- Butler, J. A. V., Dodds, E. C., Phillips, D. M. P. & Stephen, J. M. C. (1948). *Biochem. J.* **42**, 116.
- Butler, J. A. V., Dodds, E. C., Phillips, D. M. P. & Stephen, J. M. C. (1949). *Biochem. J.* **44**, 224.
- Carpenter, F. H. & Baum, W. E. (1962). *J. biol. Chem.* **237**, 409.
- Czerkawski, J. W. & Bingle, J. P. (1964). *Biochem. J.* **91**, 394.
- Dudley, H. W. (1923). *Biochem. J.* **17**, 376.
- Epstein, A. A. & Rosenthal, N. (1924). *Amer. J. Physiol.* **70**, 225.
- Field, J. B., Tietze, F. & Stetten, D., jun. (1957). *J. clin. Invest.* **36**, 1588.
- Hales, C. N. & Randle, P. J. (1963*a*). *Lancet*, i, 200.
- Hales, C. N. & Randle, P. J. (1963*b*). *Biochem. J.* **88**, 137.
- Hartley, B. S., Naughton, M. A. & Sanger, F. (1959). *Biochim. biophys. Acta*, **34**, 243.
- Hiller, A. & Van Slyke, D. D. (1922). *J. biol. Chem.* **53**, 253.
- Hoffman, W. S. (1937). *J. biol. Chem.* **120**, 51.
- Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
- Laskowski, M. & Laskowski, M., jun. (1954). *Advanc. Protein Chem.* **9**, 203.
- Linderström-Lang, K. (1949). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 117.
- McFarlane, A. S. (1958). *Nature, Lond.*, **182**, 53.
- Nicol, D. S. H. W. (1960). *Biochem. J.* **75**, 395.
- Randle, P. J. (1954). *Brit. med. J.* i, 1237.
- Randle, P. J. (1957). *Ciba Found. Colloq.: Endocrinology*, **11**, 114.
- Randle, P. J., Taylor, K. W. & Vargas, L. (1960). *J. Endocrin.* **20**, xx.
- Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
- Scott, D. A. (1925). *J. biol. Chem.* **63**, 641.
- Shonle, H. A. & Waldo, J. H. (1923). *J. biol. Chem.* **58**, 731.
- Springell, P. H. (1961). *Nature, Lond.*, **191**, 1372.
- Tomizawa, H. H., Nutley, M. L., Narahara, H. T. & Williams, R. H. (1955). *J. biol. Chem.* **214**, 285.
- Vallance-Owen, J. & Hurlock, B. (1954). *Lancet*, i, 68.
- Vallance-Owen, J. & Lukens, F. D. W. (1957). *Endocrinology*, **60**, 625.
- Witzemann, E. J. & Livshis, L. (1923). *J. biol. Chem.* **57**, 425.
- Young, F. G. (1962). *Proc. Roy. Soc. B*, **157**, 1.
- Young, J. D. & Carpenter, F. H. (1961). *J. biol. Chem.* **236**, 743.

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Purification of Pig-Kidney Diamine Oxidase and its Identity with Histaminase

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(Received 18 July 1963)

Best (1929) described an enzyme, histaminase (EC 1.4.3.6, diamine-oxygen oxidoreductase), that catalyses the oxidation of histamine and which is distributed in various animal tissues (Best & McHenry, 1930). Mann (1961) obtained a highly purified preparation of pea-seedling diamine oxidase and showed that it is a metalloenzyme containing cupric copper (Hill & Mann, 1962). Yamada & Yasunobu (1962*a, b*) suggested that crystalline plasma amine oxidase can be represented as protein-(Cu²⁺)₄-(pyridoxal phosphate)₂. The loss in activity of animal diamine oxidase after treatment with carbonyl reagents suggests that this enzyme contains a functional aldehyde or ketone group (Zeller, 1938*a*; Davison, 1956; Goryachenkova, 1956, 1959; Zeller, Stern & Wenk, 1940; Swedin, 1943). Kapeller-Adler (1949) and Kapeller-Adler & MacFarlane (1963) provided evidence that both FAD and pyridoxal phosphate are coenzymes of animal diamine oxidase. Werle & Hartung (1956) have shown that plant and animal diamine oxidases

are inhibited by chelating agents, and Mondovi, Rotilio & Costa (1963) and Goryachenkova & Ershova (1963) have provided evidence that pig-kidney diamine oxidase contains copper.

