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Biochem. J. (1961) **79**, 623

Further Purification and Properties of the Amine Oxidase of Pea Seedlings

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(Received 13 October 1960)

Although amine oxidases have been studied intensively for more than 20 years, the nature of their prosthetic groups has not been established. The amine oxidase of pea seedlings belongs to the group of amine oxidases that is inhibited by carbonyl reagents such as hydrazine and semicarbazide; the group also includes the spermine, benzylamine and diamine oxidases of animal tissues. The sensitivity of animal diamine oxidase to carbonyl reagents led to the suggestion that the enzyme contains a functional aldehyde or ketone group (Zeller, 1938). Much evidence has since been reported that the prosthetic groups both of animal diamine oxidase (Davison, 1956; Goryachenkova, 1956) and of the related plant enzyme (Werle & Pechmann, 1949; Goryachenkova, 1956) contain pyridoxal phosphate. It has also been suggested that the prosthetic group of spermine oxidase contains pyridoxal phosphate (Tabor, Tabor & Rosenthal, 1954).

Hydrogen peroxide is a product of the reactions catalysed by amine oxidases, so it has been suggested that some of these enzymes are flavoproteins. Kapeller-Adler (1949) and Goryachenkova (1956) confirmed and extended the work of Zeller, Stern & Wenk (1940) and Swedin (1943, 1944) on the flavoprotein nature of animal diamine oxidase and concluded that the prosthetic group of the enzyme contains flavinadenine dinucleotide. Goryachenkova (1956) also reported evidence that flavinadenine dinucleotide forms a part of the prosthetic group of the amine oxidase of pea and clover seedlings.

The finding that animal diamine oxidase is inhibited by cyanide suggested that the enzyme is a metalloprotein. Zeller (1940) found that other

reagents that react with heavy metals, such as sodium azide, thiourea and sodium sulphide, caused only small inhibitions, which were attributed to effects on secondary oxidations. It was concluded that animal diamine oxidase was inhibited by cyanide because this compound acts as a carbonyl reagent. The inhibition of the related plant enzyme by cyanide was attributed to the same cause by Kenten & Mann (1952). Mann (1955), working with 300-fold purified preparations of pea-seedling amine oxidase, found that the enzyme was inhibited by sodium diethyldithiocarbamate, salicylaldoxime and potassium ethyl xanthate and suggested that it might be a metalloprotein. Preliminary investigations of the heavy-metal content of the preparations showed that copper and manganese were present in possibly significant amounts. Werle & Hartung (1956) found that both the plant enzyme and animal diamine oxidase are inhibited by sodium diethyldithiocarbamate, salicylaldoxime, 8-hydroxyquinoline and dithizone. In the experiments on animal diamine oxidase, with 1:5-diaminopentane as substrate, the ammonia formation was completely inhibited by 8-hydroxyquinoline and sodium diethyldithiocarbamate. Such inhibitions cannot be attributed to effects on secondary oxidations. Suzuki (1959) reported that the plant enzyme is also inhibited by 2:2'-dipyridyl and 1:10-phenanthroline and, on the basis of the inhibition by metal-chelating agents, suggested the presence of iron, molybdenum or zinc in the prosthetic group of the enzyme.

The evidence so far obtained suggests the possibility that the enzymes contain pyridoxal phosphate, flavinadenine dinucleotide and a heavy metal in the prosthetic groups. Direct evidence for

pyridoxal phosphate or flavinadenine dinucleotide should be given by the absorption spectra of sufficiently purified preparations. Though the diamine oxidase and spermine oxidase of animal tissues have been extensively purified, no such spectrophotometric evidence has been reported. Pea seedlings provide a very rich source of plant amine oxidase. Mann (1955) described a method giving a 300-fold purification of the amine oxidase of pea-seedling extracts. The preparations so obtained were about 25 times as active, in the oxidation of 1:4-diaminobutane, as the purest preparations of animal diamine oxidase previously obtained. This suggested that evidence of the nature of the prosthetic group might be obtained more readily with the plant enzyme than with the related animal enzymes. The present work was started to test this possibility. A chromatographic procedure for further purifying the plant enzyme is reported together with preliminary spectrophotometric studies of the preparations and estimations of their content of heavy metals. Some of this work has already been briefly described (Mann, 1960).

MATERIALS AND METHODS

Calcium phosphate. Calcium phosphate (hydroxyapatite) was prepared by the method of Tiselius, Hjertén & Levin (1956).

