}$ to $8{\cdot}3 \times 10^6$ M^{-1} sec.$^{-1}$. In experiments where catalase was used the amount was 50 μg. of the crystalline preparations (Kat.f. 19 000) or equivalent amounts of the preparations of lower activity/3 ml. reaction mixtures.

Compounds. D-Lysine hydrochloride was obtained from Dr J. P. Greenstein. L-Diaminobutyric acid hydrochloride and DL-diaminopropionic acid hydrochloride were prepared by Dr S. Wilkinson. L-Hydroxylysine monohydrochloride was obtained from Dr J. G. Heathcote and DL-hydroxylysine monohydrochloride from L. Light and Co. Ltd.

Buffers. Phosphate buffers (0·2M) were prepared from solutions of KH_2PO_4 and KOH. Pyrophosphate buffers (0·2M) were prepared from solutions of $Na_2H_2P_2O_7$ and $K_4P_2O_7$.

Manometric methods. Measurements of O_2 uptake were made in air, except where otherwise stated, in the Warburg apparatus at 28°. The volume of the reaction mixtures was 3 ml., and 0·2 ml. 5N-KOH was present in the centre cups. With the pea-seedling extracts corrections for the endogenous O_2 uptakes were necessary and where experiments were done at two different pH values control measurements had to be made at both pH values. In general, no O_2 uptake was observed with the purified amine oxidase in absence of substrate and where an uptake was observed it was always too small to necessitate any correction.

EXPERIMENTAL AND RESULTS

General properties of the enzyme preparations

Activity. Table 1 shows the degree of purification obtained for six preparations. In previous attempts to purify plant amine oxidase Werle & Pechmann (1949) effected a sixfold purification of the enzyme of red clover seedlings, and the preparations of the pea-seedling enzyme obtained by Kenten & Mann (1952) were of a similar degree of purity. Tabor (1951) achieved a 660-fold purification of animal diamine oxidase to obtain a preparation of specific activity (based on mg. protein) of 670. Assuming an N content of 16% this gives a specific activity (based on mg. N) of 4200. The average specific activity of the preparations of Table 1 was 100000. The higher activity of the plant enzyme preparations was due, in part, to the fact that the pea-seedling extracts were much more active than the extracts of acetone-dried pig kidneys used for the preparation of the animal enzyme. The average specific activity of the pea-seedling extracts of Table 1 was 404, whereas that of the kidney extract was 6·4.

Specificity. The catalytic activity of the amine oxidase preparations towards a number of different substrates was tested manometrically in 0·067M phosphate buffers at pH 7 and 8·5 using a substrate

Table 1. *Purification of the amine oxidase of pea seedlings*

Activity measurements were made manometrically using 3 ml. total volume, containing 0·25 ml. seedling extract, or the amount of amine oxidase obtained from this volume of extract, in 0·067 M phosphate buffer, pH 7; they were based on the O_2 uptakes in the first 10 min. in presence of 0·01 M 1:4-diaminobutane. With the amine oxidase, but not with the extracts, catalase was added to the reaction mixtures. In experiments with the extracts the O_2 uptakes of control reaction mixtures without added substrate were subtracted. The substrate was added from the side arms. Gas phase, air; temp. 28°.

Preparation no.	Wet wt. of seedlings (g.)	Total N (mg.)		Total amine oxidase (mega-units)		Yield (%)	Specific activity		Purification Final sp. act. / Initial sp. act.
		Initial extract	Final preparation	Initial	Final		Initial	Final	
1	700	5390	9·2	1·98	0·940	47	365	102 000	279
2	400	2540	7·5	1·28	0·696	54	505	93 000	185
3	800	6600	9·6	2·64	1·22	46	400	127 000	318
4	680	4170	6·0	1·46	0·610	42	350	102 000	291
5	1080	7340	6·1	2·42	0·479	20	330	79 000	240
6	1500	7680	17·4	3·65	1·73	47	475	99 000	208

Table 2. *Comparison of the activity towards various substrates of a pea-seedling extract and the amine oxidase preparation made from it. The effect of horseradish peroxidase and a pea-seedling peroxidase fraction on the activity of the amine oxidase*

Reaction mixtures consisted of 0·5 ml. extract (0·25 ml. with 1:4-diaminobutane as substrate), or amounts of amine oxidase obtained from these volumes of extract, in 0·067 M phosphate buffer, pH 7 or 8·5. Substrate concentration 0·01 M. With the amine oxidase, but not with the extract, catalase was added to the reaction mixtures. Peroxidase, where added, was in amount equal to that in 0·5 ml. of the pea-seedling extract (45 P.G. units). In experiments with extracts the O_2 uptakes of control reaction mixtures without substrate were subtracted. These uptakes were 23 μl. O_2 at pH 7 and 13 μl. O_2 at pH 8·5. Conditions similar to those in Table 1.

Substrate	pH	Extract (μl. O_2/ 20 min.)	Amine oxidase (μl. O_2/ 20 min.)	% of original activity	Amine oxidase + horseradish peroxide (μl. O_2/ 20 min.)	% of original activity	Amine oxidase + pea-seedling peroxidase (μl. O_2/ 20 min.)	% of original activity
Ethylamine	7·0	24	4	17	2	9	6	25
Ethanolamine	7·0	24	5	21	5	21	5	21
1:4-Diaminobutane	7·0	176	86	49	84	48	90	51
Histamine	7·0	75	13	17	27	36	24	32
β-Phenylethylamine	8·5	152	44	29	85	56	86	57
Tryptamine	8·5	36	13	36	20	56	22	61
L-Lysine	8·5	24	8	33	18	75	20	83

