RECENT ATTEMPTS AT THE PURIFICATION
AND IDENTIFICATION
OF HISTAMINASE.

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CONTENTS.

SECTION I.
GENERAL INTRODUCTION 1

SECTION II.
ANALYTICAL METHODS
1. Measurement of pH 14
2. Buffers 14
3. The estimation of protein 15
4. Estimation of enzyme activity 18

SECTION III.
PURIFICATION OF PIGS' KIDNEY HISTAMINASE: 21
1. Ammonium sulphate fractionation 22
2. Thermal denaturation of inert proteins 24
3. Chromatography and re-chromatography of partly purified histaminase on DEAE-cellulose columns. 29

SECTION IV.
IDENTIFICATION OF PIGS' KIDNEY HISTAMINASE.
1(a) Electrophoresis on cellulose acetate strips at various pH's. 69
1(b) The Isoelectric Point 69
2. Starch gel electrophoresis 70
3. Specificity 78
4. Stability at various temperatures 80

SECTION V.
PROSTHETIC GROUPS OF HISTAMINASE. 82
1. Flavin adenine dinucleotide 87
2. Pyridoxal phosphate 91

SECTION VI.
GENERAL DISCUSSION AND SUMMARY 96

REFERENCES.
SECTION 1.

GENERAL INTRODUCTION.
As early as 1899, Chittenden, Mendel and Henderson observed that certain protein derivatives produce a sudden lowering of blood pressure. Osborne and Swale Vincent (1900) found that certain tissue extracts contained a pressor and depressor substance, the action of which was not abolished by atropine. Beat, Dale, Dudley and Thorpe (1927) showed that such extracts largely owed their physiological activity to the presence of histamine.

Windaus and Vegt (1907) synthesised histamine from histidine by a complicated six-step decarboxylation procedure.

Yoshimura, K. (1909/1910) obtained a base from the Japanese beverage Tamari-shoyu which had been prepared from soy beans. He did not completely identify the base but stated that it was a derivative of histidine and had the formula $C_4H_9N_3$.

In a classical work, Barger and Dale (1910) isolated histamine from the plant, ergot. In the same year, Dale and Laidlaw began extensive studies on the
physiological properties of histamine and in 1911 they showed that histamine caused a vasodilator fall of blood pressure in carnivora as well as in the monkey and in the fowl. It was in 1911 that Barger and Dale isolated histamine from intestinal mucosa.

The work of Dale and Laidlaw (1910) gave a great impetus to the study of the physiological importance of histamine. In 1912 Mellanby and Twort also observed that histamine was a constituent of intestinal mucosa and they isolated a bacillus from the intestinal contents which had the power of converting histidine into histamine. It is this work which laid the foundation for the extensive studies on the bacterial production of histamine by Koessler and Hanke (1924).

In 1919, Abel and Kubota, after several studies, claimed that histamine was the main depressory substance present in both the pituitary gland and the gastrointestinal mucosa. Many workers including Cow (1919); Jackson and Mills (1919); Hanke and Koessler (1920); Dudley (1920); Dale and Dudley (1921) and Spiro (1922) opposed Abel's views, and in 1931, Best and McHenry finally stated that Abel's claim was not entirely correct.
Beatt and McHenry (1931) reported that the signs of anaphylactic shock produced by large doses of histamine were similar to those of secondary wound shock in man. Several other workers have shown the relationship between histamine and human allergic asthma.

Dale and Laidlaw (1911) showed that histamine was inactivated by a perfusate of liver of the carnivora. Oehme (1913) reported that small intravenous doses (0.6 mg) of histamine were fatal to rabbits if given rapidly, but twenty times this amount could be given by slow infusion intravenously without harmful effects. Oehme considered the possibility that histamine could be chemically altered in the body and concluded that the animal could dispose of large amounts of histamine, if it was injected slowly.

Rustie (1915) showed that a heat-labile system, present in the pulped liver of the turkey buzzard, Carthus aur, inactivated histamine at a very high rate.

Guggenheim and Logoffler (1916) confirmed
the work of Gehme when they also found that no harm was caused to a rabbit by slow injection of 50 mg of histamine. No histamine could be detected in the rabbit's urine. When, however, the same amount of histamine was rapidly injected into the rabbit, the animal died and histamine was found in the urine. Guggenheim and Loeffler concluded that the body possessed a mechanism for inactivating histamine.

Koessler and Hanke (1924) fed histamine to dogs and guinea pigs and on account of their results they suggested that histamine may have been inactivated either by the intestinal mucosa or by the blood stream of those animals.

Best (1929) found that histamine was rendered physiologically inert when incubated with minced beef lung. In 1930, Best and McHenry reported that an enzyme system, found in various animal tissues, inactivated histamine, and they proposed the name of "histaminase" for this enzyme system. They found that this histaminase was thermolabile and
involved the oxidative deamination of histamine with an oxygen uptake and liberation of ammonia. In 1938, Zeller found that hog kidney preparations were capable of deaminating not only histaminase but also cadaverine, putrescine, agmatine and spermine. On account of these investigations Zeller declared that this enzyme was similar to Beat and McHenry’s histaminase, and therefore proposed that the name of "Diamine-oxidase" be substituted for "histaminase".

Kapeller-Adler (1944, 1949, 1951, 1953) suggested that histamine was the specific substrate of histaminase and that Zeller’s diamine-oxidase (DO) was the enzyme destroying the diamines such as cadaverine, putrescine and spermine. She urged that the term "histaminase" should be restored to the enzyme system which specifically attacked histamine, and the term "diamine oxidase (DO) should be retained for the enzyme system which inactivated the diamines mentioned above.

Enzyme systems which deaminate histamine and diamines occur very widely in nature. Marcou
and co-workers (1938) were the first investigators to observe a rise of the histamine-destroying activity in the blood of pregnant women towards the end of gestation and considered this a defence reaction. Werle (1941) has shown that the D.O. activity of Pseudomonas pyocyanea is quite high, and Gale (1942) observed also a high activity with Mycobacterium smegmatis.

Werle and von Pechmann (1948) found that D.O. does not occur in seeds. High D.O. activity, however, was encountered in pea seedlings. The enzyme is present in relatively high concentrations in clover plants. Very high activity of this enzyme is usually found in the kidney and intestinal mucosa and to a lesser extent in liver and lungs of animals. The kidney of the white rat shows only traces of the enzyme whereas the liver of birds is the site of the highest activity. (Best and McHenry, 1930).

Danforth (1939); Zeller and co-workers (1939); Birkhauser (1940); Kapeller-Adler (1941a) (1941b) (1941c); Kapeller-Adler and Adler (1943); Ahlmark
(1944) reported considerable activity of the histamine-destroying enzyme in human placenta and a sharp rise in the blood during pregnancy. The kidneys of the human adult show more D.O. activity than those of the new-born, Zeller et al (1940).

The action of diamine oxidase on diamines has been described by Zeller (1938):

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

and in the presence of catalase the $H_2O_2$ decomposed into $O_2$ and water thus:

$$RCH_2NH_2 + \frac{1}{2} O_2 \rightarrow RCHO + NH_3$$

Kiese (1940); Stephenson (1943); Laskowski et al (1945); and Kapeller-Adler (1949) have confirmed most of these steps. Best and McHenry (1930); Zeller (1938); Werle and von Pechmann (1948); Zeller (1938) reported that partially purified histaminase utilizes 1 atom of oxygen and produces 1 mol of ammonia per mol of histamine or other diamines, whereas in the crude extract in which additional enzyme systems are present, the uptake
of oxygen and the liberation of ammonia are much higher than in the above equation.

The formation of the hydrogen peroxide in the above equation has been shown by coupled oxidation, by Zeller (1938); Stephenson (1943); Friedenwald and co-workers (1942) and Kapeller-Adler (1943). The formation of the aldehyde was demonstrated by its reaction with bisulphite by Zeller and co-workers (1940) and Stephenson (1943). Kapeller-Adler and Fletcher (1959) and Kapeller-Adler (1960) confirmed the formation of iminazole acetaldehyde by isolating it as the 2,4-dinitrophenylhydrazone of iminazole acetaldehyde.

In 1949, Fleisher and Zeller pointed out that the first step in oxidative deamination involves the removal of an $\alpha$- and a $\beta$-H and that diamines without an $\alpha$- or a $\beta$-H or both should be much less actively attacked by diamine oxidase. Zeller et al (1939) and Zeller (1940, 1941) have shown that substrates like histamine and agmatine
which contain two basic groups with different affinities for the enzyme act as inhibitors at high concentration. Zeller (1938, 1942) found that carbonyl reagents such as bisulphite, dimethylcyclohexanedione, semicarbazide, hydroxylamine phenylhydrazine and marsilid inhibit diamine oxidase. This was confirmed by Werle (1940) and Zeller pointed out that this type of inhibition suggests the presence of a carbonyl (ketone or aldehyde) group in the diamine oxidase molecule.

Other reagents like pyrophosphate, McHenry and Gavin (1932): \( \text{Na}_3 \), thiourea and \( \text{H}_2\text{S} \), Zeller (1940), which react with heavy metals have negligible influence on D.O.

There is much evidence in the literature that D.O. preparations from different tissues show wide variations with regards to specificity; this is also true for D.O. preparations from the same tissue. Zeller and co-workers (1946) declared, when they measured the enzyme activity by the manometric method, that hog kidney enzyme degraded cadaverine faster than putrescine, and that putrescine was
degraded faster than histamine. Kapeller-Adler (1951) in a series of extensive studies with the aid of the microvolumetric Indigo method, first reported that partly purified histaminase from hog kidney attacks histamine faster than cadaverine while the reverse is true for preparations obtained from human placenta. Berman (1955) observed that enzyme from rabbit liver mitochondria attacked histamine faster than cadaverine but that cadaverine was attacked faster than putrescine. Kenten and Mann (1952) prepared the enzyme from pea seedlings and found that cadaverine was degraded faster than putrescine, and histamine was deaminated more slowly than putrescine. Using an enzyme preparation from Lavandula leaves, Werle and Zabel (1948) observed that putrescine was attacked with more rapidity than either cadaverine or histamine. The diamine oxidase preparation of Roulet and Zeller (1945) from Mycobacterium smegmatia seemed to attack putrescine, cadaverine and histamine in the same fashion as Werle and Zabel’s enzyme preparation from Lavandula leaves. Satake, Ando and Fujita
(1953) prepared D.O. from Achromobacter and observed that the enzyme had a higher affinity for histamine than for putrescine which was degraded much faster than cadaverine.

In manometric experiments using partly purified pigs' kidney enzyme and various concentrations of histamine and simple diamines Zeller (1938) and Kapeller-Adler (1949) reported that competition did occur between the two groups of substrates. Later (1953) Kapeller-Adler with the aid of the microvolumetric indigo method, and in better controlled experiments with more purified hog kidney preparations and with placental extracts, observed that the enzyme effects on histamine were not altered by the presence of cadaverine, putrescine and agmatine. The enzymatic oxidation of cadaverine was, however, decreased in the presence of putrescine and agmatine. Kapeller-Adler also pointed out that neither the equimolar nor the 20-fold admixture of cadaverine, putrescine or agmatine to histamine did in any way influence the oxidation of histamine by histaminase.
On the other hand, Werle (1941) reported that putrescine completely abolished the action of hog kidney extracts on histamine. When they, however, used the pea seedling preparation Werle and Hartung (1956) found an additive effect with histamine and cadaverine, measured by oxygen consumption and ammonia liberation.

Kapeller-Adler (1952), working with a partly purified pigs' kidney enzyme, observed that natural oestrogens potentiated the enzyme activity only on histamine but not on cadaverine or putrescine.

In 1958, Blaschko suggested that two separate enzymes, one present in hog kidney and similar to histaminase deaminated the di-cationic form of the histamine, while the other, present in hog serum, deaminated the mono-cationic form of histamine.

The evidence so far available indicates that most of these studies were carried out with only partly purified enzyme preparations. Many attempts at the purification of histaminase were made by various workers using different methods of purification.
(McHenry and Gavin (1935); Kiese (1948); Stephenson (1943); Laskowski et al (1945); Leloir and Green (1946); Kapeller-Adler (1949); Tabor (1951); Swedin (1956); Kapeller-Adler and Fletcher (1958) and Swedin (1958).