The purification of pig kidney diamine oxidase has been described by several authors (Stephenson, 1943; Laskowsky, 1945; Tabor, 1951; Swedin, 1958), and highly purified preparations have been obtained by Uspenskaia & Goryachenkova (1958), Mondovi (1961), Goryachenkova & Ershova (1963), Mondovi *et al.* (1963) and Kapeller-Adler & MacFarlane (1963).

Despite these numerous investigations, the identity of pig-kidney diamine oxidase with histaminase has not yet been established. Zeller (1951) and Blaschko, Friedman, Hawes & Nilsson (1959) concluded that the oxidation of histamine, and of such simple diamines as cadaverine and putrescine, was catalysed by a single enzyme that Zeller (1951) named 'diamine oxidase'. Goryachenkova (1958) arrived at similar conclusions, showing that it was

impossible to separate diamine-oxidase and histaminase activities by column electrophoresis. The identity of the two enzymes is also supported by experiments on competition between histamine and diamines (Zeller, 1938*b, c*; Kapeller-Adler, 1949) and by the results obtained with purified diamine oxidase, which is active towards histamine and diamines (Leloir & Green, 1946; Kapeller-Adler, 1949; Tabor, 1949; Mondovi *et al.* 1963; Goryachenkova & Ershova, 1963). Kapeller-Adler (1951) arrived at different conclusions and claimed that histaminase is a specific enzyme, clearly distinct from diamine oxidase. This view is based on the demonstration that histamine, in the presence of pig-kidney diamine oxidase, is oxidized much better than cadaverine and on the observation that, when FAD is added to dialysed enzyme preparations, the activity is restored only with histamine as substrate. Kapeller-Adler & MacFarlane (1963) purified a pig-kidney enzyme that is specific for histamine and is inactive, when used in small amount, on cadaverine.

The present work describes a simple method for obtaining a highly purified preparation of pig-kidney diamine oxidase and reinvestigates the problem of its identity with histaminase.

EXPERIMENTAL

Materials

Cadaverine chloride was obtained from L. Light and Co. Ltd., Colnbrook, Bucks., or Fluka A.-G. Chemische Fabrik, Buchs, Switzerland; histamine dihydrochloride, sodium borohydride, diethyldithiocarbamate and buffer salts were from E. Merck, Darmstadt, Germany; crystalline catalase was from Mann Research Laboratories Inc., Rahway, N.J., U.S.A.; pyridoxal 5-phosphate was from Hoffman-La Roche and Co. Ltd., Basel, Switzerland; DEAE-cellulose and CM-cellulose were from Serva, Heidelberg, Germany; cellulose powder (ashless) was from Whatman (W. and R. Balston Ltd., London).

Methods

Estimation of diamine-oxidase and histaminase activities. The enzyme assay was performed in parallel by two methods: (a) oxygen uptake; (b) oxyhaemoglobin oxidation (Mondovi, 1961; Mondovi *et al.* 1963).

Oxygen uptake was followed, in conventional Warburg apparatus, in the presence of cadaverine or histamine as substrate, as described by Mondovi *et al.* (1963). One unit of diamine oxidase is defined (according to the Report of the Commission on Enzymes of the International Union of Biochemistry) as the amount of enzyme that will catalyse the transformation of 1 μ mole of substrate/min. under the standard conditions, except that the substrate concentration was 17 mM for cadaverine and 1.7 mM for histamine. The rate was calculated for an incubation period of 30 min. Specific activity is expressed as units/mg. of protein.

The oxyhaemoglobin test was carried out as follows. Human oxyhaemoglobin solution (0.5%) was prepared as

described by Rossi-Fanelli & Antonini (1959). Solutions were made up containing 0.3 ml. of the sample, 1 ml. of 0.3M-potassium phosphate buffer, pH 7.4, 0.1 ml. of oxyhaemoglobin solution, 25 μ moles of substrate, and water to give a total volume of 3 ml., and were incubated for 30 min. at 38°. In the presence of the enzyme the solution turned from red to light brown owing to oxidation of haemoglobin (Zeller, 1938*b*; Stephenson, 1943; Mondovi, 1961; Mondovi *et al.* 1963). The oxidation is due to hydrogen peroxide produced by the action of diamine oxidase on the substrate. The oxidation of haemoglobin was followed by the change of extinction at 577 m μ , where the extinction of oxyhaemoglobin is much higher than that of the oxidation products (ferrihaemoglobin, etc.). This method is only semi-quantitative but is very sensitive.

Estimation of protein. Protein was determined by the biuret method of Goa (1953). Crystalline albumin from bovine serum (Sigma Chemical Co.) was used as the standard.

