which they are subjected can result in cutting into and fragmentation of the surface of the valve tips.

Finally, the 20 mm. observation tube described is not the only device that can be employed with the apparatus; the separation of the mixers from the observation tube makes it possible to use any recording device that can be introduced into the space between the mixer and the stop block. Alternative arrangements for following bioluminescent reactions (Gibson & Hastings, 1962) and fluorescence changes (Hastings & Gibson, 1963) have already been described and there is no reason why conductivity, pH or oxygen concentration should not also be followed with suitable equipment.

## SUMMARY

1. The construction of a stopped-flow apparatus for following rapid reactions with half-times from 5 msec. upwards is described.

2. Some details of performance and suggestions for operation and maintenance are given.

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# Further Properties of the Diamine Oxidase of Pea Seedlings

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Mann (1961) described a method yielding highly purified preparations of pea-seedling diamine oxidase (diamine–oxygen oxidoreductase, EC 1.4.3.6). The final preparations were pink solutions and the absorption spectra showed a band in the visible region with a maximum at about 500 mμ. Spectrophotometric investigations of the reactions of the preparations with 1,4-diaminobutane, sodium dithionite and hydrazine sulphate supported the suggestion that the pink colour is a property of the enzyme. The preparations contained 0·08–0·09% of copper which could be removed with sodium diethyldithiocarbamate; the copper-free preparations so obtained were catalytically inactive but most of the original activity was restored by adding $Cu^{2+}$ ions. Mann (1961) suggested that the copper is present in the enzyme as a complex with a carbonyl compound and that this complex forms the prosthetic group of the enzyme. A study of the inhibition of the diamine oxidase by chelating agents provided further evidence that the enzyme contains copper (Hill & Mann, 1962). Werle, Trautschold & Aures (1961) also obtained highly purified preparations of the diamine oxidase that were rose-red in concentrated solution and suggested that the colour is a property of the enzyme. These preparations contained 0·12% of copper.

The results of Mann (1961) and Hill & Mann (1962) were obtained with 1,4-diaminobutane as the substrate. Extracts of pea- and clover-seedlings, and partially purified diamine-oxidase preparations made therefrom, catalyse the oxidation not only of aliphatic diamines but also of histamine, agmatine, aliphatic monoamines, phenylalkylamines and of the dibasic amino acids lysine and ornithine (Werle & Pechmann, 1949; Kenten & Mann, 1952; Mann, 1955; Werle & Hartung, 1956; Clarke & Mann, 1959). Mann (1955) concluded that the oxidation of all these compounds was catalysed by one enzyme of wide substrate specificity. Werle *et al.* (1961) showed that many of these compounds are also oxidized by highly purified preparations of the diamine oxidase.

The present work was started to investigate further the specificity of the enzyme and the mechanism of its action.

## MATERIALS AND METHODS

*Buffers.* Orthophosphate buffers and pyrophosphate buffers were prepared as described by Hill & Mann (1962). Orthophosphate–borate buffers were prepared by mixing equal volumes of 0·4M-$KH_2PO_4$ and 0·4M-$H_3BO_3$ and adjusting to the required pH with 2N-KOH, with final dilution to twice the original volume. In the experiments on the preparation and properties of the copper-free protein the orthophosphate buffer was freed from traces of copper by extraction with diphenylthiocarbazone in $CCl_4$, as described by Hill & Mann (1962).

*o-Aminobenzaldehyde.* This was prepared by the reduction of *o*-nitrobenzaldehyde with ferrous sulphate and ammonia (Smith & Opie, 1955).

*Enzymes.* Crystalline ox-liver catalase (EC 1.11.1.6) was prepared as described by Clarke & Mann (1959). Initial preparations of diamine oxidase were made from pea seedlings by the method of Mann (1955) with some modifications. The roots of the seedlings were discarded and the remaining plant material (2–3 kg.) was minced and pressed in a tincture press (A. G. Gallenkamp and Co., London). The pulp was mixed with 0·1M-orthophosphate buffer, pH 7 (500 ml./kg. of plant material), and pressed again. The combined extracts were worked-up by the procedure of Mann (1955) to the stage where the enzyme is repeatedly precipitated at pH 5. On each occasion, immediately after adjustment to pH 5 with 0·05N-acetic acid, the suspension was centrifuged at 18 000*g* for 30 min. at 2° (15 000 rev./min. in a MSE Super-Speed 25 refrigerated centrifuge with angle head 98 300). The final precipitate was suspended in water and redissolved by adjustment to pH 7 with 0·05N-NaOH and then diluted with 10 mM-orthophosphate buffer, pH 7, so that 1 ml. was equivalent to 100 g. of plant material. These initial preparations were stored at −10°. Three or four initial preparations were combined for further purification by chromatography on calcium phosphate (hydroxyapatite) and diethylaminoethylcellulose columns by the procedure of Mann (1961). The final preparations, in 0·2M-orthophosphate buffer, pH 7, were stored at −10°.

The diamine oxidase was assayed, at 25°, by the method described by Hill & Mann (1962). A unit of diamine oxidase is defined as the amount that catalyses the oxidation of 1 μmole of 1,4-diaminobutane/min. at 25°, giving an initial rate of uptake of $O_2$ of 11·2 μl./min. The specific activity is defined as units/mg. of protein.

*Copper-free protein.* Diamine-oxidase solution (20 ml. containing 500 units/ml.) was mixed with 1 ml. of 0·1M-sodium diethyldithiocarbamate and kept at 0–5° for 15 hr. The precipitated copper–diethyldithiocarbamate complex was removed by centrifuging for 30 min. at 18 000*g* and at 2°. The supernatant solution sometimes remained slightly turbid and was clarified by filtration through a layer of acid-washed kieselguhr. The clear orange–pink solution was dialysed for 48 hr. at 0–5° against 4 × 250 ml. of 10 mM-orthophosphate buffer, pH 7. The non-diffusible material was centrifuged for 30 min. at 18 000*g* and the supernatant solution was stored at −10°.

*Oxygen uptake.* Oxygen uptake was measured in the Warburg apparatus, in air, at 25°. The volume of the reaction mixtures was 3 ml., and 0·2 ml. of 5N-KOH was present in the centre well.

*Estimation of total nitrogen and copper.* The total nitrogen and copper contents of the diamine-oxidase preparations were estimated by the procedure described by Mann (1961).

*Spectrophotometry.* Extinctions were measured with an Optica CF4DR recording spectrophotometer fitted with silica cells of 1 cm. light-path. Experiments in the absence of oxygen were made in a Thunberg-type assembly consisting of a silica cell fitted by a ground-glass joint to a special attachment containing one or two side arms and a stopcock for evacuation. Dissolved oxygen was first removed from the enzyme solution by evacuation in a Thunberg tube. The enzyme was then transferred to the cell and the substrate (or other reactant) was put in the side arm. The assembly was evacuated and the reaction started by adding the substrate from the side arm.

*Sedimentation coefficient.* The sedimentation coefficient of the diamine oxidase was determined in the Spinco model E centrifuge with the standard cell.

*Electron microscopy.* Diamine-oxidase preparations were mounted on thin carbon films by the negative-staining technique of Brenner & Horne (1959) and then examined in a Siemens Elmiskop I electron microscope at a magnification of ×80 000.

## RESULTS

### *Reactions of the holoenzyme*

*Specific activity and copper content.* The final preparations had specific activities of 48–55. The copper contents of three such preparations were 0·085, 0·086 and 0·090%. A copper content of 0·087% is equivalent to 1 g.atom of copper/73 000 g. of protein.

