The Purification and Properties of Placental Histaminase

By J. K. SMITH

University Department of Clinical Chemistry, The Royal Infirmary, Edinburgh

(Received 26 May 1966)

1. Histaminase was extracted from desanguinated human placentae and purified by salt fractionation, ion-exchange chromatography and gel filtration. The purest preparation was still contaminated with haptoglobin-methaemoglobin. 2. Histaminase activity was measured by the *o*-aminobenzaldehyde method of Holmstedt & Tham (1959), Kapeller-Adler's (1951) test and a modified spectrophotometric indigodisulphonate test of greater sensitivity. 3. Unless contaminant metal ions were removed, enzymic activity on cadaverine, but not on histamine, fell during purification. When EDTA was added to the working buffers, a constant ratio between activities towards cadaverine and histamine was maintained throughout the later stages of purification, and activities towards the two substrates could not be separated by any of the highly resolving chromatographic analyses employed. 4. The purest preparation oxidized histamine, agmatine and benzylamine more slowly than the C₄-C₆ aliphatic diamines, but mixed-substrate experiments suggested that all these amines were substrates of histaminase. 5. The substrate and inhibitor specificities of placental histaminase were compared with those of related enzymes from other sources.

Extracts of hog kidney and human placenta catalyse the oxidative deamination of histamine and several aliphatic diamines, such as cadaverine (1,5-diaminopentane), according to the overall reaction (Zeller, 1938):

Kapeller-Adler (1956a) considered that extracts from these organs contained enzymes specific for histamine and for the aliphatic diamines respectively, and called them 'histaminase' and 'diamine oxidase'. Other workers have since obtained highly purified enzyme preparations from hog kidney (Mondovi, Rotilio, Finazzi & Scioscia-Santoro, 1964), pea seedlings (Hill & Mann, 1964) and pig plasma (Buffoni & Blaschko, 1964), and these preparations apparently contain single enzymes that catalyse the oxidative deamination of both histamine and the diamines. The Commission on Enzyme Nomenclature of the I.U.B. (1961) has accepted the view that these enzymes lack complete specificity and has named them diamine-oxygen oxidoreductase (deaminating) with trivial names diamine oxidase and histaminase (EC 1.4.3.6).

Because of the differences between the findings of Kapeller-Adler (1951) and of Mondovi *et al.* (1964) and Hill & Mann (1964), it was decided to purify and reinvestigate the specificity of the enzyme or enzymes from human placenta responsible for the

oxidative deamination of histamine and the aliphatic diamines. Earlier experiments on the substrate specificity of human placental histaminase used crude preparations of the enzyme (Zeller, 1938; Kapeller-Adler, 1951, 1956a; Lindahl, 1961). For comparison of activities on histamine and cadaverine after each stage of purification a single method of assaying histaminase activity on all substrates was ideally required, and the application of column-chromatographic fractionation procedures demanded a simple and sensitive method, suitable for the assay of numerous dilute effluents. Initially, the titrimetric indigo test devised by Kapeller-Adler (1951) was used, but this proved to be unsatisfactory, and a sensitive spectrophotometric modification of the indigo test was developed.

MATERIALS AND METHODS

Materials

Agmatine sulphate, diaminobutane dihydrochloride (putrescine), diaminopentane dihydrochloride (cadaverine), *o*-aminobenzaldehyde and neuraminidase (from *Clostridium perfringens*) were obtained from Sigma Chemical Co. Ltd., London, S.W. 6. Indigodisulphonate, histamine dihydrochloride, benzylamine hydrochloride, diaminoethane dihydrochloride, aminoguanidine disulphate, semicarbazide hydrochloride, hydroxylamine hydrochloride and phenylhydrazine hydrochloride were obtained from British Drug Houses Ltd., Poole, Dorset. Isoniazid was supplied by

Mann Research Laboratories, New York, N.Y., U.S.A., and diaminopropane dihydrochloride and diamino-hexane dihydrochloride were from Eastman-Kodak Ltd., Liverpool. All inorganic reagents were A.R. grade.

Column chromatography

DEAE-cellulose (Sigma Chemical Co. Ltd.) had an exchange capacity of 1 m-equiv./g., and cellulose phosphate (Whatman P-11) had a nominal exchange capacity of 7.4 m-equiv./g. Sephadex G-200 (Pharmacia, Uppsala, Sweden) was allowed to swell in distilled water at 5°, under toluene, for several months before repeated decantation of fines. The gel bed was retained between two fixed porous piston heads in the LKB Recychrom recycling chromatography apparatus (LKB Producter AB, Stockholm, Sweden). Sample application and elution were carried out in an upwards direction with the aid of a peristaltic pump.

Starch-gel electrophoresis

This was carried out by the method of Smithies (1955) for 20 hr. at a field strength of 10 v/cm. at 5°. Horizontal slices of the completed gel were stained for protein with Nigrosine (0.1% in water) and for haptoglobin-methaemoglobin with the following solution: 0.3 M-acetate buffer, pH 4.6, 35 ml.; *oo*-dianisidine, 35 mg.; ethanol, 35 ml.; water, 80 ml.; H₂O₂ (100 vol.), 1 ml. The histaminase content of macerated serial sections of the gel was determined by the method of Holmstedt & Tham (1959) (see below). Histaminase was located in the intact gel by incubating the gel in a solution having the same composition as test solutions in the latter method.