Estimation of copper. Copper was determined by the method of McFarlane (1932) with the modification described by Smith & Krueger (1962).

Spectrophotometry. Extinctions were measured with a Beckman model DU spectrophotometer.

Column chromatography. Columns of ion-exchangers were prepared as described by Peterson & Sober (1956). These contained a 2.2 cm. $\times$ 35 cm. cellulose bed equilibrated in a cold room (0–4°) with the first buffer solution of the linear gradient used for the elution. The eluting buffer was produced with a Varigrad mixing device (Technicon Chromatography Corp., New York, U.S.A.). The rate of elution varied from 0.3 to 0.6 ml./min.; 10 ml. fractions were collected.

Column electrophoresis. Column electrophoresis was performed according to the method of Porath & Hjerten (1962). The columns contained a 3.2 cm. $\times$ 60 cm. cellulose bed equilibrated in a cold room with the buffer. The cellulose was pretreated with borohydride as described by Porath & Hjerten (1962). The rate of elution varied from 2 to 4 ml./min.; 10 ml. fractions were collected.

Preparation A: isolation of purified enzyme

Except in the first two steps, all operations were performed at 0–4°.

Extraction and preliminary purification. Frozen pig-kidney cortex (1 kg.) was homogenized in 1200 ml. of water for 10 min. at room temperature in a Waring Blender to give fraction A1 (see Table 1). The crude homogenate was placed in a beaker and stirred in a water bath at 60° for 10 min., and then the beaker was transferred and shaken in an ice-water bath to cool the contents quickly to about 25°. The cooled homogenate was centrifuged for 1 hr. at 6000*g* and the precipitate discarded. To the supernatant fluid (fraction A2), solid ammonium sulphate was added to give 33% saturation (Green & Hughes, 1955). The mixture was allowed to equilibrate for 30 min. and centrifuged off at 6000*g* for 20 min. The supernatant was brought to 60% saturation with ammonium sulphate. After 30 min. the precipitate was collected by centrifuging, and dissolved in 80 ml. of 0.01M-sodium acetate buffer, pH 5.3, to give fraction A3.

Precipitation at pH 5.3. The solution obtained in the preceding stage was divided into two portions and each was dialysed with vigorous mechanical stirring against 10 l. of

Table 1. Purification, copper content and specific activity towards cadaverine and histamine of pig kidney diamine oxidase (preparation A)

Specific activity is expressed as μ moles of substrate oxidized/mg. of protein/min. Incubations were performed under standard conditions (see the text).

Fraction	Total protein (mg.)	Copper content (μ m-moles/mg. of protein)	Total activity (with 17 mM-cadaverine) (units)	Yield (%)	Degree of purification [1]	$10^4 \times$ Sp. activity (units/mg.)			Ratio: cadaverine-oxidase activity/histamine activity
						With 17 mM-cadaverine	With 1.7 mM-histamine	With 1.7 mM-histamine	
A 1. Crude homogenate	143 000	0.39	56.48	100		3.95	2.61		1.51
A 2. Supernatant from homogenate heated at 60° for 10 min.	15 400	3.75	37.57	67	6	24.4	12.5		1.95
A 3. 33-60% saturation with $(\text{NH}_4)_2\text{SO}_4$	4 200	1.8	34.65	61	21	82.5	40.9		2.02
A 4. Precipitation at pH 5.3	700	2.12	11.45	20	41	163.6	94		1.74
A 5. Column electrophoresis	50	2.67	4.92	8.7	249	984	462		2.13
A 6. Column chromatography on DEAE-cellulose*	3.75	10.57	2.56	4.5	1726	6820	3400		2.00

* The results refer to one preparation of crude homogenate. As a rule, the product from two such preparations was used for column chromatography.

0.01 M-sodium acetate buffer, pH 5.3, for 10 hr. and then against 5 l. (with two changes) of 0.1 M-sodium acetate buffer, pH 5.3, for 24 hr. The dialysed enzyme solution was centrifuged at 20000g for 40 min. The precipitate was suspended in 80 ml. of water, dissolved by adjusting the pH to 7.2-7.4 with N-ammonia and freeze-dried to give fraction A4.