*Homogeneity of the purified enzyme.* Solutions, in 0·1M-potassium chloride, of a diamine-oxidase preparation with a specific activity of 55 were analysed in the ultracentrifuge at concentrations of 1·5, 3, 6 and 12 mg./ml. The two lower concentrations showed only a single symmetrical peak; with the higher concentrations small amounts of a faster-sedimenting material were also apparent. This probably represented not more than 5% of the total. The sedimentation coefficient, $S^0_{20}$, was 7·7 s.

In the electron microscope individual molecules were clearly visible and the preparations appeared homogeneous. In the electron micrograph the molecules appeared approximately round and were estimated to be 6·2 mμ in diameter; owing to the small size of the image it could not be decided whether the molecules were disks or spheres. If it is assumed that the molecules are spherical and have a dry density of 1·3 g./ml. a single one would weigh $1·6 \times 10^{-19}$ g., corresponding to a mol.wt. of about 96 000.

*Substrate specificity.* Amines and related compounds were tested as substrates of the diamine oxidase in reaction mixtures buffered with orthophosphate (pH 6–8·5) or orthophosphate–borate (pH 9–10). Table 1 shows the initial rates of uptake of oxygen by the reaction mixtures at pH values near the optimum for each substrate. With the αω

series of aliphatic diamines the rates of oxidation and pH optima were dependent on the chain length, as found by Kenten & Mann (1952) for pea-seedling extract. The 1,4- and 1,5-diamines were most readily oxidized, and no oxygen uptake was observed with the 1,2- and 1,3-diamines. Histamine was oxidized much less readily than 1,4-diaminobutane; spermidine was rapidly oxidized, but spermine only very slowly. With the aliphatic monoamines the rates of oxygen uptake increased with chain length from methylamine to propylamine and thereafter showed little further increase. Diethylamine and isopropylamine were not oxidized. Of the phenylalkylamines tested 2-phenylethylamine was oxidized faster than benzylamine, and 1-methylbenzylamine was not oxidized. As reported by Werle *et al.* (1961), the enzyme catalyses the oxidation of L-noradrenaline but not that of L-adrenaline. The adrenaline reaction mixtures in orthophosphate buffers, pH 7·5–8·5, showed a slight uptake of oxygen, but this was also observed in control reaction mixtures without the diamine oxidase. The increased oxygen uptake observed by Kenten & Mann (1952) when adrenaline was added to pea-seedling extract was therefore not due to catalysis by diamine oxidase.

Table 1. *Specificity of the diamine oxidase*

Reaction mixtures, of 3 ml. total volume, contained diamine oxidase and catalase (25 μg.) in 67 mM-orthophosphate buffer, pH 6–8·5, or 33 mM-orthophosphate–33 mM-borate buffer, pH 9–10. The substrate (10 mM) was added from the side arm after equilibration. The gas phase was air and the temperature 25°. The oxidase contained 50 units/mg. of protein.

| Substrate | Diamine-oxidase activity (units) | pH | Uptake of $O_2$ (μl./unit of enzyme/10 min.) |
|---|---|---|---|
| 1,2-Diaminoethane | 10 | 6–10 | 0 |
| 1,3-Diaminopropane | 10 | 6–10 | 0 |
| 1,4-Diaminobutane | 0·5 | 7·0 | 112·0 |
| 1,5-Diaminopentane | 0·5 | 7·0 | 112·0 |
| 1,6-Diaminohexane | 2 | 8·0 | 28·0 |
| 1,10-Diaminodecane | 2 | 8·0 | 21·5 |
| Spermine | 10 | 7·0 | 0·4 |
| Spermidine | 1 | 7·0 | 39·0 |
| Agmatine | 1 | 7·0 | 39·0 |
| Histamine | 10 | 7·0 | 6·0 |
| Methylamine | 10 | 8·0 | 0·7 |
| Ethylamine | 10 | 8·5 | 4·1 |
| Diethylamine | 10 | 6–10 | 0 |
| *n*-Propylamine | 5 | 9·0 | 11·2 |
| Isopropylamine | 10 | 6–10 | 0 |
| *n*-Butylamine | 10 | 9·5 | 9·0 |
| Isobutylamine | 10 | 9·0 | 3·4 |
| *n*-Hexylamine | 5 | 9·5 | 13·6 |
| *n*-Heptylamine | 5 | 9·5 | 12·7 |
| Benzylamine | 10 | 8·5 | 4·9 |
| 1-Methylbenzylamine | 10 | 6–10 | 0 |
| 2-Phenylethylamine | 1 | 8·5 | 32·0 |
| Tyramine | 1 | 8·5 | 20·0 |
| Tryptamine | 10 | 8·5 | 7·6 |
| L-Adrenaline | 10 | 6–9 | 0 |
| L-Noradrenaline | 10 | 8·5 | 1·1 |
| L-Ornithine | 10 | 8·5 | 0·7 |
| L-Lysine | 10 | 8·5 | 6·4 |

In Table 1 the concentration of all substrates was 10 mM. This concentration of 1,4-diaminobutane is sufficient to saturate the enzyme (Kenten & Mann, 1952), but with many other substrates, e.g. 2-phenylethylamine (Kenten & Mann, 1952) and lysine (Mann, 1955), higher concentrations are necessary. As was shown by Fouts, Blanksma, Carbon & Zeller (1957) for animal diamine oxidase, the plant enzyme has a lower affinity for aliphatic monoamines than for diamines. The differences in the rates of oxidation of the diamines and monoamines are therefore smaller at substrate concentrations greater than 10 mM.

*Reaction of enzyme with substrates.* Mann (1960, 1961) reported that enzyme solutions changed from pink to yellow when 1,4-diaminobutane was added under anaerobic conditions; the pink colour was restored by oxygenation of the mixture. Fig. 1 shows the effect of different concentrations of 1,4-diaminobutane on the spectrum of the enzyme. The results suggest that complete conversion of 1200 units of enzyme into the yellow product requires about 0·4 μmole of 1,4-diaminobutane. On the assumption that 1 mole of enzyme contains 1 g.atom of copper it was calculated that about 0·3 μmole of enzyme was present in each reaction mixture. Mann (1960, 1961) reported maxima at 465, 435 and 350 mμ in the absorption spectrum of the yellow product. Fig. 1 shows maxima at 466, 437·5 and 350 mμ; there is also a slight inflexion at 410–420 mμ.