Estimation of protein

The protein concentration of enzyme solutions was estimated from the extinction at 280 m μ , an *E* value of 1.0 being expressed as a protein concentration of 1 mg./ml. EDTA (0.1 mM) was included in all buffer solutions used in the purification of histaminase and in activity measurements.

Measurement of histaminase activity

Indigodisulphonate method. The procedure used was identical with the modification by Kapeller-Adler & MacFarlane (1963) of the original method of Kapeller-Adler (1951), except that 0.1 mM-EDTA was added to the buffer, and concentrations of substrates found to be required to provide optimum conditions for placental histaminase activity were different (cadaverine, 1.14 mM; histamine, 0.136 mM). Kapeller-Adler (1956) considered that the oxidative deamination of 1 mole of substrate was accompanied by the production of 1 mole of H₂O₂, which stoichiometrically oxidized part of the indigodisulphonate present, the excess of dye being estimated by titration with dilute KMnO₄ solution at the end of incubation.

In the present work, difficulty was experienced in discerning the equivalence point in the presence of different substrates and coloured proteins (cf. Burkard, 1954) with the result that activity measurements below 5 permanent units (P.U.) were subject to errors of more than

10%. Activity was linearly related to enzyme concentration only up to 15 P.U. The decolorization of the dye did not start immediately on addition of enzyme and substrate solutions but was delayed for 4–8 hr. while H₂O₂ accumulated in the solution. The extent of dye oxidation after 24 hr. incubation was sometimes much greater than could be accounted for by the oxidation of all the substrate initially present. For these reasons, a modified procedure was adopted.

Modified spectrophotometric indigodisulphonate method. Histaminase solution (0.2 ml.) was added to 2.6 ml. of 0.067 M-phosphate buffer (Sørensen, 1909), pH 6.8, containing 0.1 mM-EDTA, and 1 ml. of 0.287 mM-indigodisulphonate. The solution was warmed to 37° and the reaction started by the addition of 0.2 ml. of substrate. The final concentration of each substrate used was 2.28 mM for cadaverine or putrescine, and 34 μ M for histamine. The solutions were incubated for 24 hr., and extinction was measured in a Unicam SP.500 spectrophotometer at 610 m μ , the wavelength of maximum extinction of indigodisulphonate (ϵ 1.73 $\times$ 10⁴ mole⁻¹ cm.⁻¹). The reference cuvette contained a more-dilute (36 μ M) solution of the dye in the same buffer. One spectrophotometric unit (S.U.) of histaminase activity was the amount required to produce a decrease of 1.0 in the *E* value in 24 hr.; in practice, the concentration of the enzyme was adjusted so that the measured decrease was in the range 0.02–0.15 S.U. Indigodisulphonate was very slowly decolorized by diamines in the presence of EDTA, and controls were incubated without enzyme rather than without substrate. The concentration of protein in the assay was usually very low (less than 1 μ g./ml. for highly purified histaminase solutions) and a correction for the extinction of coloured proteins had to be applied only when assaying the crude placental extract.

The S.D. of six replicate assays, determined at four dilutions of histaminase from 0.03 to 0.15 S.U., was less than $\pm$ 5% of the mean in each case. Within this range, activity was linearly related to enzyme concentration. The linear range was of the same order for histaminase solutions at all stages of purification, and with either histamine or cadaverine as substrate. Oxygenation before incubation did not significantly increase the rate of reaction, but increased the non-enzymic oxidation of indigodisulphonate in the presence of cadaverine. Results were also less reproducible after oxygenation, and it was not adopted.

As in Kapeller-Adler's (1951) method, more dye was decolorized than could be accounted for by the stoichiometric oxidation of substrate and production of H₂O₂. This was due to a secondary oxidative mechanism of indigodisulphonate decolorization, requiring oxygen and products of the enzymic reaction, but the extent of this secondary reaction could not be estimated. For this reason, spectrophotometric units could not be converted into international units of activity. Although the incubation of the test solutions resulted in the immediate oxidation of histamine (detectable by the fluorimetric method of Shore, Burkhalter & Cohn, 1959) and production of H₂O₂ (detectable by the oxidation of *oo*-dianisidine in the presence of peroxidase), the decolorization of indigodisulphonate was delayed for 2–4 hr., after which it proceeded linearly for 24 hr. The 'lag period' in dye oxidation was not due to contaminant reducing agents in the components of the solution. It was shown that no appreciable dye oxidation occurred until a certain amount of enzymic activity had

Table 1. *Purification of placental histaminase*

Cadaverine was the standard substrate in the modified indigodisulphonate method and comparative rates of oxidation of putrescine (P/C) and histamine (H/C) are given. Units of enzymic activity are defined in the Materials and Methods section. Specific activity (S.A.) is given as enzymic units/ml./ E_{280} . The activity of stage 1 enzyme was too low to be measured by the *o*-aminobenzaldehyde method. Before assay, enzyme solutions from stages 1, 2 and 3 were dialysed against 5 mm-borate-HCl buffer, pH 8.6, to remove inhibitors. After stages 1 and 2, when inhibitors of histaminase had been removed from the preparation, the histamine/cadaverine substrate activity ratio remained approximately constant.