Buffers. Sodium phosphate buffers were prepared from Na_2HPO_4 and H_2PO_4 . Potassium phosphate buffers were prepared from KH_2PO_4 and KOH .

Catalase. Crystalline ox-liver catalase was prepared as described by Clarke & Mann (1959).

Estimation of amine oxidase. Amine oxidase was estimated manometrically, in air, in the Warburg apparatus at 28°. The volume of the reaction mixtures was 3 ml. and 0.2 ml. of 5N-KOH was present in the centre well. A unit of amine oxidase is defined as the amount which at 28° gives an uptake of 1 $\mu\text{l.}$ of O_2 /hr. (calculated from initial rates) in the presence of 10 mm-1:4-diaminobutane, catalase and 67 mm-phosphate buffer, pH 7, in a total volume of 3 ml. (Mann, 1955). The specific activity is defined as oxidase units/mg. of N.

Estimation of total nitrogen. The total nitrogen of the amine-oxidase preparations was estimated by a micro-Kjeldahl method. Samples (0.1–0.2 ml.) were digested with 1 ml. of conc. H_2SO_4 and approx. 0.2 g. of a catalyst (composition: K_2SO_4 , 80 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 10 g.; SeO_2 , 1 g.). The ammonia was distilled into boric acid solution (1%) and estimated by titration with HCl .

Metal estimations. The manganese and copper contents of the amine-oxidase preparations were estimated colorimetrically. For each estimation a sample (2–4 ml.) was digested with 2 ml. of a mixture (1:1, v/v) of conc. H_2SO_4 and redistilled HNO_3 . The manganese content of the digests was estimated by oxidation, with KIO_4 , to permanganate (Sandell, 1950); copper was estimated with sodium diethyldithiocarbamate by the procedure of Forster (1953), except that the extinction of the final solutions was measured at 436 $\text{m}\mu$. The metal contents of

two preparations were estimated spectrographically by Dr H. H. Le Riche, Soil Survey, Rothamsted Experimental Station, Harpenden, Herts. Samples (4–5 ml.) of the enzyme preparations were dialysed against 2 l. of distilled water for about 15 hr. The dialysed enzyme was washed from the sac into a platinum crucible and mixed with 5 ml. of aq. AlCl_3 soln. (equivalent to 20 mg. of Al_2O_3). The AlCl_3 used was free from spectrum lines of the trace elements under investigation. A few drops of aq. NH_3 soln. (sp.gr. 0.88) were added and the mixture was evaporated to dryness at 100° and then ignited in a muffle furnace at 450°. The residue was analysed with a Hilger large spectrograph with a d.c. carbon arc.

Spectrophotometry. Absorption was measured with a Unicam SP. 500 spectrophotometer. Anaerobic conditions, where necessary, were established by using Thunberg-type assemblies fitted to silica cells by ground-glass joints. These were specially made by Unicam Instruments Ltd.

Preparation and purification of the enzyme

Initial preparations. The amine-oxidase preparations used as starting material for purification by chromatography were made from 9-day-old pea seedlings by the method of Mann (1955), except that the roots of the seedlings were discarded. The weight of plant material used for each preparation was 1–3 kg. The volume of the enzyme preparations (in 10 mm-potassium phosphate buffer, pH 7) was such that 1 ml. was equivalent to 100 g. of the plant tissue. These enzyme preparations were stored at -10° until there was enough for the next stage in the purification.

Chromatography on calcium phosphate (hydroxyapatite). All manipulations during the chromatography of the enzyme on hydroxyapatite and in the subsequent stages of the purification were carried out at 0–5°. Five to eight of the initial oxidase preparations were combined and centrifuged. A small sample of the supernatant solution was reserved for initial estimations and the rest was applied to a hydroxyapatite column (4 cm. $\times$ 12 cm.) equilibrated with 10 mm-potassium phosphate buffer, pH 7. Elution was started with 40 mm-potassium phosphate buffer, pH 7, and continued by stepwise increments (40 mm) in the concentration of buffer up to a final concentration of 200 or 240 mm. The volume of buffer at each concentration was 100 ml. The rate of flow varied from 50 to 80 ml./hr. in different experiments. The eluate was collected in 5 ml. fractions. The fractions of the main amine-oxidase peak showing the highest amine-oxidase activity in relation to $E_{280 \text{ m}\mu}$ were combined and dialysed against 2 $\times$ 3 l. of distilled water. The ratio oxidase units/ $E_{280 \text{ m}\mu}$ in the fractions combined generally exceeded 10^4 ; fractions in which this ratio was less than 5×10^3 were rarely included.

