CORA Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

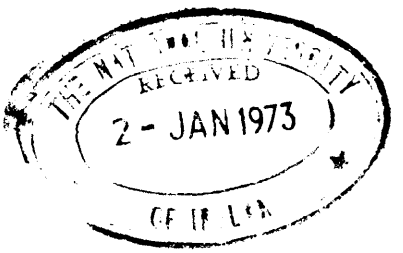
Title	The purification and characterization of the high and low molecular weight forms of rabbit intestinal adenosine deaminase
Authors	Piggott, Charles O.
Publication date	1972
Original Citation	Piggott, C. O. 1972. The purification and characterization of the high and low molecular weight forms of rabbit intestinal adenosine deaminase. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
Link to publisher's version	http://library.ucc.ie/record=b1224513~S0
Rights	© 1972, Charles O. Piggott - http://creativecommons.org/licenses/by-nc-nd/3.0/
Download date	2026-11-14 15:19:10
Item downloaded from	https://hdl.handle.net/10468/1663

UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

DP1972 PIGG
664186

THE PURIFICATION AND CHARACTERIZATION OF THE HIGH AND LOW MOLECULAR WEIGHT FORMS OF ADENOSINE DEAMINASE FROM RABBIT INTESTINE

C.O. PIGGOTT



THE PURIFICATION AND CHARACTERIZATION OF THE HIGH AND LOW MOLECULAR WEIGHT FORMS OF RABBIT INTESTINAL ADENOSINE DEAMINASE

Thesis presented for the degree of Doctor of Philosophy in the Faculty of Science, University College, Cork, National University of Ireland.

By Charles O. Piggott, B.Sc.



Department of Biochemistry * University College * Cork.

December, 1972.

"Gradually I became convinced
that the definition and purification
were all-important problems, not
only for the substances in my hands
but for the whole of biochemistry"

(A. Tiselius).

ACKNOWLEDGEMENTS

I am indebted to Professor T.G. Brady, M.Sc., Ph.D., Head of the Department of Biochemistry, University College, Cork, under whose stimulating guidance this work was carried out, for his great interest, continuous advice and valuable criticism.

I also wish to thank Dr. E.R. Tully, Dr. C.A. Ross, Dr. D.R. Headon, Mr. M. Noonan and Mr. N.P. Roring for their friendship and continuous interest while this research was going on.

My thanks are due to Mr. P. Barry, Mr. G. Coughlan, Mr. H. Keating, Miss P. Kearney, Mr. M. Kelleher and Mr. V. Neff for their ready help in technical matters.

I am grateful to Mr. J. Kenneally, Animal House attendant, U.C.C., for his help and co-operation in supplying the animals needed for this work.

A Department of Education Maintenance Grant is also acknowledged.

Finally, I wish to thank Miss E. Harrington, Physiology Department, U.C.C. for typing the manuscript.

CONTENTS

INTRODUCTION	1.
MATERIALS AND METHODS	11.
Determination of adenosine deaminase activity	12.
Determination of AMP deaminase activity	15.
Determination of alkaline phosphatase activity	15.
Determination of 5'-nucleotidase and acid phosphatase activities	16.
Determination of xanthine oxidase activity	17.
Determination of protein content	18.
Sephadex Gel-filtration	21.
Ion-exchange chromatography	22.
Concentration of adenosine deaminase solutions	25.
Thin-layer gel filtration	27.
High voltage electrophoresis	30.
Low voltage electrophoresis	32.
Location of adenosine deaminase after electrophoresis	33.
Protein staining techniques	33.
Polyacrylamide gel electrophoresis	34.
SDS-polyacrylamide gel electrophoresis	38.
Urea-polyacrylamide gel electrophoresis	40.
Photographic recording of zymograms	41.
Densitometry	41.
Radioactivity measurements	42.
Hydrolysis of proteins	42.
Amino acid analysis	43.
RESULTS	
SECTION 1. PRELIMINARY STUDIES	44.
Level of adenosine deaminase in various tissues	45.
Distribution of enzyme activity in the intestine	47.
Distribution of the two forms of the enzyme in the intestine and in other rabbit tissues	50.
Extraction of adenosine deaminase from rabbit intestine	52.

SECTION II. PURIFICATION OF THE HIGH AND LOW MOLECULAR WEIGHT FORMS OF RABBIT INTESTINAL ADENOSINE DEAMINASE 57.

Purification I 58.

Purification II 69.

Purification III 78.

Purification IV 86.

Purification V 100.

Purification VI 119.

SECTION III. PROPERTIES OF THE HIGH AND LOW Molecular WEIGHT FORMS OF RABBIT INTESTINAL ADENOSINE DEAMINASE 144.

Effect of pH on enzyme activity 145.

Effect of heating on enzyme activity 151.

Determination of activation energies 154.

Determination of isoelectric points 157.

Determination of Michaelis constants 159.

Determination of adenosine, 2'-deoxyadenosine and 3'-deoxyadenosine by the enzymes 162.

Effect of urea on enzyme activity 163.

Effect of GuHCl on enzyme activity 167.

Effect of dimethylformamide on enzyme activity 171.

Metal-ion inhibition 172.

Effect of thiol reagents on deaminase activity 176.

Photooxidation studies 184.

Effect of n-bromosuccinimide on enzyme activity 192.

Molecular weight determinations 194.

Absorbtion spectra of the purified enzymes 205.

The existence of multiple electrophoretic forms 205.

Cell fractionation studies 208.

Immunochemical characterization 211.

Effect of freezing and thawing on enzyme activity 213.

Analysis of the high molecular weight form 216.

Effect of dissociating agents on the high mol.wt. form 220.

Attempts at converting the low mol. wt. form into the high mol. wt. form 222.

Effect of sodium sulphite on the enzymes 225.

Effect of temperature as a possible means of dissociation 227.

Effect of acid and alkaline pH on the high mol.wt. form 228.