concentration of 0·01 M. The tests were made at these two pH values so that the reactions might be studied in the region of their pH optima according to the results of Kenten & Mann (1952) and of those obtained in the present work. Of the diamines tested, 1:4-diaminobutane and 1:5-diaminopentane were oxidized more readily than 1:6-diaminohexane and 1:10-diaminodecane; 1:3-diaminopropane was not oxidized. Histamine, agmatine, and spermine were all oxidized. Of the phenylalkylamines tested β-phenylethylamine was oxidized more rapidly than tyramine and tryptamine. The rate of oxidation of the aliphatic amines ethylamine and butylamine was slow in comparison with that of β-phenylethylamine as was also that of L-lysine and ethanolamine. The relative rates of oxidation of some of these substrates by a pea-seedling extract and the amine oxidase preparation obtained from it are shown in Table 2. The measurements of O_2 uptake were made over a 20 min. period since, as shown by Kenten & Mann (1952), there is a short lag period with some of the substrates. The amount of extract used in each vessel was 0·5 ml. except where 1:4-diaminobutane was the substrate when, owing to the rapidity with which this substrate was oxidized, the amount of extract used was reduced to 0·25 ml. The results show that while 49% of the activity of the original extract towards 1:4-diaminobutane was recovered in the amine oxidase preparation, only 17–36% of the original activity towards the other substrates tested was recovered. While the values for percentage recovery of activity showed considerable variations with several other preparations, this value was always much greater for 1:4-diaminobutane than for the other substrates.

The effect of peroxidase. The pea-seedling extracts had a high peroxidase activity. The amine oxidase preparations contained only traces of peroxidase. When enough peroxidase was added to amine oxidase preparations to give a mixture equal to that in pea-seedling extracts the rate of O_2 uptake was increased, with histamine, β-phenylethylamine, tryptamine or L-lysine as substrate, to about double that where the oxidations were catalysed by amine oxidase alone. Both horseradish peroxidase and the pea-seedling peroxidase fraction produced this effect (Table 2). Such additions of peroxidase caused no increase in the rate of O_2 uptake during the 20 min. period with ethylamine, ethanolamine, or 1:4-diaminobutane as substrates. With 1:4-diaminobutane, however, a small increase in the final total O_2 uptake was produced and an increase in the rate could be clearly demonstrated by the use of more peroxidase. This effect of peroxidase has not yet been systematically investigated. Such results as have been obtained suggest that peroxidase catalyses the further oxidation of the products formed by the action of the amine oxidase. Thus, with some substrates, after completion of the amine-oxidase-catalysed reaction the addition of peroxidase produced a further O_2 uptake. This occurred although the primary reactions had been completed in presence of catalase; the effect therefore was not dependent on the H_2O_2 formed in the primary reaction. With some substrates heat-treated peroxidase (15 min. in a boiling-water bath), haemin or traces of Mn^{2+} also increased the O_2 uptake. Similar effects of peroxidase on the oxidation of histamine and 1:5-diaminopentane and of haemin on the oxidation of histamine in animal diamine-oxidase-catalysed reactions have been observed by Swedin (1944) and Laskowski (1942) respectively. Kenten & Mann (1952) showed that phenylacetaldehyde was formed by the action of plant amine oxidase on β-phenylethylamine. Kenten (1953) showed that peroxidase preparations catalysed the oxidation of phenylacetaldehyde and that the rate of this oxidation was increased by addition of Mn^{2+}. In the present work it was found that, while either peroxidase or traces of Mn^{2+} increased the rate of oxidation and total O_2 uptake when added to reaction mixtures of plant amine oxidase and β-phenylethylamine, the effect produced by peroxidase and Mn^{2+} added together was not greater than the sum of their separate effects.

The results of Table 2 suggest that one enzyme of wide specificity catalyses the oxidation of the diamines, phenylalkylamines, and of L-lysine. Studies on the effect of inhibitors and on the production of H_2O_2 during the oxidations show that the enzyme catalysing the oxidation of the aliphatic monoamines has similar properties to, and is probably identical with, that oxidizing the diamines, phenylalkylamines and L-lysine.

The effect of inhibitors. Purified preparations of the plant amine oxidase were inhibited by cyanide and by carbonyl reagents (Table 3). These experiments were carried out, in general, at pH 7·5 in absence of catalase. Since β-phenylethylamine and in particular L-lysine and ethanolamine were attacked much less readily than 1:4-diaminobutane it was necessary to use comparatively large amounts of the enzyme preparation with these substrates. This may explain why, in general, the oxidation of these compounds appeared less sensitive to the inhibitors than that of 1:4-diaminobutane. Sodium azide (10^{-2} M) produced little inhibition at pH 7·5. A greater, but still only partial, inhibition was produced at pH 6. With some enzyme preparations where ethanolamine or L-lysine were used as substrates an apparent activating effect of azide developed with time. It is suggested that this was due to partial inhibition by azide of the traces of catalase present in these enzyme preparations leading to an increased accumulation of the hydrogen

Table 3. *Effect of inhibitors*

Reaction mixtures consisted of amine oxidase, with and without inhibitor, in 0·067 M phosphate buffer pH 7·5. The substrate (0·3 ml. of 0·1 M) was added from the side arm; no catalase was added. KOH was omitted from the centre cups where cyanide was used. The inhibition was calculated from the O_2 uptakes in the first 20 min. after adding the substrate. Conditions similar to those in Table 1.