Adsorption of inert material on aminoethyl- or diethylaminoethyl-cellulose. The dialysed oxidase solution was passed through a column (2 cm. $\times$ 10 cm.) of aminoethyl-cellulose (AE-cellulose) or diethylaminoethylcellulose (DEAE-cellulose), which was then washed with 25 ml. of 10 mm-potassium phosphate buffer, pH 7. To prepare the columns, Whatman AE 50 or DE 50 powder was suspended in water and adjusted to pH 7 with $\text{m-KH}_2\text{PO}_4$. The columns were equilibrated with 10 mm-potassium phosphate buffer, pH 7, before use.

Concentration on calcium phosphate (hydroxyapatite). The enzyme solutions and washings from the AE- or DEAE-cellulose column were combined and applied to a column of

Table 1. *Further purification of the amine oxidase of pea seedlings*

Preparation no.	Initial enzyme preparation				Final enzyme preparation				
	Amine oxidase				Amine oxidase				Yield (%)
	Vol. (ml.)	$10^{-3} \times$ Content (units/ml.)	N content (mg./ml.)	$10^{-3} \times$ Specific activity	Vol. (ml.)	$10^{-3} \times$ Content (units/ml.)	N content (mg./ml.)	$10^{-3} \times$ Specific activity	
1	150	40	0.450	89	16	185	0.970	191	59
2	80	61	0.625	98	10	258	1.100	235	53
3	120	46	0.438	105	20	153	0.700	219	55
4	130	54	0.500	108	14	254	0.775	328	51
5	155	43	0.575	75	20	187	0.675	277	56
6	160	50	0.514	97	14	250	0.900	278	44

hydroxyapatite (4 cm. $\times$ 4 cm.). The enzyme was eluted with 200 mM-potassium phosphate buffer, pH 7, and the active fractions of the eluate were combined and stored at -10° .

RESULTS

Purification of the enzyme

Chromatography on calcium phosphate (hydroxyapatite). Fig. 1 shows the fractionation of an initial enzyme preparation on the hydroxyapatite column (preparation 3, Table 1). Determinations of $E_{280\text{ m}\mu}$ of the fractions suggested that the proteins were partially separated into three main parts. The first two (fractions 26–70 and 70–106) were yellow and the third (fractions 106–140) was weakly pink. The secondary peak among the pink fractions was generally, though not always, observed and was probably correlated with an increase in the concentration of buffer. The fractions round the main peak (fractions 114–118) were the pinkest. In the fractions on either side of this peak the colour decreased and was progressively masked or replaced by yellow. The bulk of the enzyme was found in the pink fractions. The curve relating enzyme units to fraction number showed major and minor peaks which coincided with those of the extinction curve. The fractions of the main peak (in this experiment fractions 96–124) were combined for later purification and concentration.

Adsorption of inert material on aminoethyl- or diethylaminoethyl-cellulose. Under the experimental conditions the enzyme in the dialysed combined active fractions from the hydroxyapatite column was not adsorbed on AE- or DEAE-cellulose but much inert material, including most of the yellow pigment, was adsorbed and separated from the enzyme, particularly in the later experiments (preparations 4–6, Table 1) in which DEAE-cellulose was used.

Concentration on calcium phosphate (hydroxyapatite). When the combined active fractions (300–400 ml.) from the AE- or DEAE-cellulose columns were applied to a hydroxyapatite column (4 cm. $\times$ 4 cm.) the enzyme was associated with a pink band, a few millimetres in depth, which

Fig. 1. Fractionation of an initial amine oxidase (preparation 3, Table 1) on a hydroxyapatite column. O, $E_{1\text{ cm.}} (280\text{ m}\mu)$ of fractions; ●, amine-oxidase content of fractions.

formed at the top of the column. On elution with 200 mM-potassium phosphate buffer, pH 7, this band moved down the column and when it approached the bottom of the column the eluate was collected in 2 ml. fractions. The enzyme was found in the pink fractions, which were combined and stored at -10° .