Effect of proteolytic enzymes on the high mol. wt. form 229.

Effect of GuHCl and urea as dissociating agents 231.

Dissociation of the high mol.wt. form using SDS 234.

Effect of maleic anhydride as a dissociating agent 240.

Thiol content of rabbit intestinal adenosine deaminase 243.

Amino acid composition of rabbit intestinal adenosine deaminase 247.

SECTION IV. STUDIES ON THE RABBIT LIVER ADENOSINE DEAMINASE 254.

Purification of the enzyme 255.

Molecular weight of the enzyme 260.

DISCUSSION 261.

BIBLIOGRAPHY 276.

INTRODUCTION

Adenosine deaminase (EC 3.5.4.4) was first discovered by Schmidt (1928) during an investigation of the enzymes present in rabbit muscle. The enzyme catalyses the hydrolytic breakdown of adenosine to inosine and free ammonia without the participation of co-factors, a reaction which is distinct from the deamination of amino acids.

Since its discovery the enzyme has been shown to be widely distributed throughout the animal kingdom. The occurrence of adenosine deaminase in plants has been reported (Fiers, 1960), a finding which was later shown to be due to bacterial contamination (Brady and Hegarty, 1966). The enzyme has been isolated from Aspergillus oryzae (Kaplan, 1956) and from Escherichia coli (Koch and Vallee, 1959). Adenosine deaminases were also reported to have been found in the blow-fly (Lennox, 1941), in the larvae of Drosophilia melanogaster (Wagner, 1948), in sea-urchins' eggs (Gustafson, 1951) and in the hepato-pancreas of both crayfish and lobster (Roush, 1956). The presence of the enzyme in higher animals has been established by many workers (Conway and Cooke, 1939; Clarke, et al. 1952; Purzycka, 1962; and Brady and O'Donovan, 1965) who have also studied its distribution in various tissues. Spleen and duodenum have invariably been found to possess highest concentrations of the enzyme in most of the species investigated. In some animals, both lung and placenta have been shown to possess very high deaminase activity (Brady and O'Donovan, 1965).

The intracellular location of adenosine deaminase has also

been investigated. Although it has been demonstrated that the enzyme is present in the cytoplasm or 'soluble fraction' of the cell (Schneider and Hogeboom, 1952; Dierick et al., 1967; Muller, 1969) adenosine deaminases have also been found in other sub-cellular organelles. Stern and Mirsky (1953) detected the enzyme in nuclei isolated from calf thymus, and a latent adenosine deaminase has been shown to be associated with the mitochondrial fraction of mouse brain (Mustafa and Tewari, 1970). Rabbit brain has also been reported to have a somewhat diffuse pattern of localization. Jordan et al. (1959) found that only 63.5% of the adenosine deaminase activity of rabbit brain was in the 23,000 x g. 30 min. supernatant fraction; a total of 32.2% of the activity was recovered from the cell debri, the nuclear, the mitochondrial and the microsomal fractions. The general concept of the exclusive location of adenosine deaminase in the supernatant fraction of tissue homogenates would therefore appear to need revision.

Various suggestions have been made as regards the role of adenosine deaminase. Because of its high concentration in rabbit intestine Conway and Cooke (1939) suggested that the enzyme might be important in detoxifying the strongly vasodepressant adenosine into relatively inactive inosine. They also suggested that adenosine deamination in other tissues is a physiological mechanism for obtaining a ready supply of ammonia for use in biosynthetic reactions or for neutralizations. The latter suggestion has been further substantiated by the studies of Sanger and Mustafa (1971). They found that in rats, during NH_4CL acidosis, the renal adenosine and guanine deaminases increased by 2 and 2.5 fold respectively.

The renal DNA and RNA contents were found to decrease considerably but the protein content of the kidney did not undergo any change. Their findings suggested that both adenosine and guanine deaminases might play an important role in the renal regulation of acid-base balance.

The remarkably high deaminase activity in the lung of several species observed by Clarke et al. (1952) and Brady and O'Donovan (1965) raised the question whether adenosine deaminase might have a role in respiration. The latter authors suggested that the ammonia found in alveolar air was due to the action of the enzyme, and that this provides a subsidary route for ammonia excretion. They also attributed a detoxifying role to adenosine deaminase in both spleen and placenta (Brady and O'Donovan, 1965). Clarke et al. (1952) who studied in detail the vasodepressant activity of adenosine and some analogues also reported on the effect of adenosine deaminase as a detoxifying agent. Following these studies Baer et al. (1966) suggested that deamination of adenosine in the plasma may be the mechanism of regulation of coronary blood flow.

Although the level of adenosine deaminase in blood and plasma is normally very low (Conway and Cooke, 1939; Brady and O'Donovan, 1965), serum adenosine deaminase activity has been found to increase in tumour bearing patients (Straub et al., 1957). and in patients suffering from acute viral hepatitis or from cirrhosis of the liver (Koehler and Benz, 1962; Goldberg, 1965; and Krawczynsky et al., 1965). Galanti and Giusti (1968) and Galanti et al. (1968) described a very marked increase of serum adenosine deaminase in over one hundred patients suffering from typhoid fever and showed the increase

to be a consequence of enzyme release from damaged Peyer patches and from mesenteric lymph glands and intestinal mucosa. Recent studies by Giusti (1970) on a new group of patients have confirmed these observations, and this worker has also found an increase in serum adenosine deaminase in patients suffering from parathyroid B fever. Serum adenosine deaminase determinations therefore appear to provide a useful diagnosis of typhoid fever and other pathological conditions.

