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PURIFICATION AND IDENTIFICATION OF HOG-KIDNEY HISTAMINASE

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SUMMARY

Chromatographic, electrophoretic and spectrophotometric techniques have been combined and applied to extracts from hog-kidney cortices to isolate, purify, and identify a rather stable preparation of histaminase which appears to be homogeneous by the criteria of chromatography and analytical electrophoresis.

In a large number of electrophoretic runs evidence has been obtained that histaminase travels as a single, distinct band at pH values above 5.2 towards the anode to be located in the region between α_2- and β-globulin fractions of normal human serum. No migration of histaminase has been observed between the pH values 5.0 and 5.15 which may signify the location of the isoelectric point of histaminase. The electrophoretically pure histaminase has been found to be free from contamination with diamine oxidase. It acts specifically on histamine and on its ring-N substituted derivatives such as 1-methyl-4-(β-aminoethyl)imidazole (1,4-methylhistamine) and 1-methyl-5-(β-aminoetnyl)imidazole (1,5-methylhistamine) as substrates, but has no effect whatsoever on diamines such as putrescine, cadaverine, or hexamethylene diamine, typical substrates of diamine oxidase. The finding that histamine and 1,4-methylhistamine are both degraded by the same enzyme, histaminase, seems to add to the biological significance of histaminase in view of SCHAYER's contention that 1,4-methylhistamine is one of the main metabolites of histamine degradation *in vivo*.

Results of spectrophotometric, fluorometric, and chemical investigations of the pure enzyme point to the presence in the histaminase molecule of two prosthetic groups, FAD and pyridoxal phosphate. The reaction mechanism as well as the significance of these findings are discussed.

INTRODUCTION

Histaminase was discovered in 1929 by BEST[1], and in the following year was characterised in extensive investigations of various animal tissues by BEST AND MCHENRY[2]

Abbreviations: DO, diamine oxidase; PCMB, *p*-chloromercuribenzoate; PP, pyridoxal phosphate.

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as an enzyme system involving and oxidative deamination of histamine with the consumption of oxygen and the production of ammonia. BEST AND MCHENRY considered this system to be specific for the destruction of histamine. In 1938 ZELLER[3], however, reported that hog-kidney preparations as well as various other animal tissue extracts were capable of degrading by oxidative deamination, according to the equation:

$$RCH_2CH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

not only histamine but also diamines such as putrescine, cadaverine, and agmatine. Since the latter enzyme system appeared to ZELLER to be in many aspects similar to the enzyme described by BEST AND MCHENRY he concluded that both enzyme systems were identical and substituted the name diamine oxidase for that of histaminase. Subsequently not only the terms histaminase and DO have been interchangeably used, but also the substrates histamine, cadaverine, and putrescine, despite considerable disagreement in the literature on this point[1-7]. The matter became even more involved when it was claimed that DO degraded besides aliphatic diamines and histamine also monoamines[8-10] and that monoamine oxidase destroyed not only monoamines but also long-chained aliphatic diamines[11] as well as histamine[12-14].

If one adds to this very obscure picture of an enzyme still the ambiguity which has arisen in various laboratories over the nature of the prosthetic group (for references see ref. 15) which might be necessary for the activity of histaminase, it becomes obvious that a renewed study of this enzyme, aiming at the elucidation of the problem of its identity, specificity, and mode of action appeared to be of paramount importance.

The work described herein was undertaken in an effort to elaborate a modern technique, adequate to produce in good yield a stable preparation of pure histaminase, essential for the clarification of the problems just mentioned. A preliminary report of the work has already appeared[16].

ANALYTICAL METHODS

Protein

Protein was determined in the initial stages of enzyme purification by the biuret method, as described by GORNALL *et al.*[17], and in the later stages of purification by the method of LOWRY *et al.*[18]. Calibration curves were prepared for both methods using serial dilutions of reconstituted freeze-dried human serum (Glaxo Laboratories Ltd., Greenford, Middlesex) of known protein content.

Enzymic assays

Effect on histamine: Histaminase activity was determined by a simple microvolumetric technique[19] involving a coupled oxidation reaction in which indigo disulphonate is oxidised by the H_2O_2, formed in the primary reaction. That H_2O_2 is actually formed during the action of histaminase on histamine has been proved previously[20,21]. The assay, which is proportional to both enzyme concentration[22] and time of incubation[23] was carried out in triplicate in Pyrex test tubes (2 cm × 15 cm).

The reaction mixture consisted of 0.5 ml of a histaminase solution (10–25 units of histaminase in 0.175 M phosphate buffer (pH 6.8), 0.1 ml or 0.2 ml of substrate (5 mg histamine·2 HCl/ml phosphate buffer (pH 6.8)) and 1 ml or 2 ml of an indigo disulphonate solution (200 mg of indigo carmine "Analar" B.D.H./300 ml distilled water). The volume was made up to 10 ml with phosphate buffer (pH 6.8) and one small drop of chloroform was added as a preservative. (The final concentration of the substrate was 2.72 μmoles or 5.44 μmoles and that of the indigo carmine 1.43 μmoles or 2.86 μmoles, respectively.) Oxygen was then simultaneously passed through the 3 test tubes of each assay for 1 min (stop watch) and the test tubes were closed with rubber stoppers. Blanks, also set up in triplicate, were complete assays without the addition of the substrate. Deionised, glass-distilled water was used throughout the entire work. After an incubation for 24 h at 37°, the excess of indigo carmine, not oxidised by the H_2O_2, was titrated with 0.002 N $KMnO_4$ until the endpoint of titration was reached (*i.e.* until the blue colour of the solution had just disappeared). The amount of 0.002 N $KMnO_4$ used for the blank minus that used for the assay indicates the histaminase activity, expressed in units, one unit representing the amount of enzyme which under standard assay conditions, described above, produces the amount of H_2O_2 equivalent to 0.1 ml of 0.002 N $KMnO_4$. Since in this stoichiometric reaction one molecule of H_2O_2 is formed for each molecule of histamine oxidised[19,22], this enzymic unit corresponds to the destruction of 0.463 μg of histamine/h. To conform with recent recommendations of the Commission on Enzymes of the International Union of Biochemistry[24] this histaminase unit is now to be defined as the amount of enzyme which will under standard assay conditions catalyse the degradation of 0,0000695 μmoles (or 6.95×10^{-5} μmoles) of histamine/min.

It should be pointed out that since this reaction, like many other coupled reactions of aerobic dehydrogenases, requires the presence of high O_2 tensions[25], oxygenation should be very carefully carried out. When this estimation is performed in air, only 35% of the histaminase activity in an atmosphere of oxygen is obtained. Further, for optimal results the presence of an excess of indigo carmine throughout this coupled oxidation reaction is also essential. Catalase inhibits this reaction, for it competes with the indigo disulphonate for the H_2O_2, generated in the histaminase-substrate reaction[19,21]. This simple microvolumetric method permits very accurate serial estimations of minute amounts of histaminase in replicate with large numbers of samples being analysed simultaneously.

The specific activity of histaminase is defined as the number of enzyme units/mg of protein.

Effect on cadaverine: In the early stages of the present method of purification of histaminase, mainly during the fractionation procedures with ammonium sulphate a varying enzymic effect was obtained also with cadaverine dihydrochloride as substrate under the standard conditions of the assay.

Analytical electrophoresis on cellulose acetate paper

Electrophoretic assays were carried out in horizontal tanks according to ALBERT-RECHT[26], using cellulose acetate strips[27] (2.5 × 10 cm), various buffer solutions, μ = 0.05–0.1, and of a pH range extending from 4.6 to 8.6, with a current of approx.

0.6 mA per cm width of cellulose acetate strip, for 2½ min. The strips were stained with 0.2% Ponceau S in 6% salicylsulphonic acid for 20 min, and decolorised in four washes with 5% acetic acid. Enzyme solutions containing as little as 0.4 mg protein/ml could be successfully electrophoresed. Cellulose acetate strips were supplied by "Oxoid" Ltd. London.