Overall purification. Table 1 shows the amine-oxidase and total-N contents of the initial and final preparations from which the specific activities and yields were calculated. The specific activities of the amine oxidase in eight of the pea-seedling extracts from which the initial preparations of Table 1 were made ranged between 240 and 430, with an average value of 335. The average specific activity of the initial preparations of Table 1 was 94×10^3 . This was increased to an average specific activity of 215×10^3 in preparations 1–3, where fractionation on hydroxyapatite was followed by fractionation on AE-cellulose, and to 294×10^3 in preparations 4–6, where DEAE- replaced AE-cellulose. The total purification of the amine oxidase

of the pea-seedling extracts in the latter preparations was therefore about 880-fold. The preparations were stable; activity was not clearly decreased after storage for several weeks at -10° .

Spectrophotometric investigations

Absorption spectrum. Fig. 2 shows the absorption spectrum of the purest of the final preparations (4, Table 1). The pink colour of the preparation was associated with a band with maximum

Fig. 2. Absorption spectra of one of the final amine-oxidase preparations. ●, Preparation 4 (Table 1) containing 254 000 units/ml. (right-hand ordinate); ○, the same preparation diluted 1:5 with water (left-hand ordinate).

Fig. 3. Absorption spectra of initial and final preparations of the amine oxidase. ○, Initial preparation 3 (Table 1) containing 46 000 units/ml.; ●, final preparation 3 containing 153 000 units/ml.; □, final preparation 6 containing 250 000 units/ml.

absorption at about 500 mμ. This band was shown clearly by the undiluted preparation but it was very weak compared with the protein band at about 280 mμ ($E_{500 \text{ m}\mu}/E_{280 \text{ m}\mu} = 0.017$). Over the wavelength range investigated (260–740 mμ) these were the only two bands observed in the absorption spectra of the final preparations. Fig. 3 compares the absorption, in the visible region of the spectrum, of one of the initial preparations with two of the final preparations. In the initial preparations the presence of the pink compound was obscured by coloured impurities and the absorption spectra showed only slight inflexions in the region of 500 mμ. The 500 mμ band was shown by all the final preparations and the results of Figs. 2 and 3 suggested that its intensity was related to the concentration of amine oxidase.

Reaction with dithionite. The pink colour of the enzyme preparations was discharged by sodium dithionite and restored by bubbling O_2 through the solution. Fig. 4 shows the corresponding changes in the absorption spectrum of the enzyme solution over the wavelength range 400–700 mμ during these reactions. The absorption at 500 mμ decreased rapidly during the first few minutes after addition of the dithionite and thereafter more slowly and showed little further change after 45 min. The return of the colour when O_2 was subsequently bubbled through the solution was accompanied by an almost complete restoration of the original absorption spectrum.

Fig. 4. Reduction of a purified preparation of the amine oxidase with $Na_2S_2O_4$ and re-oxidation with O_2 . The amine oxidase was a solution, containing 0.775 mg. of N/ml., of a preparation of specific activity 328×10^3 in 200 mM-phosphate buffer, pH 7. Absorption spectra: ○, before addition of $Na_2S_2O_4$; □, 1 hr. after the addition of 1 mg. of $Na_2S_2O_4$ to 3 ml. of the enzyme solution; ●, after subsequent oxygenation, for 10 min., of the mixture of enzyme and $Na_2S_2O_4$.

Reaction with 1:4-diaminobutane. A mixture of amine-oxidase preparation and catalase was placed in the cell and the 1:4-diaminobutane was placed in the Thunberg-type hollow stopper. The assembly was then evacuated. Fig. 5 shows the changes in the absorption spectrum of the mixture. The pink colour changed to yellow on addition of the 1:4-diaminobutane and the band at $500\text{ m}\mu$ was replaced by bands with maxima at 465 , 435 and $350\text{ m}\mu$. The change from yellow to pink on oxygenation was accompanied by the disappearance of the bands at 465 , 435 and $350\text{ m}\mu$ and the re-appearance, at its original intensity, of the band at $500\text{ m}\mu$.