The level of the deaminase in certain body tissues also increases when these tissues become malignant. Nishihara et al.(1966) showed that malignant lung tissue has 3 to 7 times higher adenosine deaminase activity than corresponding normal tissue. In addition it has been shown that there is a difference in the serum adenosine isoenzyme patterns in patients with lung cancer and that this alteration may be of clinical importance in the diagnosis of lung cancer (Nishihara et al., 1970). Changes in the adenosine deaminase level of mouse liver has also been reported following the infection of mice with RICH leukemia virus (Siegler et al., 1964; Rich et al., 1965), a five-fold increase in enzyme activity being observed. Furthermore, Cory et al. (1970) have compared the properties of the deaminase in liver extracts from normal and RICH virus-infected mice, and while the enzymes had the same Michaelis constants, pH optima and activation energies, they differed in their substrate specificities and electrophoretic mobilites.

Although it was originally believed that the adenosine deaminase present in mammalian cells deaminated only adenosine and the closely related deoxyadenosine, further studies showed that the enzyme also hydrolysed 6-chloropurine ribonucleoside (Cory and

Suhadolnik, 1965) and 2-amino-6-chloropurine ribonucleoside (Baer et al., 1966) with formation of inosine and guanosine, respectively. From these observations it appeared that the basic mechanism of action was not a specific deamination but rather seemed to constitute the catalysis of a nucleophilic displacement by hydroxyl at the 6-position of the purine system. This hypothesis has been tested and it was demonstrated that the enzyme from bovine intestinal mucosa is capable of hydrolysing a number of other constituents including methylamino-, hydroxylamino- and methoxy- groups (Baer and Drummond, 1966; Baer et al., 1968). Because of the broad specificity of the deaminase an analogy has been drawn between the catalytic process and that of esterolytic enzymes.

The substrate specificity of bovine placental adenosine deaminase, purified by Sim and Maguire (1971), has also been studied in detail (Maguire and Sim, 1971) and was found to be similar to that of the intestinal enzyme. Moreover, these authors proposed a mechanism of action for the placental adenosine deaminase involving a sulphydryl, a histidine and a lysine residue at the active site which they suggest is applicable to other mammalian adenosine deaminases of similar molecular weight.

Numerous purifications of adenosine deaminase from both animal and bacterial sources have been reported. The most extensive purifications of the enzyme have been conducted in bovine tissues probably because of their ready availability and the high level of deaminase activity possessed by these tissues. Bovine intestinal mucosa was shown by Brady (1942) to be a potent source of the enzyme and since then various purifications from this source have been attempted (Zittle, 1946; Kalckar, 1947; and Klein, 1961).

In 1962 a highly purified preparation of the enzyme shown to be ultracentrifugally homogenous was obtained from bovine intestinal mucosa (Brady and O'Connell, 1962). Other bovine tissues from which the enzyme has been purified include the heart from which a chromatographically homogenous adenosine deaminase has been isolated by Rockwell and Maguire (1966). These workers demonstrated substrate inhibition and product inhibition by the enzyme. Bovine spleen adenosine deaminase has been purified by Pfrogner (1967) and O'Brien (1968) and surprisingly the preparations obtained by these workers displayed differences in their properties. Bovine lung adenosine deaminase has also been purified (Noonan, 1969).

A tissue other than that of the cow from which the deaminase has been highly purified is chicken duodenum (Hoagland and Fisher, 1967). These workers investigated the physical, chemical and catalytic properties of the enzyme and demonstrated that it closely resembled the deaminase isolated from mammalian tissues.

Procedures have also been published for the purification of adenosine deaminase from Bacillus cereus (Powell and Hunter, 1956), Escherichia coli (Koch and Vallee, 1959), bone marrow (Ipata, 1962), bovine brain (Malysheva et al., 1964), Yoshida cells (Coddington, 1965) and bovine thyroid (Dierick, 1967).

In the best characterized cases, purified adenosine deaminases have been shown to possess molecular weights in the region of 35,000 (Brady and O'Sullivan, 1967; Hoagland and Fisher, 1967; Pfrogner, 1967; Murphy, et al., 1969b; Sim and Maguire, 1971). A number of the purified enzymes, when subjected to electrophoresis, were shown to possess multiple electrophoretic components. The bovine intestinal adenosine deaminase purified by Brady and O'Connell (1962) consisted of five to six multiple forms (termed isoenzymes)

on starch gel electrophoresis. Murphy et al. (1969a), having preparatively separated the isoenzymes by ion exchange chromatography, demonstrated that they all had similar molecular weights, the only significantly detectable differences between them being differences in overall charge as evidenced by chromatographic and electrophoretic experiments. Adenosine deaminase purified from bovine serum has also been shown to exist in multiple molecular forms (Cory et al., 1967). The preparation was homogenous in the ultracentrifuge indicating that the multiple forms were of similar molecular weight as was found with the bovine intestinal enzyme.

In recent years reports have been appearing in the literature of adenosine deaminases having molecular weights greater than 35,000. These enzymes appear to have molecular weights in the region of 200,000 and 110,000 respectively. Fisher et al. (1965) presented evidence that adenosine deaminases in the livers and duodena of chickens and frogs have different properties. Following these studies Ma and Fisher (1968a) using Sephadex chromatography demonstrated the presence of a large (110,000 mol.wt.) and a small (30,000 mol. wt.) adenosine deaminase in chicken liver preparations. From their investigations they showed that the tissue specific differences between liver and duodenum in the chicken were due to the presence of the large adenosine deaminase in the liver which is not present in the duodenum. In a study undertaken to establish the basis of the tissue specific differences observed in the frog, Ma and Fisher (1968b) showed that three classes of adenosine deaminases were present in both frog liver and duodenum. The tissue specific differences appeared to be the result of different

proportions of these enzymes in the two tissues. Consequently, Fisher classified the enzymes into three groups designating them types A, B and C, which have molecular weights in the size ranges of 200,000, 100,000 and 35,000 respectively as estimated by Sephadex chromatography.