Inhibitor	Concentration (M)	Percentage inhibition with 1:4-Diamino-butane	L-Lysine	β-Phenyl-ethylamine	Ethanolamine
KCN	10^{-4}	27	0	0	0
	10^{-3}	90	0	25	0
	10^{-2}	100	79	100	92
Na azide	10^{-2}	8 (17)*	0	4	4
Semicarbazide	10^{-6}	31	23	13	17
	10^{-5}	98	94	70	87
Na diethyldithiocarbamate	10^{-4}	46	56	53	37
	10^{-3}	95	98	92	94
K ethyl xanthate	10^{-4}	44	10	24	21
	10^{-3}	94	37	58	63
Salicylaldoxime	10^{-3}	53	—	7	—
	10^{-2}	94	—	73	—

* At pH 6.

peroxide formed in the primary reaction. A fact which does not appear to have been reported previously is that the enzyme is inhibited by sodium diethyldithiocarbamate, potassium ethyl xanthate and salicylaldoxime, which are usually considered as specific inhibitors of copper-containing enzymes. The oxidation of all four substrates was inhibited by these compounds, though with salicylaldoxime the inhibition with L-lysine or ethanolamine as substrates varied to such an extent, according to the enzyme preparation used, that the results are not recorded in Table 3. Further investigations showed that the erratic results were due to the oxidation of the salicylaldoxime by H_2O_2 formed in the primary reaction. This oxidation is catalysed by traces of peroxidase in the enzyme preparations. Results demonstrating this oxidation are included in the section on H_2O_2 formation. Thiourea (10^{-2} M) and phenylthiourea ($0·8 \times 10^{-2}$ M) which are also frequently used as inhibitors of copper-containing enzymes caused little or no inhibition of any of the oxidations. In some cases an apparent activating effect developed with time when these compounds were used. This apparent activation, like that with salicylaldoxime, depends on the H_2O_2 formed in the primary reaction and is dealt with in the next section.

Hydrogen peroxide formation. Kenten & Mann (1952) proved that H_2O_2 was formed during the oxidation of 1:4-diaminobutane and β-phenylethylamine catalysed by plant amine oxidase by showing that in absence of catalase the total O_2 uptake reached 1 mol. O_2/mol. amine instead of 0·5 mol. O_2/mol. amine in presence of catalase. The increased O_2 uptake in absence of catalase was due to the accumulation of H_2O_2 since on adding catalase at the end of the reaction a rapid evolution of O_2 took place, leading to a final O_2 uptake of 0·5 mol. O_2/mol. amine. A similar method was used by Tabor (1951) to demonstrate H_2O_2 formation in oxidations catalysed by animal diamine oxidase. In the experiments with animal diamine oxidase the enzyme was inactivated during the reaction in absence of catalase. Werle & Pechmann (1949), while studying the oxidation of diamines by clover-seedling extracts, found that the catalytic activity of the extracts decreased during the oxidation, particularly when a high concentration of substrate was used. It was concluded that the amine oxidase was inactivated by H_2O_2 formed during the oxidations. With the purified amine oxidase preparations used in the present work it was found that with 1:4-diaminobutane, 1:5-diaminopentane, ethanolamine, or L-lysine as substrates the initial rate of oxidation in absence of catalase was about double that in its presence. The rate of oxidation decreased much more rapidly in absence of catalase and the final O_2 uptake was often greater with catalase present, particularly where a high concentration of substrate was used. The results of one such experiment with 1:5-diaminopentane as substrate are given in Fig. 1. In presence of catalase the oxidation proceeded to completion with an uptake of 0·5 mol. O_2/mol. amine. In the absence of added catalase the uptake almost stopped when its total was only about 70 % of that in presence of catalase. This was due to inactivation of the enzyme since, after decomposition of the accumulated H_2O_2 with catalase, a fresh addition of 1:5-diaminopentane produced little further O_2 uptake. A fresh addition of 1:5-diamino-

pentane to the reaction mixture in which catalase was initially present caused a renewed O_2 uptake though the rate of this was slow in comparison with the initial rate. These results are in agreement with the conclusions of Werle & Pechmann (1949) that the enzyme is inactivated by H_2O_2. This was tested in experiments in which the enzyme was incubated with H_2O_2 before addition of the substrate. The results of one such experiment are illustrated in Fig. 2. In this experiment samples of the enzyme in phosphate buffer at pH 7 were incubated for 30 min. at 28° in presence and absence of 0·1 ml. of 0·1 M-H_2O_2. Catalase was then added from the side arms of both vessels. When all the H_2O_2 (where present) was decomposed, 0·1 ml. of 0·1 M 1:4-diaminobutane was added from the second side arm of each vessel. The subsequent rates of O_2 uptake were about the same with both reaction mixtures showing that H_2O_2 does not inactivate the enzyme under these conditions. As a result of further investigations it was found that the enzyme in presence of its substrates, is inactivated by H_2O_2 as shown by the results illustrated in Fig. 3. In this experiment four samples of enzyme were incubated in phosphate buffer at pH 7 in Warburg vessels filled with N_2 and with yellow phosphorus in the centre cups. Two of the vessels contained in addition 0·1 ml. of 0·1 M-H_2O_2. After equilibration 0·1 ml. of 0·01 M 1:4-diaminobutane was added from the side

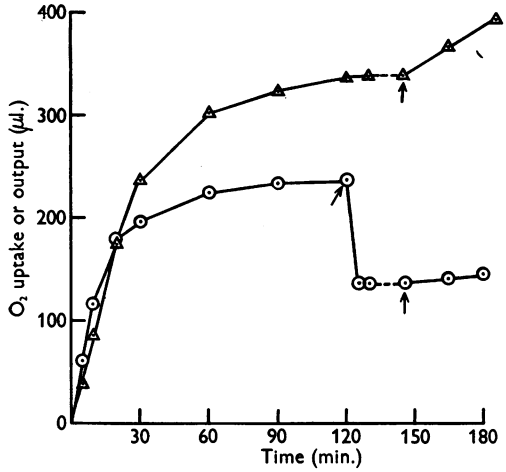

Fig. 1. Inactivation of amine oxidase during oxidation of 1:5-diaminopentane in absence of catalase. Amine oxidase in 0·067 M phosphate buffer at pH 7 with 0·3 ml. of 0·1 M 1:5-diaminopentane. △—△, catalase present initially; ⊙—⊙, catalase added at time shown by first arrow. A further 0·3 ml. of 0·1 M 1:5-diaminopentane was added to both reaction mixtures at the times shown by the second arrows. The total volume of each reaction mixture, before the second addition of substrate, was 3 ml. The substrate was added from the side arms. Gas phase, air; temp. 28°.