Column electrophoresis. A portion (2 g.) of the freeze-dried powder, corresponding to about 700 mg. of soluble proteins, was dissolved in 25 ml. of 0.01 M-potassium phosphate buffer, pH 6.5, and dialysed with stirring against 2 l. of the same buffer for 12 hr. The enzyme solution was centrifuged to eliminate the insoluble materials and was placed on an electrophoresis column previously equilibrated with the same buffer. The top of the column was connected to the cathode and the bottom to the anode. The electrophoretic run was made in 0.01 M-potassium phosphate buffer, pH 6.5, for 36 hr., the current being 18 mA at 620 v. Fig. 1 shows the elution pattern obtained by this method and illustrates that diamine oxidase is the first protein eluted from the column. After the run the column was eluted with the same buffer, and 0.5 ml. of each fraction was tested by the oxyhaemoglobin method. The enzyme activity of 1 ml. of every second active fraction was determined in the Warburg apparatus. Fractions with a specific activity higher than 0.085 were combined and freeze-dried to give fraction A5.

Column chromatography on diethylaminoethylcellulose. The freeze-dried preparations from two electrophoresis columns, corresponding to about 100 mg. of protein, were dissolved in 6 ml. of 0.05 M-potassium phosphate buffer, pH 7.2, dialysed against this buffer for 12 hr. and placed on a column of DEAE-cellulose that had been previously equilibrated with the same buffer. Three chambers of the Varigrad device were charged, in order, with 150 ml. of potassium phosphate buffer, pH 7.2, of the following concentrations: (1) 0.05 M; (2) 0.15 M; (3) 0.3 M. Fig. 2 shows the elution pattern obtained by this method. The activity was present only in the first protein peak. All fractions that were positive in the oxyhaemoglobin test were pooled and freeze-dried. The freeze-dried powder was dissolved in

Fig. 1. Elution pattern obtained by column electrophoresis of 700 mg. of protein of preparation A4. The specific activity (O) is expressed as μ moles of cadaverine oxidized/mg. of protein/min. Each fraction contained 10 ml. of eluent. —, $E_{280 \text{ m}\mu}^{1 \text{ cm}}$. Details are given in the text.

4 ml. of water, dialysed against 0.1 M-potassium phosphate buffer, pH 7.4, and concentrated again by freeze-drying to give fraction A6.

Other oxidase preparations

Preparation B. In order to have a preparation containing both diamine-oxidase and histaminase activities in a soluble form, a heated extract from 500 g. of pig kidney was

prepared as described above for fraction A2, and the proteins precipitated between 20 and 60 % ammonium sulphate saturation were collected by centrifuging after 30 min. at 4°. The precipitate was suspended in 30 ml. of water and dialysed for 60 hr. against the buffer used for column chromatography or electrophoresis. Some insoluble material was centrifuged off.

Preparation C. This preparation was obtained by the method described by Kapeller-Adler & MacFarlane (1963).

RESULTS

Properties of preparation A

The preparation of an acetone-dried powder of pig kidney (Tabor, 1951) was avoided since this causes great losses in activity. Although precipitation at pH 5.3 also involves a great loss its effects a considerable purification. The purified diamine oxidase (fraction A6) loses about 50 % of its activity after 1 month at 0–4°, but is very stable for the same period at –20°.

Overall purification. Table 1 shows the diamine-oxidase activity, the protein content, the specific activities and the yields at each stage of the purification. The specific activity of the purified enzyme (fraction A6) relative to the crude homogenate (fraction A1) indicates a purification of about 1700-fold.

Copper content. The copper content (Table 1) increased during the purification of the enzyme. Probably in the heated extract (fraction A2) some other materials containing copper are present, but they are subsequently removed. In the final preparation (fraction A6) the copper content was 10.57 $\mu\text{m-moles/mg.}$ of protein, i.e. about 0.06 %, which is similar to that observed by Mann (1961) in pea-seedling diamine oxidase (0.08–0.09 %) and to that observed by Yamada, Yasunobu, Yamano & Mason (1963) in crystalline plasma amine oxidase (0.09 %).

Effect of pyridoxal phosphate on enzyme activity. Fig. 3 shows that with the crude homogenate (fraction A1) pyridoxal phosphate has no effect on the oxidation of cadaverine, whereas with the purified enzyme (fraction A6) this coenzyme strongly increases the oxygen uptake, especially at the beginning of incubation. If the enzyme is previously heated for 10 min. at 100° the activity is completely abolished (Fig. 3).

Effect of pH and substrate concentration on diamine-oxidase and histaminase activities. Blaschko *et al.* (1959) have shown that the rate of oxidation of cadaverine is very low at pH 6.3, in the presence of pig-kidney diamine-oxidase preparations, whereas the rate of oxidation of histamine is at a maximum. Zeller, Schär & Staehlin (1939), showed that histaminase is inhibited by a high concentration of histamine. Thus the optimum conditions for

Fig. 2. Elution pattern obtained by column chromatography of 100 mg. of protein of preparation A5 on DEAE-cellulose. Each fraction contained 10 ml. of eluent. —, $E_{280 \text{ m}\mu}^{1 \text{ cm.}}$. Details are given in the text. The fractions X gave a positive reaction in the oxyhaemoglobin test.