In a study of hepatic adenosine deaminases from eleven species of amphibians (Ma and Fisher, 1968c) large amounts of both type -C and type -B enzymes were observed, the type -B enzyme having a much lower relative substrate specificity (ratio of activity with deoxyadenosine and adenosine as substrate) than the type -C enzyme. Only barely detectable amounts of the type -A enzyme were found. The livers of higher animals however showed strikingly different patterns. In a survey of hepatic adenosine deaminases in fourteen species of higher mammals including representatives from seven different orders Ma and Fisher (1969) have shown that the type -A and type -C enzymes are present, no evidence being obtained for the presence of the type -B enzyme. Both the type -A (200,000) and type -C (35,000) enzymes have the same relative substrate specificities. Moreover, the type -A enzyme appeared to partially dissociate into the type -C enzyme during ammonium sulphate concentration experiments. The authors suggested that the type -A and type -C enzymes in higher mammals actually represent one kind of enzyme which show different tendencies to aggregate in different organisms. The hepatic adenosine deaminases in a number of species of fish have also been investigated (Ma and Fisher, 1972) where the patterns resembled those found in amphibians, only type -B and type -C enzymes being present.

Adenosine deaminases of two different molecular sizes have also

been reported in human tissues (Akedo et al., 1970). The enzymes, which were designated E1 and Es, were purified from normal human lung and stomach and had molecular weights of 200,000 and 50,000 respectively. Studies on substrate specificity with respect to adenosine and deoxyadenosine revealed no apparent difference between the two enzymes suggesting that they are similar to the type -A and type -C adenosine deaminases reported by Ma and Fisher.

A large adenosine deaminase, having a molecular weight of 214,000, has also been purified from Takadiastase powder (Aspergillus orygae) by Minato (1968) and was shown to dissociate in guanidine hydrochloride into subunits possessing molecular weights of 110,000 and 29,000. Previous studies reported on the Takadiastase enzyme indicated that it was a more non-specific type of deaminase than mammalian adenosine deaminases. Wolfenden et al (1968) also compared the physical properties and amino acid composition of the Takadiastase deaminase and the bovine intestinal adenosine deaminase and demonstrated that there were differences between them.

It was reported by Buggy (1966) in this laboratory that rabbit duodenum contains two active adenosine deaminases separable by Sephadex chromatography. In a survey of the molecular weights of mammalian adenosine deaminases in a number of species using gel filtration, Murphy et al. (1969b) demonstrated that the rabbit duodenal deaminases have molecular weights of 260,000 and 34,000 respectively, the rabbit being the only species to possess a high molecular weight form of adenosine deaminase. These enzymes would appear to correspond to the type -A and type -C deaminases described by Ma and Fisher (1968b). Whereas the type -C enzymes, as already mentioned, have been purified and well characterized in a variety of species,

the type -A enzymes have not been extensively investigated and little is known about their relationship to the type -C enzymes.

It was with a view to studying this relationship that the investigations described in the present report were undertaken. The first half of the thesis is concerned with the purification of the high and low molecular weight forms of the rabbit intestinal adenosine deaminase. The latter half of the thesis involves a comparative study of the properties of the two forms and also describes attempts at converting the high molecular weight form into the low molecular weight form.

MATERIALS AND METHODS

DETERMINATION OF ADENOSINE DEAMINASE ACTIVITY

Unit of Enzyme Activity

The unit of adenosine deaminase activity is defined as that amount of enzyme, which will deaminate one micromole of adenosine, buffered at pH 7.0, per minute at 37°C.

Estimation of Enzyme Activity

The activity of the enzyme was determined by two methods:

(i) Estimation of liberated ammonia (Conway, 1939)

(ii) Measurement of decrease in adenosine concentration by spectrophotometric assay.

(i) The ammonia liberated from adenosine by the enzyme was quantitatively determined by the Conway Microdiffusion Method. No. 1 type Conway Units were set up in duplicate as follows:

Outer chamber	:	1 ml. satd. K_2CO_3
Central chamber	:	1 ml. 1% boric acid incorporating mixed indicator (0.0033% bromocresol green and 0.0066% methyl red)

The buffered substrate was prepared as follows: To 30 ml. of 1% adenosine was added 70 ml. 0.1M phosphate buffer pH 7.0(final adenosine concentration was 0.3%)

5 ml. of this mixture was pipetted into a test-tube, which was placed in a water bath at 37°C. After temperature equilibration, 1 ml. of suitably diluted enzyme (also equilibrated at 37°C) was added to the buffered substrate. The contents were rapidly mixed and replaced in the bath. After a suitable time interval, 1ml. was withdrawn and placed in the outer chamber of the Conway Unit, which

contained satd. K_2CO_3. The unit was quickly sealed by means of a glass lid with adhering fixative(paraffin wax and vaseline), rotated gently and allowed to diffuse at room temperature for 2 h. The contents of the centre chamber were then titrated to a pink colour with 0.01 N HCl using a horizontal microburette. From the titration figure, the activity of the enzyme solution can be determined.

Controls and an ammonia standard were run simultaneously.

(a) Enzyme blank. 0.6 ml. of suitably diluted enzyme was diluted with 3 ml. of water, treated in a similar manner to the analytical tubes, and 1 ml. added to the outer chamber of a Conway Unit.

(b) Adenosine blank. 0.6 ml. water added to 3 ml. buffered substrate, and treated the same as the analyticals.

(c) Absolute blank. 1 ml. distilled water added in duplicate to the outer chamber of prepared units.

(d) Ammonia standard. 1 ml. standard ammonia solution (50 µg. NH_3-N/ml.) added to the outer chamber.

(ii) Spectrophotometric assay

The spectrophotometric assay of adenosine deaminase is based on the fact that the absorption of adenosine at 265 nm is 40% greater than that of inosine at the same wavelength(Kalckar, 1947). An adenosine solution(0.1 mM) was prepared, which gave a $E^{1\ cm}_{265\ nm}$ of 1.3 on the spectrophotometer.

Procedure :

(1) 3 ml. substrate (previously incubated at 37^0C) was pipetted

into the analytical cuvette of light-path length 1 cm.