Purification of enzyme from hog kidney

Partial purification of crude histaminase by fractionation with ammonium sulphate and heat treatment. Unless otherwise stated, all procedures were carried out at room temperature.

Cortices of 24 hog kidneys were minced to a fine brei in an electric mincer giving a wet pulp of approx. 2000 g. 200 ml of 1% NaCl/100 g of wet pulp were added, and the mixture was allowed to stand for 60 min with intermittent gentle shaking, and was then filtered through muslin. The pulp was returned to the original flask and the extraction procedure repeated, using the same volume of 1% NaCl as before. The combined extracts were precipitated with finely powdered ammonium sulphate (A.R.) to 0.6 saturation by adding slowly, with mechanical stirring, 42.4 g ammonium sulphate/100 ml of solution. The precipitate was immediately filtered through Whatman No. 1 fluted filters, and the reddish filtrate discarded. The precipitate was dissolved in small portions, using approx. 200 ml of 0.02 M phosphate buffer (pH 6.8) for each portion. 50-ml aliquots of the dark brown solution, obtained, were dialysed for 60 min, at 4°, against 2000 ml of 0.02 M phosphate buffer (pH 6.8) with two changes of the buffer, then freed from the precipitate, formed, by centrifugation at 4°. The clear, yellowish-brown supernatant was then heated with mechanical stirring at 60° for 20 min in a constant temperature water bath. After rapid cooling, the very turbid solution was centrifuged at 4°, the precipitate of inert protein washed three times with small amounts of 0.02 M phosphate buffer (pH 6.8) and discarded after recentrifugation. The combined yellowish-brown, clear supernatants were reprecipitated with ammonium sulphate to 0.6 saturation. This partially purified enzyme fraction served as a starting material for chromatography on DEAE-cellulose columns. In each fractionation step the protein content and the enzyme activity were determined simultaneously.

Separation of histaminase by chromatography on DEAE-cellulose columns: Despite many attempts, made in various laboratories[28-38], the preparation of a stable, pure histaminase in a good yield with an ensuing identification of this enzyme has so far not been achieved.

In this work it was decided to try to purify histaminase by column chromatography on DEAE-cellulose[39]. SWEDIN[31] was first to attempt a purification of histaminase by chromatography on DEAE-cellulose columns. The standard conditions of his assay were, however, not very well outlined, and very few details as to the specific activity and the specificity of the chromatographed enzyme were given.

In the present work, many preliminary experiments have shown that histaminase was quantitatively adsorbed on a DEAE-cellulose column at pH 8.6, and on simple elution, at pH 5.5, the enzyme emerged from the column with virtually no loss of activity.

The method, to be described, was followed throughout this work. DEAE-

cellulose powder (Whatman DE 50) was first thoroughly washed with 0.1 N NaOH, then with distilled water to a neutral pH, and, finally, equilibrated with 0.005 M borate buffer (pH 8.6) to pack a column (2.0 cm × 23.0 cm). An aliquot of the partially purified enzyme preparation of fractionation step 3 (Table 1), obtained after heating at 60°, and after the second ammonium sulphate precipitation, was dissolved in 30 ml 0.02 M phosphate buffer (pH 6.8) and dialysed for 3 h against 2 l of 0.005 M borate–HCl buffer (pH 8.6) at 4°, with two changes of the buffer. (Throughout this work all dialysing tubings were pretreated with a 10^{-3} M Versene solution.) The slight precipitate, which usually formed after dialysis, was removed by centrifugation, and the dialysed enzyme solution, containing about 300 mg of protein,

TABLE I

PARTIAL PURIFICATION OF HISTAMINASE FROM HOG KIDNEYS

Fractionation step	Volume (ml)	Total protein (mg)	Effect on histamine		Effect on cadaverine		Specific activity on histamine in relation to the specific activity on cadaverine
			Total units	Specific activity (units/mg protein)	Total units	Specific activity (units/mg protein)	
(1) Crude extract	46.0	80 000	85 700	1.06	32 950	0.41	2.59
(2) Ammonium sulphate precipitate at 0.6 saturation; 0.02 M phosphate buffer extract; dialysed	282	4371	67 680	15.5	17 202	4.0	3.87
(3) Aliquot of (2), heated at 60° for 20 min; centrifuged; supernatant + $(NH_4)_2SO_4$ (0.6 saturation) — 2nd ammonium sulphate precipitate; 0.02 M phosphate buffer extract; dialysed (s. text)	50	265	16 900	64.0	2 484	9.36	6.83

was placed on the column, and the column developed with 75 ml of 0.005 M borate–HCl buffer (pH 8.6). Enzyme elution was accomplished with 200 ml 0.1 M acetate buffer (pH 5.5). The rate of elution was about 16 ml/h, and 15-min samples of the eluate were collected by a time-regulated fraction collector. Fractions were collected until no protein was any longer eluted from the column. It was noticed during each elution procedure that a distinct brownish band remained firmly adsorbed to the top of every column, while soon after the commencement of the elution a very pale yellow band moved down the column. The effluents, emerging from such DEAE-cellulose columns showed a pH of 7.0. Each effluent was assayed for the presence of protein and histaminase. Effluents with a high specific activity were subjected, for analytical purposes, to electrophoresis on cellulose acetate strips. Effluents from several individual columns, showing the same high degree of purity, as indicated by their specific activity as well as by their electrophoretic patterns, were pooled, and

for maximal purification were subjected either to rechromatography on DEAE-cellulose or to a precipitation with ammonium sulphate to 0.6 saturation.

The method of rechromatography of histaminase on DEAE-cellulose columns was essentially the same as for chromatography, as described above. 5–10 chromatographic effluents of a similar degree of purity, usually obtained from 3 columns, were combined into one fraction (approx. 35 ml) and dialysed for 3 h against 2 l 0.005 M borate–HCl buffer (pH 8.6) at 4°. The slight precipitate, which formed, was separated by centrifugation, and the clear, yellowish enzyme solution was applied to the DEAE-cellulose column.

For precipitation with ammonium sulphate, effluents from several columns with a similar degree of purity were pooled, and solid ammonium sulphate was added under mechanical stirring to give an 0.6 saturation. The precipitate separated by centrifugation, was dissolved in a small amount of 0.02 M phosphate buffer (pH 6.8) and the solution obtained was freed by centrifugation from the small insoluble residue, consisting mainly of inert protein. The clear pale yellow supernatant was dialysed against 0.02 M phosphate buffer (pH 6.8) for 30 min at 4° and, after dilution, was investigated for its histaminase and protein contents.

Concentration of chromatographed effluents

The pooled fractions from the chromatographic runs were concentrated in the early part of the work by ultrafiltration according to ALBERT-RECHT AND STEWART[34], using Visking tubings at a high positive pressure (10 atm). In the later stages of the present work, however, it was found to be more convenient to concentrate chromatographic eluates by osmosis, using a simple, very little time consuming technique, described by HSIAO AND PUTNAM[35]. A dialysing casing, containing the chromatographic eluate was covered with dry, powdered sucrose for 1 h. The sucrose was then removed from the dialysing tubing by washing with water, and dialysis of 20 min against 0.02 M phosphate buffer (pH 6.8). The 3–4-fold concentration of each chromatographic effluent, thus achieved, gave a final concentration of about 1–2 mg of protein per ml of solution, suitable for most analytical purposes.

RESULTS AND DISCUSSION

Partial purification of the enzyme by fractionation with ammonium sulphate and thermal denaturation of inert protein

Typical results of a partial purification procedure, repeatedly carried out on various batches of crude hog-kidney extracts are summarised in Table I. In each step the enzyme activity was investigated simultaneously on two substrates, histamine and cadaverine, no enzymic effect in any of the purification stages having been obtained with hexamethylene diamine, or putrescine as substrates. It is apparent from the specific activities, shown in Table I, that an about 60-fold purification of the enzyme with histamine as substrate, as compared with the crude extract, was achieved on fractionation with ammonium sulphate, and heating the enzyme solution at 60° for 20 min. With cadaverine as substrate, however, not only was the initial activity of the crude extract much lower than that with histamine as substrate, but also a barely 22-fold purification was achieved in the same purification procedure.