We have also investigated the spectral changes that occur when 1,10-diaminodecane, ethylamine, benzylamine, noradrenaline, histamine, spermine, spermidine or lysine is added to the enzyme. In each experiment 1 μmole of the substrate was added to 3 ml. of enzyme (1200 units). All compounds reacted with the enzyme to give yellow solutions with absorption spectra apparently identical with that of the enzyme–1,4-diaminobutane product. When the mixtures were oxygenated the pink colour returned. Fig. 2 shows the results with histamine. We suggest that the yellow products formed in the anaerobic reactions between the enzyme and its substrates are enzyme–substrate complexes. Some of the substrates reacted so rapidly that the velocities of formation of the yellow complexes could not be measured. Noradrenaline, spermine, lysine and ethylamine reacted more slowly. Fig. 3 shows that the reaction of 3 ml. of the enzyme (1200 units) with 1 μmole of 1,4-diaminobutane to form the yellow complex, as measured by the increase in extinction at 466 mμ, was complete within 15 sec. With 1 μmole of

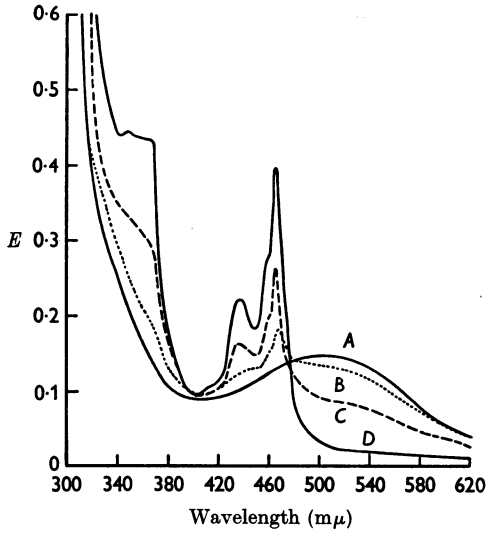


Fig. 1. Reaction of diamine oxidase with 1,4-diaminobutane under anaerobic conditions. Absorption spectra of solutions of diamine oxidase (1200 units) in 3 ml. of 0·2M-orthophosphate buffer, pH 7, were determined after the addition, under anaerobic conditions, of the following amounts of 1,4-diaminobutane: *A*, none; *B*, 0·1 μmole; *C*, 0·2 μmole; *D*, 1 or 2 μmoles.

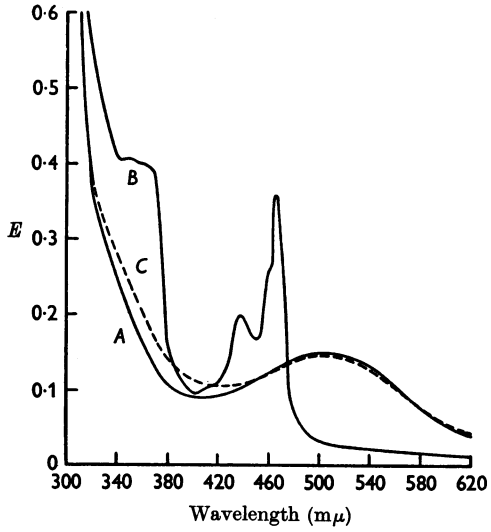


Fig. 2. Reaction of diamine oxidase with histamine. Absorption spectra of solutions of diamine oxidase (1200 units) in 3 ml. of 0·2M-orthophosphate buffer, pH 7, were determined: *A*, before the addition of histamine; *B*, after the addition, under anaerobic conditions, of 1 μmole of histamine; *C*, after the oxygenation of the mixture of enzyme and histamine (25 μg. of catalase was added to the mixture before oxygenation).

L-lysine or of ethylamine there was a lag period before the extinction increased. The reaction velocities were increased and the lag periods partially or completely eliminated by increasing the concentrations of ethylamine and L-lysine. These experiments were all made at pH 7 which is near the optimum for the oxidation of 1,4-diaminobutane; the oxidations of L-lysine and ethylamine were optimum at pH 8·5. Since the enzyme has a comparatively low affinity for ethylamine and L-lysine, it would be expected that at low concentrations of these substrates the rates of the overall enzyme reactions would be limited by the rates of formation of the enzyme–substrate complexes.

*Effect on the spectrum of the enzyme of compounds related structurally to substrates.* No change in the absorption spectrum of the enzyme was observed when 1 μmole of aniline, 1-methylbenzylamine, diethylamine, isopropylamine or adrenaline was added under anaerobic conditions. The short-chain diamines 1,2-diaminoethane and 1,3-diaminopropane, which in the manometric experiments were found not to be substrates, both reacted with the enzyme to give yellow products. Fig. 4 shows that the absorption spectrum of the product with 1,2-diaminoethane closely resembled that of the enzyme–substrate complexes, but the product was much more stable in oxygen. The spectrum determined immediately after oxygenation for 15 sec. was little changed, whereas oxygenation for 5 sec. was sufficient to oxidize all the enzyme–1,4-diaminobutane complex. The yellow colour slowly

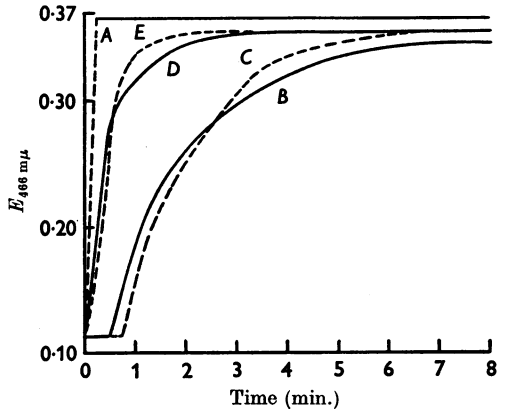


Fig. 3. Rates of formation of enzyme–substrate complexes. The substrates were added, under anaerobic conditions, to 1200 units of diamine oxidase in 3 ml. of 0·2M-orthophosphate buffer, pH 7, and the subsequent rates of increase of extinction at 466 mμ were measured. The substrates added were: *A*, 1 μmole of 1,4-diaminobutane; *B*, 1 μmole of ethylamine; *C*, 1 μmole of L-lysine; *D*, 5 μmoles of ethylamine; *E*, 5 μmoles of L-lysine.

faded during subsequent standing in air for 30 min. but the pink colour did not return. After further oxygenation of the reaction mixture for 2 min. complex changes slowly occurred in the spectrum. We suggest that the yellow product is a complex of the enzyme with 1,2-diaminoethane and that the inability of the enzyme to catalyse the oxidation of 1,2-diaminoethane is due to the relative stability to oxygen of this complex. Fig. 5 shows the spectrum of the yellow product formed by the enzyme with 1,3-diaminopropane. The bands at 466, 437·5 and 350 mμ are apparent but at relative intensities different from those of the enzyme–substrate complexes. After oxygenation the solution remained yellow; the 466 and 437·5 mμ bands disappeared but the 350 mμ band increased in intensity. The intensity of this band slowly decreased in air and after 24 hr. at room temperature had disappeared with a partial reappearance of the pink colour.

*Effect of chelating agents on the enzyme.* Hill & Mann (1962) suggested that the primary reaction involved in the inhibition of diamine oxidase by chelating agents is combination of the reagents with enzyme-bound copper to form inactive enzyme–inhibitor complexes. They found that the inhibitions produced by all the chelating agents tested, except sodium diethyldithiocarbamate, could be reversed by adding metal ions with which the chelating agents form complexes. The enzyme–diethyldithiocarbamate complex dissociated to give the copper-free protein and copper-diethyldithiocarbamate complex, and this type of inhibition was reversible only by $Cu^{2+}$ ions. The present work showed that when sodium diethyldithiocarbamate was added to the enzyme solution the colour rapidly changed from pink to orange–yellow and a new band appeared in the absorption spectrum, with maximum extinction at 436 mμ, resembling that of the copper–diethyldithiocarbamate complex. This colour rapidly intensified and precipitation of the copper–diethyldithiocarbamate complex started within a few minutes. 1,10-Phenanthroline caused little change in the absorption spectrum of the enzyme but prevented the formation of the yellow complex of the enzyme with 1,4-diaminobutane. When $Ni^{2+}$ ions were subsequently added from a second side arm to the mixture of enzyme, 1,10-phenanthroline and 1,4-diaminobutane, the yellow complex slowly appeared.