Fig. 6 shows the absorption spectra of pyridoxal phosphate and of mixtures of pyridoxal phosphate with 1:4-diaminobutane at pH 7. The absorption spectrum of pyridoxal phosphate at pH 7 shows bands with maxima at 330 and $388\text{ m}\mu$. Addition of 1:4-diaminobutane intensified the yellowness of the solution. This change was correlated with a shift of the $388\text{ m}\mu$ band to longer wavelengths; the $330\text{ m}\mu$ band was reduced in intensity and a new

band appeared with maximum absorption at about $275\text{ m}\mu$. These changes, which may be attributed to the formation of the Schiff's base (Matsuo, 1957), were pronounced only when the concentration of

Fig. 6. Absorption spectra of pyridoxal phosphate and of mixtures of pyridoxal phosphate and 1:4-diaminobutane. Reaction mixtures contained 0.05 mm -pyridoxal phosphate, with and without 1:4-diaminobutane, in 100 mm -phosphate buffer, pH 7. The mixtures were allowed to stand for 1 hr. before the absorption spectra were determined. $\circ$, Without 1:4-diaminobutane; $\bullet$, 0.33 mm -1:4-diaminobutane; $\square$, 3.3 mm -1:4-diaminobutane; $\triangle$, 10 mm -1:4-diaminobutane.

Fig. 5. Reaction of a purified amine-oxidase preparation with 1:4-diaminobutane. The amine oxidase was a solution (2.5 ml.), containing 0.900 mg. of N/ml. , of a preparation of specific activity 278×10^3 in 200 mm -phosphate buffer, pH 7. Catalase ($25\text{ }\mu\text{g.}$) was added to this solution. Absorption spectra: $\circ$, before addition of 1:4-diaminobutane; $\bullet$, after addition, under anaerobic conditions, of 0.1 ml. of 10 mm -1:4-diaminobutane to 2.5 ml. of the enzyme solution; $\square$, after subsequent oxygenation of the mixture of enzyme and 1:4-diaminobutane.

Fig. 7. Reaction of a purified amine-oxidase preparation with hydrazine. The amine oxidase was a solution, containing 0.900 mg. of N/ml. , of a preparation of specific activity 278×10^3 in 200 mm -phosphate buffer, pH 7. Absorption spectra were determined before ($\circ$) addition of hydrazine sulphate and 10 min. ($\bullet$), 3 hr. ($\square$) and 6 hr. ($\triangle$) after addition of 0.1 ml. of 10 mm -hydrazine sulphate to 3 ml. of the enzyme solution.

1:4-diaminobutane was very high in relation to that of the pyridoxal phosphate.

Reaction with hydrazine. Fig. 7 shows the changes in the absorption spectrum of the final enzyme preparation produced by addition of hydrazine sulphate. When the hydrazine was added, the mixture changed from pink to yellow and the band at 500 $m\mu$ was replaced by bands at 420 and 330 $m\mu$. On standing in air at room temperature the yellow slowly faded but the mixture did not again become pink. The decrease in the intensity of the yellow was correlated with a decrease in the intensity of the band at 420 $m\mu$, which, after 3 hr., was no longer detectable in the absorption spectrum of the solution. The band at 330 $m\mu$ also decreased in intensity, though more slowly, on standing; it was still apparent in the absorption spectrum after 6 hr.

Investigations on the nature of the essential metal

Metal content. Spectrographic analysis of one of the final preparations (1, Table 1) showed that manganese and copper were the trace elements present in highest concentrations. Zinc and iron were present in lower, but possibly significant, amounts. Table 2 shows the results of colorimetric analyses of the copper and manganese contents of the initial and final preparations. The initial preparations contained more manganese than copper but during purification the copper content increased whereas the manganese content decreased. In the final preparations (4-6), the copper content rose to 0.08-0.09% while the manganese content fell to about 0.01%. These results were confirmed by a spectrographic analysis of preparation 6, which gave a copper content of 0.085% and a manganese content of 0.006%. The spectrographic analysis also showed that, except for zinc, the concentration of which was estimated to be <0.06%, other trace elements, including iron and molybdenum, were present at concentrations <0.004%.

Inactivation by dialysis against diethyldithiocarbamate and reactivation by Cu^{2+} ions. The

potassium phosphate buffer and 1:4-diaminobutane used in these experiments were freed from traces of copper by extraction with a solution of diphenylthiocarbazone (dithizone) in CCl_4 , as described by Hewitt (1952), and then freed from traces of dithizone by extraction with CCl_4 .

A sample (4 ml.) of one of the final preparations (5) was dialysed, at 0-5°, in Visking cellulose tubing against 40 ml. of 10 mM-sodium diethyldithiocarbamate in 200 mM-phosphate buffer, pH 7. The colour of the enzyme preparation inside the sac changed from pink to golden yellow within 15 min., and a yellow precipitate was slowly deposited, leaving the supernatant solution pink. After 24 hr. the precipitate (A) and the supernatant solution (B) were separated by centrifuging.