Stage	Vol. (ml.)	E_{280}	Enzyme activity						$10^3 \times \text{S.A. (b)} / \text{S.A. (a)}$ ratio
			(a) Modified indigo-disulphonate method		(b) <i>o</i> -Aminobenzaldehyde method				
			Substrate: cadaverine		Substrate: putrescine		Substrate: putrescine		
			$10^{-3} \times \text{Total S.U.}$	S.A.	P/C ratio	H/C ratio	S.A.		
1. Crude extract	9620	11.95	46	0.40	0.50	0.99	—	—	
2. Pptn. at 0.65 satn. with $(\text{NH}_4)_2\text{SO}_4$	3000	17.8	36	0.67	0.34	0.57	0.035	52	
3. Pptn. at 0.35–0.65 satn. with $(\text{NH}_4)_2\text{SO}_4$	223	47.6	216	20.4	0.33	0.16	0.086	4.2	
4. Chromatography on DEAE-cellulose	344	11.05	123	32.3	0.49	0.23	0.154	4.8	
5. Chromatography on cellulose phosphate	440	1.06	46	99.0	0.39	0.21	0.528	5.3	
6. Gel filtration on Sephadex G-200	130	1.16	47	314.0	0.46	0.21	1.53	4.9	
7. Recycling gel filtration on Sephadex G-200	345	0.24	18	206.0	0.54	0.23	0.93	4.7	

taken place and, presumably, a critical concentration of H_2O_2 had formed. Where an experiment demanded that the initial velocity of histaminase activity be known, this was measured as the rate of decolorization of the dye in the period of linear velocity immediately after the initial delay.

o-Aminobenzaldehyde method. Histaminase solution was incubated at 37° with 2.5 mM *o*-aminobenzaldehyde and 1.0 mM-putrescine in a total volume of 4 ml. in 0.067 M-phosphate buffer (Sørensen, 1909), pH 6.8, containing 0.1 mM-EDTA, as described by Holmstedt & Tham (1959). Controls containing all components except substrate were incubated in the same way. The increase in extinction of the tests at $430\text{m}\mu$ was measured at the end of the incubation period. Initial velocity was not maintained for more than 1 hr., but tests were sometimes incubated for longer periods when precise measurement of initial velocity was not required. One unit of activity in this method was defined as the amount of enzyme required to produce an increase in the *E* value of 1.0 in 1 hr.

RESULTS

Purification of placental histaminase

For the purification of the enzyme, a combination of ammonium sulphate precipitation and fractionation on columns of DEAE-cellulose, cellulose phosphate and Sephadex was used. The purification attained at each stage is shown in Table 1. The procedure finally adopted was as follows.

Stage 1. Normal human placentae were obtained within 30 min. of delivery, the umbilical cord was cut off at the base and the amnion was removed. The vascular bed of each placenta was perfused from the foetal side with 0.9% sodium chloride for $\frac{1}{2}$ –1½ hr., with 3–9 l. of solution, until the maternal and foetal surfaces were pink. The chorion and insoluble blood clots were cut away and the remaining tissue was stored at -15° . Five organs prepared in this way were thawed and ground in an electric mincer, and the mince was then homogenized twice with 2 vol. of sodium chloride solution for 30 min. After each extraction, the mince was filtered through muslin and the pooled filtrates were centrifuged. The mince and extract were kept cool throughout all these operations, and the next stage was started within 12 hr. of extraction. Desanguination of the placentae at stage 1 was always incomplete, and the preparation remained heavily contaminated with haemo-proteins.

Stage 2. The filtrate from stage 1 was brought to 0.65 saturation with ammonium sulphate and the precipitate was collected and dissolved in 0.02 M-phosphate buffer, pH 6.8. Stage 2 resulted in little purification, but served to concentrate the preparation and protect it from bacterial degradation, which otherwise set in rapidly, even at $0-5^\circ$.

Stage 3. Ammonium sulphate was added to give

0.35 saturation, and the precipitated protein discarded. More ammonium sulphate was added to the filtrate to give 0.65 saturation. This suspension could be stored at $0-5^\circ$ for at least 6 months without loss of activity. The precipitate was collected and dissolved in 5 mM-borate-hydrochloric acid buffer, pH 8.6, and dialysed for a total of 36 hr. against three 5 l. changes of the same buffer. A comparison of the specific activities of stage 2 and stage 3 enzyme as measured by the two methods showed that the second precipitation stage removed non-diffusible substances (possibly peroxidases) that inhibited histaminase activity measured by the modified indigodisulphonate method, but not by the *o*-aminobenzaldehyde method.

Stage 4. The solution from stage 3 was applied to a column (5 cm. $\times$ 80 cm.) of DEAE-cellulose, previously equilibrated with 5 mM-borate-hydrochloric acid buffer, pH 8.6, and washed in with 2 l. of the same buffer containing 0.08 M-sodium chloride; red haemoproteins were eluted. Elution of histaminase, together with brown haemoproteins, was effected with 0.17 M-phosphate buffer, pH 6.8, containing 0.5 M-sodium chloride; 20 ml. fractions were collected, and those containing most histaminase activity (Fig. 1a) were pooled and dialysed for 24 hr. at 4° against three 3 l. changes of 0.02 M-phosphate buffer, pH 6.8. Stage 4 did not resolve histaminase from haemoproteins as effectively as small-scale chromatography at the same pH under a shallow salt gradient, but a very large protein load could be processed quickly without great dilution of the preparation.