Moreover, the ratio of the specific activity on histamine in relation to that on cadaverine was not constant at all, but differed significantly from one purification step to another. It should be also mentioned here that during the purification procedures of different batches of hog-kidney extracts the enzyme activity on cadaverine varied considerably, since in some batches the enzyme effect on the latter substrate was already lost before the thermal treatment of the enzyme solution, whereas in some other batches some enzymic effect on cadaverine could be still detected after the heating step.

This observation of a missing relative constancy at various stages of enzyme purification of the enzyme effect on histamine to that on cadaverine confirms previous findings[5,22]. KOBAYASHI[36] in his recent attempts to purify hog-intestine DO has isolated several fractions which metabolised both histamine and cadaverine. This author also found that the ratio of enzymic activity on histamine to that on cadaverine varied considerably among the different purification fractions. Consideration of these findings, such as the varying ratio of the enzymic degradation of histamine in relation to that of cadaverine, the fact that in the later stages of purification as will be seen later, enzymic fractions were isolated which were highly active on histamine and completely inactive on cadaverine, and finally, the observation made in the present paper as well as in previous work[22], that no enzymic activity on putrescine was encountered at any of the purification stages lends no support to the claim of ZELLER[3] that hog-kidney histaminase and DO are a single enzyme.

Chromatography of histaminase on DEAE-cellulose followed by electrophoresis of chromatographic effluents on cellulose acetate strips

The method designed in this work for the purification of histaminase by chromatography on DEAE-cellulose was used in 55 independent assays, each of them comprising 3 or 4 individual columns.

Fig. 1 represents a typical chromatogram of an enzyme preparation, obtained from step 3 of the partial purification procedure (Table I), containing 315 mg protein and 22 500 enzyme units, corresponding to a specific activity of 71.4, with histamine as substrate, and with no activity on cadaverine. On the basis of the elution pattern this diagram has been divided into 3 parts: A, B, and C. To each of these 3 sections an electropherogram, typical of the relevant part, has been attached, each of them showing in its upper part an electropherogram of normal human serum proteins, used in each run as a marker. Electropherogram D in the right upper corner represents the electrophoretic pattern of the enzyme material before its chromatography on DEAE-cellulose, showing two diffuse bands in the globulin fraction, and one well defined band in the albumin region, with some protein at the point of application. It can be seen from this diagram that the total enzyme activity emerged from the column as a single, well resolved, almost symmetrical peak, shown in section B, and it follows from the electropherogram of the peak effluent of this section that the enzyme protein moved as a single, distinct band towards the anode, located at pH 6.8 of the electrophoretic runs in the region between the α_2- and β-globulin fractions of normal human serum. The effluents of this section showed a very pale yellow colour, and displayed a distinct greenish yellow fluorescence under

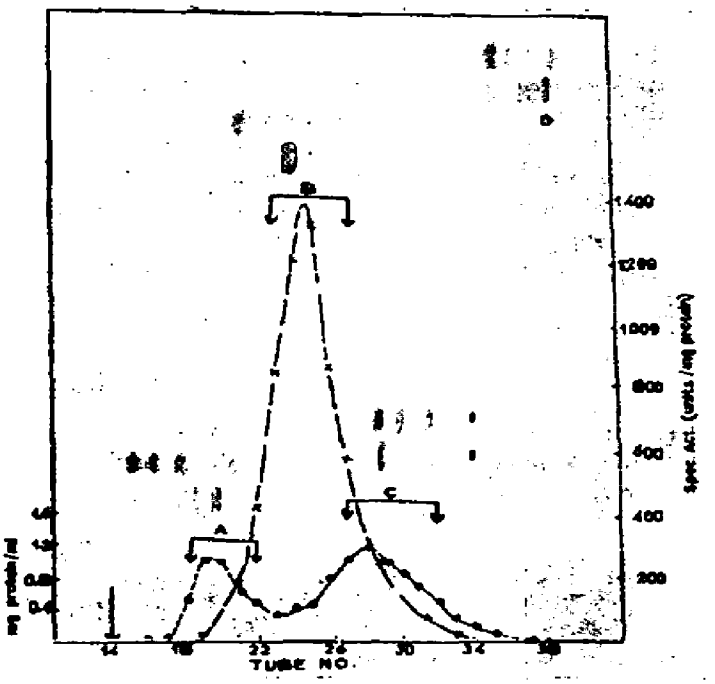


Fig. 1. Elution diagram obtained on chromatography of histaminase on DEAE-cellulose columns O– – –O, protein (mg/ml); ×– – –×, specific activity of histaminase (units/mg protein). At the arrow elution was started. Electrophoresis was carried out on cellulose acetate strips with an ionic strength of the phosphate buffer of 0.05, at pH 6.8. A current of 0.6 mA/cm width of strip was used for 4 h. The strips were stained with Ponceau S. Electropherograms above sections A,B, and C as well as the electropherogram D show in their upper parts patterns of normal human serum proteins.

ultraviolet light. The peak effluent of fraction B showed a specific activity of 1400, indicating a 20-fold purification as compared with the enzyme material before chromatography. It can be further seen from Fig. 1 that part A, with a negligible amount of histaminase, consisted merely of inert protein, shown in the electropherogram of this section to have travelled as a broad diffuse band with some protein remaining at the point of application. Likewise, section C contained very little histaminase activity but comparatively large amounts of protein, shown in the corresponding electropherogram as a broad diffuse band in the globulin fraction in addition to a distinct band with the mobility of albumin. On comparison of the electropherograms of these 3 chromatographic sections with the electropherogram D of the partially purified histaminase preparation before chromatography the great resolution power of DEAE-cellulose becomes obvious. With regard to the numerical data of this chromatographic assay, the total histaminase activity eluted from the column, amounted to 34 594 units and the total protein to 101.65 mg. This corresponds to a recovery of 154% of the histaminase activity and to a recovery of only 32.4% of the protein content of the enzyme solution before chromatography on DEAE-cellulose.

It should be emphasized that in most of the 55 chromatographic assays the

yield of histaminase activity, recovered from a DEAE-cellulose column, ranged from 125 to 190% of the enzyme activity of the material applied to a column with a recovery of only 30–34% of the total protein content before chromatography. This points towards a considerable selective separation ability of DEAE-cellulose, indicating that the bulk of histaminase inhibitors is retained on the column.

To investigate the possibility of a further purification of histaminase, effluents of various DEAE-cellulose columns showing highest specific activity and, electrophoretically, the same degree of purity, were pooled, dialysed, and either rechromatographed on DEAE-cellulose or precipitated with $(NH_4)_2SO_4$ to give an 0.6 saturation.