Since, for the solution of the problem concerning the identification, specificity and prosthetic group of histaminase, a pure, homogenous preparation of histaminase appeared to be of paramount importance, it was decided to attempt the purification of histaminase by modern chromatographic techniques.
SECTION II.

ANALYTICAL METHODS.
1). Measurement of pH.

Throughout this study only glass distilled water was used and pH was estimated with a Dynacap glass electrode pH meter. All reagents were of analytic grade and all glass apparatus was cleaned with chromic cleaning solution. Rubber stoppers were boiled before use.

2). Buffers.

pH 2.2 - pH 8.0. A mixture of 0.2 M disodium phosphate and 0.1 M citric acid was prepared according to McIlvain (1921).

pH 5.0 - pH 9.6. (Ellis and Simpson (1956)). These buffers were used as follows:

- pH 5.0 0.1 M NaOAc - 0.048 M HOAc
- pH 5.5 0.1 M NaOAc - 0.015 M HOAc
- pH 6.5 0.021 M Na₂HPO₄ - 0.037 M NaH₂PO₄
- pH 7.5 0.030 M Na₂HPO₄ - 0.005 M NaH₂PO₄
- pH 8.6 0.026 M Na₂B₄O₄ - 0.022 M HCl
- pH 9.6 0.026 M Na₂B₄O₇ - 0.02 M NaOH
- pH 6.8 0.153 M NaH₂PO₄·2H₂O -
  0.174 M Na₂HPO₄·12 H₂O. Sorensen (1909).
pH 8.6  0.05 M Sodium barbitone. Owen (1956).
This range of buffers were prepared from a mixture
of 0.1 M Na₂CO₃ - 0.1 M sodium borate.
pH 11.0 - 12, were made from a mixture of 0.1 M
Na₂HPO₄ - 0.1 M NaOH. Kolthoff and Vleeschhouwer
(1927).

3). THE ESTIMATION OF PROTEIN.

(a). The biuret reaction. Gornall, Bardawill and David
(1949) was used for the estimation of enzyme protein
only in the very initial stages of fractionation of
the pig’s kidney histaminase when the protein concen-
tration was very high.

REAGENTS.

1.5 gm of cupric sulphate and 6 gm. of sodium
potassium tartrate were dissolved in 500 ml. of
distilled water in a 1 litre volumetric flask. 300 ml.
of 10% NaOH was added with constant stirring and the
volume made up to a litre. The biuret reagent was
stored in a polythene bottle.
METHOD.

The total protein solution, in a pyrex test tube, was made up to a total volume of 2 ml. with 0.9 % NaCl, 8.0 ml. biuret reagent was added, the tubes closed with rubber bungs, shaken and allowed to stand for 30 minutes. The developed colour was read in a Unicam photoelectric colorimeter at 540 nm using a blue filter. The protein concentration was estimated from standard curve which was prepared from serial dilutions of freeze dried serum (Glaxo Laboratories Ltd.) with known protein content.

b) **Method of Lowry and co-workers (1951)**.

**REAGENTS.**

Reagent A 2% Na$_2$CO$_3$ in 0.1 N NaOH  
Reagent B 0.5% cupric sulphate in 1% sodium potassium tartrate (made up fresh each time).  
Reagent C alkaline copper solution. Mix 50 ml. of reagent A with 1 ml of reagent B.
Reagent D  Diluted Folin Ciocalteu phenol reagent. Titrate Folin Ciocalteu phenol reagent with NaOH to a phenolphthalein end point on the basis of this titration dilute the reagent to make it 1 N in acid.

The Folin Ciocalteu phenol reagent was diluted 1 in 2.2, stored in a brown bottle and kept in the refrigerator.

**METHOD.**

To a sample of 5 to 100 micrograms of protein in 0.2 ml or less in a 5 ml. test tube, 1 ml of reagent C was added. The solution was instantly mixed and allowed to stand for 10 min at room temperature. 0.1 ml. of reagent D was added very rapidly and mixed immediately. After 30 min, 0.5 ml of the developed blue coloured solution was transferred to a micro cell and read in the Unicam spectrophotometer S.P. 500. For a range of 5 - 25 μg. protein per ml. of final volume readings were made at a wave length of 750 μm. For higher concentrations readings were
made at a wavelength of 500 μm.

Standard curves were plotted using reconstituted human serum of known protein content. When the enzyme preparation was too concentrated, it was diluted with 0.01 M phosphate buffer to bring it within the limits of the curve.


This method, which is a modification of Zeller's qualitative indigo test is based on the fact that during the action of histaminase 1 mol of \( \text{H}_2\text{O}_2 \) is formed for each molecule of substrate oxidised (Zeller, 1938), and is an example of a coupled oxidation reaction whereby the \( \text{H}_2\text{O}_2 \) generated in the enzymic reaction is utilised for the oxidation of the indigo disulphonate. The excess of indigo disulphonate, not oxidised by the enzymatically formed \( \text{H}_2\text{O}_2 \) is determined by titration with 0.002 N \( \text{KMnO}_4 \).

REAGENTS
REAGENTS:

a) Phosphate buffer pH 6.8.

b) Substrates: 200 mg histamine dihydrochloride (B.D.H.) was dissolved in 40 ml of the above buffer. 400 mg cadaverine dihydrochloride or putrescine dihydrochloride (B.D.H.) in 40 ml of phosphate buffer pH 6.8.

c) Indigo solution was made by dissolving 200 mg. of indigo carmine, (B.D.H.) in 300 ml distilled water and kept in a brown bottle in the refrigerator.

d) 0.002 N KMnO₄ freshly made before each titration was prepared from a stock solution of 0.1 N KMnO₄.

METHOD.

To 0.5 ml. of the histaminase extract in a test tube was added 8.4 ml. of buffer, 0.1 ml. of substrate and 1 ml. of indigo carmine solution, with one small drop of chloroform as preservative. The final volume of the reaction mixture was 10 ml. The tube was closed
with a rubber stopper and the solution mixed by gentle shaking. Blanks without the addition of substrate and assays with the addition of substrate were both set up in triplicate. Oxygen was passed through the tubes for one minute, the tubes being then again shaken gently and incubated at $37^\circ C$ for 24 hr. After incubation, the excess of indigo carmine in the blank and assay tubes was titrated with $0.002 \text{ M } \text{KMnO}_4$ until the end point, at which the blue colour has just faded, was reached. The difference in titration value between the assay and the blank is indicative of the amount of $\text{H}_2\text{O}_2$ formed in the reaction and thus of the enzyme activity. The activity is expressed in permanganate units P.U. 1 P.U. indicates the amount of enzyme, which after 24 hr. requires an end titration of $0.1 \text{ ml.}$ of $0.002 \text{ N } \text{KMnO}_4$. Since the microvolumetric technique mentioned above is based on the theory that during the action of histaminase on histamine 1 molecule of $\text{H}_2\text{O}_2$ is formed for every molecule of histamine oxidised, 1 P.U. corresponds to the destruction of $0.46 \mu g$ of histamine and to the uptake of $0.05 \mu l$ of oxygen per 60 min. (Kapeller-Adler, 1951).
SECTION III.

PURIFICATION OF PIGS' KIDNEY HISTAMINASE.
The first attempts to purify histaminase were made in 1935 by McHenry and Gavin when they obtained a preparation with 20 times more activity than the crude minced hog kidney. Kiese (1940) and Stephenson (1943) purified histaminase by fractionation with ammonium sulphate. Laskowski and co-workers (1945) found that no loss of histaminase activity occurred and the degree of purification of their hog kidney enzyme was even doubled after the crude 1% NaCl enzymic extract had been heated at 65°C. The enzyme was then further purified by fractionation with ammonium sulphate, followed by adsorption on calcium phosphate gel. Leloir and Green (1946) also purified the pigs' kidney enzyme by precipitation with ammonium sulphate and they improved their purification method by adsorbing the enzyme on tricalcium phosphate and alumina C by isoelectric precipitation and finally by electrophoretic separation.

Kapeller-Adler (1949) extracted the enzyme from the acetone powder prepared from hog kidney with 1% NaCl, heated it to 62°C, precipitated it at 0.5 saturation with solid ammonium sulphate and finally
adsorbed it on calcium phosphate gel. Tabor (1951), Kapeller-Adler and Fletcher (1958) employed acid pH precipitation methods to improve the purity of the enzyme.

The method which was developed and used in this work for the purification of pigs' kidney histaminase is described in detail below and the purification scheme is shown in Table 1.

1). AMMONIUM SULPHATE FRACTIONATION.

Hog kidneys were obtained from the local slaughter house immediately after killing. The connective tissue, fat and medullas were removed and rejected. The cortices were minced to a fine brei in an electric mincer.

The weighed, minced brei was then transferred to a conical flask, and 200 ml. of 1% NaCl per 100 g. of minced pulp were added. This mixture was allowed to stand for 60 min, with intermittent, gentle shaking, then filtered through muslin. The pulp was returned to the original flask and the
process repeated, using the same volume of 1% NaCl as before. The pulp was then discarded and the total volume of the 1% NaCl extract measured. (Fraction 1).

The 1% NaCl extract was transferred to a beaker and 42.4 g/100 ml of finely powdered solid ammonium sulphate were slowly added while stirring with an electric stirrer. The precipitate was immediately filtered through Whatman No. 1 fluted filters. The reddish filtrate was discarded. The precipitate was dissolved in 0.02 M phosphate buffer pH 6.8 and precipitated to 0.2 saturation (14.1 g/100 ml) with finely powdered solid ammonium sulphate under mechanical stirring. This was filtered and the dark brown filtrate, (Fraction 11) was made 0.6 saturated with ammonium sulphate when it was precipitated by 28.2 g/100 ml of ammonium sulphate. The precipitate was redissolved three times and fractionated three times to 0.6 saturation with solid ammonium sulphate. This was filtered through Whatman No. 1 fluted filters and the filtrate discarded. The precipitate was dissolved in 1050 ml of 0.20 M
phosphate buffer pH 6.8 and reprecipitated to 0.6 saturation with ammonium sulphate. Usually, 100 ml aliquots of this precipitate were filtered and redissolved in 0.02 M phosphate buffer, pH 6.8 and dialysed for 60 min, then heated at 60°C as described below.

2) THERMAL DENATURATION OF INERT PROTEINS

100 ml of the crude histaminase which was fractionated with ammonium sulphate, as described above, was dialysed for one hour against 0.02 M phosphate buffer pH 6.8 then centrifuged at 4°C for 15 min at 2000 r.p.m. and the precipitate discarded. The clear, yellowish brown supernatant was heated with constant swirling at 60°C for 20 min in a constant temperature water bath. After cooling under cold running tap water the turbid enzyme solution was centrifuged at 4°C for 15 min at 2000 r.p.m. and the supernatant separated from the precipitate. The precipitate was washed three times with 0.02 M phosphate buffer pH 6.8 and recentrifuged. The insoluble precipitate was discarded but the washings
TABLE I.

SUMMARY OF PURIFICATION OF PIGS' KIDNEY HISTAMINASE.

(Details of the purification are given in the Text.)

Pulp from 24 pigs' kidney cortices extracted with 1% NaCl.
(200 ml. 1% NaCl/100 g. of tissue and procedure repeated twice).

\[ \text{FRACTION I} \]

1% NaCl extract precipitated at 0.6 saturation with \((\text{NH}_2)_2\text{SO}_4\) (42.4 g. \((\text{NH}_2)_2\text{SO}_4/100 \text{ ml})\)
precipitate dissolved in 0.02 M phosphate buffer pH 6.8, then
dialysed 1 hr. against phosphate buffer pH 6.8

\[ \text{FRACTION II} \]

50 ml. aliquot of Fraction II heated for 20 min. at 60°C,
insoluble precipitate of inert protein washed twice with 0.02 M phosphate buffer pH 6.8 and
washings added to the brown enzyme solution

\[ \text{FRACTION III} \]

(This enzyme solution was the starting material for chromatography).
were combined with the supernatant, (Fraction III).

The purification scheme is outlined in Table I. In each of these fractionation stages the protein and the enzyme activity were assayed simultaneously in order to throw some light on the purification procedure.

RESULTS.

The scheme followed during the fractionation of the pigs' kidney histaminase with ammonium sulphate is shown in Table I. This procedure was carried out repeatedly on different batches of pigs' kidney extracts. A typical example of the results obtained in such a purification procedure is shown in Table II. In the early stages of the purification procedure the protein content was estimated by the Biuret method, then later, by the Lowry method, when the protein content had been decreased.