Stage 5. The solution from stage 4 was applied to a column (5 cm. $\times$ 80 cm.) of cellulose phosphate, previously equilibrated with 0.02 M-phosphate buffer, pH 6.8, and washed in with 300 ml. of the same buffer. A linear gradient of 0–3 M-sodium chloride over 4 l. in equilibrating buffer was used to elute histaminase. Histaminase activity in the breakthrough peak was rejected, and the fractions of highest specific activity (Fig. 1b), eluted between about 0.2 M- and 0.4 M-sodium chloride, were pooled. Stage 5 partly resolved histaminase from methaemoglobin; the loss of enzyme by association with the latter protein in the breakthrough peak could be diminished by dividing the sample load between two columns, but the resulting increase in volume of the effluent was not offset by significant improvements in yield and specific activity.

Stage 6. The pooled fractions from stage 5 were concentrated to 20 ml. by osmosis against Carbowax (polyethylene glycol, mol. wt. 20 000; Union Carbide Ltd., London, W.1), and pumped upwards through a column (3 cm. $\times$ 100 cm.) of Sephadex G-200. The buffer used in packing and elution of the column was 5 mM-borate-hydrochloric acid, pH 8.6, containing 0.1 M-sodium chloride. Hist-

Fig. 1. Chromatographic elution patterns at different stages during the purification of placental histaminase. (a) Enzyme from stage 3 of the scheme was added to a column of DEAE-cellulose in 5mm-borate-HCl buffer, pH 8.6; after development with 2l. of 0.08M-NaCl in buffer, histaminase was eluted with 0.17M-phosphate buffer, pH 6.8, containing 0.5M-NaCl, starting at $\downarrow$. (b) Enzyme from the previous stage was applied to a column of cellulose phosphate in 0.02M-phosphate buffer, pH 6.8, and eluted under a gradient of 0.3M-NaCl over 4l. of buffer, starting at $\downarrow$. (c) Enzyme from the previous stage was concentrated and filtered once through Sephadex G-200. (d) Enzyme from the previous stage was concentrated and recycled twice through Sephadex G-200. Details of conditions are given in the Materials and Methods section. —, Histaminase activity (S.U./ml.); ----, protein (E_{280}); ····, methaemoglobin and haptoglobin-methaemoglobin (E_{408}); - - - - , $[Na^+]$ in effluents (M).

aminase was eluted in the first of three protein peaks (Fig. 1c), and the fractions of highest specific activity were pooled. Stage 6 resolved histaminase from the last traces of haemoglobin and methaemoglobin, but the enzyme remained contaminated with haptoglobin-methaemoglobin and possibly some lipoprotein.

Stage 7. Stage 6 enzyme was concentrated to small volume against Carbowax, and 3ml., containing 55mg. of protein, was subjected to recycling gel filtration (Porath & Bennich, 1963) on the column of Sephadex G-200 used in stage 6. The patterns of protein and histaminase activity eluted from the column after two cycles are shown in Fig. 1(d). Stage 7, in which the preparation was exposed to room temperature for about 3 days, resulted in a decrease in specific activity of histaminase, but the effluent was purer than that from stage 6 in that fewer contaminating proteins were present,

and this preparation was used in specificity studies. Recycling gel filtration resolved the enzyme from some minor contaminants, including the complex between methaemoglobin and the lower-molecular-weight complexes of haptoglobin types 2-1 and 1-1. The most-active fractions from stage 7 were concentrated and subjected to starch-gel electrophoresis at pH 8.6, and slices of the gel stained for protein, haptoglobin-methaemoglobin and histaminase. All bands developing in the protein stain could be identified as haptoglobin-methaemoglobins by the *oo'*-dianisidine reaction. Histaminase activity was spread across the entire region occupied by the protein bands, and no single protein band corresponding to histaminase could be discerned. The spreading of histaminase stain was not due simply to diffusion of the histaminase stain, since narrow serial sections of the gel, cut along the path of migration immediately after electro-

phoresis, showed a comparable diffuse pattern of histaminase activity.

Further purification. Many attempts were made to resolve histaminase from its last contaminants by exploiting possible differences in their electrical properties over the entire pH range in which the enzyme is stable. Experiments with other ion-exchangers, attempted modification of the haptoglobin contaminants with neuraminidase and preparative electrophoresis on starch blocks at pH 8.6 were unsuccessful. By elution of stage 6 enzyme from DEAE-cellulose at pH 5.5, under a shallow salt gradient, histaminase could be separated from contaminant haptoglobin complexes, but the enzyme was inactivated by up to 95%. This inactivation was not reversible by change in pH or the addition of possible coenzymes.

Effect of EDTA. After stages 1 and 2, when inhibitors of histaminase had been removed from the preparation, the histamine/cadaverine substrate activity ratio remained approximately constant (Table 1). The substrate activity ratio for the immediate homologues, putrescine and cadaverine, showed variations of the same order of magnitude. If EDTA was omitted from the buffers used in the purification scheme and in activity measurements, the putrescine/cadaverine substrate activity ratio varied from one placental preparation to another, and between successive stages, and the histamine/cadaverine substrate activity ratio increased after stage 1, until almost no activity on cadaverine could be detected after stage 4. This virtual loss of activity on cadaverine, which could be reversed by the addition of EDTA, was attributed to the variable concentration of metal ions in different placental extracts, and the reagents added to them.