Rechromatography of pooled chromatographic effluents on DEAE-cellulose columns

Fig. 2 is a typical elution diagram, obtained on rechromatography on DEAE-cellulose of a histaminase solution with 57 mg protein and 21 900 enzymic units,

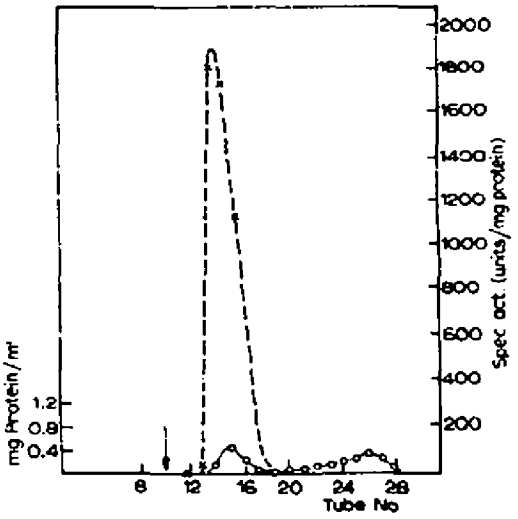


Fig. 2. Elutions diagram of a histaminase preparation after rechromatography on a DEAE-cellulose column. At the arrow elution was started. ○ - - - ○, protein (mg/ml); × - - - ×, specific activity of histaminase (units/mg protein).

corresponding to a specific activity of 384 with histamine as substrate, and no effect on cadaverine. As seen in this figure, the total histaminase activity emerged from the column almost immediately after the commencement of elution as a single, very well resolved, symmetrical peak, well separated from a small tail of inert protein. The total histaminase eluted from DEAE-cellulose amounted to 18 625 units, corresponding to a recovery of 85% of the histaminase activity of the enzyme solution before rechromatography. The total protein eluted was 25.2 mg of which only 13.39 mg, *i.e.* 23.5% of the protein content before rechromatography were histaminase protein. The specific activity of the enzyme peak was 1800 indicating that a 4.7-fold purification of the enzyme preparation was achieved on rechromatography, with an overall 1800-fold purification of histaminase as compared to the

crude enzyme preparation. In some other chromatographic assays an about 2000-fold purification was obtained on rechromatography, probably indicating maximal purity of the enzyme , since the specific activity did not change on further rechromatography. The effluents from rechromatography, showing peak enzyme activity were individually concentrated in dialysing tubings with powdered sucrose without any loss of enzyme activity.

Fig. 3. Two typical electropherograms of an effluent after rechromatography on DEAE-cellulose. Electrophoresis carried out on cellulose acetate strips, at an ionic strength of 0.05 in phosphate buffer (pH 6.8) (top strip) and 0.1 (bottom strip). Normal human serum used as a marker.

Fig. 3 shows two electropherograms, typical of such a concentrated effluent. The electrophoresis was carried out at pH 6.8 and an ionic strength of the phosphate buffer of 0.05 (top strip), and of 0.1 (bottom strip). In both instances the enzyme travelled towards the anode as a single, distinct band, located in the region between α_2- and β-globulin fractions of normal human serum (top part in each strip). As was to be expected, the protein mobilities were greater at the lower ionic strength of the buffer with a wider separation of the bands, whereas at the higher ionic strength lower mobilities but more symmetrical and sharper boundaries were observed.

Further purification of pooled effluents of DEAE-cellulose columns by precipitation with ammonium sulphate to 0.6 saturation

TABLE II

PRECIPITATION OF POOLED EFFLUENTS OF DEAE-CELLULOSE COLUMNS WITH AMMONIUM SULPHATE TO 0.6 SATURATION AT pH 6.8

Histaminase	Total activity (units)	Total protein (mg)	Specific activity
Before precipitation with $(NH_4)_2SO_4$	836	2.970	282
After precipitation with $(NH_4)_2SO_4$	756	0.278	2700

As can be seen from this table an almost 10-fold purification of the chromatographic effluents was achieved by precipitation with ammonium sulphate. The purified enzyme showed a specific activity of 2700, and was recovered in a yield of 89.7%. After concentration with sucrose, these pure histaminase solutions were used along with pure histaminase solutions, obtained by rechromatography on DEAE-cellulose, for the study of the behaviour of this enzyme.

Clearly, the results obtained on purification of histaminase by chromatography on DEAE-cellulose columns substantiate previous suggestions[5-7, 38] in demonstrating that it is possible to obtain histaminase completely free of DO activity.

Properties of purified histaminase

Electrophoretic mobility: Varying minute amounts of purest enzyme fractions, homogeneous by the criterion of chromatography were concentrated by osmosis with sucrose, then subjected to electrophoresis on cellulose acetate strips over a pH range extending from 4.6 to 8.6, and with the ionic strength of the buffer varying between 0.05 and 0.1. Normal human serum was used as a marker.

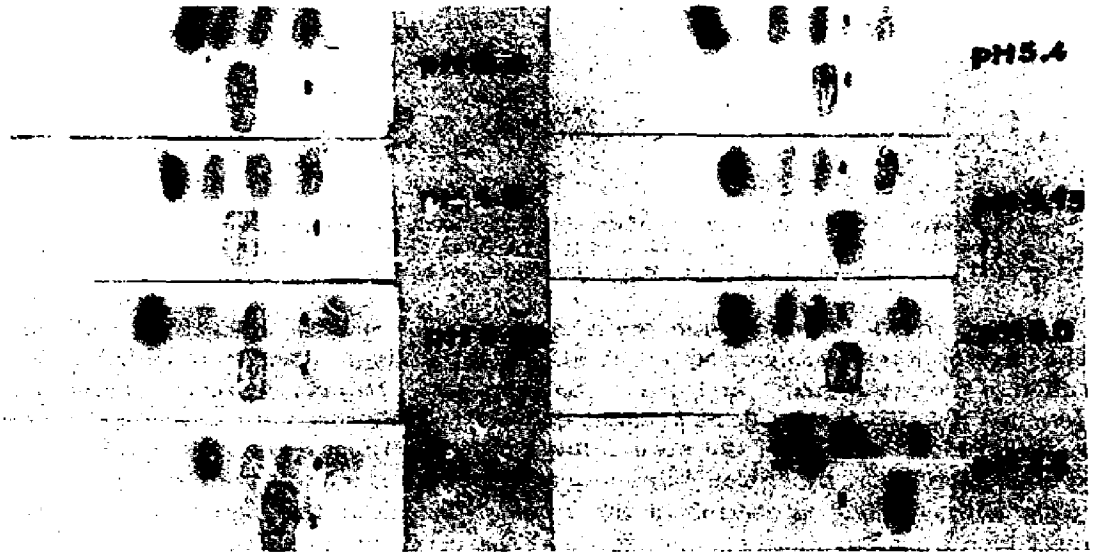


Fig. 4. Electropherograms of pure histaminase obtained on cellulose acetate strips, at pH values ranging from 4.6 to 8.6, with the ionic strength of the buffer solutions varying between 0.05 and 0.1. A current of 0.6 mA/cm width of strip used for 4 h. Strips stained with Ponceau S. Normal human serum used as a marker.

Fig. 4 illustrates the patterns, obtained, from which it can be seen that pure histaminase travelled at all pH values as a single, apparently homogeneous band, in most instances towards the anode, to be located at pH 6.8 and 8.6 in the region between α_2- and β-globulin fractions of human serum. Whereas, at pH 4.6 the enzyme migrated towards the cathode, no migration of histaminase was seen between pH 5.0 and pH 5.15, which would place the isoelectric point of histaminase between these two pH values. The fact that varying the amount of enzyme protein, or the ionic strength, or the pH value of the buffer did not reveal more than one component in the resulting ionograms seems to indicate electrophoretical homogeneity of pure histaminase preparations.

Stability: Preparations of pure histaminase were stable for weeks at 4°, and for months in the deep-freeze cabinet (−18° to −20°) with almost no loss of activity. Heating, up to 62°, did not affect the enzyme activity.

Effect of substrate concentration: As mentioned above, the optimal substrate concentration for a histaminase content of 10–25 units was 2.72–5.44 μmoles of histamine dihydrochloride/10 ml. It should be emphasized that in the presence of superoptimal histamine concentrations a considerable inhibition of histaminase

activity was always observed. The significance of this finding will be discussed later.

Effect of pH: Fig. 5 shows the reaction velocity of histaminase as a function of pH. It can be seen that the activity of pure histaminase appears optimal at pH 6.8 with a rather steep decline below and above this pH value.