(2) 0.1 ml. suitably diluted enzyme was added to the substrate, the contents rapidly mixed, and the spectrophotometer allowed to record continuously over a period of 2 min. 3 ml. phosphate buffer pH 7.0 was placed in the blank cell.

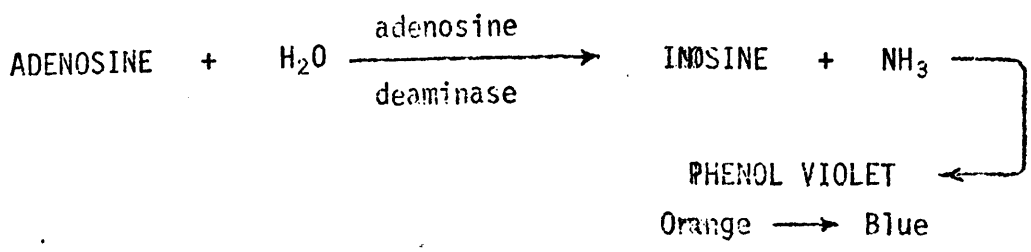
(3) The ΔE/min. was measured in the initial part of the curve, and enzyme activity determined using the following equation:

$$\text{e.u./ml.} = \frac{\Delta E/\text{min.} \times 3.1}{0.1 \times 8.1}$$

The activity obtained with this method of assay represented only 65% of the true activity obtained by the microdiffusion technique. The concentration of adenosine used in the spectrophotometric assay method (0.1 mM) was too low to give zero order kinetics and maximum velocity. However, this method is very rapid, and was used instead of the microdiffusion assay, where speed rather than accuracy was required.

A RAPID QUALITATIVE METHOD FOR RECOGNITION OF ADENOSINE DEAMINASE ACTIVITY

This assay method is based on the colour change observed when alkali is added to a solution of phenol violet indicator (orange → blue). The alkali in this case was provided by the ammonia liberated by the action of the enzyme on adenosine.



Procedure :

To 1.0 g. Agar, 1.0 g. adenosine and 25 ml. distilled water was added 25 ml. saturated phenol violet solution. The entire mixture was heated to 90°C with constant stirring. It was then poured into a shallow dish, and allowed to set in a uniform layer. On application of the analytical solution to the gel, the appearance of a dark blue spot indicated enzyme activity, the length of time taken for the spot to appear being inversly proportional to the enzyme activity. This method was very useful for detecting regions of enzyme activity during elution of columns.

DETERMINATION OF AMP DEAMINASE ACTIVITY

AMP deaminase activity was estimated essentially by the method of Kalckar (1947). 200µM AMP was made up in 0.1M potassium succinate pH 6.5. To 3 ml. of this buffered substrate solution was added 0.1 ml. enzyme in a 1 cm. cuvette. The decrease in absorbance at 265 nm was measured spectrophotometrically at 30°C. The rate of change in optical density at that wavelength gave a measure of the enzyme activity.

DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY

Alkaline phosphatase activity was assayed by incubating 0.1 ml. enzyme with 3 ml. 1mM p-nitrophenyl phosphate in 1.0 M Tris buffer pH 8.0, which is the pH optimum for the reaction (Garen, 1960). The increase in absorption at 410 nm, resulting from the dephosphorylation of p-nitrophenylphosphate to yield p-nitrophenol, was followed

spectrophotometrically with a cuvette of 1 cm. path length.

The molar absorbancy for p-nitrophenol in 1M Tris pH 8.0 = 1.62×10^4.

Specific activity was calculated using the following equation:

$$\text{Sp. Activity (units/mg. protein)} = \frac{\Delta E/\text{min.} \times 1{,}000}{1.62 \times 10^4 \times \text{mg. enzyme/ml. reaction mixture}}$$

DETERMINATION OF 5'-NUCLEOTIDASE AND ACID PHOSPHATASE ACTIVITIES

The estimation of these enzyme activities was carried out according to Itoh (Itoh, 1967).

The 5'-nucleotidase was assayed in the following incubation mixture : 100 µmoles of Tris-Maleate-NaOH buffer pH 6.5, 10 µmoles of $MgCl_2$, 3 µmoles of 5'-IMP and enzyme preparation in a total volume of 1.0 ml.

The reaction mixture for the assay of acid phosphatase contained in 1 ml., 100 µmoles of acetate buffer pH 5.5, 10 µmoles of $MgCl_2$, 3 µmoles of disodium phenylphosphate and enzyme.

The incubation was carried out at 37°C for 10 min. and the reaction terminated by the addition of trichloroacetic acid to a final concentration of 5%. The precipitated protein was removed by centrifugation and the supernatant fluid used for Pi determination by the method of Fiske and Subbarow (Fiske and Subbarow, 1925). To the supernatant fluid was added 5 ml. molybdate reagent (0.02M ammonium molybdate in 3.3 N sulphuric acid) and 1 ml. 0.25% aminonaphthol-sulphonic acid and the final volume brought to 25 ml. A control was

also included using glass distilled water instead of the supernatant fluid. Both the control and the analytical were incubated at 37°C for 5 min., allowed to cool, and the absorbance of each measured at 750 nm.

Using a standard phosphate solution, a graph was constructed relating absorbance to Pi concentration. From this graph the Pi concentration of the analytical was estimated.

A unit of activity corresponds to the liberation of 1 µmole of Pi per hour.

DETERMINATION OF XANTHINE OXIDASE ACTIVITY

Xanthine oxidase activity was estimated by measuring the increased absorbancy at 290 nm when hypoxanthine is converted to uric acid. A unit of activity is the amount of enzyme which forms one micromole of urate per minute at 25°C.