Fig. 3. Action of pyridoxal phosphate on the diamine-oxidase activity of crude homogenate (preparation A1) and of purified enzyme (preparation A6). Incubations were carried out under standard conditions (see the text), with 34 mM-cadaverine as substrate. ●, Crude homogenate (200 mg. of protein) tested in the presence of 10 $\mu\text{g.}$ of pyridoxal phosphate; ○, crude homogenate (200 mg. of protein) tested in the absence of pyridoxal phosphate; ▲, purified enzyme (109 $\mu\text{g.}$) tested in the presence of 10 $\mu\text{g.}$ of pyridoxal phosphate; △, purified enzyme (109 $\mu\text{g.}$) tested in the absence of pyridoxal phosphate; □, purified enzyme (109 $\mu\text{g.}$) heated for 10 min. at 100° and tested in the presence of 10 $\mu\text{g.}$ of pyridoxal phosphate.

the study of the oxidation of cadaverine and of histamine by this enzyme differ. Fig. 4 illustrates the effect of concentration of substrate (histamine and cadaverine) on the rate of oxidation by purified enzyme (fraction A6) at three pH values. Very similar results were obtained with fraction A2.

Fig. 4. Effects of pH and substrate concentration on diamine-oxidase and histaminase activities of purified diamine oxidase (preparation A6). Oxygen uptakes were measured during 1 hr. under standard conditions (see the text), except that the molarity of buffer was 0.15. Each vessel contained 70 μ g. of enzyme. $\square$, Cadaverine, pH 7.4; $\triangle$, cadaverine, pH 6.7; $\circ$, cadaverine, pH 6.3; $\blacksquare$, histamine, pH 7.4; $\blacktriangle$, histamine, pH 6.7; $\bullet$, histamine, pH 6.3.

Attempts to separate diamine-oxidase and histaminase activities

Ammonium sulphate fractionation. Stepwise ammonium sulphate fractionation was performed by adding the salt to 20 ml. samples of fraction A2; the proteins precipitated at 0–20, 20–40, 40–60, 60–80 and 80–100% saturation with ammonium sulphate were collected after 30 min. at 4°, dissolved in 5 ml. of 0.1M-potassium phosphate buffer, pH 7.4, and dialysed against the same buffer overnight. Table 2 shows that the maximum diamine-oxidase and histaminase activities are present in the protein fraction precipitated at 40–60% saturation.

Table 2. Ammonium sulphate fractionation of preparation A2

Specific activity is expressed as μ moles of substrate oxidized/mg. of protein/min. Incubations were performed under standard conditions (see the text).

Percentage saturation with $(\text{NH}_4)_2\text{SO}_4$	$10^4 \times \text{Sp. activity}$	
	With 17 mM-cadaverine	With 1.7 mM-histamine
0–20	0	0
20–40	13.9	7.9
40–60	91.5	44.5
60–80	5.7	2.7
80–100	0	0

Fig. 5. Chromatography on DEAE-cellulose of 400 mg. of pig-kidney extract (preparation B). Three chambers of the Varigrad were charged, in order, with 200 ml. of potassium phosphate buffer, pH 7.2, of the following concentrations: (1) 0.01 M; (2) 0.1 M; (3) 0.3 M. Diamine-oxidase and histaminase activities are reported as follows: (a) oxygen uptake (upper right-hand scale) after incubation for 1 hr. under standard conditions by using 2 ml. of each fraction, with 17 mM-cadaverine ($\circ$) or 17 mM-histamine ($\triangle$); (b) oxyhaemoglobin test (lower right-hand scale) expressed as $\Delta E_{577 \text{ m}\mu}$ after incubation for 30 min. under standard conditions, by using 0.3 ml. of each fraction in the presence of 8.5 mM cadaverine ($\bullet$) or 8.5 mM-histamine ($\blacktriangle$). —, $E_{280 \text{ m}\mu}^{1 \text{ cm}}$. Details are given in the text.