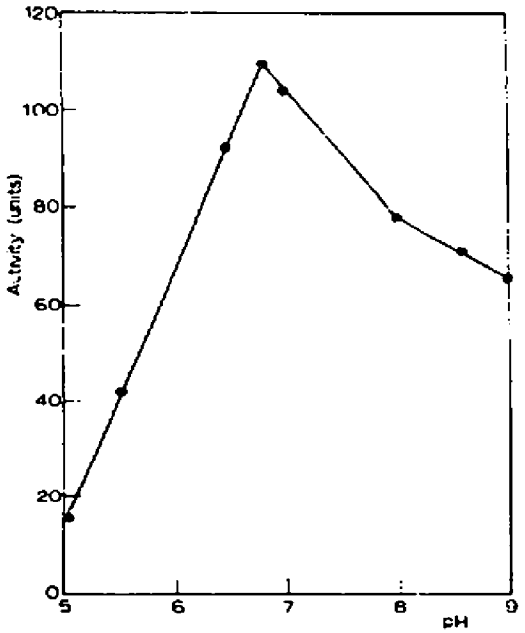


Fig. 5. Effect of pH on the activity of histaminase. Acetate buffers were used for pH values of 5.0 and 5.5, phosphate buffers for pH values of 6.0 and 8.0, and borate buffers for pH values of 8.6 and 9.0.

Nature of the reaction product of the histaminase action on histamine

In paper chromatographic assays previous findings[37] were confirmed. On incubation at 37° for 60 min pure histaminase acts on histamine with the formation of only one reaction product, imidazole acetaldehyde, which is oxidised by further incubation with added xanthine oxidase at 37° for 60 min to imidazole acetic acid.

Substrate specificity

As pointed out above, minute amounts of pure histaminase preparation were found to be highly active on histamine, but completely inactive on putrescine, cadaverine, and hexamethylene diamine as substrates. Two methyl derivatives of histamine, however, with substitution in the imidazole nucleus, the 1-methyl-4-(β-aminoethyl)-imidazole (1,4-methylhistamine) and the 1-methyl-5-(β-aminoethyl)-imidazole (1,5-methylhistamine) proved to be, as can be seen in Table III, very good substrates of pure histaminase.

Furthermore, in mixtures of histamine with either of these 2 compounds no additive effect, but competition was obtained which indicates that a single enzyme was operative during the degradation of the substrate mixtures. Substitution in the

TABLE III

EFFECT OF PURE HISTAMINASE ON HISTAMINE, 1,4-METHYLHISTAMINE, AND 1,5-METHYLHISTAMINE, AND ON MIXTURES OF HISTAMINE WITH THESE COMPOUNDS

Substrate (approx. 2.7 μmoles)	*Histaminase activity (units)*
Histamine	10.7
1,4-Methylhistamine	7.6
1,5-Methylhistamine	6.5
Histamine + 1,4-methylhistamine	8.8
Histamine + 1,5-methylhistamine	9.7

imidazole nucleus of histamine does apparently not interfere with histaminase action. These results substantiate previous findings[38,39,40].

From exhaustive studies, carried out with many coworkers on the catabolism of histamine *in vivo*, SCHAYER[41] concluded that one of the major pathways of histamine metabolism involves an enzymic methylation in the histamine molecule of the imidazole nitrogen, remote from the side chain, to give 1-methyl-4-(β-aminoethyl)-imidazole (1,4-methylhistamine) (see ref. 42). The fact that pure histaminase is able to degrade not only histamine but also its physiological metabolite, 1,4-methylhistamine, seems to add to the biological significance of this enzyme.

Effect of various typical enzyme inhibitors on histaminase

Histaminase is inhibited to a varying extent by carbonyl reagents such as cyanide, bisulphite, hydroxylamine, semicarbazide, isoniazide, aminoguanidine, and phenylhydrazine which is in agreement with ZELLER's[43] work. The enzyme is, however, not inhibited by sodium azide, thiourea, and H_2S which seems to indicate that heavy metal is not required for histaminase action[43]. Moreover, histaminase is insensitive towards iodoacetate, PCMB, and allicin which points to the absence of SH-groups[44], essential for its enzymic action.

Nature of the prosthetic group(s) of histaminase

Whereas it has been repeatedly suggested and experimental evidence has been presented by various workers[4,20,45] that histaminase is a flavoprotein, FAD being involved as a prosthetic group in the oxidative deamination process of histamine by histaminase, no such evidence for the presence of a flavin compound in the histaminase molecule was found by LASKOWSKI *et al.*[46] and LELOIR AND GREEN[47]. Since the histaminase preparations, which had been found to contain FAD, had been only partially purified it could be argued that the presence of FAD in those preparations could have been due to a contamination. Hence, it appeared to be essential to re-examine this question with electrophoretically pure histaminase preparations, obtained in this work. Different pure histaminase solutions in 0.02 M phosphate buffer (pH 6.8) containing between 0.4 and 1.4 mg of protein/ml, were subjected to a spectrophotometric investigation in the Unicam, SP 500, spectrophotometer. All these histaminase preparations showed a distinct greenish yellow fluorescence under

ultraviolet light. A solution of authentic FAD (L. Light & Co Ltd., Colnbrook, England) containing 0.5 μg of FAD/ml of 0.02 M phosphate buffer (pH 6.8) and showing the characteristic absorption maxima at 375 mμ and 450 mμ was used as a marker. It was very disappointing to find in many investigations that none of the pure histaminase preparations showed the absorption peaks, characteristic of FAD. Since, however, as mentioned above, pure histaminase solutions displayed a greenish yellow fluorescence under ultraviolet light, it was decided to investigate histaminase solutions in a spectrofluorometer by means of which, as is well known, absorption characteristics of fluorescent organic compounds can be obtained at concentrations as low as 0.1 μg/ml (see ref. 48).

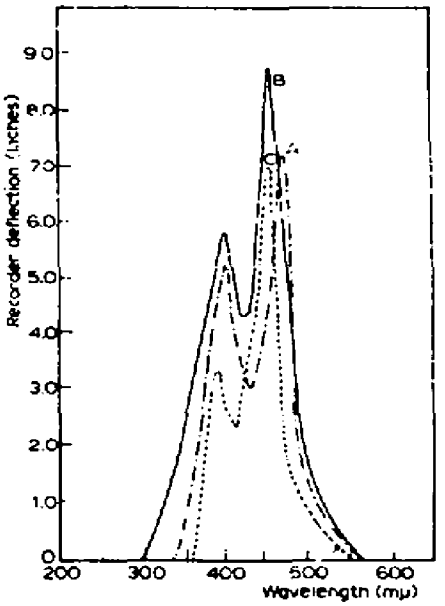


Fig. 6. Activating spectra of pure histaminase before and after acidification with 0.1 N HCl, and of authentic FAD at the fluorescence emission maximum at 530 mμ. —·—·—, pure histaminase in 0.02 M phosphate buffer (pH 6.8) with 0.44 mg protein/ml; ———, the same enzyme solution after acidification with 0.1 N HCl; . . . ., authentic FAD in 0.02 M phosphate buffer (pH 6.8), 0.2 μg FAD/ml.

Fig. 6 shows the activating spectra of the electrophoretically pure histaminase (Curve A), and the authentic FAD (Curve C), at pH 6.8, obtained in the Aminco-Bowman spectrofluorometer, when the apparatus was set at the fluorescent wavelength of 530 mμ, corresponding to the known maximum fluorescence of FAD[49]. The maxima of activation, obtained for FAD at 375 mμ and 450 mμ, as seen in Curve C, compare well with references in the literature[49]. Curve A shows that two absorption maxima, very similar to those give by authentic FAD, were obtained with the pure histaminase preparation. A slight shift towards the longer wavelengths occurred, especially apparent at the absorption maximum at 450 mμ which moved to 458 mμ. Such shifts of absorption maxima have been discovered in many flavoproteins[48]. Finally, Curve B illustrates the activating spectrum, obtained from pure histaminase on acidification with 0.1 N HCl. It can be clearly seen from this

curve that, on acidification, FAD must have been liberated from the enzyme, since the two absorption maxima were found after acidification at the same points as those of authentic FAD. Moreover, on acidification the fluorescence of the solution increased to such an extent that the absorption peaks could be only recorded after the sensitivity of the apparatus had been reduced about 30-fold. Fig. 7 shows two electropherograms, obtained from the same histaminase preparation before and after acidification with 0.1 N HCl, using normal human serum as a marker. Whereas, before acidification the enzyme migrated to its usual position between the α_2- and β-globulin fractions (lower parts of both electropherograms), no migration whatsoever took place after acidification (upper part of top electropherogram), indicating that a denaturation of the enzyme must have taken place. It should be noted here that very similar spectrofluorometric and electrophoretic results were obtained when the pure enzyme was heated at 80° for 60 sec.