The precipitate (A) was dissolved in 10 ml. of CCl_4 . The absorption spectrum of the resulting yellow solution resembled solutions of the copper

Fig. 8. Inactivation of amine oxidase by dialysis against sodium diethyldithiocarbamate, as described in the text, and reactivation with Cu^{2+} ions. The amount of enzyme preparation used in each vessel was 0.0025 ml. of the undialysed preparation (5) or an equivalent volume of the preparation after dialysis. O, Undialysed preparation. Dialysed preparation: ●, no added Cu^{2+} ions; Δ, 0.1 μM - Cu^{2+} ions; □, 1 μM - Cu^{2+} ions; ×, 10 μM - Cu^{2+} ions.

Table 2. Copper and manganese contents of the initial and final preparations

Results are based on the protein contents of the preparations, which were calculated from the N contents by using the factor of 6.25.

Preparation no.	Initial preparation			Final preparation		
	$10^{-3} \times$ Specific activity	Cu (%)	Mn (%)	$10^{-3} \times$ Specific activity	Cu (%)	Mn (%)
1	89	0.040	0.069	191	0.063	0.059
2	98	0.046	0.074	235	0.062	0.049
3	105	0.045	0.066	219	0.057	0.038
4	108	0.053	0.067	328	0.082	<0.01
5	75	0.031	0.072	277	0.079	<0.01
6	97	0.042	0.063	278	0.092	0.014

diethyldithiocarbamate complex in CCl_4 , and showed a band with maximum absorption at $436\text{ m}\mu$. Assuming the extinction at $436\text{ m}\mu$ was entirely due to the copper complex it was calculated that the solution contained $12.5\text{ }\mu\text{g.}$ of copper; the copper content of the sample of enzyme used in the experiment was $13.4\text{ }\mu\text{g.}$

The supernatant solution (B) was dialysed for 16 hr. at $0-5^\circ$ against 200 ml. of 10 mM-phosphate buffer, pH 7. Fig. 8 shows that the dialysed preparation retained little of the activity of the original preparation; most of the lost activity was restored by the addition of Cu^{2+} ions (as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$). Maximum reactivation was given by Cu^{2+} ions at a concentration of $1\text{ }\mu\text{M}$. Copper ions at concentrations of $0.1-10\text{ }\mu\text{M}$ did not increase the activity of the original preparation but at $10\text{ }\mu\text{M}$ produced an inhibition which increased during the course of the oxidation. With this concentration of Cu^{2+} ions an inhibiting effect was also observed with the dialysed enzyme, after an initial activation (Fig. 8). Zinc ions (added as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) at concentrations of $0.1-10\text{ }\mu\text{M}$ had no activating effect on the dialysed preparation.

DISCUSSION

The purest preparations of pea-seedling amine oxidase hitherto reported were made by Uspenskaia & Goryachenkova (1958) by electrophoretic fractionation of initial preparations obtained by the method of Mann (1955). The combined methods gave a 550-fold purification of the enzyme but the final preparations were unstable and lost all their activity within 24 hr. The method now described, involving chromatographic fractionation on hydroxyapatite and DEAE cellulose, yields purer and more stable preparations.

These preparations of the plant enzyme have activities which are much higher than those so far reported for the purified preparations of the related animal diamine oxidase, though exact comparison is rarely possible because different methods are used to express the specific activities. The highly purified preparations of the animal enzyme obtained by Tabor (1951) had a specific activity (enzyme units/mg. of protein), calculated from the rate of oxidation of 1:4-diaminobutane at 37.5° , of 670. The preparations of the plant enzyme obtained by the present method had an average specific activity (enzyme units/mg. of nitrogen), calculated from the same reaction at 28° , of 294×10^3 . Taking into account the fact that the plant enzyme catalyses the oxidation of 1:4-diaminobutane about twice as rapidly at 37.5° as at 28° , and assuming a nitrogen content of 16%, it may be calculated that the plant preparations were about 140 times as active as the animal pre-

parations. The purest preparations of animal diamine oxidase so far obtained appear to be those of Uspenskaia & Goryachenkova (1958), which were claimed to be about three times as active as those of Tabor (1951).

During preliminary investigations of the substrate specificity of the enzyme it was found that the oxidation of di- β -phenylethylamine was not catalysed either by the final preparations or by pea-seedling extracts. The oxidation observed by Kenten & Mann (1952) may thus have been that of an impurity in the sample of di- β -phenylethylamine used.