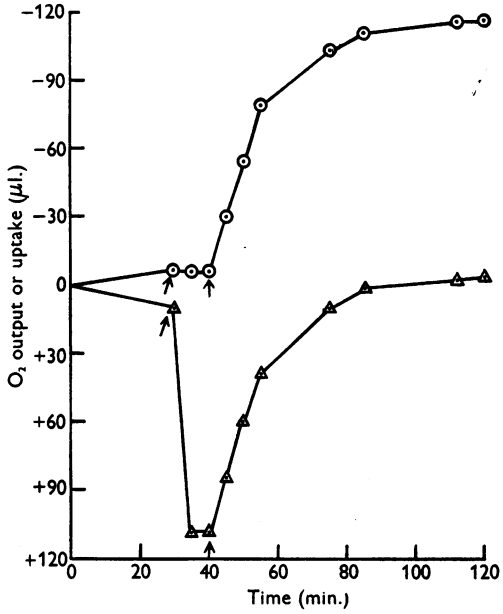

Fig. 2. Stability of amine oxidase in presence of hydrogen peroxide. Amine oxidase in 0·067 M phosphate buffer at pH 7. ⊙—⊙, in absence of hydrogen peroxide; △—△, in presence of 0·1 ml. of 0·1 M hydrogen peroxide. Catalase was added to both reaction mixtures at the time shown by the first arrows. Substrate (0·1 ml. of 0·1 M 1:4-diaminobutane) was added at the time shown by the second arrows. Conditions similar to those in Fig. 1.

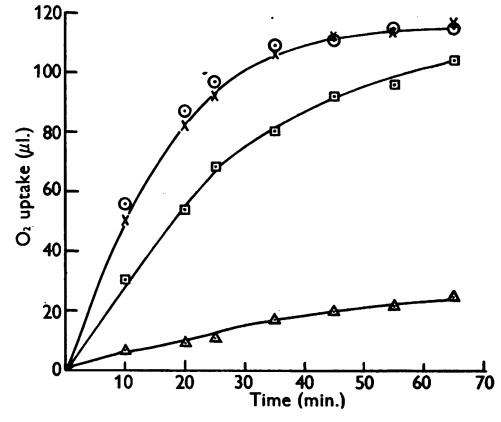

Fig. 3. Inactivation of amine oxidase in presence of hydrogen peroxide and substrate. Amine oxidase in 0·067 M phosphate buffer pH 7 was first incubated for 30 min. in N_2. ⊙—⊙, with no further addition; ×—×, with 0·1 ml. of 0·01 M 1:4-diaminobutane; ⊡—⊡, with 0·1 ml. of 0·1 M hydrogen peroxide; △—△, with 0·1 ml. of 0·01 M 1:4-diaminobutane and 0·1 ml. of 0·1 M hydrogen peroxide. After incubation for 30 min. catalase was added to each vessel, the N_2 was replaced by air and 0·1 ml. of 0·1 M 1:4-diaminobutane was added from the side arms. The curves show the subsequent O_2 uptakes under conditions similar to those in Fig. 1.

arms of one of each of the two pairs of vessels. After 30 min. incubation catalase was added to all the reaction mixtures from the second side arms. When the H_2O_2 (where present) had all been decomposed the yellow phosphorus was removed from the centre cups and the vessels were filled with air. After equilibration, 0·1 ml. of 0·1 M 1:4-diaminobutane was added to each reaction mixture. The subsequent O_2 uptakes showed that the enzyme had been almost completely inactivated by the previous incubation with 1:4-diaminobutane and H_2O_2 together. Previous incubation with 1:4-diaminobutane alone or H_2O_2 alone had comparatively little inactivating effect. In further experiments of this type a partial inactivation of the enzyme was produced with concentrations of H_2O_2 as low as $3{\cdot}3 \times 10^{-5}$ M. It is suggested that H_2O_2 attacks the reduced form of the enzyme produced by interaction with the substrate.

On the basis of these results on the formation of H_2O_2 and its inhibiting effect it is possible to explain the activating effect of thiourea and the erratic results with salicylaldoxime described in the section on inhibitors. An example of the apparent activating effect of thiourea is illustrated in Fig. 4. In this experiment 0·3 ml. of 0·1 M 1:4-diaminobutane was used as substrate in absence of catalase. The theoretical O_2 uptake was 672 μl. In absence of thiourea the O_2 uptake stopped at 95 μl. owing to inactivation of the enzyme by the accumulated H_2O_2. In presence of thiourea, at this point, the O_2 uptake which had not completely stopped had reached 158 μl. The apparent activation by thiourea is, therefore, due to partial protection of the enzyme from inactivation. When catalase was added from the side arms the presence of H_2O_2 was demonstrated in the reaction mixture without thiourea but not in that with thiourea present. Thiourea does not prevent the decomposition of H_2O_2 by catalase under these conditions. Similar results have been obtained with phenylthiourea. It is known that thiourea is oxidized by H_2O_2 in neutral solution to thiourea dioxide (Barnett, 1910; Boëseken, 1936*a*, *b*). Thiourea therefore protects the enzyme by removing H_2O_2.