Properties of the purified enzyme

Determination of K_m . The determination of K_m for the activity of histaminase on different substrates was handicapped by the lack of a suitable assay method applicable to all substrates. An attempt was made to measure K_m for three substrates by the modified indigodisulphonate method, by using the rate of dye oxidation during the linear phase as an approximation to the initial velocity of the enzyme-substrate reaction.

The concentration of histamine giving maximal activity in this method was 20–40 μM ; higher concentrations resulted in significantly lower activities. No substrate optima were observed for cadaverine and putrescine, up to 6 mM. Although there was no direct evidence that the rate of indigodisulphonate oxidation was a reliable measure of initial rates of substrate oxidation, data for these substrates showed a linear relationship between $[S]/v$ and $[S]$ (where $[S]$ is the substrate concentration and v the

'initial' rate of dye oxidation). The apparent K_m values for cadaverine and putrescine were of the same order, 0.2 mM. The results for histamine (except those at greater-than-optimum concentrations) showed a linear relationship between $1/[S]$ and $1/v$, and an apparent K_m of 6 μM was calculated.

The more direct *o*-aminobenzaldehyde method was used to determine K_m for putrescine. An optimum of activity between 0.5 mM- and 1.0 mM-putrescine was observed; an apparent K_m of 0.07 mM was derived from the linear plot of $1/v$ against $1/[S]$ for substrate concentrations less than 0.5 mM.

Effect of pH. The rate of activity of histaminase was measured by the modified indigodisulphonate method in phosphate buffers (Sørensen, 1909) of pH values from 5.6 to 8.0 (measured at room temperature) in the presence of 0.1 mM-EDTA and near-optimum concentrations of several substrates. Rates of indigodisulphonate oxidation during the linear phase were measured, as an approximation to the initial velocity of the enzyme-substrate reaction. The pH optimum of a model reaction, in which enzyme solution was replaced by 0.05 μmole of synthetic hydrogen peroxide, was measured simultaneously. The pH optimum for the action of histaminase on putrescine was measured, in the

Fig. 2. Dependence of histaminase activity on pH. The rate of histaminase activity was measured in phosphate buffers of pH values 5.6 to 8.2, as described in the text. The top curve (▲) was obtained by using the *o*-aminobenzaldehyde method with putrescine as substrate. The curves with open symbols were obtained by using the modified indigodisulphonate method: ○, cadaverine; Δ, putrescine; □, histamine. The concentration of enzyme, from stage 7 of the purification, varied with the substrate used. The broken line (●) shows the pH-dependence of a non-enzymic model reaction measured in the same buffers, in which 0.05 μmole of synthetic H_2O_2 oxidized indigodisulphonate in the presence of cadaverine.

same range of buffers, by the *o*-aminobenzaldehyde method; the results are compared in Fig. 2. Curves obtained by the modified indigodisulphonate method showed different pH optima for cadaverine, putrescine and histamine. The curves obtained for putrescine by the two methods were similar. The pH-dependence of the non-enzymic oxidation of indigodisulphonate by synthetic hydrogen peroxide in the presence of cadaverine was distinct from that of the enzymic oxidation of cadaverine, in the modified indigodisulphonate method.

Substrate specificity. The rates of placental histaminase (stage 7) activity on a number of diamines are compared in Table 2. Because the linear phase of indigodisulphonate oxidation was delayed for slightly different periods in the presence of different substrates, the velocities of dye oxidation during the linear phase were measured, rather than the total extinction change after 24 hr. incubation. The rate of oxidative deamination of the homologous aliphatic diamines by histaminase increased from C₂ to C₅, and decreased again at C₆. Histamine and agmatine were oxidized at about the same rate as 1,3-diaminopropane, but benzylamine was oxidized only very slowly.

Substrate competition. Since some diamines were oxidized only very slowly, compared with the C₄-C₆ aliphatic diamines, the ability of the former to compete with near-saturating concentrations of putrescine for the active centre of histaminase was tested in mixed-substrate experiments. Stage 7 enzyme was incubated with each substrate alone, and with an equimolar mixture of putrescine and the substrate, by using the modified indigodisulphonate method. Extinction changes after 24 hr. incubation are shown in Table 3. 1,2-Diaminoethane, histamine and agmatine, which had

been oxidized much less rapidly than putrescine in Table 2, appeared to compete with putrescine for the active centre of histaminase.

Since benzylamine was oxidized much more slowly than putrescine (Table 2), more-direct methods were used in investigating the possibility that these two substrates could compete with each other for the same enzyme. The oxidation of benzylamine to benzaldehyde by purified histaminase, in the absence and presence of the test solutions, was measured by extinction changes at 250 m μ (Tabor, Tabor & Rosenthal, 1954). Test solutions containing histaminase and substrates (2.28 mM) in a total volume of 4 ml. in 0.067 M-phosphate buffer, pH 6.8, and control solutions without substrate, were incubated for 24 hr. and substrate was added to the controls immediately before extinction measurements were made (Table 4). Putrescine inhibited the oxidative deamination of benzylamine.

The *o*-aminobenzaldehyde method was also used to detect substrate competition between putrescine and less-rapidly oxidized amines. Extinction

Table 2. *Relative rates of oxidation of amines by highly purified placental histaminase (modified indigodisulphonate method)*

The concentration of each substrate was 2.28 mM, and 0.2-1.0 ml. of appropriately diluted enzyme from stage 7 of the purification scheme was used. The rate of indigodisulphonate decolorization was measured during the linear phase.