The result is consistent with the finding of Hill & Mann (1962) that $Ni^{2+}$ and other metal ions reverse the inhibition of the enzyme by 1,10-phenanthroline. We suggest that the $Ni^{2+}$ ions remove the 1,10-phenanthroline combined with the enzyme-bound copper, thus liberating the enzyme, and that the copper in the enzyme takes part in the formation of the yellow enzyme–substrate complex. The yellow colour of the enzyme–substrate complex was rapidly discharged by 1,10-phenanthroline; the

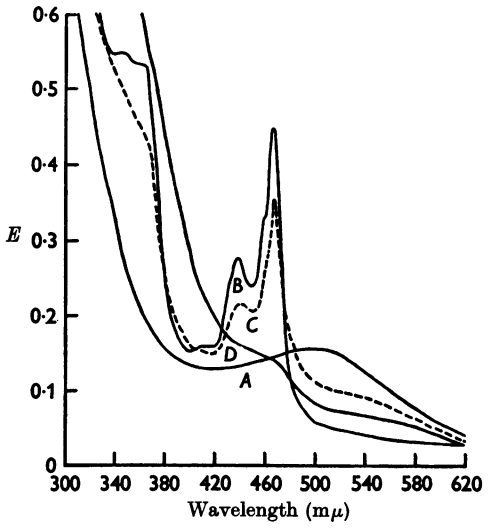


Fig. 4. Reaction of diamine oxidase with 1,2-diaminoethane. Absorption spectra of diamine oxidase (1200 units) in 3 ml. of 0·2M-orthophosphate buffer, pH 7, were determined: *A*, without 1,2-diaminoethane; *B*, with 1 μmole of 1,2-diaminoethane under anaerobic conditions; *C*, after oxygenation of the mixture for 15 sec.; *D*, after subsequent standing in air for 30 min.

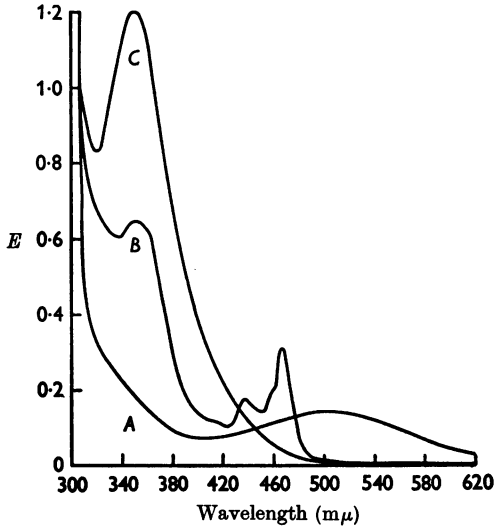


Fig. 5. Reaction of diamine oxidase with 1,3-diaminopropane. Absorption spectra of diamine oxidase (1200 units) in 3 ml. of 0·2M-orthophosphate buffer, pH 7, were determined: *A*, without 1,3-diaminopropane; *B*, with 1 μmole of 1,3-diaminopropane under anaerobic conditions; *C*, after oxygenation of the mixture of diamine oxidase and 1,3-diaminopropane (25 μg. of catalase was added to the mixture before oxygenation).

disappearance of the yellow was not accompanied by reappearance of the pink colour (Fig. 6). This suggests that the yellow colour comes from a copper complex.

*Reactions of the copper-free protein*

*Reactivation of enzyme by cupric ions.* Mann (1961) and Hill & Mann (1962) showed that the copper-free protein obtained by incubating the enzyme with sodium diethyldithiocarbamate had lost the property of catalysing the oxidation of 1,4-diaminobutane; however, most of the lost activity was restored by adding $Cu^{2+}$ ions. Table 2 shows similar results with other substrates. The possibility that the uptake of oxygen in such systems may not always accurately assay diamine oxidase is suggested by the results of Bruns & Stüttgen (1951), who showed that $Cu^{2+}$ ions increased both the rate and the total uptake of oxygen by the system of placental diamine oxidase and histamine. This was attributed to catalysis by $Cu^{2+}$ ions of the oxidation of imidazol-3-ylacetaldehyde, the product of the enzyme-catalysed reaction. The optimum concentration of $Cu^{2+}$ ions varied with the substrate from 1 $\mu$M with 2-phenylethylamine to 10 $\mu$M with histamine (Table 2). The activity decreased when the optimum $Cu^{2+}$ ion concentrations were exceeded because the enzyme was inactivated by $Cu^{2+}$ ions during the oxidations. This inactivation, previously reported with 1,4-diaminobutane as substrate (Mann, 1961; Hill & Mann, 1962), was also observed with the substrates shown in Table 2. The concentration of $Cu^{2+}$ ions required to produce such inactivation varied with the substrate and was larger with histamine than with 2-phenylethylamine. Under the conditions given in Table 2, with 1,4-diaminobutane as substrate no reactivation of the copper-free protein was observed with $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Co^{2+}$, $Ni^{2+}$, $Zn^{2+}$ or molybdate ions, at concentrations between 1 and 100 $\mu$M.

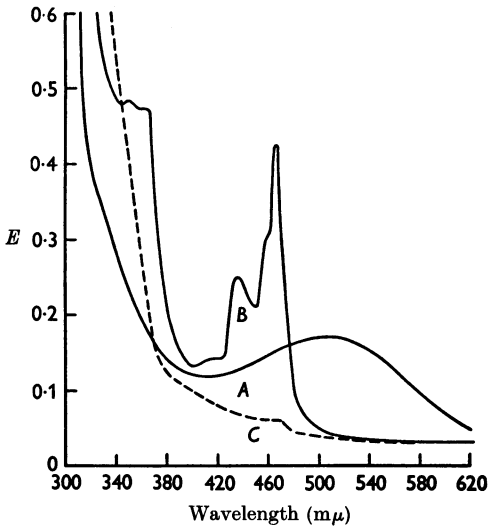


Fig. 6. Effect of 1,10-phenanthroline on the yellow enzyme–substrate complex. Absorption spectra: *A*, diamine oxidase (1200 units) in 3 ml. of 0·2M-orthophosphate buffer, pH 7; *B*, after the addition, under anaerobic conditions, of 1 $\mu$mole of 1,4-diaminobutane; *C*, after the addition to the mixture of 2 $\mu$moles of 1,10-phenanthroline from the second side arm.

*Time-course of reactivation.* Hill & Mann (1962) used pyrophosphate buffer to prevent inactivation of the enzyme by $Cu^{2+}$ ions. Fig. 7 shows that sodium ethylenediaminetetra-acetate (EDTA) is more effective. Pyrophosphate and EDTA also prevented the reactivation of the copper-free protein by $Cu^{2+}$ ions. Fig. 8 shows this effect of EDTA: the reactivation by 3·3 $\mu$M-$Cu^{2+}$ ion was almost completely prevented by 6·6 $\mu$M-EDTA. The copper-free protein was initially almost completely inactive but its activity slowly increased during incubation without added $Cu^{2+}$ ions. This increase in activity was prevented by EDTA and was probably due to the presence in the reaction medium of traces of copper not extracted by dithizone. Table 3 shows the time-course of the

Table 2. *Reactivation of the copper-free protein by cupric ions*

The copper-free protein and catalase (25 $\mu$g.) were added to orthophosphate buffer and to mixtures of orthophosphate buffer with $CuSO_4$. The substrate (10 mM) was added from the side arm after equilibration for 30 min.