Attention should be drawn to the fact that throughout the entire purification procedure of pigs' kidney histaminase attempted in this work, no enzymic activity in any of the purification stages was observed
<table>
<thead>
<tr>
<th>FRACTION AND TREATMENT</th>
<th>VOLUME (ml)</th>
<th>TOTAL PROTEIN (mg.)</th>
<th>HISTAMINASE ACTIVITY</th>
<th>CADAVERINASE ACTIVITY</th>
<th>Specific Activity on histamine in relation to the specific activity on cadaverine</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl extract of pulp</td>
<td>4600</td>
<td>80,000</td>
<td>85,700</td>
<td>1.06</td>
<td>32,950 0.41 2.59</td>
</tr>
<tr>
<td>Precipitate to 0.6 saturation with (NH₄)₂SO₄ filtered, and dissolved in 0.02 M phosphate buffer, dialysed 1 hr. against 0.02 M phosphate buffer pH 6.8.</td>
<td>282</td>
<td>4,371</td>
<td>67,680</td>
<td>15.5</td>
<td>17,202 4.0 3.87</td>
</tr>
<tr>
<td>After the 50 ml. aliquot was heated 30 min. at 60°C, insoluble pptate washed, then discarded.</td>
<td>50</td>
<td>265</td>
<td>16,900</td>
<td>64</td>
<td>2,484 9.37 6.83</td>
</tr>
</tbody>
</table>
when putrescine was used as substrate, whereas Tabor (1951), by his method, could achieve a purification of the enzyme effect on putrescine double that on histaminase. Attempts to achieve an enzymatic effect on putrescine by varying the concentration of both enzyme extract and putrescine remained unsuccessful. At each stage of the purification procedure, the enzyme activity on histamine and on cadaverine was estimated, using the same volume of the enzyme fraction, or the conditions were altered by varying the amount of fraction volume in relation to substrate concentration.

In the case of the enzyme activity on histamine, the purification procedure up to the stage of heating at 60°C, yielded a 40 - 64-fold increase in specific activity of the enzyme as compared to the crude sodium chloride extract which had an initial specific activity of 1.06, (see Table II).
The reaction mixture with cadaverine showed a low initial specific activity, and the yield of the enzymic activity towards cadaverine, in contrast to histamine, decreased considerably at each purification stage. Table II shows that up to the heating stage there was a 60.4-fold increase of enzymic activity towards histamine whereas there was only a 22.9-fold increase of enzymic activity towards cadaverine.

It must be emphasised that in the purification of different batches of pigs' kidneys, there were some variations in the values obtained for the enzyme activity towards histamine even at the same purification stage, but the variations were greater for the enzymic activity towards cadaverine. In most cases the activity towards cadaverine was completely lost before the stage of heating the enzyme extract, whereas in others only a negligible amount of cadaverinase activity could be detected.
after heating.

During the purification procedure the ratio of the specific activity of the enzyme towards histamine in relation to that of cadaverine was never constant, e.g. Table II shows that the ratio of the specific activity on histamine to the specific activity on cadaverine varied from 2.59 to 6.83. If it were one and the same enzyme which acted on both histamine and cadaverine, then the ratio of these specific activities should always be constant. A constant ratio of the enzyme activity towards histamine and cadaverine was never found. These results agree with those of Kapeller-Adler and Fletcher (1958).

Since the purification of histaminase by Fractionation with ammonium sulphate followed by heating did not yield a very satisfactory result, and since Kapeller-Adler and Fletcher (1958) have shown in preliminary experiments that a higher degree of purification could be achieved by adsorbing the crude histaminase on "Amberlite" CG-50, it was decided to attempt to purify this
enzyme by a modern chromatographic technique.

3) CHROMATOGRAPHY AND RECHROMATOGRAPHY OF PARTLY PURIFIED HISTAMINASE ON DEAE-CELLULOSE COLUMNS.

As early as 1912, Harrison observed that on passing a solution of NaCl through purified cotton, hydrochloric acid appeared in the eluate. Several workers confirmed the cation exchange properties of cellulose and Hartmann (1930) prepared diethylaminoethyl cellulose as a cellulose derivative which was initially aimed at improving the dyeing characteristics of textile fibres. It is this compound, sometimes referred to as DEAE-cellulose or DE 50, a weakly basic monofunctional anion exchanger, along with the cation exchanger, carboxyl methyl cellulose, CM-cellulose, which was introduced as a purifying agent in protein chemistry by Peterson and Sober (1956).

In the first experiments by Sober and Peterson (1956) 900 mg of plasma proteins were applied to a column of about 50 x 2.5 cm. of DEAE-cellulose equilibrated with sodium phosphate buffer of 0.005 M
ionic strength and of pH 7.0. A combined salt and pH gradient system was used to elute the plasma proteins from the column. Most of the protein components of the serum emerged from the column in order of decreasing isoelectric points.

The introduction of DEAE-cellulose, CM-cellulose and phosphorylated cellulose as purifying agents in protein chemistry by Peterson and Sober has met with great success and gave a great impetus to the study of the chromatography of proteins. Since then, several anionic and cationic derivatives of cellulose have been prepared. The cation exchangers, sulfomethyl and sulfoethyl cellulose, and the anion exchangers, triethylaminoethyl cellulose, and the epichlorohydrin-triethanolamine cellulose and para-aminobenzyl cellulose have been used to purify several different types of proteins.

Various hormones have been purified by chromatography on columns of cellulose derivatives. Ellis and Simpson (1956) purified growth hormones by adsorption on both DEAE-cellulose and CM-cellulose columns and eluted the adsorbed growth hormone by gradient elution.
Using the same technique, Hlis in 1960, was able to obtain an electrophoretically homogenous pituitary proteinase by chromatography on DEAE-cellulose and a 6-fold purification of parathyroid hormone was achieved by Friedman and Munson (1959). When they eluted the adsorbed hormone from a column of CM-cellulose. The thyroid stimulating hormone (TSH) was partially purified on cellulose columns by Condliffe and Bates (1956). The cation exchanger CM-cellulose and the anion exchanger DEAE-cellulose were both used by Farmer in 1959 to purify adrenocorticotrophin.

Serum has been examined chromatographically on the anion exchanger, DEAE-cellulose, by means of a gradient of decreasing pH and increasing salt concentration. Coloured bands (buff, red, tan, yellow and blue) were formed and could be followed down the column. The effluent fractions were examined electrophoretically, spectrophotometrically and, in some cases, immunologically and chemically (Sober and co-workers, 1956). Using the DEAE anion-cellulose chromatographic procedure, Humphrey and
Porter (1957) and Fahay (1959) were able to separate $\alpha$-globulin into five different fractions.

The cellulose ion exchanger, carboxymethyl cellulose was found to be an important adsorbent for the separation and identification of numerous abnormal forms of human and of animal haemoglobins (Huisman 1958).

Speer and co-workers (1959) studied blood group antibodies with the aid of chromatography on DEAE-cellulose and Hagan et al (1960) reported the partial purification of human plasminogen by chromatography on CM-cellulose column.

In addition to hormones and other serum proteins numerous enzymes have been successfully purified by chromatography on cellulose columns.

Haver et al (1954) and Sober and Peterson (1954) independently separated ribonuclease and deoxyribonuclease activity by chromatography on DEAE-cellulose. A 2-to 10-fold purification was achieved by Mitz and Yanari (1956) when they chromatographed liver catalase and kidney cathepsin on columns of DEAE-cellulose, and Lewis...
and Thiele (1957) fractionated pancreatic insulinae on the same medium.

The simple purification method as applied to leucine amino peptidase is a good example of the usefulness of DEAE-cellulose anion exchanger, in enzyme purification, (Folk et al, 1959). This particular protein mixture was chromatographed on a small (0.9 x 25 cm) column of DEAE-cellulose equilibrated with the eluting buffer of low ionic strength, (0.005 M) and of a pH 8.0. The protein was eluted from the column by a linear gradient of NaCl from 0 to 0.3 M, added to the same buffer used for the equilibration.

Brown and Ward (1958) reported the partial purification of tyrosinase by chromatography on DEAE-cellulose column and in 1960, Press, Porter and Cabra isolated and studied the properties of cathepsin D after chromatography on both CM- and DEAE-cellulose. Chromatography and rechromatography on DEAE-cellulose was used by Dixon, Kornberg and Lund (1960) and Yamamoto and Beavers (1961) in their attempts to purify malate synthetase. When threonine
synthetase was chromatographed on DEAE-cellulose. Flavin and Slaughter (1960) achieved a 500-fold purification.

According to ultracentrifugal and electrophoretic analyses, a 90% homogenous glutamic acid decarboxylase was obtained by Shukuya and Schwert (1960) when the enzyme was chromatographed on DEAE-cellulose. This same medium was used by Jakoby and Fredericks (1960) to purify erythrol dehydrogenase.

Mann (1961) reported a method in which he adsorbed pea seedling amino oxidase on calcium phosphate, and passed the dialysed effluent through columns of DEAE-cellulose column.

As early as 1930, Beat and McHenry first observed that histaminase could be purified by selective adsorption on an adsorbing agent. Since then, Laskowski, Lamley and Keith (1945) and Kapellen-Adler (1949) adsorbed histaminase on calcium phosphate and alumina. Even though some degree of purification of the histaminase was achieved in every case, the total yield was low. Swedin and co-workers (1956) adsorbed histaminase on
columns of Dowex 50 resin, and eluted it with a dilute phosphate buffer.

Later Swedin (1958) reported on two chromatographic methods for the purification of histaminase. He adsorbed histaminase preparation on either alumina columns or DEAE-cellulose columns, and eluted the enzyme in both procedures by means of salt gradients. Swedin claimed to have obtained satisfactory results with both chromatographic methods. The conditions of his published experiments are, however, not very clear. Since reports in the literature concerning the purification of other enzymes on DEAE-cellulose columns appeared very promising, it was decided to subject the histaminase preparation, which had been purified in this work by fractionated precipitation with ammonium sulphate and freed from inert protein by thermal denaturation, to chromatography on DEAE-cellulose columns. It was hoped to find adequate experimental conditions for the preparation of a pure homogenous histaminase.

EXPERIMENTAL METHODS/
EXPERIMENTAL METHODS.

The anion exchanger, diethyl aminoethyl (Whatman DEAE) – cellulose powder, DE 50, was used throughout this work.

PRELIMINARY OBSERVATIONS.

20 g. of Whatman DEAE-cellulose resin was placed in a 2-litre beaker and to this was added 2 litres of freshly prepared 0.1 N NaOH. This mixture was stirred for 20 min. with an electric stirrer, allowed to settle for 10 min, then the supernatant fluid containing the unsettled resin particles was decanted. The remaining NaOH was removed from the resin by suction through a Buchner funnel. The whole process was repeated three times then the DEAE-cellulose was washed to a neutral pH with glass distilled water, then equilibrated with the required buffer as described below. Preliminary experiments were then carried out to study (a) the optimum pH for the adsorption of the crude hog kidney histaminase on DEAE-cellulose and (b) the optimum conditions at which the adsorbed histaminase
was eluted from the cellulose resin:

**OPTIMUM pH FOR THE ADSORPTION OF HISTAMINASE ON DEAE-CELLULOSE.**

The adsorption of histaminase was studied by the batch technique with constant protein concentration and a constant quantity of DEAE-cellulose. Parallel adsorption experiments were carried out in the following buffers of pH 5.0 to 9.6 at 0.1 ionic strength (Ellis and Simpson):

- **pH 5.0**: 0.1 M NaOAc - 0.06 M HOAc
- **pH 6.5**: 0.21 M Na₂HPO₄·12H₂O - 0.037 M NaH₂PO₄·H₂O
- **pH 7.5**: 0.03 M Na₂HPO₄·12H₂O - 0.005 M NaH₂PO₄·2H₂O
- **pH 8.6**: 0.026 M Na₂B₄O₇ - 0.02 M NaOH

2.0 g portions of the DEAE which was treated with 0.1 N NaOH as described above were placed in a beaker and carefully equilibrated with one of the equilibrating buffers. Equilibration was carried out by stirring the resin with successive quantities of the equilibrating buffer until the pH of the supernatant of the DEAE was exactly the same as that of the equilibrating buffer. The supernatant buffer was removed by decantation and suction
through a Buchner funnel. After the DEAE-cellulose was well equilibrated it was transferred to a graduated centrifuge tube.