Substrate	Comparative rate (cadaverine=100)
1,2-Diaminoethane	5
1,3-Diaminopropane	23
1,4-Diaminobutane (putrescine)	54
1,5-Diaminopentane (cadaverine)	100
1,6-Diaminohexane	40
Histamine	23
Agmatine	17
Benzylamine	5

Table 3. *Substrate competition for highly purified placental histaminase (modified indigodisulphonate method)*

Test solutions contained 2.28 mM-substrate, with and without the admixture of 2.28 mM-putrescine. The same volume of diluted enzyme from stage 7 of the purification scheme was used in each test, and the total extinction change after 24 hr. incubation recorded. Control solutions were incubated without enzyme. Histaminase activity is 87% of $V_{\max}$ at 2.28 mM-putrescine, and 92% of $V_{\max}$ at 4.56 mM-putrescine.

Substrate	Enzymic activity (S.U.)	
	2.28 mM-Substrate alone	2.28 mM-Substrate + 2.28 mM-putrescine
Putrescine	0.096	0.101
1,2-Diaminoethane	0.013	0.078
Histamine	0.038	0.050
Agmatine	0.029	0.039

Table 4. *Competition between putrescine and benzylamine for highly purified placental histaminase*

The conditions of assay are described in the text.

Substrate(s)	Activity ($\Delta E_{250}/24$ hr.)
Benzylamine	0.425
Putrescine	0.055
Benzylamine + putrescine	0.255

changes after 3 hr. incubation are shown in Table 5. In all cases, the activity in the presence of both the substrate and putrescine was lower than in the presence of putrescine alone.

Inhibitor specificity. Table 6 summarizes the effect of increasing concentrations of histaminase inhibitors on the activity of the enzyme on putrescine, measured by both methods. Control assays, containing all components except enzyme, were incubated similarly. Indigodisulphonate was decolorized by some of the inhibitors, presumably by

reduction, and an asterisk marks those tests in which this non-enzymic decolorization was very pronounced.

DISCUSSION

Comparison of assay methods. The measurement of Δ^1 -pyrroline produced by the oxidative deamination of putrescine (Holmstedt & Tham, 1959) offers a more direct measure of histaminase activity than indigometric methods. However, both methods gave consistent results in most applications. After stage 3 of the purification scheme, when inhibitors specific for the indigometric method had been removed, the ratio of specific activities obtained by both assay methods remained constant. The pH-dependence of histaminase activity on putrescine was the same, measured by either method, and the pH-dependence of a model reaction, in which indigodisulphonate was oxidized by synthetic hydrogen peroxide rather than by hydrogen peroxide produced enzymically, was distinct from the pH-dependence of the enzymic reaction on all three substrates. This finding supports the view that pH optima derived from indigometric data reflect the true pH-dependence of the enzyme-substrate reactions. Enzymic activity in both assays was subject to inhibition by the same compounds, and often to a similar extent, although high concentrations of some inhibitors had specific effects on the secondary reactions measuring product formation. Different K_m values were obtained by using the indigodisulphonate method and the *o*-aminobenzaldehyde method, the latter showing an inhibition of histaminase by high

Table 5. *Substrate competition for highly purified placental histaminase (o-aminobenzaldehyde method)*

The conditions of assay are given in the Materials and Methods section. The concentration of all substrates was 1.14 mM, and the same volume of diluted enzyme from stage 7 was used in each test. Extinction changes after 3 hr. incubation were measured. The enzyme is saturated by 1.14 mM-putrescine in this method, and is slightly inhibited by excess of substrate at 2.28 mM.

Substrate	Activity ($\Delta E_{430}/3 \text{ hr.}$)	
	1.14 mM-Substrate alone	1.14 mM-Substrate + 1.14 mM-putrescine
Putrescine	0.416	0.385
1,2-Diaminoethane	0.003	0.244
1,3-Diaminopropane	0.004	0.340
1,6-Diaminohexane	0.253	0.372
Histamine	0.004	0.034
Agmatine	0.026	0.067
Benzylamine	0.004	0.365

Table 6. *Inhibitors of placental histaminase*

Diluted enzyme from stage 7 of the purification scheme was incubated with putrescine for 24 hr., under the conditions of the modified indigodisulphonate method (a) and the *o*-aminobenzaldehyde method (b), with and without the addition of inhibitors. Controls contained all components except enzyme solution.

Concn. of inhibitor.....	Inhibition (%)					
	1 mM		10 μM		0.1 μM	
	(a)	(b)	(a)	(b)	(a)	(b)
Sodium azide	0	0	2	0	2	0
Ammonium chloride	100	0	43	0	6	0
Sodium cyanide	33	98	0	0	6	0
Aminoguanidine	100	100	100	100	63	63
Isoniazid	44*	78	24	53	0	7
Diethyldithiocarbamate	100*	66	44	12	13	0
Semicarbazide	100*	100	56	61	2	14
Hydroxylamine	—*	100	100	100	42	40

* Controls severely affected by inhibitor.

concentrations of putrescine that was not evident with the former. Placental histaminase may be usually sensitive to inhibition by the dihydroquinazolinium compounds formed in the test. The oxidative deamination of putrescine in both assay methods was inhibited by the same substrates (Tables 3 and 5).