| Substrate | Copper-free protein ($\mu$g.) | pH | Uptake of $O_2$ ($\mu$l./10 min.) Concn. of $CuSO_4$ ($\mu$M) 0 | 0·1 | 0·3 | 1·0 | 3·0 | 10·0 | 30 |
|---|---|---|---|---|---|---|---|---|---|
| 1,4-Diaminobutane | 20 | 7·0 | 9 | 20 | 32 | 52 | 71 | 64 | 39 |
| 1,10-Diaminodecane | 80 | 8·0 | 6 | 20 | 34 | 60 | 63 | 47 | 34 |
| Spermidine | 40 | 7·0 | 3 | 9 | 18 | 29 | 46 | 45 | 25 |
| Histamine | 300 | 7·0 | 0 | 4 | — | 19 | 40 | 60 | 54 |
| Ethylamine | 300 | 8·5 | 0 | 4 | 9 | 21 | 42 | 26 | 14 |
| 2-Phenylethylamine | 40 | 8·5 | 2 | 11 | 22 | 33 | 26 | 9 | 8 |
| L-Lysine | 300 | 8·5 | 0 | 13 | 23 | 41 | 71 | 70 | 64 |

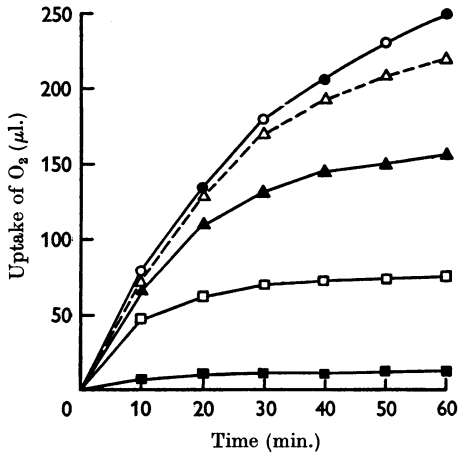


Fig. 7. Inactivation of diamine oxidase by $Cu^{2+}$ ions and its prevention by pyrophosphate buffer and EDTA. The control reaction mixture, of 3 ml. total volume, contained diamine oxidase (0·75 unit) and catalase (25 μg.) in 67 mM-orthophosphate buffer, pH 7. Other reaction mixtures contained, in addition, 0·3 mM-$CuSO_4$ with and without pyrophosphate buffer, pH 7, or EDTA. The substrate (10 mM-1,4-diaminobutane) was added from the side arm after equilibration. The additions were: ○, none (control reaction mixture); ■, 0·3 mM-$CuSO_4$; □, 0·3 mM-$CuSO_4$ and 0·3 mM-pyrophosphate buffer, pH 7; ▲, 0·3 mM-$CuSO_4$ and 0·6 mM-pyrophosphate buffer, pH 7; △, 0·3 mM-$CuSO_4$ and 1·2 mM-pyrophosphate buffer, pH 7; ●, 0·3 mM-$CuSO_4$ and 0·6 mM-EDTA. Other conditions were as given in Table 1.

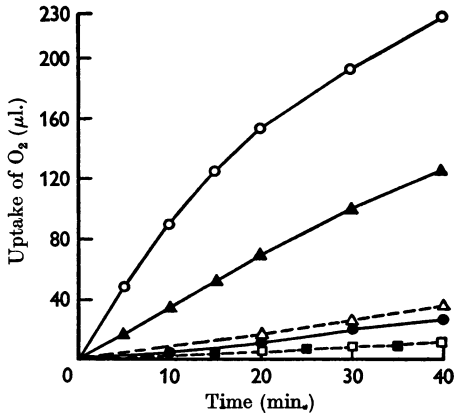


Fig. 8. Activation of the copper-free protein by $Cu^{2+}$ ions and its prevention by EDTA. The control reaction mixture, of 3 ml. total volume, contained copper-free protein (15 μg.) and catalase (25 μg.) in 67 mM-orthophosphate buffer, pH 7. Other reaction mixtures contained, in addition, $CuSO_4$ or $CuSO_4$ and EDTA. The substrate (10 mM-1,4-diaminobutane) was added from the side arm after equilibration for 30 min. The additions were: ●, none (control reaction mixture); □, 3 μM-EDTA; ○, 3 μM-$CuSO_4$; ▲, 3 μM-$CuSO_4$ and 3 μM-EDTA; △, 3 μM-$CuSO_4$ and 6 μM-EDTA; ■, 3 μM-$CuSO_4$ and 1 mM-EDTA. Other conditions were as given in Table 1.

reactivation of the copper-free protein by $Cu^{2+}$ ions. The copper-free protein was incubated at 25°, in the presence and absence of added $Cu^{2+}$ ions, for the times indicated. EDTA was then added to stop reactivation and to prevent inactivation by $Cu^{2+}$ ions during the subsequent assay. Incubation in the absence of added $Cu^{2+}$ ions produced a slow partial activation. The rate of reactivation was increased by adding 0·1 μM-$Cu^{2+}$ ion and was then almost complete in 3 hr. The enzyme contains 0·087% of copper, so complete reactivation of 10 μg. of copper-free protein requires combination with 8·7 μmg. of $Cu^{2+}$ ions or about 1·4 ml. of 0·1 μM-$Cu^{2+}$ ion. With 10 μM-$Cu^{2+}$ ion reactivation was complete within 5 min.

*Absorption spectrum.* Fig. 9 shows the absorption spectrum of a solution of the copper-free protein compared with that of the original diamine oxidase preparation. The copper-free protein was orange–pink and the colour was weaker than that of the enzyme solution. When $Cu^{2+}$ ions were added the colour changed to pink and intensified and the maximum shifted from 480 to about 500 mμ.

*Reaction with 1,4-diaminobutane.* Fig. 9 also shows that 1,4-diaminobutane under anaerobic conditions discharged the colour of the copper-free protein but caused little or no formation of the yellow enzyme–substrate complex; the absorption curve of the mixture showed only a slight inflexion at 466 mμ. The orange–pink colour was not restored by oxygenation; prolonged oxygenation caused a slight turbidity which increased the background absorption.

Table 3. *Time-course of the reactivation of the copper-free protein by* $Cu^{2+}$ *ions*

The copper-free protein (10 μg.) and catalase (25 μg.) were added to orthophosphate buffer and to mixtures of orthophosphate buffer and $CuSO_4$. The mixtures were incubated for the times indicated; EDTA (1 mM) was then added from the side arm and 5 min. later the substrate (10 mM-1,4-diaminobutane) was added from the second side arm. Other conditions were as given in Table 1.

| Time of pre-incubation with $CuSO_4$ (min.) | Uptake of $O_2$ (μl./10 min.) Concn. of $CuSO_4$ (μM) | | | | |
|---|---|---|---|---|---|
| | 0 | 0·1 | 1 | 10 | 100 |
| 0 | 2 | 2 | 2 | 2 | 0 |
| 1 | 4 | 4 | 9 | 14 | 38 |
| 5 | 5 | 8 | 17 | 36 | 53 |
| 15 | 7 | 14 | 27 | 47 | 48 |
| 30 | 11 | 23 | 37 | 49 | 49 |
| 60 | 15 | 34 | 44 | 50 | — |
| 120 | 22 | 38 | 46 | — | — |
| 180 | 30 | 44 | 47 | — | — |

Fig. 10 shows that, when the copper-free protein was first reactivated by incubation with $Cu^{2+}$ ions, and EDTA was then added to prevent inactivation by $Cu^{2+}$ ions during catalytic activity, the subsequent addition of 1,4-diaminobutane resulted in the formation of the yellow enzyme–substrate complex. The pink colour was restored by oxygenation. In an experiment similar to that of Fig. 10 except that $Ni^{2+}$ ions were used in place of $Cu^{2+}$ ions, the colour of the mixture was bleached by 1,4-diaminobutane but no yellow complex was formed. These results provide further evidence that the enzyme–substrate complex is a yellow copper complex.