2.0 ml aliquot of the crude enzyme extract containing a total of 1.10 mg of protein was added to each batch of resin. This was well mixed with the aid of a stirring rod for 3 min, and then centrifuged at 2000 r.p.m. at 4°C for 15 min. 0.1 ml of the supernatant was then removed from each tube and tested for protein by the Lowry Method.

RESULTS.

At pH 5.0, only 29% of the enzyme protein were found to be adsorbed on the DEAE-cellulose, but at pH 6.5 as well as at pH 7.5 90% of the enzyme protein was adsorbed. However, at pH 8.6 and 9.6 the enzyme protein was quantitatively adsorbed on DEAE-cellulose resin. Further experiments showed that optimum results were obtained on the DEAE-cellulose columns equilibrated with 0.05 M borate-HCl buffer pH 8.6. These conditions were adhered
to throughout all the experiments.

It was then decided to find out whether the adsorbed histaminase could be eluted quantitatively from the DEAE-cellulose column by means of simple elution.

**COLUMN CHROMATOGRAPHY AND SIMPLE ELUTION.**

In order to study the optimum conditions for simple elution parallel experiments were then carried out, using four different eluting buffers, each of 0.1 ionic strength but of different pH:

1). pH 4.0  0.026 M Na borate + 0.2 M HCl
2). pH 5.5  0.10 M NaOAc + 0.015 M HOAc
3). pH 6.5  0.021 M Na$_2$HPO$_4$ + 0.037 M NaH$_2$PO$_4$
4). pH 7.5  0.030 M Na$_2$HPO$_4$ + 0.0015 M NaH$_2$PO$_4$

A slurry was made of the DEAE-cellulose which had been well equilibrated with 0.005 M borate-HCl buffer, pH 8.6, and this was poured into four separate glass columns, each 0.9 x 9.5 cm. The resin was then allowed to settle under flow conditions induced by gravity. After gravity settling was almost complete,
the DEAE-cellulose was packed by the application of oxygen pressure until a flow rate of 20 ml/hr. was obtained. 1 ml of the enzyme extract which was dialysed against the equilibrating buffer, and which contained exactly 6.0 mg of protein and 560 enzyme units with a specific activity of 93.4, was pipetted on to the surface of each of the four columns. After the enzyme was adsorbed on the resin, the inner sides of the columns were washed 5 times, each time with 1 ml. of the equilibrating buffer. Each column was then connected to a separatory funnel which contained 50 ml of the 0.005 M borate-HCl buffer pH 8.6. 10 ml. effluent fractions were collected manually every half hour. Each column was then eluted with 100 ml. of one of the above mentioned eluting buffers. Each effluent was then analysed for protein and histaminase activity.

**RESULTS.**

The results of these preliminary parallel experiments with simple elution are shown in Table
TABLE III.

PARALLEL EXPERIMENT OF SIMPLE ELUTION
WITH BUFFERS OF DIFFERENT pH.

Specific Activity of Histaminase before adsorption
on DEAE-cellulose = 93.7.

<table>
<thead>
<tr>
<th>pH of eluting buffer</th>
<th>% of adsorbed protein eluted</th>
<th>% of adsorbed Histaminase eluted</th>
<th>spec. act. of effluents</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>11.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5.5</td>
<td>35.4</td>
<td>85</td>
<td>422</td>
</tr>
<tr>
<td>6.5</td>
<td>40.0</td>
<td>72</td>
<td>250</td>
</tr>
<tr>
<td>7.5</td>
<td>32.7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
III. No histaminase activity was found in the effluents from the column which was eluted with the borate-HCl buffer pH 4.0. Nor was there any histaminase activity in the effluents from the column which was eluted with the phosphate buffer pH 7.5. 72% of the total histaminase activity was, however, eluted by phosphate buffer pH 6.5, and 85% of the total histaminase activity was eluted by the buffer of pH 5.5. Moreover, it can be seen from Table III that elution of the adsorbed histaminase from the DEAE column with the buffer of pH 5.5 increased the specific activity of histaminase from 93.4 to 422.

Since the highest specific activity and the highest yield were obtained on simple elution of the histaminase adsorbed on DEAE-cellulose with the buffer of pH 5.5, this buffer was chosen as the eluting agent throughout this work.

These preliminary observations form the basis of the method which was developed and standardised in this work for the purification of histaminase by chromatography on DEAE-cellulose.
columns. This method is described in detail below:

**PREPARATION OF THE DEAE-CELLULOSE RESIN.**

To 20.0 gm of Whatman DEAE-cellulose in a beaker was added 2.0 litres of freshly prepared 0.1 N NaOH. The mixture was stirred with an electric stirrer for 20 min. and allowed to settle for 15 min. Non-sedimenting material was then removed by decantation. The excess of NaOH was removed by suction through Whatman No 42 filter paper in a Buchner funnel. The treatment with 0.1 N NaOH was repeated 3 times, then the DEAE-cellulose was washed several times with glass distilled water until the pH of the supernatant of the resin was neutral. (The pH checked with glass electrode). For equilibration of the resin to PH 8.6, 1500 ml of the 0.005 M borate-HCl buffer were added to the DEAE-cellulose powder under mechanical stirring which was continued for 20 min. The solution was then allowed to settle down and the supernatant whose pH was checked with the glass electrode was
then removed by decantation and suction. This procedure was repeated four times using the same amount of 0.005 M borate-HCl buffer pH 8.6 each time. The equilibration of the resin was considered to be completed when the pH of the supernatant after repeated cycling was 8.6.

The equilibrated DEAE-cellulose was stored suspended in 1500 ml. of borate-HCl buffer at pH 8.6 at 4°C. The whole process was essentially the same when the DEAE-cellulose was being regenerated.

PREPARATION OF THE DEAE-CELLULOSE COLUMNS.

In the course of this work it was found that glass condensers were particularly suitable as column containers. Two different sizes, 0.9 x 15 cm and 2 cm x 23 cm condensers were used. The lower constricted end of each column was loosely plugged with glass wool.

A slurry of 2 g. of the equilibrated DEAE-cellulose resin was made in about 20 ml. of the 0.005 M borate-HCl buffer for the smaller column
(0.9 x 15 cm), whereas a slurry of 7 g. in about 80 ml. of equilibrated buffer was prepared for the larger column (2 x 23 cm). The suspended resin was well stirred with an electric stirrer in order to dispose of air bubbles and to obtain an even mixture. The slurry was then poured into the column container and allowed to settle under flow conditions produced by gravity. The DEAE-cellulose in the column was then further compressed by application of oxygen pressure until the desired flow rate was achieved. Best results were obtained with a flow rate of 16 ml/hr. A small piece of glass wool, placed on the surface of the resin in the column, prevented any disturbance of the DEAE-cellulose when any solution was added to the column. The packed adsorbent column was then mounted above a fraction collector and was ready for use. A separatory funnel, clamped directly above the column, served as the reservoir for both the developing buffer and for the eluting buffer. At no time was the column allowed to run dry.

CHROMATOGRAPHY OF HISTAMINASE ON COLUMNS OF DEAE-CELLULOSE.
CHROMATOGRAPHY OF HISTAMINASE ON COLUMNS OF DEAE-CELLULOSE.

For the dialysis of the histaminase solution before its application to the DEAE-cellulose column, Versene pre-treated dialysis tubing was used (Massey, Palmer and Bennett, 1961). The dialysis tubing was soaked overnight in $10^{-3}$ M Versene and then washed thoroughly several times both with tap water followed by glass distilled water.

10 ml aliquots of the partially purified histaminase which had been fractionated with ammonium sulphate followed by heating at $60^\circ$C were dialysed in Versene pre-treated dialysis tubing against 2 litres of 0.005 M borate-HCl buffer pH 8.6 for 3 hours at $4^\circ$C. It was found to be absolutely essential to dialyse the enzyme for 3 hours before applying it to the column. If this procedure were not strictly observed, then the enzyme was not quantitatively adsorbed on the DEAE-cellulose. The slight precipitate which usually formed after dialysis, was separated by centrifugation and the clear, yellowish supernatant solution was applied to
the top of the column and allowed to enter the adsorbent under flow conditions induced by gravity. 10 ml. of the dialysed enzyme solution usually containing about 100 mg. of protein was chromatographed on columns of 0.9 x 15 cm but when larger columns (2.0 x 23 cm) were used, 30 ml. of the dialysed enzyme solution containing 300 mg. of protein was chromatographed. Three or four individual columns were run in parallel each time. The specific activity of the enzyme extract to be chromatographed was always estimated immediately before chromatography.

DEVELOPING THE COLUMN.

On the application of the light brown enzyme solution to the top of the adsorbent column a distinct brownish-yellow band soon formed. After the histaminase solution was adsorbed on the resin, the inner sides of the column were washed 5 times, each time using 1 ml of the developing buffer. The column was then connected to the separatory funnel which contained the developing buffer, 0.005 M borate-HCl buffer pH 8.6. The chromatogram was then
developed with 70 ml. of that buffer.

**ELUTION.**

After having developed the column, simple elution was then carried out using 200 ml. of 0.1 M acetate buffer pH 5.5. The eluting buffer was introduced when only a 2 cm. layer of the developing buffer remained above the surface of the DEAE-cellulose in the column. Effluent fractions were collected every 15 min. by means of an automatic fraction collector. Fractions were collected until no protein was 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, whereas soon after the commencement of the elution a very pale yellow band moved down the column. The brownish band, probably containing contaminants remained adsorbed to the top of the column even after the elution had been completed. The effluents which emerged from each DEAE-cellulose column showed a pH of 7.0.
EXAMINATION OF THE EFFLUENTS.

The volume of each effluent was measured and noted. It was observed that comparatively soon after the elution had begun the effluents displayed a pale yellow colour. The protein concentration in each effluent was then estimated by the Lowry Method and the histaminase activity was estimated by the microvolumetric method and the specific activity (expressed as units per mg protein) of each effluent was determined. Effluents with a high specific activity were subjected to zone electrophoresis on cellulose acetate strips for the inspection of their homogeneity.

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 subjected to rechromatography for further purification.

RECHROMATOGRAPHY OF HISTAMINASE ON DEAE-CELLULOSE COLUMNS.

The method for rechromatography of histaminase on DEAE-cellulose was essentially the same as for
chromatography, as described above. Simple elution with 0.1 M acetate buffer pH 5.5 was carried out as described above.

**POOLING THE EFFLUENTS FOR RECHROMATOGRAPHY.**

5 to 10 effluent fractions of a similar degree of purity usually from three columns were combined into one fraction. (Approximately 35 ml.) and dialysed for 3 hrs against the 0.005 M borate-HCl buffer pH 8.6 in Versene pre-treated dialysis tubing. The slight precipitate which formed after dialysis was separated by centrifugation and the clear yellowish enzyme solution was applied to the top of the column after its protein content, histaminase activity and specific activity had been determined. Incidentally, the sometimes larger volume (50 ml) of the dialysed enzyme solutions that were applied to the DEAE-cellulose columns for rechromatography had no effect whatsoever on subsequent results (Tombs and co-workers, 1961).

The column was developed and eluted as before and effluent fractions were collected every 15 min.
on the automatic fraction collector. The specific activity of the individual effluents was determined in the usual way.

After rechromatography the protein content of the effluents with the highest specific activity was usually very low and therefore not suitable for electrophoretic assays. It was found that 0.4 mg. protein per ml. of solution was the lowest amount for a successful electrophoresis. Hence, concentration of the highly purified enzyme solution had to be carried out.

**CONCENTRATION OF THE EFFLUENT FRACTION AFTER RECHROMATOGRAPHY.**

Different techniques of concentration were applied to the effluents after rechromatography:
(a) ultrafiltration, (b) dialysis against polyvinyl pyrrolidone (mol. wt. 11,000) and (c) concentration by dialysis against powdered sucrose.

(a) **ULTRAFILTRATION.** The method of concentrating the effluents by ultrafiltration was carried out according to the method designed in our Laboratories.
by Albert-Recht and Stewart (1960) whereby the pooled effluents were ultra-filtered through cellophane dialysing tubing at high positive pressure of 10 atmospheres.