Zeller (1965) has objected to indigometric histaminase assays on the grounds that histamine, but not the aliphatic diamines, catalyses the oxidation of indigodisulphonate by synthetic hydrogen peroxide, which otherwise proceeds very slowly, and he has attempted to explain in this way the failure of Kapeller-Adler & MacFarlane (1963) to detect activity towards aliphatic diamines in highly purified hog-kidney histaminase. It was observed during the present work that the ratio of placental histaminase activities towards histamine and cadaverine remained constant, and less than unity, throughout purification of the enzyme. Moreover, even in model experiments in which synthetic hydrogen peroxide replaced enzymically produced hydrogen peroxide, cadaverine proved to be a much better catalyst of indigodisulphonate oxidation than histamine.

The modified indigodisulphonate test was adequate for the determination of histaminase concentrations, e.g. in chromatographic effluents, under strictly comparable conditions, but the lack of a close relationship between substrate oxidation and decolorization of the dye, especially during the early stages of the reaction, made indigometric tests unsuitable for precise kinetic studies. However, no adequate alternative method applicable to all substrates, and of comparable sensitivity, was available. The modified spectrophotometric indigodisulphonate test was about 50-fold more sensitive than the methods of Kapeller-Adler (1951) and Holmstedt & Tham (1959).

Effect of metal ions. In the early stages of this work, substrate-specific inhibition of histaminase by metal ions almost led to the conclusion that at least two oxidative enzymes were present in the preparation. However, activity towards cadaverine was never detected in any fraction free from activity towards histamine.

It is possible that metal ions form co-ordinate bonds with the nitrogen atoms of diamines, and so alter their spatial and electronic configuration that the complexes are less readily accepted at the enzyme's active centre than the native diamines. This differential action of EDTA-reversible inhibitors of histaminase activity on its different substrates may explain the conflicting reports on the specificity of related enzymes. Kapeller-Adler & MacFarlane (1963) and Uozumi, Nakahara, Higashi & Sakamoto (1964) reported that preparations of hog kidney, originally active on both

histamine and cadaverine, gradually lost activity towards cadaverine during purification, whereas Mondovì *et al.* (1964) achieved 1700-fold purification of hog-kidney histaminase without appreciable change in the histamine/cadaverine substrate activity ratio. With the exception of adaptive bacterial enzymes (Gale, 1942; Satake, Ando & Fujita, 1953), specific for histamine and for putrescine, almost all other reports have suggested that plant and mammalian diamine oxidases oxidize histamine as well as diamines at an appreciable rate (e.g. Zeller, 1938, 1963; Hill & Mann, 1964; Waton, 1956; Lindahl, 1961; Blaschko, Friedman, Hawes & Nilsson, 1959).

pH-activity curves. The pH optimum for the action of the enzyme on histamine supports the contention of Blaschko *et al.* (1959) that mammalian diamine oxidases attack the dicationic form of this substrate. Differences in pH optima are not due solely to the dissociation constants of the substrates' ionic groups; Kenten & Mann (1952) found that the optima of pea-seedling diamine oxidase for cadaverine and putrescine were even more widely divergent than observed here with placental histaminase, although both diamines are completely dicationic below pH 8.

Specificity of placental histaminase. Since histaminase was not completely purified, the substrates oxidized by even the purest preparation could not be assumed to have been oxidized by a single enzyme, without additional evidence. Although some of the diamines tested were oxidized much more slowly than the C₄-C₆ aliphatic diamines, mixed-substrate experiments presented in Tables 3-5 showed that the C₂ and C₃ aliphatic diamines, agmatine, benzylamine and histamine inhibited the oxidation of putrescine. Table 3 showed that non-additive values of indigodisulphonate oxidation were obtained in the presence of an equimolar mixture of each substrate with a near-saturating concentration of putrescine. The results of Table 5, obtained by a more direct assay method, showed that other substrates (including benzylamine) inhibited the action of histaminase on an optimum concentration of putrescine; the fact that a twice-optimum concentration of putrescine alone is inhibitory does not affect the conclusion that the other substrates were partially effective in replacing putrescine at the active centre of histaminase. The simplest explanation of these results is that each substrate competes with putrescine at the active centre of the same enzyme. The alternative possibility, that a number of distinct enzymes are present in the purest preparation, each specific for a single substrate and inhibited by other diamines, can be proved only by physical separation of these enzymes.

The evidence that histamine, cadaverine and

putrescine are oxidized by the same enzyme is as follows:

(a) When precautions were taken to remove inhibitors from the preparation, the histamine/cadaverine substrate activity ratio at successive stages of purification varied within the limits of experimental error, and did not tend to approach zero or infinity.

(b) Histamine markedly inhibited the oxidation of putrescine by histaminase as measured by two independent methods.

(c) Histamine oxidation could be estimated indigometrically or by a specific fluorimetric method in the presence of the purest preparation of histaminase. The extent of histamine oxidation could not be accounted for by contamination with any of the other enzymes known to metabolize histamine (see below).