*Instability of the enzyme–substrate complex in the presence of cupric ions.* The yellow enzyme–substrate complex formed from the enzyme and 1,4-diaminobutane was stable provided that anaerobic conditions were maintained. When 1,4-diaminobutane was added to mixtures of the copper-free protein with 0·33 mM-$Cu^{2+}$ ion the complex made only a transitory appearance unless EDTA was present, as in the experiments of Fig. 10. This was found to be due to the instability of the yellow complex in the presence of $Cu^{2+}$ ions. Fig. 11 shows

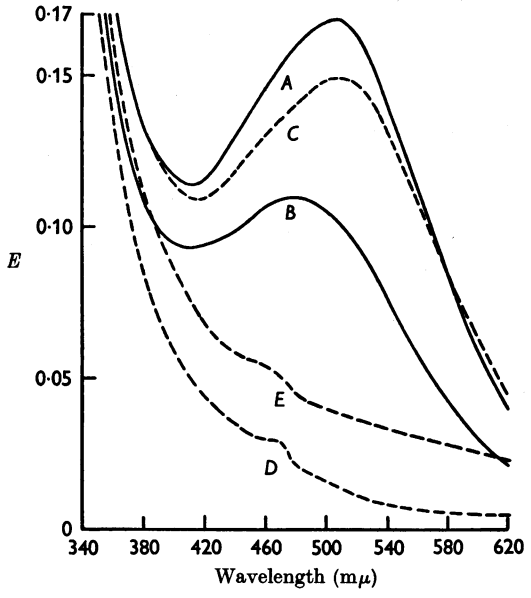


Fig. 9. Effects of $Cu^{2+}$ ions and of 1,4-diaminobutane on the absorption spectrum of the copper-free protein. The absorption spectra of the following were determined: *A*, diamine oxidase (1200 units; 22 mg. of protein) in 3 ml. of 0·2M-orthophosphate buffer, pH 7; *B*, the copper-free protein (22 mg.) in 3 ml. of 0·01M-orthophosphate buffer, pH 7; *C*, as for *B* 30 min. after the addition of 1 μmole of $CuSO_4$; *D*, as for *B* after the addition of 1 μmole of 1,4-diaminobutane under anaerobic conditions; *E*, as for *D* after oxygenation for 2 min.

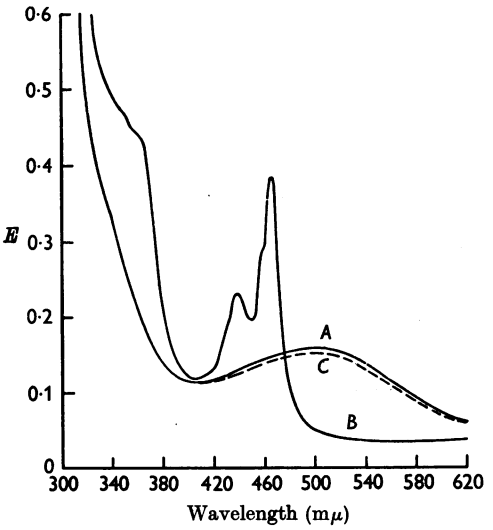


Fig. 10. Reaction of the reactivated copper-free protein with 1,4-diaminobutane. The copper-free protein (22 mg.) in 3 ml. of 0·01M-orthophosphate buffer, pH 7, was mixed with 1 μmole of $CuSO_4$ and kept at 0–5° for 16 hr.; 2 μmoles of EDTA were then added. The absorption spectra of the following were determined: *A*, the reactivated protein; *B*, as for *A* after the addition of 1 μmole of 1,4-diaminobutane under anaerobic conditions; *C*, as for *B* after oxygenation.

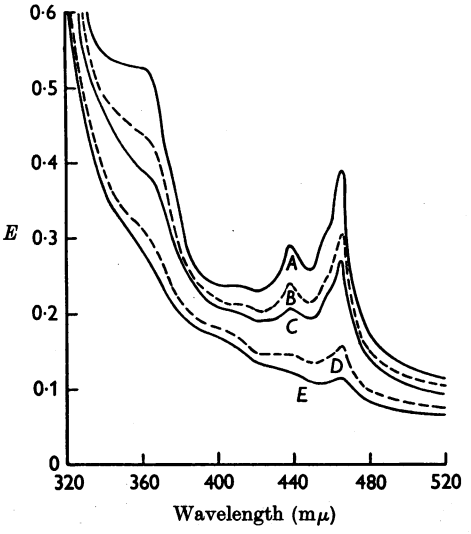


Fig. 11. Instability of the enzyme–substrate complex in the presence of $Cu^{2+}$ ions. $CuSO_4$ (0·1 μmole) was mixed with 3 ml. (1200 units) of diamine oxidase in 0·2M-orthophosphate buffer, pH 7. Absorption spectra were determined immediately after the addition of 1 μmole of 1,4-diaminobutane to the mixture under anaerobic conditions (*A*), and then after 10 min. (*B*), 30 min. (*C*), 2 hr. (*D*) and 4 hr. (*E*).

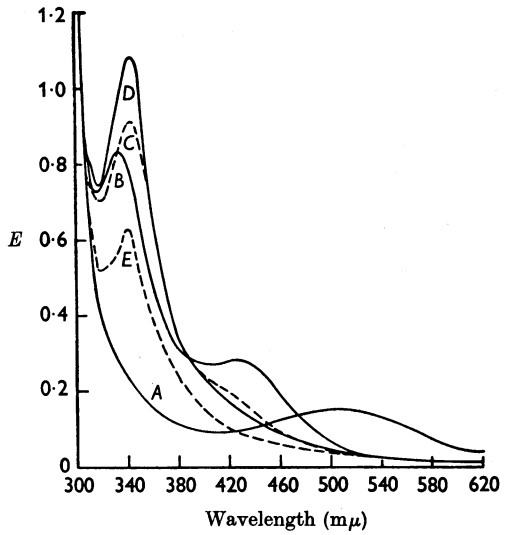


Fig. 12. Reaction of diamine oxidase with hydrazine. Absorption spectra of solutions of diamine oxidase (1200 units; 22 mg. of protein) in 3 ml. of 0·2M-orthophosphate buffer, pH 7, were determined before (*A*) the addition of 1 μmole of hydrazine sulphate, and then 1 min. (*B*), 1·5 hr. (*C*), 5·5 hr. (*D*) and 27 hr. (*E*) after the addition. The mixture was kept at 0–5° for the last 21·5 hr.