The ultrafiltration apparatus consisted mainly of the dialysing tubing which was pulled over a central core, made of stainless steel. The lower end of the dialysing tubing was tied off, whereas the upper end was stretched over a rubber sleeve washer on the conically expanded upper end of the cone. The outside surface of the cellophane was wrapped in a strip of nylon which served as a protection for the cellophane. This assembly was carefully introduced into a stainless steel casing which was capable of withstanding pressures up to 20 atm. The central core assembly and the casing were fitted together tightly at both ends by means of bolts and wing nuts.

The cellophane bag between the cone and the casing with the upper end closed off by the rubber washer, and the lower end closed off by an expanded plug, was filled with the pooled effluent through
a vent hole in the top of the core. The ultrafiltration apparatus was then connected to an oxygen cylinder by a length of armoured tubing. The initial introduction of oxygen pressure was very gradual and the ultrafiltrate from the enzyme solution was allowed to escape from the instrument through a series of small holes drilled in the lower end of the steel casing. This particular ultrafiltration apparatus which we used for concentrating the pooled effluents had a capacity for holding 10 ml. of solution, however successive serial additions of the pooled effluent through the vent hold in the top of the core made it possible to concentrate pooled effluents with initial volume up to 65 ml. The effluents were usually concentrated 5 to 10 times then assayed for protein content and histaminase activity. Electrophoresis on cellulose acetate strips was then carried out on the concentrated effluent. Concentration of the effluents by ultrafiltration was mainly used in the early part of this work.
(b) Concentration of the effluents by dialysis against a 20% solution of polyvinyl pyrrolidone (Moret and co-workers, 1955) was tried but was unsuccessful since it seemed to inhibit the activity of the enzyme. Tombs and co-workers (1961) working with polyvinyl pyrrolidone found that it contaminated his $\alpha$-2 globulin solution when he attempted to concentrate it with polyvinyl pyrrolidone.

(c) Working with papain, Putnam (1961) found that his enzyme preparation was not inhibited when it was concentrated by powdered sucrose. The dialysis tubing containing the dilute enzyme solution was covered for 60 min. with dry powdered sucrose. After dialysis the dialysing tubing was freed from sugar by washing the dialysing sac. Putnam found that by this method a 10-fold concentration of the protein was obtained.

Concentrating the effluents by the method described by Putnam (1961) was found to be successful. The technique of concentrating the effluents by sucrose was found to be less time-consuming that the
ultrafiltration method and was therefore used in all subsequent experiments.

After concentration against sucrose, the effluents were dialysed against 0.02 M phosphate buffer pH 6.8 for 30 min. A 3–4-fold concentration of each effluent was found to be adequate in order to achieve a suitable final protein concentration of 1–2 mg. of protein per ml. of solution. The protein content and histaminase activity were again determined and electrophoresis on cellulose acetate strips carried out to determine the homogeneity of these fractions.

**ELECTROPHORESIS ON CELLULOSE ACETATE STRIPS.**

The technique of zone electrophoresis is now a common method as one of the means of judging the purity of an enzyme preparation. The pioneer work of Tiselius (1937) on the migration of protein in an electric field gave the first indication of the value of this phenomenon in the characterisation of proteins and enzymes. Mills and Smith (1951) studied the enzyme, β-glucuronidase by paper electrophoresis and Gillespie and co-workers (1952) were able to
separate the enzymes of *Aspergillus oryzae* by this technique, using barbitone buffer pH 8.6. Levy and Marzia (1953) were able to construct a graph of the enzyme activity against protein concentration and elegantly revealed the area of alkaline phosphatase in the paper electrophoretic strip. Ahmed and co-workers (1959) used filter paper electrophoresis to study the purity of alkaline phosphatase from different sources. They observed that the enzyme activity of all the preparations to be in the γ-globulin region. Using free boundary electrophoresis at pH 4.0, 5.5 and 6.8 as a criterion for the purity of his preparation, Ellis (1960) reported that his proteinase preparation, purified by chromatography on DEAE-cellulose was essentially homogenous.

Hamberg and Rocha Silva (1954) were the first to subject histaminase to paper electrophoresis. These workers adopted the method of Kunkel and Tiselius (1951) by which they applied a partly purified histaminase to Whatman No. 20 filter paper and the electrophoresis was carried out in veronal buffer, pH 8.6. Under these conditions they found that the enzyme activity travelled towards the anode.
Swedin et al (1956) investigated a histaminase preparation before and after adsorption on Dowex 50 by paper electrophoresis at a pH of 8.6 but whereas before adsorption three fractions travelling towards the anode were revealed on the electrophoretic strips of which only one showed enzymatic activity towards histamine, after adsorption on the ion exchange resin only one fraction containing histaminase activity was obtained.

Kapeller-Adler and Fletcher (1958) studied the electrophoretic mobilities of a partly purified preparation of histaminase on Whatman No. 3 filter paper at pH 5.0, 6.8 and 8.6. They observed that at pH 6.8 the histaminase activity was to be found in a well-defined boundary whereas at pH 8.6 the histaminase activity was located in a very broad diffuse band. There was no mobility of the enzyme protein at pH 5.0.

**METHOD.**

Cellulose acetate strips were supplied by "Oxoid" Ltd., London. Since the protein content
of each effluent obtained after chromatography of histaminase on columns of DEAE-cellulose was relatively low even after concentration by sucrose, it was found to be very convenient to use cellulose acetate strips as the supporting medium for electrophoresis.

Cellulose acetate strips originally developed from bacteriological cellulose acetate membrane filters was first introduced as a supporting medium for zone electrophoresis for serum by Kohn (1957), who found this medium to have many advantages over ordinary filter paper. Moreover, a rapid separation of very small quantities of protein, even as small as 5 μg per 0.1 ml, in the form of well-defined bands has been achieved, (Kohn, 1960).

BUFFERS.

Electrophoresis of the effluents was carried out at this stage in 0.05 M phosphate buffer pH 6.8 (Sorensen, 1909):

\[
\text{pH 6.8} = 0.153 \text{ M NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} - 0.174 \text{ M Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}.
\]
SIZE OF STRIPS.

The size of the cellulose acetate strips as supplied by the manufacturer was 5 x 20 cm. The strips were cut with a sharp scalpel to the required size, 2.5 x 10 cm, and then impregnated with the required buffer.

IMPREGNATION OF THE STRIPS.

This was performed by floating the strips on the surface of the buffer pH 6.8 in a plastic sandwich box, allowing the strips to soak up the buffer from underneath. This was essential, as quick submersion of the strips trapped air, and created opaque spots which took a long time to soak up the buffer (Kohn, 1960). After the strips became completely impregnated with the buffer, they were then placed between two porcelain plates then submerged in the buffer in the sandwich box until ready for use. It was found to be essential to soak the strips overnight in the required buffer before they were used (Kohn, 1957).
THE ELECTROPHORETIC APPARATUS.

The apparatus used for electrophoresis was designed in this laboratory by Albert-Recht (1959) and consisted chiefly of the trough assembly and the strip chamber. Six cellulose acetate strips each 2.5 x 10 cm which had been soaked overnight in the required buffer were blotted between two layers of filter paper to remove the excess buffer. The strips were then placed horizontally across the strip chamber, which consisted of thin polythene sheeting supported by a pad of plastic foam, provided efficient heat insulation and prevented excessive water condensation on the walls of the tank. The lid of the chamber was securely fastened down at each end with wing nuts. The access of buffer solution to the strips was facilitated by Whatman No 3 filter paper wicks which dipped into the buffer reservoir. The ends of the acetate strips on the chamber were sandwiched between the ends of the connecting filter paper wicks and a bar of perspex. This prevented the strips from sagging. The buffer troughs were filled with the required buffer solution.
THE ELECTRODES.

Four electrodes, made of platinum, were placed in the electrode vessel at each end of each buffer trough. The narrow portion of the electrode vessel dipping into the buffer in the trough was closed off by a plug of pulped tissue paper. This arrangement reduced the diffusion of decomposed buffer from the vessel into the main trough.

THE CURRENT.

A constant current of 8 - 9 mA was supplied by a Shandon power pack. With the current switched on, the apparatus was equilibrated for 15 min. before the sample was applied.

APPLICATION OF SAMPLE.

The current was switched off and the effluents were applied to the well-equilibrated strips, 3 cm from the cathodic end of each strip. Vaccine capillary tubes were found to be particularly suitable for applying the effluent to the strips. The application of the effluent was effected through a narrow slit in the lid which was closed during the
run by a plastic adhesive strip. The electrophoretic determinations were made on 5 - 8 µl of each effluent in three successive streaks. 5 µl of human serum was run as a guide beside each effluent on each strip. One half the width of the strip was used for the effluent, whilst the other half was used for the serum.

**THE ELECTROPHORETIC RUN.**

After the application of the effluents, the current was again switched on. The whole assembly was then enclosed within a Perspex hood. Each run lasted exactly 4 hr. After the electrophoretic run was completed, the strips were removed one by one by means of forceps then immediately stained.

**STAINING AND DRYING THE CELLULOSE ACETATE STRIPS.**

The cellulose acetate strips were stained according to the method of Kohn (1960). The staining solution consisted of 0.2% Ponceau S dye for electrophoresis (obtained from George T. Gurr Ltd., London) in 6% salicyl sulphonic acid. Immediately after the electrophoretic run, the cellulose acetate strips
Elution diagram of histaminase chromatographed on DEAE-cellulose columns (2 x 23 cm) at pH 8.6. Enzyme solution (specific activity 71.6) contained before chromatography 315 mg of protein. At the "arrow" simple elution commenced with the 0.1 M acetate buffer pH 5.5. For convenience, the diagram is divided into 3 parts: Parts A and C represent effluents with the lowest specific activity. Part B is representative of effluents with the highest specific histaminase activity.

0---0 mg protein/ml.
X---X specific activity units/mg protein.
were placed in the Ponceau S staining solution. After 20 min. the strips were removed from the dye then washed 4 times with 5% acetic acid solution and dried between two layers of filter paper under light pressure. The entire procedure was carried out at room temperature.

RESULTS.

The method developed in this work for the purification of histaminase by chromatography and rechromatography on DEAE-cellulose was used in 55 independent assays, each of them comprising three or four individual columns. Typical results of these experiments are shown in Tables IV, V and in Figs. I, II and III.

Fig. 1 illustrates a typical elution diagram obtained when the dialysed enzyme solution was chromatographed on the anion exchanger DEAE-cellulose. At the effluent marked by an arrow simple elution with 0.1 M acetate buffer, pH 5.5, was instituted. For convenience, the elution diagram in Fig. 1 has been divided into three parts, marked A, B and C. Part A which contained mainly inert protein with negligible histaminase activity is followed by Part B which contained very little protein.
FIG. II. Illustration of results presented in Fig. I by electrophoretic patterns. Above each Part (A, B and C) an electrophoretic pattern, typical of effluents of this part is shown. D is the electrophoretic pattern obtained from the enzyme material (Spec. Act. 71.6) before application to chromatography on DEAE-cellulose column. The electrophoresis was carried out at pH 6.8 on cellulose acetate strips. A current of 0.6 mA/cm width of strip was used, the electrophoretic run lasted exactly 4 hr. The strips were stained with Ponceau S.
but 80% - 90% of the total histaminase activity applied to the column followed by Part C which like Part A contained very little histaminase activity but relatively large amounts of protein. Fig. I shows that soon after the commencement of elution with the acetate buffer pH 5.5, (marked in the diagram by the arrow) the entire histaminase activity was eluted in a single symmetrical peak (Part B). The effluents of Part B, with the highest histaminase activity, showed a very pale yellow colour and a distinct greenish yellow fluorescence when examined under the Woods lamp.

The elution diagram shown in Fig II is similar to that shown in Fig. I. Above each part, A, B, C of the elution diagram shown in Fig II typical electropherograms obtained from the effluents of the respective parts are presented. The electropherogram above Part B as compared with those above Parts A and C shows that this part contains not only the highest histaminase activity but it distinctly appears to be electrophoretically purer than the effluents in Parts A and C. D, presented on top of
Typical results obtained after Chromatography of histaminase on DEAE-cellulose column (2 x 23 cm) equilibrated with 0.005 M borate-HCl buffer pH 8.6. Flow rate of column was 16 ml/hr, and effluents collected every 15 min. The heated enzyme, Fraction III (See Tables 1 and 11) was dialysed for 3 hr. against 0.005 M borate-HCl buffer pH 8.6 before being chromatographed. The solution containing 315 mg protein and 22,500 enzyme units with a specific activity of 71.5 was applied to the column. Simple elution with 0.1 M acetate buffer pH 5.5 was started at Tube No. 14.