The results of the spectrophotometric investigations of the final enzyme preparations suggest that the enzyme itself is pink; the intensity of the band with a maximum at $500\text{ m}\mu$ is related to the concentration of enzyme in these preparations. The pink compound is easily reduced by sodium dithionite and the reduced form is auto-oxidizable; it may function as a hydrogen carrier in the enzyme-catalysed reactions.

The fact that the pink colour of the preparations is discharged by 1:4-diaminobutane under anaerobic conditions, and is restored by subsequent oxygenation, could mean that the pink compound is involved in the catalytic activity of the enzyme and that it may undergo a cycle of reduction and re-oxidation during such activity. The plant amine oxidase belongs to the group of amine oxidases which are inhibited by hydrazine, so the fact that the colour is discharged by hydrazine provides further evidence that the enzyme is pink. This reaction suggests that the pink compound may be a carbonyl compound; the primary reaction product is yellow and absorbs maximally at $420\text{ m}\mu$, which indicates that an aromatic carbonyl compound is involved. Some of the Schiff's bases of pyridoxal phosphate, which are yellow and absorb maximally at $415\text{ m}\mu$, are unstable and gradually change to colourless forms absorbing maximally at $330-335\text{ m}\mu$ or $278-285\text{ m}\mu$ (Christensen, 1958). The resemblance between the behaviour of these Schiff's bases and that of the product of the reaction of hydrazine with the enzyme preparations supports the suggestion that an aromatic carbonyl compound is involved in this reaction. If this carbonyl compound is pyridoxal phosphate, it is not split from the enzyme during the reaction with hydrazine, because the hydrazone of pyridoxal phosphate is stable in phosphate buffer, pH 7, and absorbs maximally at 284 and $320\text{ m}\mu$ (Curry & Bulen, 1960). Assuming that the catalytic activity of the enzyme depends on the oxidation and reduction of an aromatic carbonyl compound present in its prosthetic group, the spectrum of the mixture of the enzyme preparation and 1:4-diaminobutane, under anaerobic conditions, may

be regarded as that of a Schiff's base formed between the reduced enzyme and the substrate. The fact that the absorption spectrum of the enzyme was changed greatly by low concentrations of 1:4-diaminobutane suggests that this enzyme-substrate compound is largely undissociated. The spectrophotometric investigations of the Schiff's base formed by 1:4-diaminobutane with free pyridoxal phosphate suggests that this compound is largely dissociated at pH 7.

The absorption spectra of the final enzyme preparations are not typical of an enzyme containing flavinadenine dinucleotide or pyridoxal phosphate in its prosthetic group. Enzymes which contain flavinadenine dinucleotide in their prosthetic groups show the characteristic absorption bands of this compound, whose maxima, at 375 and 450 $m\mu$, are usually only shifted some 5–15 $m\mu$, generally towards longer wavelengths, by combination with the apoenzymes. Thus the absorption maxima are at 377 and 455 $m\mu$ with glucose oxidase (notatin) (Keilin & Hartree, 1948) and at 389 and 465 $m\mu$ with the L-amino acid oxidase of moccasin venom (Singer & Kearney, 1950). In the absorption spectra so far described of enzymes containing pyridoxal phosphate, the position of the absorption band attributed to this compound varies with pH between about 330 and 430 $m\mu$ (e.g. glutamic acid decarboxylase; Shukuya & Schwert, 1960). When such enzymes are treated with 0.1N-sodium hydroxide, the pyridoxal phosphate is split off and the absorption spectrum then shows the band with maximum at 388 $m\mu$ characteristic of pyridoxal phosphate in neutral or alkaline solution. The pinkness of the amine-oxidase preparations is little affected by changes of pH over the range pH 5–10. The colour is discharged by 0.1N-sodium hydroxide but no evidence has so far been obtained from the absorption spectra that this change is correlated with the formation of free pyridoxal phosphate.