The erratic results obtained with salicylaldoxime as inhibitor were also due to the oxidation of salicylaldoxime by H_2O_2 formed in the primary reaction but in this case the oxidation, unlike that of thiourea, was catalysed by traces of peroxidase. This is shown by the results illustrated in Fig. 5. Four reaction mixtures were used containing amine oxidase, or amine oxidase together with peroxidase, and catalase in 0·067 M phosphate buffer at pH 7 in presence and absence of 0·01 M salicylaldoxime. The substrate (0·1 ml. of 0·1 M 1:4-diaminobutane) was added from the side arms. A comparatively large amount of amine oxidase was used. Under these conditions the inhibition produced by the salicylaldoxime was only partial and the 1:4-diaminobutane was almost all oxidized in the experimental

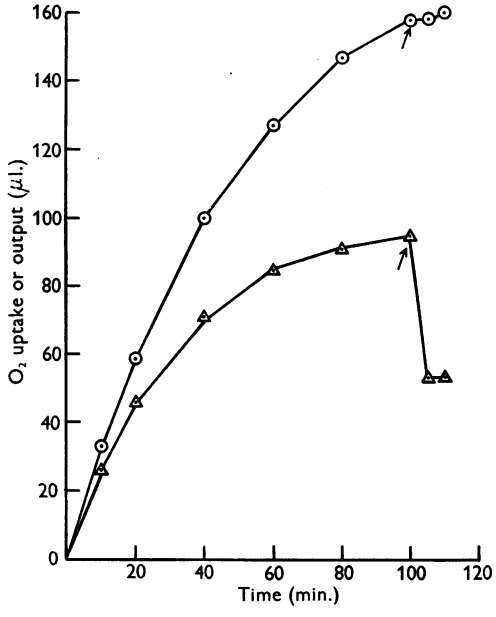

Fig. 4. The effect of thiourea in preventing inactivation of amine oxidase by hydrogen peroxide. Amine oxidase in 0·067 M phosphate buffer, pH 7, with 0·3 ml. of 0·1 M 1:4-diaminobutane. ⊙—⊙, 0·3 ml. of 0·1 M thiourea; △—△, no thiourea. Catalase was added at the times shown by the arrows. Conditions similar to those in Fig. 1.

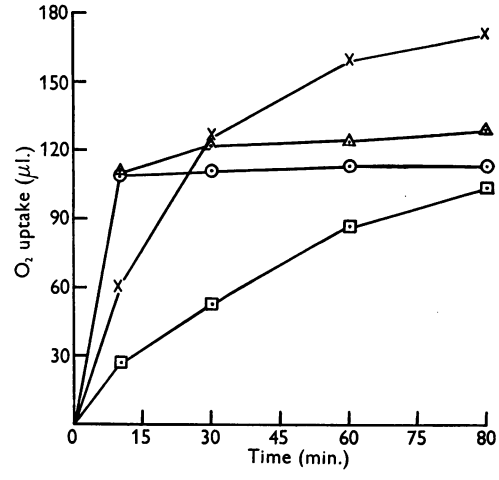

Fig. 5. Oxidation of salicylaldoxime by the amine oxidase system coupled with horseradish peroxidase. Amine oxidase and catalase in 0·067 M phosphate buffer, pH 7, with 0·1 ml. of 0·1 M 1:4-diaminobutane. ⊙—⊙, no further addition; △—△, peroxidase (76 P.G. units) present; ⊡—⊡, 0·6 ml. of 0·05 M salicylaldoxime; × — ×, peroxidase and salicylaldoxime present. Conditions similar to those in Fig. 1.

time though much more slowly than in absence of inhibitor. Where peroxidase was present in addition to inhibitor the final O_2 uptake was much greater than that required for the amine-oxidase-catalysed oxidation of the 1:4-diaminobutane and the reaction mixture was strongly yellow at the end of the experiment. Heat-treated peroxidase (30 min. at 100°) produced a much smaller effect.

Effect of oxygen tension. Werle & Pechmann (1949) found that the oxidation of 1:5-diaminopentane, catalysed by clover-seedling extract, was much more rapid in O_2 than in air. In the present work with purified preparations of amine oxidase when the amount of enzyme used was such as to give rates of O_2 uptake of up to 300 μl./hr. the rates were the same in air as in O_2. With larger amounts of enzyme giving rates of O_2 uptake in air, of about 1000 μl./hr. the uptake was much faster in O_2 than in air. But this is to be expected since at such rates the diffusion of O_2 into the reaction mixtures becomes the limiting factor (Umbreit, 1945). It is suggested that this factor and not a low affinity of this enzyme for O_2 may have been responsible for the results of Werle & Pechmann (1949).

Manometric studies of the oxidation of L-, D- *and* DL-*lysine*

Effect of pH. The variation of activity with pH was tested in 0·067 M phosphate buffers in presence of catalase with 0·01 M L-lysine as substrate. At reactions outside the buffering range of phosphate the initial pH was measured using separate reaction mixtures and the final pH with the experimental reaction mixtures. The greatest differences observed were less than 0·2 pH unit. The average of these initial and final pH values were used in plotting the pH curve (Fig. 6) which was based on the O_2 uptake over the first 20 min. The curve shows an optimum between pH 8·3 and 8·8 falling off steeply on both sides.

Effect of substrate concentration. The effect of substrate concentration on the rate of oxidation was tested with L-, D- and DL-lysine in 0·067 M phosphate buffer, pH 8·0, in presence of catalase (Fig. 7). D-lysine was only tested at low concentrations since the amount of this amino acid available was small. The rate of its oxidation was about 85% that of L-lysine. DL-Lysine was oxidized at a rate intermediate between that of L- and D-lysine. The highest concentrations of L- and DL-lysine used (0·066 M) were insufficient to saturate the enzyme. The affinity of the enzyme for these amino acids is, therefore, much lower than its affinity for 1:4-diaminobutane or β-phenylethylamine (Kenten & Mann, 1952).