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<tr>
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<td>0</td>
<td></td>
</tr>
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</tr>
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<tr>
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</table>

Total protein eluted = 100.38 mg = 31.8% of total protein applied.
Total Histaminase eluted = 34,974 enzyme units = 155% of total histaminase applied. Electrophoresis at pH 6.8 was carried out on all effluents containing enzymic activity (See Fig II). For convenience, the Table is divided into 3 parts, A, B and C, similar to those shown in Fig. I and II. The effluents comprising Part B which contains the electrophoretically purest fractions and highest histaminase activity were individually concentrated by sucrose and placed into deep freeze in order to study the stability of highly purified histaminase.
Fig. II shows electrophoretic pattern of the crude enzyme protein before column chromatography. The electropherogram of normal human serum is shown in the upper portion of each electrophoretic strip, served only as a guide. The typical bands of serum globulin (\(\gamma, \beta, \gamma_2\) and \(\alpha_1\)) and of serum albumin (marked above strip D) can be seen in each serum electropherogram. The electropherogram of Part A, which consists mainly of inert protein, shows a very broad diffuse band with a relatively large amount of protein remaining at the point of application. Furthermore, this diffuse band of protein occupied the entire region between the \(\alpha_2\) globulin and the point of application. The electrophoretic pattern of Part B shows that the protein moved in a distinct band which appears between the \(\alpha_2\) and the \(\beta\)-globulin region. The electropherogram above Part C shows in addition to a broad diffuse band of inert protein in the globulin fraction a distinct zone in the albumin region.

Table IV represents the numerical data of the typical chromatogram represented in Fig. I and Fig. II. From this Table it can be seen that the
effluent containing the peak histaminase activity showed a specific activity of 1400 which indicates an almost 20-fold purification in comparison to the specific activity of 71.5 of the starting material. Furthermore, this Table shows not only that the enzyme was quantitatively recovered but also that 155% of the histaminase activity contained in the starting material has been obtained. This points towards a considerable selective purifying ability of DEAE-cellulose resin. Since not only the total histaminase was recovered but there was even an increase of 55% suggests that the bulk of the histaminase inhibitors remained on the column. In all the chromatographic assays the yield of histaminase activity ranged from 150 - 190% but the total protein recovered was between 30 - 34% and even those chromatograms where the degree of purification was relatively low, the yield of histaminase activity was always above 125%.

The purification achieved in some chromatograms was sometimes lower than others: for example, the specific activity in one column of a chromatogram
FIG. III. Elution diagram of a histaminase preparation after rechromatography on a DEAE-cellulose column. Effluents of a chromatogram similar to effluents of Part B, Fig. I and II (highest specific activity and purity) were pooled and rechromatographed under conditions as in Fig. I. The specific activity of the enzyme solution before rechromatography was 364. At the "arrow" elution was started.

$0 - O$ mg protein/ml.

$X - X$ specific activity units/mg protein.
increased from 56 before chromatography to a peak activity of 600 after chromatography, in another column of this chromatogram the specific activity increased from 56 to above 900. Variation in the degree of purification achieved on column chromatography may have been due to: (a) failure to have dialysed the starting material properly, (b) the DEAE-cellulose having not been well equilibrated, (c) poor packing of the columns or (d) the fact that the cellulose resin varied from batch to batch. When all these conditions were strictly observed a 20-fold or even larger purification of the enzyme material applied to the column could be achieved.

Fig. III is a typical elution diagram obtained after rechromatography. Here it can be seen that the total histaminase activity was concentrated in a very few tubes almost immediately after simple elution was started (at the point marked by the arrow in the diagram). The total histaminase activity emerged from the column as a single well-resolved symmetrical peak, separated from the inert protein seen in the latter part of
FIG. IV. Two typical electropherograms obtained from different columns after rechromatography of histaminase on DEAE-cellulose columns. The histaminase boundaries lying between the $\alpha_2$ and the $\beta$-globulin regions have been marked for illustration. Each effluent was concentrated by sucrose, then dialysed for 20 min against 0.02 M phosphate buffer pH 6.8 before being applied to the cellulose acetate strip. The serum on the upper part of each strip served only as a guide for the electrophoretic run. The electrophoresis was carried out in 0.05 M phosphate buffer pH 6.8. A current of 0.6 mA/cm width of strip was used and the electrophoretic run lasted exactly 4 hr. The strips were stained with Ponceau S.
Typical results obtained after Rechromatography of histaminase on DEAE-cellulose column (2 x 23 cm), equilibrated with 0.005 M borate-HCl buffer pH 8.6. Flow rate of the column was 16 ml/hr. and effluent fractions collected every 15 min. The effluents of a chromatogram similar to that presented in Table IV containing highest specific activity as well as electrophoretically purest fractions (similar to Part B of Table IV) were pooled, then dialysed for 3 hr. against borate-HCl buffer pH 8.6, and applied to the column. The enzyme solution contained 57 mg. protein and 21,900 enzyme units with a specific activity of 384. Simple elution with 0.1 M acetate buffer pH 5.5 was started at Tube No. 10.

<table>
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<th>Tube No.</th>
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<td></td>
<td>28</td>
<td>1.15</td>
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</table>

Total protein eluted = 25.10 mg = 44% of total protein applied. Total histaminase eluted = 18,625 enzyme units = 85.0% of total histaminase applied. Histaminase protein eluted = 13.39 mg. i.e. 53.4% of the total protein eluted.
the elution diagram.

The electropherograms of two typically highly-purified effluents obtained after rechromatography are shown in Fig. IV. As stated before, the normal human serum on the upper portion of each strip served as a guide for the electrophoretic run. Both electropherograms in Fig. IV showed that the highly-purified histaminase enzyme migrated as a distinct homogenous boundary to a position between the $\alpha_2$ and $\beta$-globulin region and it can be seen that no protein has remained at the point of application.

Table V represents the numerical data of the rechromatogram presented in Fig. III. From this Table it can be seen that highest histaminase activity was concentrated in the five tubes shortly after elution was started. The specific activity increased from 384 before rechromatography to a maximum of 1800 after rechromatography. In some rechromatograms the specific activity increased to 2000 or even more. Table V shows also that the total yield of histaminase activity after rechromatography was due to the fact that the histaminase inhibitors remained adsorbed on
the DEAE-cellulose column after the elution had been completed. As indicated in Table V, the protein content of the fractions with the highest histaminase activity eluted was found to be only 53.4% of the total protein eluted from the column after rechromatography.
SECTION IV.

IDENTIFICATION OF PIGS' KIDNEY HISTAMINASE.
Electropherograms obtained from highly purified histaminase with specific activity of 2000 at various pH. The histaminase zone on each strip has been marked for illustration. The serum on the upper part of each electrophoretic strip served only as a guide for the electrophoretic run. A current of 0.6 mA/cm width of strip was used and the electrophoretic run in each case lasted exactly 4 hr. The strips were stained with Ponceau S.
(1a) **ELECTROPHORESIS ON CELLULOSE-ACETATE STRIPS AT VARIOUS pHs.**

Electrophoresis on cellulose-acetate strips was carried out according to the method described in Part 3 of Section III.

**BUFFERS:**

(1) pH 5.0 - pH 8.0 was produced by mixing different quantities of 0.2 M Na₂HPO₄ and 0.1 M citric acid according to the method of Mollvaine (1921).

(2) pH 8.6 - 0.05 M sodium barbitone (Owen, 1956).

**RESULTS.**

Fig. V shows the electrophoretic patterns at different pH values of the purified histaminase. All the electrophoregrams show a single distinct zone and at pH 6.8 and pH 8.6 the widely purified histaminase migrated as a single zone located between the α₂ and β-globulin regions.

(1b) **THE ISOELECTRIC POINT.**

Fig. V also shows that the migration of the
highly purified histaminase zone decreased with decreasing pH and at pH 5.0 and 5.15 no migration is to be observed. It can also be seen in Fig. Y that at pH 4.6 the enzyme band starts migrating towards the cathode. The isoelectric point was thus estimated to be between pH 5.0 and pH 5.15; no corrections having been made for electroendosmotic flow.

(2) **STARCH GEL ELECTROPHORESIS.**

An attempt was made to study the migration pattern of the purified histaminase by zone electrophoresis in starch gel. The method which we used was mainly that described by Smithies (1955) and is as follows:

**EXPERIMENTAL METHOD.**

Wherever possible Analytical grade reagents were used.

(a) **BUFFERS.** Two different buffers were used:

(1) borate buffer pH 8.48

The borate buffer in which the starch gel was
made consisted of 0.03 M boric acid and 0.012 M NaOH/litre, and a 2% NaCl in this was used as the electrode buffer. The bridge buffer contained 0.3 M boric acid and 0.06 M NaOH/litre.

(11) phosphate buffer pH 7.0.

Starch gel electrophoresis was also carried out in phosphate buffer pH 7.0. This buffer was prepared as follows.

260 ml. of 0.2 M Na₂HPO₄ was added to 220 ml. of 0.2 M NaH₂PO₄·2H₂O (Bodman, 1960). For starch gels this buffer was diluted 1:5.

(b) THE STARCH.

The hydrolysed starch for making the gels was obtained from Connaught Medical Research Laboratories, University of Toronto, Canada.

(c) UNHYDROLYSED POTATO STARCH.

30 g. of B.D.H. (British Drug Houses Ltd.) potato starch was washed by suspending it in acetone, and after allowing the suspension to stand for 30 min, the acetone was poured off. This was done
three times after which the acetone was removed by drying the starch at 50°C overnight.

(d) THE APPARATUS.

This was made mainly of perspex and consisted primarily of two parts: (i) two buffer troughs and (ii) the trays for holding the gel.

(i) The buffer trough. Each buffer trough consisted of two distinct separate compartments: compartment A, in which was placed the electrophoretic buffer, and compartment B, in which was placed the electrode buffer. During the electrophoretic run these two compartments were connected by means of a thick wad of Whatman No. 3 filter paper. Compartment A of both buffer troughs were connected by means of a narrow bore glass tubing which also served as the buffer levelling mechanism.

(ii) The trays. The tray (0.5 x 7 x 20 cm) into which the starch gel was poured for electrophoresis was also made from perspex.
PREPARING THE STARCH GEL.

A 12.0% starch suspension was prepared by adding 30 g. of the hydrolysed starch to 250 ml. of the buffer in a conical pyrex vacuum filtration flask. The suspension was well mixed by vigorous swirling then heated over a flame. Swirling was continued throughout the heating process as this prevents the formation of lumps in the heated starch. The starch suspension was heated until it became a viscous translucent liquid just short of boiling. The Flask was then quickly stoppered with a rubber bung and negative pressure applied with a filter pump. Under this negative pressure the starch was allowed to boil for exactly 1 min.

The hot starch solution was then poured into the perspex tray (0.5 x 7 x 20 cm) and immediately covered with a flat piece of perspex, previously coated on its underside with liquid paraffin, and which was gently lowered in such a manner as to exclude air bubbles. The thin perspex cover was then pressed home, thus excess starch was pressed out leaving a gel of uniform thickness. The gel
was allowed to cool for 30 min. at room temperature, then kept for 2 hr. at 4°C. The starch gel was then ready for use. Gels were used on the same day as they were prepared.

APPLICATION OF THE PURIFIED HISTAMINASE.

Using a solid-backed safety razor blade, three slots each 0.2 x 1 cm were cut in the gel, 4 cm from the cathodic end. Two of these slots were used for the concentrated enzyme solution whereas the third slot was used for human serum. Care was taken to make the slots as narrow as possible as this gave better resolution. 0.3 ml. of the purified enzyme solution to be electrophoresed was added to 4 or 5 mg. of potato starch in a beaker and carefully mixed. This suspension was then taken up into a Pasteur pipette and applied to the slot in the gel. Human serum was always run as a guide in each electrophoretic run.

Each slot was then sealed with a glass microscopic slide previously coated with a uniform film of liquid paraffin. The surface of the gel was then covered with a thin piece of polythene sheet.
also coated with liquid paraffin.

THE ELECTROPHORETIC RUN.