Chromatography on diethylaminoethylcellulose and carboxymethylcellulose. Fig. 5 illustrates a chromatogram of 400 mg. of proteins (preparation B) obtained by using a concentration gradient of potassium phosphate buffer at pH 7.2. Diamine-oxidase and histaminase activities emerged in the same area of the chromatogram. From a replicate chromatogram, which gave similar results, the pooled contents of tubes 1-20, 21-35 and 35-50 (Fig. 5) were freeze-dried, dissolved in a little water and dialysed against 0.1M-potassium phosphate buffer, pH 7.4, for 12 hr.; oxygen uptakes of the samples were determined in the presence of 3.4 mM-histamine or 34 mM-cadaverine during a period of 13 hr. Table 3 illustrates that some activity was present in the third sample but that both cadaverine and histamine were oxidized. Other chromatograms were performed with DEAE-

cellulose as follows: four chambers of the Varigrad device were charged, in order, with potassium phosphate buffer of the following concentrations and pH values: (1) 0.01M, pH 8.5 (150 ml.); (2) 0.01M, pH 8.5 (75 ml.) + 0.01M, pH 5.5 (75 ml.); (3) 0.05M, pH 5.5 (150 ml.); (4) 0.1M, pH 5.5 (150 ml.). The results were similar to that described in Fig. 5. In order to elute all the proteins from the column, an elution with 0.5M-potassium chloride was carried out after the column chromatography. Some activity was present in the proteins that were more strongly absorbed but both cadaverine and histamine were oxidized. In a run with CM-cellulose (Fig. 6) the enzymic activities were again coincident.

Column electrophoresis. Column electrophoresis of 400 mg. of protein (preparation B) was performed at pH 6.5 (in 0.01M-potassium phosphate buffer for

Table 3. *Oxidation of cadaverine and histamine in the presence of the proteins eluted from a column chromatography on diethylaminoethylcellulose*

Activity is expressed as oxygen uptake under standard conditions (see the text). Each vessel contained 5 mg. of protein, with 34 mM-cadaverine or 3.4 mM-histamine as substrate. One drop of chloroform was added as preservative. Samples *A* corresponds to fractions 1-20, sample *B* to fractions 21-35 and sample *C* to fractions 35-50, obtained by chromatography on DEAE-cellulose as reported in Fig. 5. Other details are given in the text.

Time of incubation (hr.) ...	O ₂ uptake (μl.)					
	Sample <i>A</i>		Sample <i>B</i>	Sample <i>C</i>		
	2	13		2	13	
Substrate						
Cadaverine	10	12	690	17	170	
Histamine	0	0	220	13	126

Fig. 6. Chromatography on CM-cellulose of 400 mg. of pig-kidney extract (preparation B). Five chambers of the Varigrad were charged, in order, with 100 ml. of potassium phosphate buffer of the following concentrations and pH values: (1) 0.002M, pH 6.5; (2) 0.01M, pH 6.5; (3) 0.01M, pH 7; (4) 0.1M, pH 7.4; (5) 0.1M, pH 7.4. Diamine-oxidase and histaminase activities are reported as follows: (a) oxygen uptake (upper right-hand scale) with cadaverine (○) and histamine (Δ) as substrate; (b) oxyhaemoglobin test (lower right-hand scale) with cadaverine (●) and histamine (▲) as substrate. —, $E_{280\text{ m}\mu}^{\text{cm}}$. Details are given in the text and Fig. 5.

20 hr. at 24 mA and a potential of 700 v) and at pH 7.6 (in 0.01 M-potassium phosphate buffer for 9 hr. at 30 mA and a potential of 700 v). Both diamine-oxidase and histaminase activities were present in the same area and were well separated from the bulk of inactive proteins.

Ratio of cadaverine-oxidase to histaminase activity during the purification of diamine oxidase. Table 1 shows the specific activities with the two substrates during the purification of diamine oxidase (preparation A). In this case 1.7 mM-histamine and 17 mM-cadaverine were employed as substrates, at pH 7.4. The ratio of specific activities remained constant throughout the purification.

Oxidase activities of enzyme preparation C. Incubations were performed in Warburg vessels for 6 hr. in 0.175 M-potassium phosphate buffer, pH 6.8, with 1 mg. of the enzyme in the presence of 1 mM-cadaverine or 1 mM-histamine. In this case also both substrates were readily oxidized.

DISCUSSION

The copper content of the purified enzyme and the increase in copper content during the purification of the enzyme indicate that pig-kidney diamine oxidase may be a copper-containing protein. This suggestion is supported by the absorption spectrum of the purified enzyme in the presence of diethyldithiocarbamate (Mondovi *et al.* 1963), this being typical of copper-containing proteins (Mahler, Hübscher & Baum, 1955). The state and the role of copper have not yet been established.