The substrate and inhibitor specificities of placental histaminase resemble those of diamine oxidases from hog kidney and pea seedling (Zeller, 1963; Hill & Mann, 1964). The enzyme differs from the benzylamine oxidase of human serum (McEwen, 1965) in its susceptibility to diethyldithiocarbamate and in the comparative rates of oxidation of histamine, benzylamine and putrescine, and it differs from mitochondrial monoamine oxidase (Blaschko, 1963) in its sensitivity to carbonyl reagents. During the present investigation, unsuccessful attempts were made to detect FAD, pyridoxal phosphate and copper by fluorimetric and spectrophotometric methods similar to those of Kapeller-Adler & MacFarlane (1963) and Yamada & Yasunobu (1962). The only evidence for the presence of copper or pyridoxal phosphate was the inhibition of the enzyme by diethyldithiocarbamate and carbonyl reagents, which are by no means specific in their action.

Another placental enzyme, imidazole *N*-methyltransferase, may be more important than histaminase in protecting the mother against foetal histamine (Bjüro, Lindberg & Westling, 1964; Lindberg, Lindell & Westling, 1963a,b; Lindberg, 1963a,b; Lindberg & Törnqvist, 1966). This suggests that placental histaminase, which, as we have seen, can oxidize several aliphatic diamines faster than histamine, may be concerned with the destruction of these substances rather than histamine, though their function in normal metabolism is not as yet understood.

The author is indebted to Professor L. G. Whitby and Dr D. W. Moss for encouragement and criticism of this work, and gratefully acknowledges the provision of a Scholarship by the Medical Faculty of the University of Edinburgh. Professor R. J. Kellar kindly permitted the use of placenta from the Simpson Memorial Maternity Pavilion, Edinburgh.

REFERENCES

- Bjüro, T., Lindberg, S. & Westling, H. (1964). *Acta obstet. gynec. scand.* **43**, 206.
- Blaschko, H. (1963). In *The Enzymes*, 2nd ed., vol. 8, p. 337. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
- Blaschko, H., Friedman, P. J., Hawes, R. & Nilsson, K. (1959). *J. Physiol.* **145**, 384.
- Buffoni, F. & Blaschko, H. (1964). *Proc. Roy. Soc. B*, **161**, 153.
- Burkard, W. (1954). *Contribution à l'Étude de l'Histaminase*. Paris: R. Foulon et Cie.
- Commission on Enzyme Nomenclature of the I.U.B. (1961). *Report of the Commission on Enzyme Nomenclature of the I.U.B.* Oxford: Pergamon Press Ltd.
- Gale, E. F. (1942). *Biochem. J.* **36**, 64.
- Hill, J. M. & Mann, P. J. G. (1964). *Biochem. J.* **91**, 171.
- Holmstedt, B. & Tham, R. (1959). *Acta physiol. scand.* **45**, 152.
- Kapeller-Adler, R. (1951). *Biochem. J.* **48**, 99.
- Kapeller-Adler, R. (1956a). *Ciba Found. Symp.: Histamine*, p. 356. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.
- Kapeller-Adler, R. (1956b). *Biochim. biophys. Acta*, **22**, 391.
- Kapeller-Adler, R. & MacFarlane, H. (1963). *Biochim. biophys. Acta*, **67**, 542.
- Kenten, R. H. & Mann, P. J. G. (1952). *Biochem. J.* **50**, 360.
- Lindahl, K. N. (1961). *Ark. Kemi*, **16**, 1.
- Lindberg, S. (1963a). *Acta obstet. gynec. scand.* **42**, Suppl. 1, 3.
- Lindberg, S. (1963b). *Acta obstet. gynec. scand.* **42**, Suppl. 1, 26.
- Lindberg, S., Lindell, S. E. & Westling, H. (1963a). *Acta obstet. gynec. scand.* **42**, Suppl. 1, p. 35.
- Lindberg, S., Lindell, S. E. & Westling, H. (1963b). *Acta obstet. gynec. scand.* **42**, Suppl. 1, p. 49.
- Lindberg, S. & Törnqvist, A. (1966). *Acta obstet. gynec. scand.* **45**, 131.
- McEwen, C. M. (1965). *J. biol. Chem.* **240**, 2003.
- Mondovi, B., Rotilio, J., Finazzi, A. & Scioscia-Santoro, A. (1964). *Biochem. J.* **91**, 408.
- Porath, J. & Bennich, H. (1963). *Arch. Biochem. Biophys.* Suppl. 1, 152.
- Satake, K., Ando, S. & Fujita, H. (1953). *J. Biochem., Tokyo*, **40**, 299.
- Shore, P. R., Burkhalter, A. & Cohn, V. A. (1959). *J. Pharmacol.* **127**, 182.
- Smithies, O. (1955). *Biochem. J.* **61**, 628.
- Sörensen, S. P. L. (1909). *C.R. Lab. Carlsberg*, **8**, 1.
- Tabor, C. W., Tabor, H. & Rosenthal, S. M. (1954). *J. biol. Chem.* **208**, 645.
- Uozumi, K., Nakahara, I., Higashi, T. & Sakamoto, Y. (1964). *J. Biochem., Tokyo*, **56**, 601.
- Watson, N. G. (1956). *Brit. J. Pharmacol.* **11**, 119.
- Yamada, H. & Yasunobu, K. T. (1962). *J. biol. Chem.* **237**, 3077.
- Zeller, E. A. (1938). *Helv. chim. Acta*, **21**, 880.
- Zeller, E. A. (1963). In *The Enzymes*, 2nd ed., vol. 8, p. 314. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
- Zeller, E. A. (1965). *Fed. Proc.* **24**, 766.