The tray containing the gel was then placed on the two buffer troughs. Both ends of the tray were connected to the electrophoresis buffer in Compartment A of the buffer troughs by means of thick wads of Whatman No. 3 filter paper which had been well soaked in the bridge buffer. Compartment B of each trough containing the electrode buffer was connected to Compartment A also by means of thick wads of Whatman No. 3 filter paper.

The gel was cooled by circulating tap water through a coil of cellophane dialysing tubing placed to cover the entire surface of the starch gel. The whole apparatus was then covered with a perspex hood, then the current switched on. Each electrophoretic run lasted 18 to 22 hr.

THE CURRENT.

The electrodes made of platinum wire, dipped into the electrode buffer in Compartment B. A
constant current of 40 mA at a voltage of about 200 volts was applied.

SLICING THE GEL.

After the run was complete the tray was removed, the surface of the gel exposed, then blotted with soft tissue to remove excess fluid.

With the aid of a spatula the sides and bottom of the gel was freed from the walls of the perspex tray. A piece of flat perspex having the same dimensions was then placed on the surface of the gel. The tray was then inverted, thus releasing the gel onto the perspex sheet, which was then transferred on to another tray, slightly larger than the original one. The gel then stood clear of the tray and was ready for slicing. Using the edges of the tray as a guide, the gel was then sliced with the tensioned wire of a cheese knife, drawn through the gel with one gentle sweep.

STRAINING THE GEL.

The tray was then placed in a developing
**FIG. VI. Starch gel electropherogram.**

A is the highly purified histaminase after rechromatography.

B is the crude histaminase before chromatography.

C is human serum for comparison.

The starch gel electrophoresis was carried out in borate buffer pH 8.48 (Smithies, 1955). The origin is marked by the arrow. Current 40 mA/20 hr. Amidoschwartz 10 B was used as a dye.
dish which contained teepol–acetic acid–water (25:25:100), (Bodman, 1960). After standing in this for about 60 sec. the upper slice of the gel was then gently floated off from the lower one leaving the cut surfaces exposed. Both slices of the gel were then transferred to another dish, blotted with tissue paper to remove the excess fluid, then stained.

The gel was stained for 60 sec. with a saturated solution of amido schwartz in methanol–acetic acid–water (50:10:50). After staining, the starch gel was washed with the same solvent as that in which the amidoschwartz was dissolved. 4 or 5 washings were necessary to clear the gel, the gel was stored in 5% acetic acid solution.

RESULTS.

Starch gel electrophoresis at both pH 8.48 and 7.0 has been used also to study the migration pattern of a highly purified histaminase. Fig. VI shows a diagram of the starch electropherogram at pH 8.48. A, is the diagram of the highly purified histaminase. B, represents the electropherogram of the crude
histaminase before column chromatography, and C, the electrophoretic pattern of normal human serum, served as a guide for the electrophoretic run. The serum zones described are according to Smithies (1955).

As shown in Fig VI, the same highly purified histaminase showed a single distinct zone between the \( \gamma_2 \) and \( \beta \)-globulin when electrophoresis at several different pH was carried out on cellulose acetate strips. However, when this preparation was submitted to electrophoresis in starch gel, at both pH 7.0 and pH 8.48, the histaminase migrated as a single diffuse band located between the Fast \( \gamma_2 \) and Slow \( \gamma_2 \) -globulin with the suggestion of a zone in the Fast \( \gamma_2 \) -globulin region as shown in Fig VI (A). Fig. VI (B) shows that the crude enzyme has not only a distinct zone in the albumin region but also a diffuse zone throughout the globulin region with some protein remaining at the point of application.

3. SPECIFICITY.

As mentioned above, the crude enzyme preparation showed no effect whatever when putrescine was used as substrate.
<table>
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<th>Chromatogram and column no.</th>
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<th>HISTAMINASE BEFORE CHROMATOGRAPHY</th>
<th>HISTAMINASE AFTER CHROMATOGRAPHY</th>
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<th>CADAVERINASE AFTER CHROMATOGRAPHY</th>
<th>Spec. Act. on histamine in relation to Spec. Act. on Cadaverine</th>
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</thead>
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</tbody>
</table>
From Table II, it can be seen that the ammonium sulphate fractionation did not affect the enzyme reaction on cadaverine to the same extent as that on histamine. A 64-fold purification was achieved when histamine was used as substrate, but only a 20-fold purification was achieved when cadaverine was used as substrate, as seen in Fraction III of the purification scheme in Table II. Moreover, if one and the same enzyme was concerned in the reaction on both substrates, the ratio of the specific activity on histamine to the specific activity on cadaverine should remain constant in all stages of the purification. It can be seen in Table II that this ratio varied widely. This effect was previously noticed by Kapeller-Adler (1953) and Kapeller-Adler and Fletcher (1958).

It is noteworthy that after heating some enzyme fractions (Fraction III of Table I) to 60°C, the enzyme did not have any activity on cadaverine at all.

In Table VI, results obtained before and after chromatography on DEAE-cellulose columns are presented. In each case, the starting material had been fraction-
ated with ammonium sulphate then heated to 60°C (See Fraction III of Table I) and both histamine and cadaverine were used as substrates in parallel experiments. It can be seen in Table VI that in most of these assays, no effect whatever on cadaverine was obtained. In those experiments where an effect on cadaverine was obtained after chromatography, the yield was a very poor one as compared to the effect on histamine. After chromatography, the yield of the activity on cadaverine was only between 17.6% and 35.8% whereas the yield of the activity on histamine was always above 125%. The increased yield of histaminase activity seems to be due to retention of histaminase inhibitors in the DEAE-column. Fig. VI also shows that the ratios of the specific activity on histamine in relation to the specific activity on cadaverine (H/C) varied from chromatogram to chromatogram ranging from 9.72 to 17.0. It should be further mentioned that neither with the crude enzyme nor with the highly purified enzyme was any activity obtained when hexamethylene diamine was used as substrate.

**STABILITY.**

It was found that throughout this work, after
concentration by sucrose, the highly purified histaminase solution could be kept for weeks at 4°C without considerable loss of specific activity.

It also appeared interesting to study the effect of very low temperatures on the stability of the enzyme, so it was decided to investigate whether the highly purified histaminase solution could be kept in the deep freeze without loss of activity after reconstitution. Using three different highly purified preparations, it was found that after 30 days in the deep freeze at -18 to -20°C at least 70% of the histaminase activity was recovered in all the preparations. These results are being further investigated. Swedin (1958) and Mann (1961) also found that their preparations of pigs' kidney and plant histaminase were also stable at low temperatures (-10 to -20°C).
SECTION V.

PROSTHETIC GROUPS OF HISTAMINASE.
As early as 1940, Zeller, Stern and Wenk suggested that the prosthetic group of D.O. was flavin adenine dinucleotide (FAD). Swedin (1943, 1944), Kapeller-Adler (1949), working independently, found that the effect of histaminase on histamine was diminished by dialysis. Swedin could restore the enzyme activity by addition of a methanol extract to the enzyme, and Kapeller-Adler restored the enzyme activity in various ways, i.e. by the addition of either (a) flavin adenine dinucleotide prepared from yeast, (b) acetone extract of the intact enzyme or, finally, (c) authentic flavin adenine dinucleotide preparation made from D-amino acid oxidase. On the basis of these results, Swedin (1943, 1944) and Kapeller-Adler (1949) suggested that the prosthetic group of histaminase was flavin adenine dinucleotide. Kapeller-Adler (1949) showed that when a partly purified preparation of hog kidney histaminase was dialysed (for 24 - 72 hr.) against tap water or distilled water in the cold, its action on histamine was decreased, but that on cadaverine and putrescine increased. These results,
however, were reversed when flavin adenine dinucleotide was added to the dialysed enzyme.

Laskowski and co-workers (1945) were unable to reanimate the histaminase preparations with FAD after attempting to split the enzyme by the method of Warburg and Christian (1938). They concluded that if histaminase was a flavoprotein its protein moiety must be more unstable towards acid than that of other flavoproteins.

Leloir and Green (1946) rejected all previous claim that FAD was the prosthetic group of "histamine oxidase" on the grounds that the solution of their partially purified hog kidney enzyme was colourless.

Werle and von Pechmann (1949) working with diamine oxidase of plant origin put forward the hypothesis that both flavin adenine dinucleotide and pyridoxal phosphate may be the prosthetic groups of this enzyme.

On account of the well known extreme sensitivity of histaminase to carbonyl reagents, and of experiments carried out on rats deficient in pyridoxine, Sinclair (1952) claimed that pyridoxal phosphate was the
prosthetic group of histaminase. In 1953, however, Sinclair rejected this hypothesis since in later experiments he could not establish any satisfactory connection between the effect of Vitamin B₆ deficiency and a lowered histaminase activity.

Davison (1956) reported that pyridoxal phosphate was probably the prosthetic group of diamine oxidase because pyridoxal phosphate was able to reverse the inhibition of this enzyme caused by isonicotinic acid hydrazide.

Goryachenkova (1956) working with both plant and animal diamine oxidase suggested that both flavin adenine dinucleotide and pyridoxal phosphate were the prosthetic groups of this enzyme. With purified D.O. preparations of animal tissue and plant origin, she failed, however, to find net transamination between diamines and added pyridoxal phosphate or free pyridoxal.

More recently, Mann (1961) working with highly purified preparations of amine oxidase from pea seedlings suggested that a copper complex of a carbonyl
compound formed the prosthetic group of the enzyme.

It appeared, therefore, essential to study the problem of the prosthetic group of histaminase on a highly purified preparation of this enzyme obtained with the chromatographic methods described in this work. It was intended to subject this preparation to an investigation for a possible occurrence of the two prosthetic groups flavin adenine dinucleotide and pyridoxal phosphate mentioned in the literature. The two synthetic compounds flavin adenine dinucleotide (FAD) and pyridoxal phosphate needed for comparison were obtained from L. Light & Co. Ltd., Coln Brook, England, and from Roche Products Ltd., respectively.

The purified histaminase solutions obtained from all the chromatographic experiments showed a yellowish green fluorescence when examined under the Woods lamp. It was decided to use both the Unicam S.P. 500 spectrophotometer and the Aminco-Bowman spectrofluorometer to study the absorption spectrum and fluorescence spectrum of the purified histaminase.

It is a well known fact that the sensitivity
of the Unicam S.P. 500 spectrophotometer is much less than that of the Aminco-Bowman spectrofluorometer which is able to measure as little as 0.1 μg/ml of an organic compound which fluoresces (Bowman, 1955). Theorell (1958) confirmed that fluorometry is much more sensitive than spectrophotometry, $10^{-6}$ M concentration being on the average the lowest concentration that can be accurately measured by the spectrophotometer, whereas this lowest limit for spectrophotometry roughly coincides with the highest concentration suitable for fluorometry. Furthermore, spectrophotometry at low absorbances is complicated by even a trace of turbidity. In fluorometry stray light from the particles or reflected light will disturb only if the exciting light contains a wave length able to pass through the filter or monochromater of the recorder.

In this study it was also observed that no absorption spectrum could be obtained in solutions containing 0.1 μg FAD/ml in the Unicam S.P. 500 spectrophotometer, whereas a solution of the same concentration of FAD gave a high fluorescence spectrum in the Aminco-Bowman spectrofluorometer.
FIG. VII. Activating spectra resulting from fluorescence emission maximum at 530 mp light.

- •- purified histaminase in 0.02 M phosphate buffer pH 6.8. Protein concentration 1.33 mg/ml.

- - - - Authentic FAD in 0.02 M phosphate buffer pH 6.8, 0.5 μg of FAD/ml.
1) **FLAVIN ADENINE DINUCLEOTIDE.**

Fig. VII shows the activating spectra of both the highly purified histaminase and of authentic FAD at pH 6.8, obtained when the apparatus was set at the maximum fluorescence of FAD, 530 μ (Beinert, 1960). It can be seen in Fig. VII that the activating maximum obtained in this work for authentic FAD, 375 μ and 450 μ, compare well with the results in the literature. Fig. VII also shows that the second activating maximum of the purified histaminase was to be found at 458 μ, indicating that a small shift has occurred. **Massey and co-workers** (1961) have found that the absorption peaks of crystalline d-amino acid oxidase shifted to 378 μ and 462 μ. The claim that most flavoproteins are non-fluorescence does not therefore apply to the pure intact histaminase preparation, since all pure preparations obtained in this work showed a yellowish green fluorescence when examined under the Woods lamp, and also showed the typical FAD activating spectrum as discussed in Fig. VII above.