The absorption spectrum of the purified enzyme shows only a single maximum at 280 m μ , as described by Mondovi *et al.* (1963). Yamada & Yasunobu (1962*a, b*) have demonstrated that one prosthetic group of plasma amine oxidase is pyridoxal phosphate, but this was revealed only in the presence of diethyldithiocarbamate. Only after this substance has removed the copper does the spectrum of pyridoxal phosphate appear. However, in pig-kidney diamine oxidase the copper is strongly bound to the protein and it is not removed by diethyldithiocarbamate (Mondovi *et al.* 1963). The presence of pyridoxal phosphate is indeed suggested by the sensitivity of diamine oxidase to such inhibitors as isoniazid, sulphite, cyanide and cysteamine, but the specificity of these substances is not absolute. Goryachenkova & Ershova (1963) observed a low content of pyridoxine in purified pig-kidney diamine oxidase, and Kapeller-Adler & MacFarlane (1963) demonstrated the fluorescence spectrum of pyridoxal phosphate in purified histaminase. We find that the activity of our purest enzyme (fraction A6) is increased by the addition of pyridoxal phosphate. Although this activation may be unspecific, it does suggest that pyridoxal

phosphate is a coenzyme of pig-kidney diamine oxidase.

Our efforts to separate diamine-oxidase and histaminase activities have all been unsuccessful. When the proteins of a crude pig-kidney extract (fraction A2) were separated by ammonium sulphate fractionation, by column chromatography and by column electrophoresis, the maximum activity was coincident towards both cadaverine and histamine. Further, the ratio of diamine-oxidase to histaminase activity was constant at all steps of purification. Only with crude homogenate (fraction A1) was the ratio lower, but this may be because the products obtained by the action of diamine oxidase on histamine are oxidized by other enzymes (Tabor, 1951). Moreover, histaminase prepared according to the method of Kapeller-Adler & MacFarlane (1963) and diamine oxidase purified as described in the present paper were active towards both substrates.

The effects of pH and substrate concentration on the oxidation of cadaverine and histamine (which are the same for a crude preparation and the purified enzyme) may explain some of the discrepancies described in the literature. With regard to the pH-dependence, it can be assumed that with both cadaverine and histamine the real substrate is the dicationic form of the diamine (Blaschko *et al.* 1959). Since there is a large difference in the dissociation constants of the second basic group in histamine and cadaverine (Blaschko *et al.* 1959; Albert, 1955), one would expect, as indeed it is found, that the pH-dependence of the activity is different with the two substrates. The activities towards cadaverine and histamine cannot therefore be directly compared at any pH.

The effect of substrate concentration is also different for the two substrates. Histamine, in fact, is oxidized at an optimum rate at a concentration of 1 mM, whereas higher concentrations inhibit. With cadaverine, on the other hand, the oxidation rate increases with substrate concentration even for concentrations as high as 17 mM. It was concluded that, in the pig-kidney cortex, diamine-oxidase and histaminase activities are present in a single enzyme and that some of the discrepancies in the literature may be explained by the experimental conditions used by different authors.

SUMMARY

1. Pig-kidney diamine oxidase was purified by controlled heat denaturation, pH precipitation, ammonium sulphate fractionation, column electrophoresis and column chromatography on DEAE-cellulose. The procedure gave 1700-fold purification.
2. The copper content of the enzyme preparations increased during purification to about 0.06 %.

It is suggested that pig-kidney diamine oxidase is a copper-containing protein.

3. The purified enzyme was strongly activated by the addition of pyridoxal phosphate.

4. The effects of pH and substrate concentration on diamine-oxidase and histaminase activities were investigated with both a crude preparation and the purified enzyme.

5. A pig-kidney-cortex extract containing diamine-oxidase and histaminase activities was fractionated by column chromatography on DEAE-cellulose and CM-cellulose and by column electrophoresis. The ratio of cadaverine-oxidase activity to histaminase activity remained about constant at all stages of purification. It is concluded that pig-kidney diamine-oxidase and histaminase activities are present in the same enzyme.

This work is part of a programme supported by 'Impresa di Enzimologia del Consiglio Nazionale delle Ricerche'.