Fig. 7. Electropherograms of a histaminase solution (see Fig. 6) before acidification (lower part of both strips) and after acidification with 0.1 N HCl (upper part of top strip). Phosphate buffer (pH 6.8), ionic strength 0.05. Current of 0.6 mA/cm width of strip/4 h.

For further identification of these optical findings, pointing towards the presence of FAD in the histaminase molecule, the effect of the reducing agent sodium dithionite on histaminase was examined in the spectrofluorometer, and the results are shown in Fig. 8. It is seen from Curve B of this diagram that $Na_2S_2O_4$ considerably affected the spectral behaviour of histaminase (Curve A), since, on its action on the enzyme, the peak at 375 mμ appeared to be greatly reduced and that at 458 mμ almost completely disappeared. Curve C shows that oxygenation for only 60 sec of the histaminase solution, which had been reduced by dithionite, caused a rapid reappearance of both absorption maxima, typical of oxidised FAD (Curve D). The behaviour of histaminase towards dithionite with the apparent formation of a leucoflavin derivative and the rapid reoxidation of the latter by molecular oxygen seems to be a further indication of the involvement of a flavin group in the molecule of this enzyme. According to SINGER AND KEARNEY[60] the reoxidation of reduced flavoenzymes by molecular oxygen gives rise to H_2O_2 formation; hence, the finding of peroxide among the reaction products of a biological oxidation reaction is a reliable indication of the presence of a flavoenzyme. As mentioned above, the formation of one molecule of H_2O_2 per molecule of substrate during the action of histaminase was independently discovered by various workers already in the very early studies of this enzyme[3,20,21].

Incidentally, results similar to those observed with dithionite were obtained on the addition of histamine to a pure histaminase solution. A distinct bleaching of the pale yellow colour was observed. Owing, however, to a rapid reoxidation of

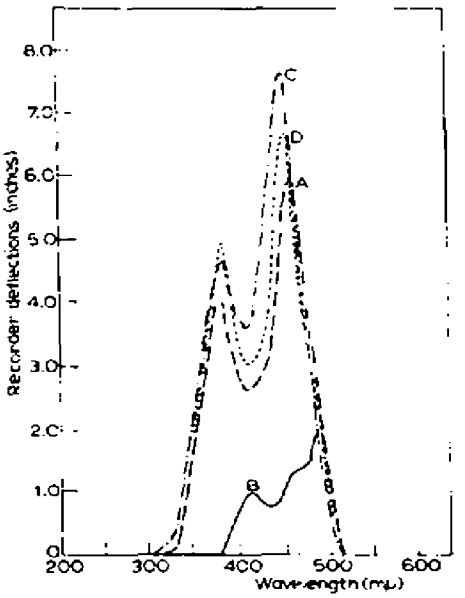


Fig. 8. Activating spectra, obtained at the fluorescence emission maximum at 530 mμ of pure histaminase before and after treatment with dithionite, followed by reoxidation of the reduced enzyme with molecular O_2. A, Histaminase in 0.02 M phosphate buffer (pH 6.8) with 0.44 mg protein/ml; B, the same enzyme solution after the addition of 0.2 ml of 4% $Na_2S_2O_4$ in phosphate buffer (pH 6.8); C, B after reoxidation with molecular oxygen; D, authentic FAD in 0.02 M phosphate buffer (pH 6.8) (0.2 μg FAD/ml).

the reduced enzyme, which could not be fully controlled, the fluorometric changes on addition of histamine were less dramatic as compared with those, seen with dithionite. MORELL[51] had also observed that hypoxanthine decreased the absorption of xanthine oxidase at 450 mμ much more slowly than did sodium dithionite.

Although pure histaminase preparations had failed to show in the Unicam, SP 500, spectrophotometer the absorption peaks at 375 mμ and 450 mμ, typical of FAD, two other absorption maxima, however, a very small one at 330 mμ and a much larger one at 405 mμ were regularly observed on spectrophotometric examinations of this enzyme. Since workers such as WERLE AND PECHMANN[52], SINCLAIR[53], DAVISON[54], and GORYACHENKOVA[55] had previously claimed that histaminase was a PP-dependent enzyme, and because of the well known fact, that carbonyl reagents are strong inhibitors of histaminase activity, it was decided to investigate whether the absorption maxima, observed in the Unicam, SP 500, spectrophotometer could be possibly related to the existence of PP in the histaminase molecule. It is known[56] that synthetic PP shows, at pH 7.0, two absorption maxima, one at 330 mμ and another one at 388 mμ.

Fig. 9 shows the absorption spectra of a pure histaminase preparation in 0.02 M phosphate buffer solution, at pH 6.8, as well as at pH 6.1, and also the absorption spectrum of authentic PP (Roche Products Ltd., Welwyn Garden City, Herts.) at pH 6.8. It can be seen from this figure that at both pH 6.1 and pH 6.8 the absorption pattern of histaminase greatly resembled the spectrum, shown by authentic PP. The intensity of the absorption maxima of histaminase appeared to be greater at

pH 6.1 compared with that at pH 6.8. SHUKUYA AND SCHWERT[57] made similar observations with glutamic acid decarboxylase when they found that a change in the absorption maxima depended upon the pH value of the solution.

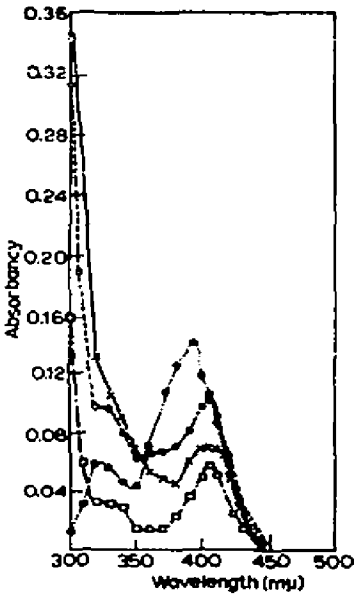


Fig. 9. Absorption spectra of pure histaminase and of authentic PP with 0.72 mg/ml enzyme protein. □ ·-·- □, Histaminase in phosphate buffer (pH 6.8); ○- - -○, histaminase in phosphate buffer (pH 6.1); × — × , histaminase after addition of 50 μg histamine as substrate; ● · · · · · ●, authentic PP in phosphate buffer (pH 6.8) (10 μg/ml).

It can be further seen from Fig. 9 that a shift towards the longer wavelengths occurred at the larger absorption peak of histaminase at both pH 6.1 and pH 6.8, from 388 mμ to 405 mμ. A spectral change of this type has been observed with many PP-dependent enzymes[58,59]. Finally, an addition of histamine (50 μg) to the histaminase solution in phosphate buffer (pH 6.1) caused a significant alteration of the absorption spectrum, in so far, as the absorption maximum at 405 mμ greatly decreased in intensity, and the absorption peak at 330 mμ was completely obliterated. Similar spectrophotometric results were previously obtained with PP-dependent enzymes[56,58,59], and it is generally believed that these changes, probably due to the intermediate formation of a Schiff base between PP and amino compounds, are involved in reaction mechanisms, catalyzed by this type of enzymes.

JENKINS AND SIZER[60] observed that PP exhibited at pH 7.0 a strong fluorescence at 400 mμ, whereby the activating maximum was located at 322 mμ, and a very weak fluorescence at 495 mμ with an activating maximum at 395 mμ.

Fig. 10 shows the activating spectra of a histaminase solution as well as of a solution of authentic PP, at pH 6.8, obtained with the spectrofluorometer set at the fluorescent maximum of PP at 400 mμ. In agreement with references in the literature, the activating peak of authentic PP was found to be at 322 mμ, while the activating peak of histaminase moved towards the shorter wavelength of 300 mμ.