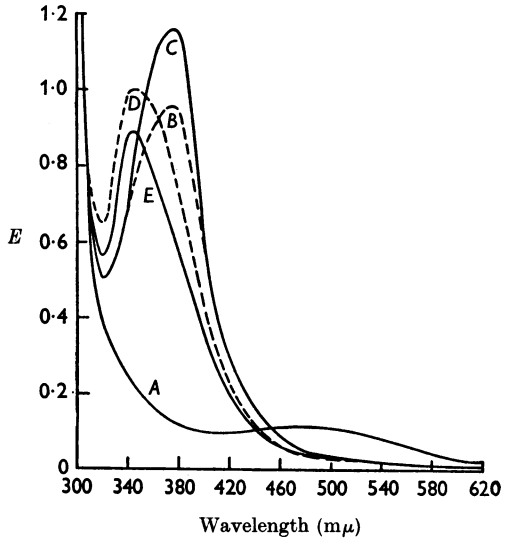


Fig. 13. Reaction of the copper-free protein with hydrazine. Absorption spectra of solutions of the copper-free protein (22 mg.) in 3 ml. of 0·01M-orthophosphate buffer, pH 7, were determined before (*A*) the addition of 1 μmole of hydrazine sulphate and then 6 min. (*B*) and 20 hr. (*C*) after the addition. $CuSO_4$ (1 μmole) was then added to the mixture and absorption spectra were determined after a further 2 hr. (*D*) and 5 hr. (*E*).

that a slower breakdown of the complex was caused by 33 μM-$Cu^{2+}$ ion. The addition of the copper sulphate to the enzyme solution initially caused a slight turbidity which slowly disappeared on standing, as shown by the decrease in the background absorption. The yellow complex formed by adding 1 μmole of 1,4-diaminobutane to the mixture had almost completely disappeared after 4 hr.; its disappearance was not accompanied by reappearance of the pink colour and this was only partially restored by oxygenation. Subsequent assay of the reaction mixture showed that most of the enzyme had been inactivated.

*Reactions of the holoenzyme and copper-free protein with hydrazine*

Mann (1961) described the changes in the absorption spectrum of a diamine-oxidase solution produced by hydrazine sulphate. Fig. 12 shows the results subsequently obtained with the recording spectrophotometer. On the addition of hydrazine the colour of the enzyme changed from pink to yellow and the initial change was the replacement of the 500 mμ band by a band with a sharp maximum at 333 mμ and a broader less-intense band with a maximum at 415–430 mμ. On standing in air at room temperature the solution slowly lost its yellow colour, and after 90 min. the 415–430 mμ band had disappeared and the maximum of the other band had shifted to 342 mμ and intensified. This band reached a maximum in 5–6 hr. and then slowly decreased. Mann (1961) reported a more rapid fall in the intensity of this band but did not observe the shift.

Fig. 13 shows the changes in the absorption spectrum of the copper-free protein when hydrazine was added. The colour changed from pink to yellow and the band with a maximum at 480 mμ was replaced by a band with a maximum at 372 mμ. The intensity of this band increased rapidly during the first few minutes after mixing and then remained stable for at least 20 hr. When $Cu^{2+}$ ions were added to the mixture the band shifted to 342 mμ and its intensity slowly decreased. These results suggest that the hydrazine derivative of the enzyme is unstable because of the presence of copper.

## DISCUSSION

The results show that the oxidations of monoamines, diamines, polyamines and amino acids studied are all catalysed by one enzyme. As originally suggested by Werle & Pechmann (1949), the enzyme resembles in substrate specificity the diamine oxidase of animal tissues which, as shown by Fouts *et al.* (1957), catalyses the oxidation of both mono- and di-amines. As with the animal

enzyme (Zeller, 1938*b*), the rates of oxidation of aliphatic diamines by the plant enzyme depend on the chain length, but, though both enzymes attack preferentially the short-chain diamines 1,4-diaminobutane and 1,5-diaminopentane, the oxidation of 1,3-diaminopropane is catalysed only by the animal enzyme. Secondary amines are not oxidized by either enzyme. Monoamine oxidase (EC 1.4.3.4) oxidizes both primary and secondary amines (Blaschko, Richter & Schlossmann, 1937; Randall, 1946), but, of the aliphatic series of diamines, oxidizes only the long-chain members (Blaschko & Duthie, 1945).

In view of the long controversy about the identity of histaminase and animal diamine oxidase the finding that plant diamine oxidase catalyses the oxidation of histamine is of special interest. Zeller (1938*a*) showed that preparations of histaminase also catalysed the oxidation of diamines and concluded that one enzyme, diamine oxidase, was responsible. This was generally supported by later work (e.g. Tabor, 1951; Goryachenkova, 1958), and histaminase has been used as a trivial name for diamine oxidase. Other workers maintained that two enzymes were involved and Kapeller-Adler & Macfarlane (1962, 1963) have now reported the isolation from pig-kidney cortex of an histaminase catalysing the oxidation of histamine and its ring-nitrogen derivatives but without action on diamines, and conclude that histaminase and animal diamine oxidase are separate enzymes. The possibility that animal diamine oxidase also catalyses the oxidation of histamine is supported by our results.

Mann (1961) estimated that the activity of plant diamine oxidase as a catalyst of the oxidation of 1,4-diaminobutane was about 50 times that of the purest preparations of animal diamine oxidase previously reported. The rate of the catalysed oxidation of 1,4-diaminobutane is about 20 times that of histamine. But this comparatively small activity of the plant enzyme with histamine appears to be much greater than that of the histaminase obtained by Kapeller-Adler & Macfarlane (1963). Thus histaminase was reported to have a specific activity (units/mg. of protein), at 37°, of 2700. A unit of histaminase was calculated to be the amount catalysing the oxidation of 0·463 $\mu$g. of histamine/hr. By taking an enzyme unit as the amount catalysing the transformation of 1 $\mu$mole of substrate/min., as in the present work, the recalculated specific activity is about 0·2. The purest preparations of plant diamine oxidase so far obtained have a specific activity, at 25°, of 55 with 1,4-diaminobutane or about 3 with histamine. The plant enzyme oxidizes histamine twice as rapidly at 37° as at 25°; its activity as a catalyst of the oxidation of histamine seems therefore to be about 30 times that of histaminase.

Our results support the suggestion of Mann (1961) that the pink colour of the plant diamine-oxidase preparations is a property of the enzyme and that the prosthetic group contains copper and a carbonyl group. Further work is necessary to establish the nature of the carbonyl compound. The group of amine oxidases inhibited by carbonyl reagents, such as hydrazine and hydroxylamine, includes the plant and animal diamine oxidases, histaminase, spermine oxidase (EC 1.5.3.3) and benzylamine oxidase (Bergeret, Blaschko & Hawes, 1957). Much evidence has been obtained by other workers using partially purified preparations of plant and animal diamine oxidases that the carbonyl compound is pyridoxal phosphate. Tabor, Tabor & Rosenthal (1954) purified spermine oxidase and suggested that the prosthetic group contained pyridoxal phosphate. Gorkin (1962) showed that highly purified preparations of the enzyme were inhibited by chelating agents and concluded that the enzyme contains zinc. Yamada & Yasunobu (1962*a*) further purified and crystallized spermine oxidase. The absorption spectrum of the pink protein showed a band with maximum absorption at about 480 m$\mu$. The colour was discharged by substrate under anaerobic conditions and restored by oxygenation. Yamada & Yasunobu (1962*b*) showed that the enzyme contains copper and concluded that valency change of the copper did not occur during catalytic activity. This was subsequently confirmed by electron-spin-resonance spectroscopy (Yamada, Yasunobu, Yamano & Mason, 1963). Yamada & Yasunobu (1962*b*) reported that the absorption spectrum of the copper-free protein showed a band with maximum absorption at 380 m$\mu$. In a preliminary note Yamada & Yasunobu (1962*c*) concluded that this absorption band is due to pyridoxal phosphate and that the prosthetic group of spermine oxidase is a copper complex of pyridoxal phosphate. Kapeller-Adler & Macfarlane (1962, 1963) reported that the prosthetic group of histaminase contains pyridoxal phosphate and FAD. The absorption spectrum of the pale-yellow enzyme solution showed bands with maxima at 330 and 405 m$\mu$ which were attributed to pyridoxal phosphate. From the absorption and fluorescence spectra reported by Kapeller-Adler & Macfarlane (1963) it can be calculated that 1 mole of pyridoxal phosphate is present in 40 000–50 000 g. of the preparation, and 1 mole of FAD in 1 000 000–2 000 000 g. This suggests that further evidence is necessary to exclude the possibility that the FAD is present as an impurity.