Reagents :

Substrate : 10 mg. hypoxanthine in 500 ml. H_2O
Buffer : 50 mM phosphate pH 7.5
Enzyme preparation

Procedure :

Into 1 cm. cuvettes pipetted the following:

	Test	Control
Buffer	1.9 ml.	1.9 ml.
Water	-	1.0 ml.
Enzyme	0.1 ml.	0.1 ml.
Substrate (at zero time)	1.0 ml.	-

The rate of increase in absorbance at 290 nm was then measured

over a period of several minutes. The rate is proportional to enzyme concentration within limits of 0.01 to 0.02 units per test.

The molar absorbancy of uric acid = 1.22×10^4 cm^{-1} (Westerfield, 1959).

$$\text{Sp. Activity (units/mg. protein)} = \frac{\Delta E/\text{min.} \times 1{,}000}{1.22 \times 10^4 \times \text{mg./ml. reaction mixture}}$$

DETERMINATION OF PROTEIN CONTENT

Two methods were used for determining protein concentration.

(a) Biuret Method
(b) Ultraviolet absorption at 280 nm

(a) Biuret Method.

100 mg. purified egg albumen (thoroughly dried in a dessicator) was accurately weighed out and dissolved in 10 ml. 2% NaCl (protein concentration = 10 mg/ml.). A series of protein solutions were prepared from this, ranging in concentration from 0-10 mg./ml. (Final volume = 1 ml.). To each solution 4 ml. Biuret Reagent was added and allowed to stand at room temperature for 30 min. The absorbance of each tube was then measured at 555 nm using tube 0 as a blank. Absorbance was plotted against protein concentration (mg./ml.) to obtain a standard graph (Fig. 1). To determine the protein concentration of a test solution, 1 ml. was treated as above and the absorbance measured at 555 nm. From the standard graph the concentration could be directly read off.

This method could not be used to obtain an absolute determination of the protein concentration of crude protein extracts because different

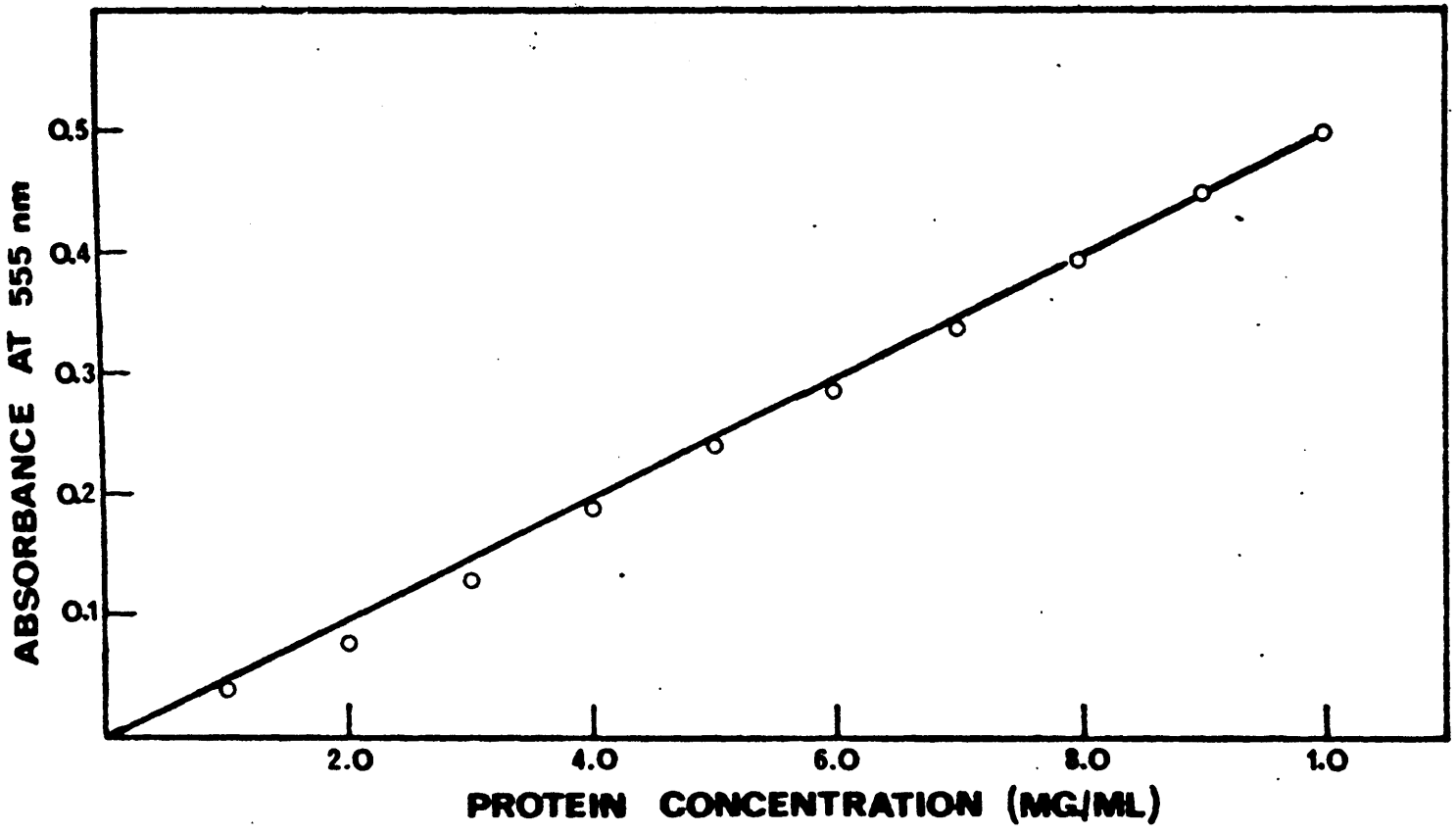


FIG. 1. Standard graph for measurement of protein concentration using the Biuret Method. Solutions of egg albumen (0 - 10 mg./ml.) were incubated with 4 ml. of Biuret reagent, and the absorbance at 555 nm measured after 30 min.

proteins give different colour yields so that exact values cannot be determined.