Total oxygen uptake. The oxidation of 1:4-diaminobutane by the purified amine oxidase preparations in presence of catalase in phosphate buffer at pH 7 proceeded with the uptake of 0·5 mol. O_2/mol. 1:4-diaminobutane. With L- or D-lysine as substrates, however, in 0·067 M phosphate buffer at pH 8·5 the O_2 uptake continued slowly after a value of 0·5 mol. O_2/mol. substrate had been reached and, if sufficient enzyme preparation was used, reached double this value. Evidence has been obtained that this is due to the further oxidation of the primary oxidation products, formed by the action of amine oxidase, probably catalysed by traces of metals and of peroxidase in the enzyme preparation. When pyrophosphate was used in place of phosphate buffer this secondary oxidation

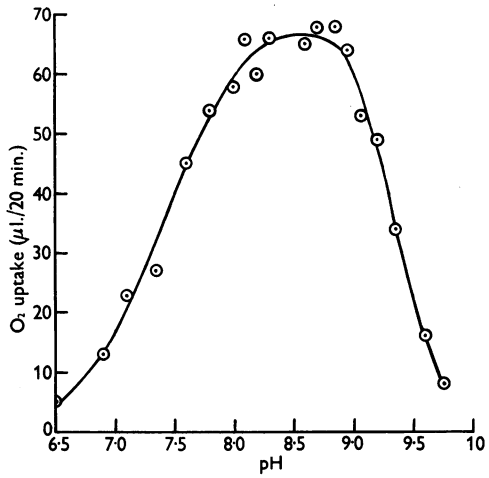

Fig. 6. Effect of pH on the rate of oxidation of L-lysine. Amine oxidase, catalase, and 0·01 M L-lysine in 0·067 M phosphate buffers. Conditions similar to those in Fig. 1.

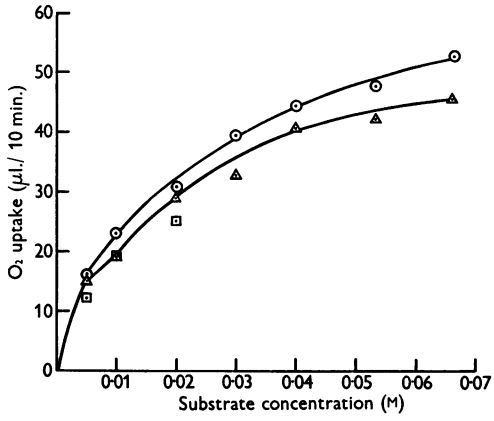

Fig. 7. Effect of concentration of L-, D- and DL-lysine on the rates of oxidation. Amine oxidase and catalase in 0·067 M phosphate buffer, pH 8. ⊙—⊙, L-lysine; △—△, DL-lysine; ⊡—⊡, D-lysine. Conditions similar to those in Fig. 1.

was partially inhibited and the rate of O_2 uptake became very slow once a value of 0·5 mol. O_2/mol. L-lysine had been reached. This is shown by the results illustrated in Fig. 8. In these experiments the oxidation of 0·3 ml. of 0·1 M L- or D-lysine was followed in 0·067 M phosphate and pyrophosphate buffers at pH 8·5 in presence of catalase. At the points marked by arrows peroxidase was added to the reaction mixtures from the side arms. An immediate rapid O_2 uptake took place which was much greater with the reaction mixtures in pyrophosphate than with those in phosphate, and caused in all cases a final O_2 uptake approaching 1 mol. O_2/mol. L- or D-lysine. It is suggested that pyrophosphate partially protects the primary oxidation products from further oxidation by impurities in the enzyme preparation.

Amino acids tested. DL-Diaminopropionic acid, L-diaminopropionic acid, L-ornithine, DL- and L-hydroxylysine, DL-α-aminocaproic acid (norleucine) and ε-aminocaproic acid were tested as substrates for plant amine oxidase at concentrations of 0·01 M in 0·067 phosphate buffers at pH 5·0, 6·0, 7·0, and 8·5 in presence of catalase. L-Hydroxylysine was only tested at pH 8·5 at a concentration of 0·001 M owing to the small amount of this amino acid available. With amounts of amine oxidase giving an O_2 uptake with L-lysine (0·01 M) of 100–200 μl. O_2/hr. no uptake was observed with any of these acids except L-ornithine. One sample of L-ornithine gave a slow rate of O_2 uptake under these conditions which was increased by increasing the concentration of L-ornithine. This observation could not be confirmed subsequently with different samples of L-ornithine and was therefore attributed to the presence of impurity.

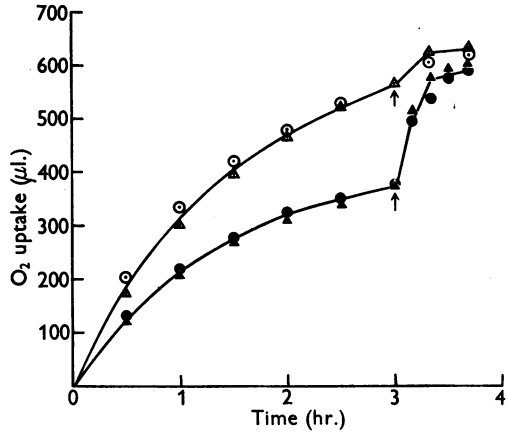

Fig. 8. Total oxygen uptake with L- and D-lysine (0·3 ml. of 0·1 M) in 0·067 M phosphate and pyrophosphate buffers, pH 8·5, in presence of catalase. The effect of horseradish peroxidase. ⊙—⊙, L-lysine in phosphate; △—△, D-lysine in phosphate; ●—●, L-lysine in pyrophosphate; ▲—▲, D-lysine in pyrophosphate. Peroxidase (76 P.G. units) was added at the times shown by the arrows. Conditions similar to those in Fig. 1.