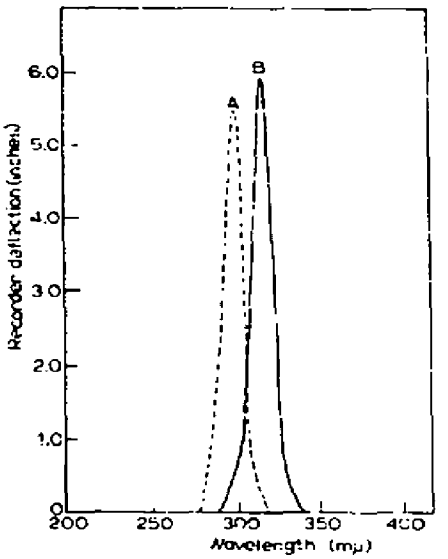


Fig. 10. Activating spectra of pure histaminase and of authentic PP at the fluorescence emission maximum, at 400 mμ. - - - - -, Histaminase in 0.02 M phosphate buffer (pH 6.8) (enzyme protein 0.72 mg/ml); ——— , authentic PP in 0.02 M phosphate buffer (pH 6.8) (10 μg/ml).

The foregoing data seem to present good evidence to accept that both FAD and PP are prosthetic groups of histaminase, thus substantiating early relevant suggestions by WERLE AND PECHMANN[52], and more recent ones by GORYACHENKOVA[55]. The question, however, why pure histaminase solutions with a similar, very low protein content should have allowed one of its two prosthetic groups, PP, to be easily revealed in the not very sensitive spectrophotometer, Unicam SP 500, whereas for the detection of the other prosthetic group, FAD, the 100-times more sensitive spectrofluorometer, Aminco-Bowman, had to be used, is at present, only a matter of speculation. It may well be that more molecules of PP than of FAD are attached to the apoenzyme of histaminase. In this connection it is relevant to mention that SINGER AND KEARNEY[50] have stressed the fact that most flavoproteins in very reliable measurements have been found to contain only 1 molecule of flavin/molecule of protein (about 65 000–75 000 g). More work will have to be devoted to further studies concerning the problem of the attachment of FAD and PP to the apoenzyme of histaminase.

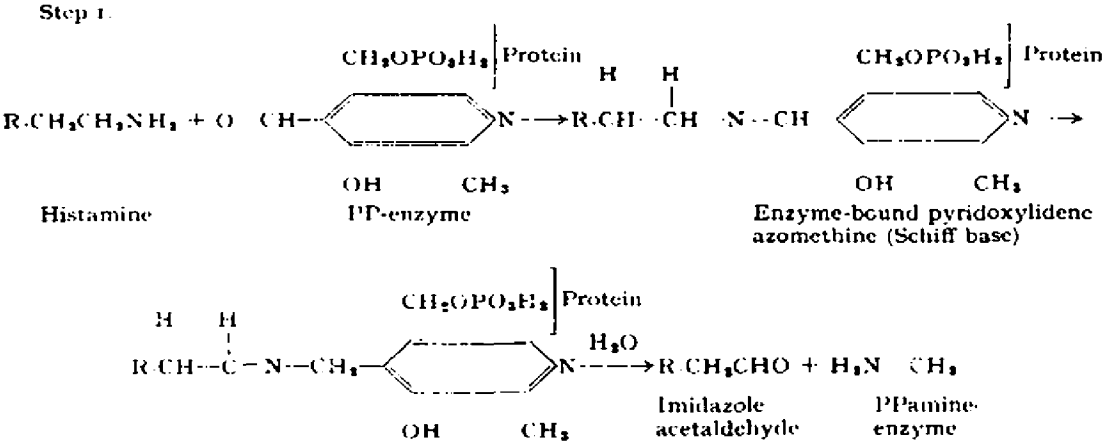
Consideration of reaction mechanisms involved

In Scheme 1 and Scheme 2 two reaction mechanisms are suggested, either of which may be operative when pure histaminase acts on histamine.

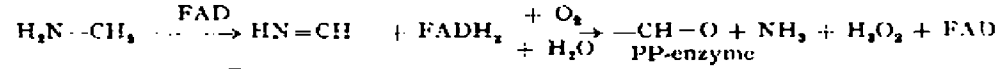
In Scheme 1, which is similar to that recently proposed by BRAUNSTEIN[15] for the action of DO, a two-step reaction mechanism is presented, involving in its first step a transamination between the substrate and the PP-form of histaminase with the formation of a Schiff base, the enzyme-bound pyridoxylidene azomethine. Electronic displacements may result in dissociation of the α-hydrogen atom and shift of double bonds, leading to the tautomeric azomethine. This is hydrolyzed to

Scheme 1

Step 1.



Step 2.



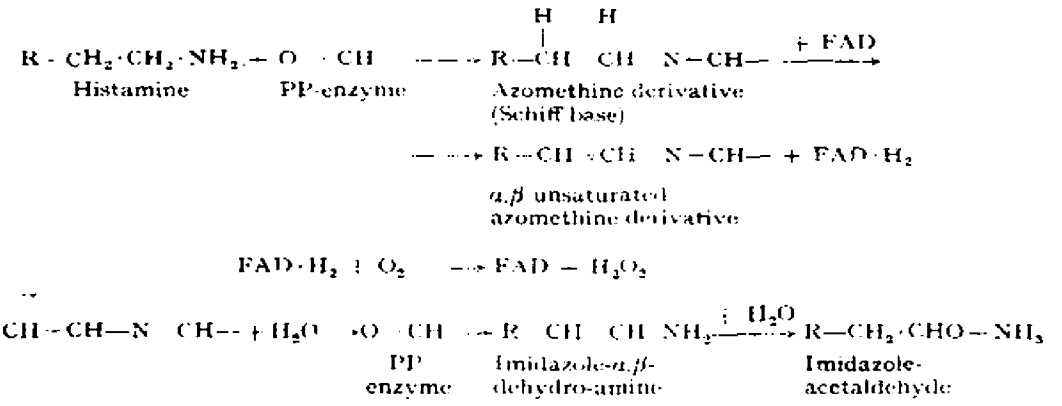
R, imidazole; PP, pyridoxal-5-phosphoric ester; PPamine, pyridoxamine-5-phosphoric ester.

imidazole acetaldehyde and the pyridoxamine phosphate–enzyme. In the second step of this mechanism the pyridoxamine phosphate form of the enzyme may undergo dehydrogenation by the second prosthetic group of histaminase, FAD, to the enzyme-bound pyridoxylimino phosphate compound, and the reaction mechanism is completed by reoxidation of the leucoflavin by oxygen to FAD, and hydrolysis of the enzyme-bound pyridoxylimino phosphate to the PP-form of histaminase with the formation of NH_3 and H_2O_2.

With purified DO preparations from hog kidney and clover seedlings GORVACHENKOVA[81] failed to achieve net transamination between diamines and added PP or free pyridoxal. It will be shown later, however, that *in vitro*, enzymic experiments with added PP and diamines, especially histamine, should be assessed only with greatest reserve because of the possible formation of not dissociable cyclic condensation products.

In Scheme 2 a reaction mechanism is shown, similar to that proposed in 1949 by WERLE AND PECHMANN[82] for the action of plant DO on its substrates. Thus, the enzymic deamination of histamine may, according to this scheme, proceed first by condensation of histamine with the PP-form of histaminase with the formation of a Schiff base which, however, does not undergo tautomeric rearrangement, but is directly dehydrogenated by the flavin prosthetic group, FAD, to the α,β-unsaturated azomethine complex with the formation of the leucoflavin, $FAD \cdot H_2$. The reaction cycle is completed by reoxidation of the leucoflavin by O_2 with the formation of H_2O_2, and stepwise hydrolysis of the α,β-unsaturated azomethine complex to the

Scheme 2



R, imidazole; PP, pyridoxal-5-phosphoric ester.