Fig. VIII (a) shows the activating spectra before and after the acidification of the purified
FIG. VIII (a). Activating spectra resulting from fluorescence emission maximum at 530 μm light.

--- purified histaminase in 0.02 M phosphate buffer pH 6.8. Enzyme protein concentration, 0.44 mg/ml.

--- same enzyme solution which has been acidified with 0.1 N HCl.

.... Authentic FAD in 0.02 M phosphate buffer pH 6.8, 0.2 μg FAD/ml.

FIG. VIII (b). Electropherogram of the histaminase solutions of Fig. VIII (a): before and after HCl was added to these solutions. The electrophoresis was carried out in 0.05 M phosphate buffer pH 6.8. A current of 0.6 mA per cm width of strip was applied for 4 hr. The strips were stained with Ponceau S.
histaminase with 0.1 N HCl. It should be mentioned here that after the acidification, a liberation of FAD from the histaminase must have taken place, since the fluorescence of the solution increased to such an extent that the absorption maximum could only be recorded when the sensitivity of the apparatus was very much reduced (about 30 times lower than in Fig. VII).

This is in agreement with the work of Bessey, Lowry and Love (1949) who observed that as the pH of an aqueous solution of FAD is decreased, the fluorescence increased reversibly to a maximum at pH 2.9.

Fig. VIII (b) shows the electropherogram of the purified histaminase before and after acidification with HCl. It is seen here that the purified enzyme has migrated as before (i.e. between the $\alpha_2$- and $\beta$-globulin regions), but no migration took place after the enzyme had been acidified and the protein remained at the point of application, indicating that the enzyme was denatured after having been acidified with HCl. Hence on acidification FAD
Activating spectra resulting from fluorescence emission maximum at 530 mp light.

(-- -) Purified histaminase in 0.02 M phosphate buffer pH 6.8; enzyme protein concentration 0.44 mg/ml (Curve A).

(---) The same enzyme solution, reduced by the addition of 0.2 ml of 4% Na₂S₂O₄ in phosphate buffer pH 6.8 (Curve B).

(-----) Reoxidation of the reduced histaminase with oxygen. (Curve C).

(.....) Authentic FAD in 0.02 M phosphate buffer pH 6.8 0.2 µg FAD/ml. (Curve D).
seemed to have been released with an apparent denaturation of the histaminase apoprotein. Here it should be noted that a similar phenomena occurred after the enzyme had been heated at 80°C for 60 sec.

(a) **REDUCTION OF HISTAMINASE BY SODIUM DITHIONITE.**

For further identification of the optical findings concerning FAD the reduction of the purified histaminase was studied by following the almost complete disappearance of the activating spectra on addition of sodium dithionite to the enzyme solution and their full reappearance on oxygenation. The yellowish colour of the histaminase solution was bleached by the addition of Na₂S₂O₄. Fig. IX shows the activating spectra of the pure histaminase before and after the reduction by Na₂S₂O₄. Curve A is the activating spectrum of the pure enzyme before the addition of sodium dithionite; Curve B is the activating spectrum obtained exactly 20 sec after 0.2 ml. of a 4% Na₂S₂O₄ in 0.02 M phosphate buffer pH 6.8 had been
added to the enzyme solution. Curve C is the spectrum after the enzyme solution had been reoxidised by bubbling oxygen through the reduced enzyme solution for 60 sec. and Curve D is the activating spectrum of 0.2 μg FAD/ml. It can be seen from Curve B that a rapid decrease of the activating maxima occurred after Na₂S₂O₄ was added to the enzyme solution. However, after oxygen was bubbled into the solution, the FAD was reoxidised and therefore the intensity of the activating maxima increased considerably (Curve C) and was even much higher than before the addition of Na₂S₂O₄ (Curve A).

These results agree with those of Ball (1939), Corran and co-workers (1939), Horecker and Heppel (1949) and Morrel (1952) who, working with xanthine oxidase found that 50 - 72% of the total absorption at 450 μm was reduced by Na₂S₂O₄ but was fully restored by air.

(b) REDUCTION OF HISTAMINASE BY HISTAMINE.

Results similar to those mentioned above were obtained on the addition of histamine to a highly purified enzyme solution. A distinct bleaching of the yellow colour of the enzyme was observed. However,
FIG. X. Absorption spectra of purified histaminase and authentic pyridoxal phosphate.

α——α Highly purified histaminase in buffer pH 6.8.

○——○ Highly purified histaminase in buffer pH 6.1.

X——X Purified histaminase + histaminase substrate. (The same concentration of enzyme protein was used throughout).

•——• Authentic pyridoxal phosphate in buffer pH 6.8.
owing to rapid reoxidation of the enzyme in experiment which could not be fully controlled the fluorometric spectrum decreased much more slowly than in the case of sodium dithionite. Morrel (1952) observed that hypoxanthine decreased the absorption of xanthine oxidase at 450 m\(\mu\) to the same extent, but much slower than did sodium dithionite.

2) **PYRIDOXAL PHOSPHATE.**

Synthetic pyridoxal phosphate shows two absorption maxima, one at 330 m\(\mu\), and the other at 388 m\(\mu\) (Matsuo, 1957). Fig. X shows the absorption spectra of the purified histaminase at pH 6.1 and pH 6.8 and also the absorption spectrum of authentic pyridoxal phosphate pH 6.8. The intensity of the absorption maxima of the purified histaminase was greater at the pH 6.1 as compared with that at pH 6.8. Shukuya and Schwert (1960) made similar observations with glutamic acid decarboxylase when they found that changes in absorption maximum depend upon the pH of the solution.

From Fig. X it can be seen that at both pH
6.1 and pH 6.8 the absorption pattern of the highly purified histaminase resemble that shown by authentic pyridoxal phosphate. A shift, however, towards the longer wavelength occurs at the second absorption peak of the purified histaminase at both pH 6.1 and pH 6.8, since the absorption spectra of the latter occurs at 405 μm as compared with the absorption maximum of synthetic pyridoxal phosphate at 388 μm. At 330 μm where the first absorption peak of synthetic pyridoxal phosphate was located, the absorption maximum of the purified histaminase at pH 6.1 as well as at pH 6.8 appeared to be very similar to that of genuine pyridoxal phosphate. A considerable change of the absorption pattern was observed on addition of the substrate, histamine to the purified histaminase solution when it was observed that the peak at 330 μm completely disappeared and the peak at 405 μm was found to be greatly reduced.

Jenkins and Sizer (1960) observed that pyridoxal phosphate exhibits a strong fluorescence at 400 μm, whereby the activating maximum was located at 322 μm. They observed, however, a very weak fluorescence
**FIG. XI.** Activating spectra resulting from fluorescence emission maximum at 400 μm light.

- Purified histaminase in 0.02 M phosphate buffer pH 6.8.
- Authentic pyridoxal phosphate in 0.02 M phosphate buffer pH 6.8.
maximum at 495 μ when the activating maximum was at 395 μ. The highly purified histaminase showed a strong fluorescence maximum at 400 μ with an activating maximum at 300 μ, no other fluorescence peak having been obtained with the enzyme. This may be explained on the grounds that the highly purified enzyme solutions investigated showed a very low concentration and, further, as mentioned above, that the fluorescence intensity of the second peak is a weak one. Fig. XI shows both activating maxima of purified histaminase solution as well as of synthetic pyridoxal phosphate at the fluorescence maximum of 400 μ. It can be seen from this Fig. that a shift of the activating maximum toward the shorter wavelength occurred with the enzyme.

The results obtained here confirm the previous suggestions (Werle and von Pechmann (1949) and Goryachenkova (1956)), and indicate that flavin adenine dinucleotide and pyridoxal phosphate are both prosthetic groups of histaminase. With reference to the mechanism for the action of both these prosthetic groups of histaminase it should be
pointed out here that in 1949, Werle and von Pechmann developed a theory concerning the combined action of both flavin adenine dinucleotide (FAD) and pyridoxal phosphate. According to their scheme, the enzymic destruction of histamine can be explained as follows:

\[
\begin{align*}
(1) & \quad R - CH_2 - CH_2 - NH_2 + \text{Enzyme} - CH = O - H_2 O & \quad R - CH_2 - CH_2 - N \\
& \quad \text{Enzyme} \quad CH & \quad \text{Enzyme} \quad CH \\
& \quad \text{(Schiff's base)} & \quad \text{(Schiff's base)}
\end{align*}
\]

\[
\begin{align*}
(2) & \quad R - CH_2 - CH_2 - N & \quad R - CH - CH - N \\
& \quad \text{Enzyme} \quad CH & \quad \text{Enzyme} \quad CH \\
& \quad + \text{FAD} & \quad + \text{FADH}_2 \\
& \quad \text{(Schiff's base)} & \quad \text{(Schiff's base)}
\end{align*}
\]

\[
\begin{align*}
(3) & \quad + 2H_2 O + O_2 & \quad \text{Enzyme} \quad \overset{\text{H}}{\longrightarrow} & \quad + R - CH_2 - CHO + NH_3 + H_2 O_2 + \text{FAD}
\end{align*}
\]

In Equation I, the carbonyl group of the pyridoxal phosphate of the enzyme reacts with the substrate histamine and forms a Schiff's base.
In Equation 2, flavin adenine dinucleotide (FAD above) takes up 2 hydrogens from the Schiff's base, the FAD itself being reduced to FADH₂.

In Equation 3, due to the presence of molecular oxygen and water, the enzyme is reoxidised with the formation of iminazole acetaldehyde + ammonia + oxidised flavin adenine dinucleotide and hydrogen peroxide.
SECTION VI.

GENERAL DISCUSSION AND SUMMARY.
The aim of this work was to attempt a purified histaminase preparation in order to study problems such as the specificity of the enzyme and the prosthetic groups of this enzyme.

The method developed in this work for the purification of histaminase appears to be satisfactory. A highly purified preparation of histaminase was obtained by chromatography and rechromatography of the extract obtained from fractionation with ammonium sulphate followed by thermal denaturation of the inert protein of the crude histaminase. The enzyme was adsorbed on the DEAE-cellulose columns at pH 8.6 and it was eluted from the columns by means of simple elution with 0.1 M acetate buffer, pH 5.5. Using this method it was found that the histaminase emerged from the DEAE-cellulose column almost immediately after simple elution had been started, as a single well-resolved symmetrical peak. After chromatography, the effluents were subjected to rechromatography. An over-all 2000-fold purification of histaminase activity as compared to that of the crude sodium chloride extract could be finally achieved.
The results of electrophoretic experiments carried out on cellulose acetate strips at several different pH's seemed to indicate that the enzyme obtained in the purification procedure described was apparently pure. At all the pH's studied, the histaminase migrated as a single distinct band toward the anode. At pH 6.8 and pH 8.6 this enzyme zone could be located in the region between the $\alpha_2$- and $\beta$-globulin of human serum. With decreasing pH the mobility of the enzyme zone also decreased and no migration of the enzyme band occurred at pH 5.0 and pH 5.15. Hence, it may be suggested that the isoelectric point of histaminase is between pH 5.0 and pH 5.15. At pH 4.6 the enzyme travelled as a single, distinct band toward the cathode. Since only one criterion of purity, electrophoresis, has been so far applied it cannot, however, be said with certainty that the enzyme preparation obtained in this work, is indeed homogenous.

The results obtained in starch gel electrophoresis with a highly purified histaminase preparation were unfortunately not clear-cut. The enzyme travelled
as a faint, diffuse zone toward the anode. A faint, distinct band, however, could be observed in the region of the Fast$\alpha_2$-serum globulin.

The highly purified histaminase was stable for weeks at 4°C and most of the histaminase activity was recovered after the enzyme had been kept in the deep freeze at -18 to -20° for 30 days.

It seems to follow from the present work that histaminase is the specific enzyme for the destruction of histamine, no enzyme activity whatsoever having been observed when cadaverine, putrescine and hexamethylenediamine were used as substrates. It appears, therefore, essential that the term "histaminase" should be reserved for the enzyme which specifically destroys histamine.

Finally, it has been found here that both flavin adenine dinucleotide and pyridoxal phosphate are the prosthetic groups of the highly purified histaminase, thus confirming previous suggestions of Werle and von Pechmann (1949) and Goryachenkova (1956).
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