DISCUSSION

The highly purified preparations described retain the property of catalysing the oxidation not only of aliphatic diamines but also that of phenylalkylamines, aliphatic monoamines, and of L- and D-lysine. The relative rates at which these substrates are attacked differ according to whether the purified enzyme preparations or the seedling extracts are used as catalysts. It has been shown that this is due, at least in part, to the fact that the oxidations with the pea-seedling extract are not confined to the primary reactions catalysed by the amine oxidase. During the purification of the enzyme the factors catalysing the secondary oxidations are largely removed. The oxidations studied are all accompanied by hydrogen peroxide formation and are similarly affected by the inhibitors tested. The evidence so far obtained suggests, therefore, that only one enzyme is involved which is less specific than animal diamine oxidase. While the aliphatic diamines are the substrates most readily attacked, it seems best to retain for the enzyme the name plant amine oxidase suggested by Kenten & Mann (1952). The fact that both D- and L-lysine are attacked suggests that the oxidation of these substrates takes place at the ε-amino group. The enzyme does not catalyse the oxidation of α- or ε-aminocaproic acids. It is probable, therefore, that both amino groups of lysine are necessary for the formation of an active enzyme–substrate compound. Of the diamino acids tested, only lysine was oxidized. As with the diamines the length of the hydrocarbon chain may be the deciding factor.

The affinity of the enzyme for the diamines is much greater than that for the phenylalkylamines (Kenten & Mann, 1952) and this again is much greater than that for L-lysine. The rate of oxidation of L-lysine is very slow in comparison with that of the diamines and phenylalkylamines. Nevertheless, the property of the amine oxidase of catalysing the oxidation of L-lysine may be of importance in plant metabolism. Thus Mann & Smithies (unpublished) have obtained evidence that the reaction product cyclizes to form a compound which on catalytic hydrogenation gives pipecolinic acid.

The fact that hydrogen peroxide formation accompanies oxidation with both the plant amine oxidase and the animal diamine oxidase has led to the suggestion that both enzymes are flavoproteins. Some evidence has been advanced that a flavin is the prosthetic group of the animal enzyme (Zeller, Stern & Wenk, 1940; Swedin, 1944; Kapeller-Adler, 1949) but no conclusive proof of this has yet

been obtained. The activity of the plant enzyme is not dependent on an easily dissociable prosthetic group. Thus little or no loss of activity was found on prolonged dialysis. Werle & Pechmann (1949), using extracts of clover seedlings to catalyse the oxidation of 1:5-diaminopentane, found a partial loss of activity on prolonged dialysis. The lost activity could be restored either by the addition of the concentrated outer liquid from the dialysis or by boiled clover-seedling extracts. The results were interpreted as evidence for the existence of a co-enzyme. In the results reported the activity was never more than doubled by the addition of the dialysates or boiled extracts. The total O_2 uptake with the extracts was 1 mol. O_2/mol. 1:5-diamino-pentane whereas with the purified amine oxidase used in the present work the uptake was 0·5 mol. O_2/mol. 1:5-diaminopentane. It is suggested, therefore, that the loss of activity on dialysis was due to the removal either of a factor catalysing the further oxidation of the primary reaction product or of compounds capable of oxidation by the hydrogen peroxide formed in the primary reaction. It is well known that hydrogen peroxide formed in enzyme reactions may be used for secondary or coupled oxidations catalysed by peroxidase or catalase.

In the absence of catalase the enzyme is rapidly inactivated during the oxidations which it catalyses. Inactivation is also produced by adding hydrogen peroxide but only when a substrate of the enzyme is also present. It is suggested that a reduced form of the enzyme is attacked by hydrogen peroxide. The inactivation is prevented by thiourea and phenylthiourea in so far as these inhibitors, rather than the enzyme, are oxidized by the hydrogen peroxide formed. In this connexion it is of interest that Bernheim & Bernheim (1942) found that the inhibition of tyrosinase produced by phenylthiourea and thiourea disappeared during the course of the enzyme-catalysed oxidations. The possibility that these compounds were destroyed by the enzyme was considered but it was shown that the inhibition period was not shortened when the inhibitors were incubated with the enzyme before adding the substrate. These results do not exclude the possibility that the inhibitors are oxidized when both enzyme and substrate are present. Reaction inactivation is a characteristic of the copper-containing oxidases. Such an inactivation of ascorbic acid oxidase, which is prevented by catalase and peroxidase, is not attributed to hydrogen peroxide itself but to a 'hydrogen peroxide precursor' formed as an intermediate in the ascorbic acid oxidase reaction (Powers & Dawson, 1943). It is suggested that a compound resembling hydrogen peroxide in that it can oxidize phenylthiourea and thiourea may be formed in tyrosinase-catalysed reactions.

The inhibition of animal diamine oxidase by cyanide was attributed by Zeller (1940) to its action as a carbonyl reagent. The slight inhibitions reported with heavy metal reagents such as pyrophosphate, sodium azide, thiourea and hydrogen sulphide have been attributed to their action on the further oxidation of the primary reaction products. The inhibition of plant amine oxidase by sodium diethyldithiocarbamate, salicylaldoxime, and potassium ethyl xanthate reported in the present work cannot be due to effects on secondary oxidation. Thus the oxidation of 1:4-diaminobutane catalysed by the enzyme preparation, in presence of catalase requires only 0·5 mol./mol. 1:4-diaminobutane and is, therefore, presumably confined entirely to the amine oxidase-catalysed reaction. Yet the inhibition produced approaches 100%. The results suggest that the plant enzyme may be a metalloprotein. In preliminary experiments, the three plant amine oxidase preparations so far examined for heavy metals have been found to contain both copper and manganese in amounts of 0·03–0·06% based on the protein content. No proof has yet been obtained that the presence of either of these metals is essential for the activity of the enzyme.

SUMMARY

1. A method is described by which the amine oxidase of pea-seedling extracts can be purified up to 300-fold.

2. The purified enzyme preparations catalysed the oxidation not only of diamines but also, though less readily, that of phenylalkylamines, aliphatic monoamines and of L- and D-lysine.

3. L- and DL-Hydroxylysine, DL-diaminopropionic acid, L-diaminobutyric acid, L-ornithine and L-histidine were not oxidized.

4. Hydrogen peroxide was formed during the oxidation of all the substrates. The inactivation of the enzyme by hydrogen peroxide has been confirmed and the conditions of this inactivation have been investigated.