PP-form of histaminase, NH_3 and imidazole acetaldehyde, the latter formed via the imidazole-α,β-dehydroamine.

Only further studies will help to decide which of the two reaction mechanisms, described, is indeed operating during the action of histaminase on histamine.

Some additional evidence, that both FAD and PP are the prosthetic groups of histaminase, was obtained in many different assays leading to a successful reversible partial resolution of histaminase, followed by reactivation of the enzyme on addition of FAD or PP. Partial resolution of histaminase was achieved either by prolonged dialysis, or by acidification of pure histaminase solutions to pH 5.2 in the presence of $(NH_4)_2SO_4$ (0.6 saturation). Optimal reactivation results were obtained on incubation of relevant enzyme solutions with added 0.029 μmole of FAD or 0.166 μmole of PP.

Effect of prolonged dialysis on the activity of pure histaminase and effects of added FAD or PP or of a mixture of FAD and PP on the activity of the dialysed enzyme

It can be seen from Table IV, which is representative of results obtained in such dialysis experiments, that on prolonged dialysis (22 h) 15.8% of the original histaminase activity were lost, probably due to the partial removal of one or both of the prosthetic groups. Furthermore, this table shows that on incubation of the dialysed enzyme with the added, optimal amount of FAD there was not only a complete recovery of the enzyme activity, lost on dialysis, but there was even a gain in activity, whereas after incubation of the dialysed enzyme with the optimal amount of added PP the enzyme activity, lost on dialysis, was to a large extent, but not completely regained. Finally, it is seen that on incubation of the dialysed enzyme with an added mixture of optimal amounts of both FAD and PP only about 50% of histaminase activity, removed by dialysis, were restored. These results may indicate that FAD and PP are involved in the same reaction mechanism.

TABLE IV

EFFECT OF PROLONGED DIALYSIS ON THE ACTIVITY OF HISTAMINASE, AND EFFECTS OF ADDED FAD, OR PP, OR OF A MIXTURE OF BOTH COMPOUNDS ON THE DIALYSED ENZYME

2 ml of a histaminase solution (specific activity 1000) in 0.02 M phosphate buffer (pH 6.8) dialysed against 2 l of 0.02 M phosphate buffer (pH 6.8) for 22 h at 4° with one change of buffer. Preincubation of 0.2-ml aliquots of dialysed enzyme with 0.1 ml FAD (0.029 μmole) or 0.1 ml PP (0.166 μmole) or with a mixture of 0.1 ml FAD + 0.1 ml PP in an endvolume of 1 ml of 30 min at 37°. The histaminase activity is then determined as described in the methodical part.

Effect of prolonged dialysis on histaminase	Percentage of decrease in enzyme activity
	15.8
Preincubation of dialysed enzyme with added	Percentage of increase in enzyme activity
FAD	26.0
PP	11.7
Mixture of FAD and PP	8.0

Partial reversible resolution of pure histaminase by acidification to pH 5.2 in the presence of $(NH_4)_2SO_4$ (0.6 saturation)

To 30 ml of a histaminase solution (specific activity 1440) in 0.02 M phosphate buffer (pH 6.8) cooled in an ice bath, 0.1 N, ice-cold, acetic acid was dropwise added with mechanical stirring until pH 5.2 was reached. While mechanical stirring continued, solid ammonium sulphate was added to achieve 0.6 saturation. The turbid solution was left over night in the refrigerator at 4°. The precipitate, formed, was separated by centrifugation in a refrigerated centrifuge, and was dissolved in about 10 ml of 0.02 M phosphate buffer (pH 6.8). The insoluble residue, probably consisting of denatured protein, was eliminated by centrifugation. The clear supernatant was made up to 30 ml with 0.02 M phosphate buffer (pH 6.8) and in 0.2-ml aliquots the specific activity of histaminase was estimated. 0.2-ml aliquots of the clear supernatant were then preincubated with either 0.1 ml of FAD (0.029 μmole) or 0.1 ml of PP (0.166 μmole) or with a mixture of 0.1 ml FAD and 0.1 ml PP in an endvolume of 1 ml for 30 min at 37°. The histaminase activity was then estimated, as usual.

TABLE V

EFFECT OF ACIDIFICATION TO pH 5.2 ON THE ACTIVITY OF HISTAMINASE AND EFFECTS OF ADDED FAD, OR PP, OR OF A MIXTURE OF FAD AND PP ON THE ACTIVITY OF THE PARTIALLY RESOLVED ENZYME

Particulars of the experiments are given in the text.

Acidification of histaminase to pH 5.2 in the presence of $(NH_4)_2SO_4$ (0.6 saturation)	Percentage of decrease in enzyme activity
	31.7
Preincubation of the partially resolved enzyme with added	Percentage of increase in enzyme activity
FAD	21.1
PP	17.9
Mixture of FAD and PP	14.6

Table V shows the results obtained, from which one can see that on acidification to pH 5.2 in the presence of ammonium sulphate about 30% of enzyme activity were lost. Unlike in experiments with prolonged dialysis, however, incubation with added FAD did not restore completely the original activity, 66% of the lost enzyme activity having been only regained. This seems to indicate that, whereas on dialysis the enzyme may have been reversibly split, on acidification to pH 5.2 a small proportion of histaminase might have been denatured. On incubation of the partially resolved enzyme with added PP, even less of the lost activity was restored. Finally, when a mixture of both FAD and PP was incubated with the enzyme after acidification, barely half of the lost activity was regained, which is in agreement with similar observations, made in the dialysis experiments, mentioned above, and adds weight to the hypothesis that FAD and PP may compete for the same active site of the enzyme.

An important feature of the PP catalysis of the histaminase-histamine reaction, not to be disregarded, became apparent during the study of the effects of added PP on histaminase preparations, which had been subjected to prolonged dialysis. When attempting to determine the optimal amounts of FAD and of PP, to be added to the dialysed enzyme, in order to achieve best reactivation of the enzyme, it was found that, unlike FAD, PP showed a very distinct concentration optimum which, if greatly overstepped, could cause even an inhibition of histaminase action on histamine. At this point it should be also recalled that, as mentioned above, a very sharp substrate optimum was essential in order to achieve best histaminase action on histamine, since in the presence of superoptimal concentrations, histamine strongly inhibited its own enzymic degradation. Thus, it appears that both the specific substrate of histaminase, histamine, and one of its prosthetic groups, PP, may, under not fully controlled experimental conditions, act as inhibitors of the reaction mechanism in which they are fundamentally involved.

This interaction between histaminase and its substrate as well as one of its prosthetic groups may well find its explanation, when one considers, that histamine belongs to the very small group of compounds which are capable of reacting irreversibly with PP with the formation of stable, non dissociable cyclic condensation products[62]. Owing to this reaction mechanism, excessive amounts of histamine may cause a strong histaminase inhibition by removing the PP, thus rendering it unavailable to the apoenzyme. Conversely, superoptimal amounts of PP may condense with histamine, and hence may deprive the enzyme, at least partly, of its substrate. These considerations seem to support the view that optimal experimental conditions should be first established before trying to assess the reaction mechanism *in vitro* of histaminase.

JAKOBY AND BONNER[63], when working with the PP-dependent enzyme kynureninase found that this enzyme was also inhibited by its prosthetic group as well as by its substrate, kynurenine.

The finding that PP may inhibit histaminase seems to raise the interesting question as to the biological significance of the presence of PP in the histaminase molecule. It may be that the importance of PP as a prosthetic group of histaminase lies just in its inhibitory properties, thus enabling it to regulate the action of histaminase on its specific substrate, histamine. It may be of interest to recall here results, quoted above in Table IV, showing that on incubation of dialysed histaminase with

an, added, small amount of FAD more activity was recovered than had been lost on dialysis. This gain in histaminase activity may be possibly explained by the lack of interaction on the part of PP, which might have been partially removed by dialysis. PP may, thus, constitute one of the regulatory factors in the biological histamine formation and destruction.

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