(b) Ultraviolet absorption at 280 nm.

For solutions, whose protein concentration could not be determined by the Biuret Method (e.g. solutions containing ammonium sulphate), ultraviolet absorption at 280 nm was used.

With impure protein solutions (containing nucleic acids and their derivatives) the following equation was employed (Layne, 1957).

$$\text{Protein conc. (mg./ml.)} = 1.55 \times E^{1cm}_{280\ nm} - 0.76 \times E^{1cm}_{260\ nm}$$

This is a revised form of an equation used by Warburg and Christian (1942).

COLUMN CHROMATOGRAPHY

Two types of column chromatography were used :

1. Gel-filtration using Sephadex G-75 and Sephadex G-200.
2. Ion-exchange chromatography using DE-52 cellulose.

SEPHADEX GEL FILTRATION

Sephadex gel filtration, first introduced in 1959 by Porath and Flodin, is a separation method based on differences in molecular size. It is a rapid and gentle procedure, and therefore specially suitable for the separation of labile biological substances such as proteins and enzymes. This technique has been used for desalting and concentration of macromolecules (Flodin, 1960) and in many purification procedures. It has also been used with success in determining molecular weights of protein molecules (Andrews, 1964).

Construction of Sephadex Columns

The dry Sephadex was stirred into a large conical flask, and allowed to swell in an excess of water for a period of time. Fines were removed by repeated decantation, and the gel suspension deareated using a suction pump. It was then allowed to equilibrate over night against the desired buffer. A glass column 60 cm. x 2.8 cm. was mounted vertically, and fitted with an extension tube. The bottom of the column was packed with a glass wool disc, and some glass beads. The buffer was passed up through the column to a height of about 10 cm., to avoid air bubbles being trapped in the orifice.

The measured buffer-Sephadex slurry was then poured into the extension tube and the Sephadex particles allowed to settle under gravity, while the buffer was allowed to drain off slowly through the orifice. When the required bed-height of Sephadex was reached, the extension tube was removed. A circular paper was allowed to settle on top of the bed. The column was then attached to a reservoir and buffer passed through the column at a flow rate of 1 ml./min.(approx.) for 48 hours.

During the latter stages of the work reported in this thesis, commercial Sephadex columns with plastic fittings were used.

Application of proteins to the Sephadex columns

Proteins were applied to the columns in two ways. In the first procedure the meniscus was allowed to run almost into the gel i.e. just to the top of the gel bed. The protein sample was then added slowly, allowed to sink into the bed, and washed down with 1 ml. of equilibrating buffer. The column was then filled with buffer to the correct height above the bed and elution begun.

The second procedure used was the "layering" technique. The density of the protein solution was increased by adding sucrose in solid form. On application to the columns, this formed a layer between the Sephadex-gel bed and the eluting buffer. The layer was allowed to seep into the gel, and then elution was begun. This method of application avoided damage to the surface of the Sephadex bed and was preferred to the former procedure for this reason.

ION-EXCHANGE CHROMATOGRAPHY

Ion-exchange chromatography, as opposed to gel-filtration, allows

the separation of proteins on the basis of difference in charge, rather than molecular size.

Treatment of the ion-exchange material

The material used was the microgranular anion exchange, Whatman DEAE cellulose DE 52. This was pre-swollen material, which did not require cycling with acid and alkali. About 500 ml. 0.03 M sodium citrate buffer pH 5.8 was added to 50 g. DEAE cellulose. The mixture was stirred and allowed to stand for a few minutes. The buffer was filtered off, and the process repeated until the slurry had attained a pH of 5.8. The material was then washed with excess water, the fines removed by decantation, and allowed to equilibrate in 0.002M sodium citrate buffer pH 5.8.

Construction of the column

A pyrex K 15/30 Sephadex column with plastic fittings was used. The base of the column consisted of a porous disc encassed in a screw-off plastic fitting. The column dimensions were 30 cm. x 1.5 cm. The ion-exchange buffered slurry was poured into the column, and allowed to settle out under gravity. This was repeated until the desired bed height was reached, and the column equilibrated over night with a slow flow of 0.002 M sodium citrate buffer pH 5.8..

Application of protein to the column

The buffer was allowed to drain off until it had almost reached the top of the DEAE bed. The protein solution was then placed in a reservoir and allowed to flow on to the column. Unabsorbed protein was removed with the starting buffer and an ionic gradient applied

to elute the enzyme.

Gradient elution

A reservoir containing 0.03 M sodium citrate buffer pH 5.8 was connected to a 300 ml. mixing chamber containing 0.002 M sodium citrate pH 5.8, and provided with a magnetic stirrer. This gave an increasing gradient which resulted in the elution of adsorbed protein.

The DEAE cellulose had to be regenerated before use again. This was accompanied by washing with IN HCl until pH 4.0 was reached, followed by extensive washing with water. It was then treated with IN NaOH to bring it to pH 10.0, followed by further extensive washing with water. The ion-exchanger was then ready for equilibration as previously described.



CONCENTRATION OF ADENOSINE DEAMINASE SOLUTIONS

Three procedures were used to concentrate solutions of the enzyme. In one the deaminase solution was concentrated by freeze drying (lyophilization). In this procedure a solution of the enzyme was frozen in a thin layer around the walls of a round bottomed flask by immersion in a mixture of acetone and dry-ice. The flask was then attached to a Quickfit lyophilizer and evacuated using an A.E.I. (Associated Electrical Industries) Metrovac Rotary Vacuum Pump Type GDR 210 (Fig. 2). Since the deaminase was invariably denatured when concentrated by this method, other procedures were used which gave little or no loss in enzyme activity.

In the first of these procedures the deaminase solution was applied to a DEAE cellulose column (3 cm. x 1 cm.) and eluted with a small volume of 2 M NaCl. This method was capable of effecting a ten fold concentration of the enzyme.