REFERENCES

- Albert, A. (1955). *Brit. J. Pharmacol.* **10**, 314.
 Best, C. H. (1929). *J. Physiol.* **67**, 256.
 Best, C. H. & McHenry, E. W. (1930). *J. Physiol.* **70**, 349.
 Blaschko, H., Friedman, P. J., Hawes, R. & Nilsson, K. (1959). *J. Physiol.* **145**, 384.
 Davison, A. N. (1956). *Biochem. J.* **64**, 546.
 Goa, J. (1953). *Scand. J. clin. Invest.* **5**, 218.
 Goryachenkova, E. V. (1956). *Biokhimiya*, **21**, 247.
 Goryachenkova, E. V. (1958). *Dokl. Akad. Nauk S.S.S.R.* **123**, 898.
 Goryachenkova, E. V. (1959). *Vitamin Akad. Nauk Ukr. S.S.R.* **4**, 15.
 Goryachenkova, E. V. & Ershova, E. A. (1963). In *Chemical and Biological Aspects of Pyridoxal Catalysis*, p. 447. Ed. by Snell, E. E., Fasella, P. M., Braunstein, A. & Rossi-Fanelli, A. Oxford: Pergamon Press Ltd.
 Green, A. A. & Hughes, W. L. (1955). In *Methods in Enzymology*, vol. 1, p. 76. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Hill, J. M. & Mann, P. J. C. (1962). *Biochem. J.* **85**, 198.
 Kapeller-Adler, R. (1949). *Biochem. J.* **44**, 70.
 Kapeller-Adler, R. (1951). *Biochem. J.* **48**, 99.
 Kapeller-Adler, R. & MacFarlane, H. (1963). *Biochim. biophys. Acta*, **67**, 542.
 Laskowsky, M. (1945). *Arch. Biochem.* **6**, 105.
 Leloir, L. F. & Green, D. E. (1946). *Fed. Proc.* **5**, 144.
 McFarlane, W. D. (1932). *Biochem. J.* **26**, 1022.
 Mahler, H. R., Hübscher, G. & Baum, H. (1955). *J. biol. Chem.* **216**, 625.
 Mann, P. J. G. (1961). *Biochem. J.* **79**, 623.
 Mondovi, B. (1961). *Proc. 5th int. Congr. Biochem., Moscow, Abstr. Commun.* vol. 5, pp. 102, 1294.
 Mondovi, B., Rotilio, G. & Costa, M. T. (1963). In *Chemical and Biological Aspects of Pyridoxal Catalysis*, p. 415. Ed. by Snell, E. E., Fasella, P. M., Braunstein, A. & Rossi-Fanelli, A. Oxford: Pergamon Press Ltd.
 Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
 Porath, J. & Hjerten, S. (1962). *Meth. biochem. Anal.* **9**, 193.
 Rossi-Fanelli, A. & Antonini, E. (1959). *Arch. Biochem. Biophys.* **80**, 291.
 Smith, J. L. & Krueger, R. C. (1962). *J. biol. Chem.* **237**, 1121.
 Stephenson, N. R. (1943). *J. biol. Chem.* **149**, 169.
 Swedin, B. (1943). *Acta med. scand.* **64**, 209.
 Swedin, B. (1958). *Acta physiol. scand.* **42**, 1.
 Tabor, H. J. (1949). *Fed. Proc.* **8**, 335.
 Tabor, H. J. (1951). *J. biol. Chem.* **188**, 125.
 Uspenskaia, V. D. & Goryachenkova, E. V. (with Mogilevskaya, Z. G. & Poliakov, V. P.) (1958). *Biokhimiya (Engl. translation)*, **23**, 199.
 Werle, E. & Hartung, G. (1956). *Biochem. Z.* **328**, 228.
 Yamada, H. & Yasunobu, K. T. (1962a). *J. biol. Chem.* **237**, 1511.
 Yamada, H. & Yasunobu, K. T. (1962b). *Biochem. biophys. Res. Commun.* **3**, 387.
 Yamada, H., Yasunobu, K. T., Yamano, T. & Mason, H. S. (1963). *Nature, Lond.*, **198**, 1092.
 Zeller, E. A. (1938a). *Naturwissenschaften*, **26**, 578.
 Zeller, E. A. (1938b). *Helv. chim. acta*, **21**, 880.
 Zeller, E. A. (1938c). *Helv. chim. acta*, **21**, 1645.
 Zeller, E. A. (1951). In *The Enzymes*, vol. 2, part 1, p. 536. Ed. by Sumner, J. B. & Myrback, K. New York: Academic Press Inc.
 Zeller, E. A., Schär, B. & Staehlin, S. (1939). *Helv. chim. acta*, **22**, 837.
 Zeller, E. A., Stern, R. & Wenk, M. (1940). *Helv. chim. acta*, **23**, 3.