Our preliminary experiments to demonstrate the presence of pyridoxal phosphate in plant diamine oxidase have so far been unsuccessful. The original suggestion that the enzyme contains pyridoxal

phosphate and FAD was made by Werle & Pechmann (1949), who showed that when plant saps containing diamine oxidase were dialysed for several days, with the object of removing the prosthetic group, the subsequent rates of oxygen uptake by the dialysed saps in the presence of 1,5-diaminopentane were increased by adding pyridoxal hydrochloride. Goryachenkova (1956) reported similar increases, both in the uptake of oxygen and the formation of ammonia by the systems, when pyridoxal phosphate and FAD were added. Werle & Hartung (1956) found that pyridoxine, pyridoxamine and a number of apparently unrelated compounds, including phenols, ascorbic acid, 2-oxoglutaric acid, cysteine and glutathione, were also effective. Hydrogen peroxide, which is a product of the diamine-oxidase reactions, inactivates the enzyme (Werle & Pechmann, 1949; Mann, 1955). The possibility that the effective compounds act by removing hydrogen peroxide was considered but rejected by Werle & Hartung (1956). Preliminary investigations of these apparent activations (A. J. Clarke, J. M. Hill & P. J. G. Mann, unpublished work) show that peroxidase, which is plentiful in the plant saps, catalyses the oxidation of pyridoxine and related compounds by hydrogen peroxide. These compounds can therefore increase the uptake of oxygen by systems of plant saps and amines by consuming the hydrogen peroxide which is formed in the diamine-oxidase reaction and which is usually decomposed by catalase. In view of this the possibility that some, at least, of the apparent activations reported by Werle & Hartung (1956) may be due to prevention of the destruction of the diamine oxidase by hydrogen peroxide is under investigation. The results of Goryachenkova (1956) cannot be explained in the same way, because they were obtained with catalytic amounts of pyridoxal phosphate and FAD.

Most of the reactions of amino acids catalysed by enzymes containing pyridoxal phosphate can also be catalysed by model systems of pyridoxal and metal ions. This led Metzler, Ikawa & Snell (1954) to postulate reaction mechanisms for the enzyme reactions involving complexes of the enzyme protein with a metal chelate of the Schiff base formed from the amino acid and pyridoxal phosphate. Our results suggest that the reaction mechanism of the diamine oxidase may be similar. Aldehydes other than pyridoxal function in the model systems (Ikawa & Snell, 1954), and $Cu^{2+}$ ions are generally the most effective metal ions (Longenecker & Snell, 1957). We suggest that the yellow products formed by the reaction between the diamine oxidase and its substrates are enzyme–substrate complexes, probably copper complexes of Schiff bases. The existence of these coloured intermediate products, stable under anaerobic conditions, provides an unusual opportunity to study the mechanism and kinetics of the enzyme reactions.

The inactivation of the enzyme by hydrogen peroxide, formed as a product of the catalysed reactions, can be partially prevented by catalase. Mann (1961) and Hill & Mann (1962) found that the enzyme was rapidly inactivated by $Cu^{2+}$ ions during the catalysed reactions; this inactivation was not prevented by catalase. Our results suggest that $Cu^{2+}$ ions cause an abnormal breakdown of the enzyme–substrate complex resulting in the inactivation of the enzyme. Since this occurs under anaerobic conditions it is independent of hydrogen peroxide. Mann (1955) showed that in absence of substrate the enzyme is stable to hydrogen peroxide concentrations which inactivate it when substrate is present. This suggests that the inactivating effect of hydrogen peroxide, like that of $Cu^{2+}$ ions, is produced by reaction with the enzyme–substrate complexes.

The fact that plant diamine oxidase and spermine oxidase both contain copper suggests the possibility of its presence in other amine oxidases. Gorkin (1959) and Barbato & Abood (1963) reported that monoamine oxidase is inhibited by chelating agents and conclude it contains a heavy metal; Werle & Hartung (1956) reported the inhibition of animal diamine oxidase by 8-hydroxyquinoline and sodium diethyldithiocarbamate. Suggestions that neither animal diamine oxidase (Zeller, 1940) nor histaminase (Kapeller-Adler & Macfarlane, 1963) contains heavy metals are based on the lack of inhibition by sodium azide, thiourea and sodium sulphide. Werle & Hartung (1956) found that sodium sulphide inhibits plant diamine oxidase, but Mann (1955) reported that 10 mM-sodium azide produced only a slight inhibition at pH 7·5; a greater but still only partial inhibition was produced at pH 6. Thiourea at the same concentration did not inhibit and, in absence of catalase, appeared to activate. This apparent activating effect was found to be caused by the oxidation of the thiourea by the hydrogen peroxide formed as a product of the primary reaction; it was suggested that the thiourea partially protects the enzyme from inactivation by hydrogen peroxide. It therefore appears that the evidence so far reported is insufficient to exclude the possibility of the presence of copper, or other heavy metal, in animal diamine oxidase and histaminase.

## SUMMARY

1. Purified preparations of pea-seedling diamine oxidase catalysed the oxidation not only of aliphatic diamines but also that of aliphatic monoamines, phenylalkylamines, histamine, spermidine, agmatine and the amino acids lysine and ornithine.

2. The diamine-oxidase preparations were pink solutions with maximum absorption, in the visible region, at about 500 mμ. When substrate was added under anaerobic conditions the colour changed to yellow and the absorption band with maximum at 500 mμ was replaced by bands with maxima at 466, 437·5 and 350 mμ. The pink colour was restored by oxygenation. These colour changes were produced by all the substrates tested but not by related compounds that are not substrates. Evidence was obtained that the yellow products are enzyme–substrate complexes.

3. 1,10-Phenanthroline, which inhibits the enzyme, prevents the formation of the yellow products and discharges their colour. It is suggested that the yellow products are copper complexes. The inhibition of the formation of the complexes by 1,10-phenanthroline is reversed by $Ni^{2+}$ ions.

4. The copper-free protein, obtained by incubating the diamine oxidase with sodium diethyldithiocarbamate, was catalytically inactive. The activity towards all the substrates tested was restored specifically by $Cu^{2+}$ ions.

5. The copper-free protein was orange–pink with maximum absorption at about 480 mμ. Substrate, added under anaerobic conditions discharged the colour but yellow complexes were not formed. The pink colour was not restored by oxygenation. When the copper-free protein was first reactivated by $Cu^{2+}$ ions the addition of substrate produced the yellow complex and subsequent oxygenation restored the pink colour.

6. The yellow complexes were stable under anaerobic conditions but $Cu^{2+}$ ions caused a rapid abnormal breakdown resulting in inactivation of the enzyme. This breakdown was prevented by ethylenediaminetetra-acetate.

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