The copper content of the final preparations and the increase in copper content during the final purification indicate that the amine oxidase may be a copper-containing enzyme. This suggestion is supported by the results of the preliminary attempts to prepare the copper-free enzyme, by precipitation of the copper with diethyldithiocarbamate, and to demonstrate its activation by Cu^{2+} ions. The magnitude of this effect of Cu^{2+} ions has varied widely in different experiments, and conditions have not yet been established in which complete inactivation and reactivation of the enzyme always occurs. Further work is necessary to establish such conditions and to prove that the observed restoration of activity by Cu^{2+} ions is a true reactivation and not a de-inhibition, and that this reactivation is brought about specifically by

Cu^{2+} ions. The copper is apparently present in the enzyme in the cupric form; the possibility that the catalytic activity involves a valency change of the copper has not yet been investigated. Most complexes of cupric copper are blue or green but the only absorption band shown by the enzyme preparations in the visible part of the spectrum is that from the pink compound. It is therefore suggested that the copper occurs as a complex with the carbonyl compound and that this complex forms the prosthetic group of the enzyme.

SUMMARY

1. The amine oxidase of pea-seedling extracts was further purified by column chromatography on hydroxyapatite and diethylaminoethyl cellulose. This procedure, together with the preliminary fractionation previously described, gave an 880-fold purification.

2. Spectrophotometric investigations were made of the purified enzyme and of its reactions with sodium dithionite, 1:4-diaminobutane and hydrazine.

3. The final preparations were pink solutions with maximum absorption at about 500 $m\mu$; this was the only band detected in the visible part of the spectrum.

4. The colour was discharged by sodium dithionite and restored by oxygenation.

5. The enzyme solution changed from pink to yellow when 1:4-diaminobutane was added in anaerobic conditions. The pink was restored by oxygenation of the mixture.

6. The colour of the enzyme solution changed from pink to yellow when hydrazine was added. On standing in air the yellow slowly faded but the mixture did not again become pink.

7. The copper content of the enzyme preparations increased during purification to 0.08–0.09%. The copper was removed by precipitation with sodium diethyldithiocarbamate. The supernatant solution, after dialysis to remove sodium diethyldithiocarbamate, retained little of the activity of the original preparation. Most of the lost activity was restored by the addition to the dialysed solution of Cu^{2+} ions.

8. It is suggested that the pink colour is due to a carbonyl compound and that a copper complex of this compound forms the prosthetic group of the enzyme and undergoes a cycle of reduction and reoxidation during the catalytic activity.

I thank Dr H. H. Le Riche for the spectrographic analyses of the purified enzyme preparations and Unicam Instruments Ltd. for the gifts of the Thunberg-type assemblies used in the spectrophotometry.

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The Liver-Lipid Constituents of Male and Female Rats

1. EFFECTS OF THE FAT-DEFICIENCY SYNDROME

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(Received 26 August 1960)

Green, Horner, Lowe & Morton (1957), studying the effect of feeding cholesterol to rats, noted a sex difference in that the treatment decreased liver vitamin A reserves in males but not in females.

It is well established that male rats have a much higher requirement for linoleic acid than females (see, for example, Greenberg, Calbert, Savage & Deuel, 1950). Linoleate is the predominant ester of cholesterol in the plasma of healthy rats (Klein, 1957). Peifer & Holman (1955) and Holman & Peifer (1960) have shown that dietary cholesterol accelerates the onset of fat deficiency.

On such evidence, Green *et al.* (1957) suggested that the sex difference which they had observed might be no less closely linked with the intake of polyunsaturated fatty acids than with that of cholesterol.

The experiments to be described were planned to reveal any sex difference in respect of liver vitamin A stored by fat-deficient rats. The paper immediately following (Horner & Morton, 1961) is concerned with similar experiments in which fat-deficient rats were fed with cholesterol.

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Liver lipids other than vitamin A were also determined. The cholesteryl esters, triglycerides and phospholipids were separated on silicic acid columns, and the polyunsaturated acids from these fractions were analysed by alkali-isomerization. These procedures were first applied by Mead & Fillerup (1954) to the study of rat plasma lipids. They have since been used by several workers to observe the incorporation into the esters of liver and plasma of the polyunsaturated fatty acids supplied by various edible fats.

In the present work the emphasis is on differences between the sexes and between fat-deficient animals and controls given the minimum amount of linoleic acid believed to be essential for good growth.

EXPERIMENTAL

Animals and diets. Hooded rats were fed on the following basal fat-free diet from weaning: sucrose, 73%; casein (Genatosan), 21%; salts (Hubbell, Mendel & Wakeman, 1937), 4%; Monotype paper punchings, 2%. The vitamin mixture (Table 1) was added (3 g./kg. of diet).

Procedure. Nine male rats and nine females selected from four litters born over a period of 5 days were given the fat-free diet from weaning. Three days after the youngest