5. The enzyme was inhibited not only by cyanide and semicarbazide, as previously reported, but also by diethyldithiocarbamate, salicylaldoxime and potassium ethyl xanthate.

6. Salicylaldoxime and thiourea were oxidized by the hydrogen peroxide formed in the primary reaction. This oxidation of salicylaldoxime was catalysed by peroxidase.

7. It is suggested that one enzyme—plant amine oxidase—catalyses the oxidation of diamines, phenylalkylamines, aliphatic amines and of L- and D-lysine and that this enzyme may be a metalloprotein.

Gifts of D-lysine hydrochloride, by Dr J. P. Greenstein, L-diaminobutyric acid, hydrochloride and DL-diamino-

propionic acid hydrochloride, by Dr S. Wilkinson and Dr T. S. G. Jones, and of L-hydroxylysine hydrochloride, by Dr J. G. Heathcote, are gratefully acknowledged.

REFERENCES

Barnett, E. de B. (1910). *J. chem. Soc.* **97**, 63.
Bernheim, F. & Bernheim, M. L. C. (1942). *J. biol. Chem.* **145**, 213.
Blaschko, H. & Duthie, R. (1945). *Biochem. J.* **39**, 478.
Blaschko, H. & Hawkins, J. (1950). *Brit. J. Pharmacol.* **5**, 625.
Boëseken, J. (1936*a*). *Rec. Trav. chim. Pays-Bas*, **55**, 1040.
Boëseken, J. (1936*b*). *Proc. Acad. Sci. Amst.* **39**, 47.
Bonnichsen, R. K. (1947). *Arch. Biochem.* **12**, 83.
Chance, B. & Herbert, D. (1950). *Biochem. J.* **46**, 402.
Cromwell, B. T. (1943). *Biochem. J.* **37**, 722.
Hirsch, J. G. (1953). *J. exp. Med.* **97**, 345.
Kapeller-Adler, R. (1949). *Biochem. J.* **44**, 70.
Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
Kenten, R. H. (1953). *Biochem. J.* **55**, 350.
Kenten, R. H. & Mann, P. J. G. (1952). *Biochem. J.* **50**, 360.
Kenten, R. H. & Mann, P. J. G. (1954). *Biochem. J.* **57**, 347.
Laskowski, M. (1942). *J. biol. Chem.* **145**, 457.
Powers, W. H. & Dawson, C. R. (1943). *J. gen. Physiol.* **27**, 181.
Swedin, B. (1944). *Ark. Kemi Min. Geol.* **17A**, 27.
Sumner, J. B. & Dounce, A. L. (1937). *J. biol. Chem.* **121**, 417.
Tabor, H. (1951). *J. biol. Chem.* **188**, 125.
Tabor, C. W., Tabor, H. & Rosenthal, S. M. (1954). *J. biol. Chem.* **208**, 645.
Tsuchihashi, M. (1923). *Biochem. Z.* **140**, 63.
Umbreit, W. W. (1945). In *Manometric Methods and Tissue Metabolism*, p. 9. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess. Publ. Co.
Werle, E. & Pechmann, E. (1949). *Liebigs Ann.* **562**, 44.
Werle, E. & Raub, A. (1948). *Biochem. Z.* **318**, 538.
Werle, E. & Roewer, F. (1950). *Biochem. Z.* **320**, 298.
Werle, E. & Roewer, F. (1952). *Biochem. Z.* **322**, 320.
Willstätter, R. & Stoll, A. (1918). *Liebigs Ann.* **416**, 21.
Zeller, E. A. (1938). *Helv. chim. acta*, **21**, 880.
Zeller, E. A. (1940). *Helv. chim. acta*, **23**, 1418.
Zeller, E. A., Stern, R. & Wenk, M. (1940). *Helv. chim. acta*, **23**, 3.

The Sedimentation Behaviour of Mixtures of Hyaluronic Acid and Albumin in the Ultracentrifuge

By J. P. JOHNSTON*
Department of Biochemistry, University of Oxford, and Department of Physiology, Charing Cross Hospital Medical School

(*Received* 20 *October* 1954)

The work described here arose from an investigation of the synovial fluid of human knee joints: its other aspects are described by Johnston (1955*a*, *b*). In several samples from normal and nearly normal joints, it was observed that the lines representing the variation of the rate of sedimentation of the hyaluronic acid with concentration differed considerably from one sample to another. Since these differences appeared to be correlated with the concentration of protein in the sample, the mutual effects of hyaluronic acid and albumin on the sedimentation of the other were investigated further.

Johnston & Ogston (1946) showed that at a given concentration one protein sediments at a different rate (usually more slowly) in the presence of a second protein than it does alone. If this protein is the more slowly sedimenting of the two, its concentration is increased behind the sedimenting boundary of the faster component; the faster component undergoes an apparent (but unreal) fall of concentration. This increase in concentration of the slower component would be expected to cause a reduction of its rate of sedimentation. In the systems studied by Johnston & Ogston (1946) the last effect was unimportant, since the sedimentation rates of the proteins studied did not vary rapidly with concentration. In the present case, however, the sedimentation rate of hyaluronic acid varies rapidly with its concentration (Ogston & Stanier, 1950, 1952) and it is shown that the effect of albumin on the sedimetantion rate of hyaluronic acid can be explained by the operation of this effect, which increases the concentration of the hyaluronic acid when it is the slower component.

The rate at which hyaluronic acid sediments in the region of the cell in which albumin is present, and the converse case, have also been investigated, and an attempt has been made to explain the results obtained.

* Present address: Medical Division, Chemical Defence Experimental Establishment, Porton, Wilts.

METHODS

Materials. Both ox and human synovial fluids were used. Knee-joint fluid from freshly slaughtered cattle was collected and stored as described by Ogston & Stanier (1950). Human synovial fluid obtained at operation at the Wingfield-Morris Orthopaedic Hospital, Oxford, was supplied by