In the second procedure Sephadex G-15 (coarse grade) was used. The enzyme solution was conveniently concentrated by adding a known amount of Sephadex G-15 (water uptake - 1.5 ml. per g. dry Sephadex) to it. Water and low molecular weight substances were then absorbed by the swelling Sephadex beads, while the enzyme remained in the external solution. After 15 min. the gel grains were removed by centrifugation and the supernatant retained. This procedure is simple and rapid and was especially useful when small volumes had to be concentrated.

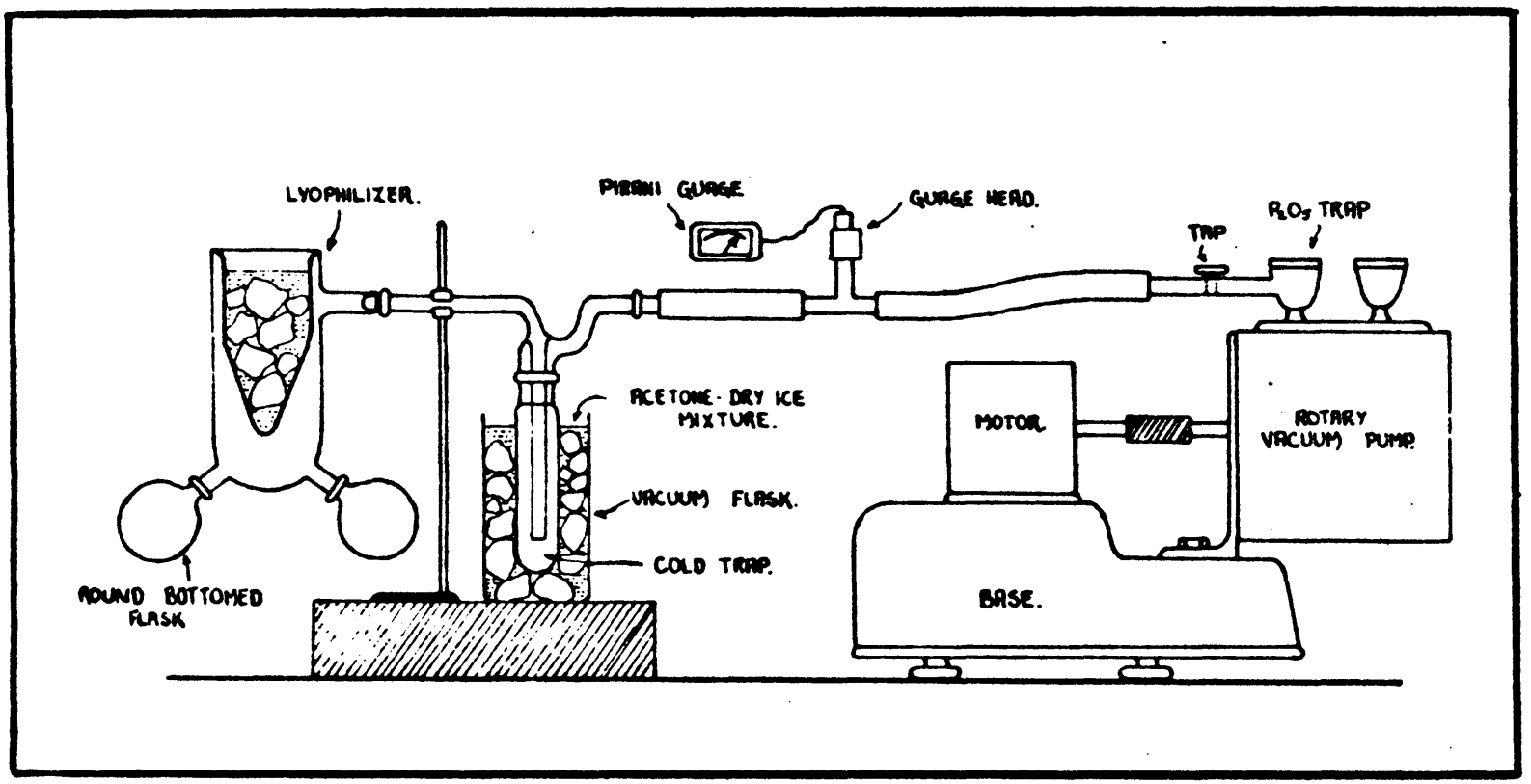


FIG. 2. Schematic representation of apparatus used for freeze drying adenosine deaminase solutions.

THIN-LAYER GEL FILTRATION

A schematic representation of the apparatus used in this technique is shown in Fig. 3.

Procedure

The Sephadex (G-75 superfine grade) was pre-swollen in buffer for 24 h. using 15 ml. of buffer per gram of Sephadex. (When using G-200, 20 ml. buffer per gram was added, the swelling time being 3 days). The glass plates had dimensions 20 cm. x 10 cm. and were thoroughly cleaned with detergent and distilled water before use. The clean dry plates were then coated with the swollen gel, using a Miller-Kirchner type spreader (supplied by CAMAG, Switzerland). The thickness of the layer was 0.5 mm.

The plates were run by descending chromatography in an air-tight chamber (polythene basin with glass cover). The solvent reservoirs were connected, both to the upper and lower ends of the plate, by filter paper wicks (Whatman 3 mm.), the upper wick having dimensions 10 cm. x 10 cm. and the lower wick 10 cm x 6 cm. The flow rate through the gel was regulated by the angle of the plate to the horizontal which was usually kept between 10 and 20 degrees. The flow rate was measured by applying a 1% solution of Blue Dextran. This compound proved to be useful, because it has the same Rf value as high molecular weight adenosine deaminase, both macromolecules being completely excluded from the gel. The exact position of this form of the enzyme was therefore known at the end of a run. Each run usually took 6-8 h., the flow rate being approximately 1.5 cm./h. Before

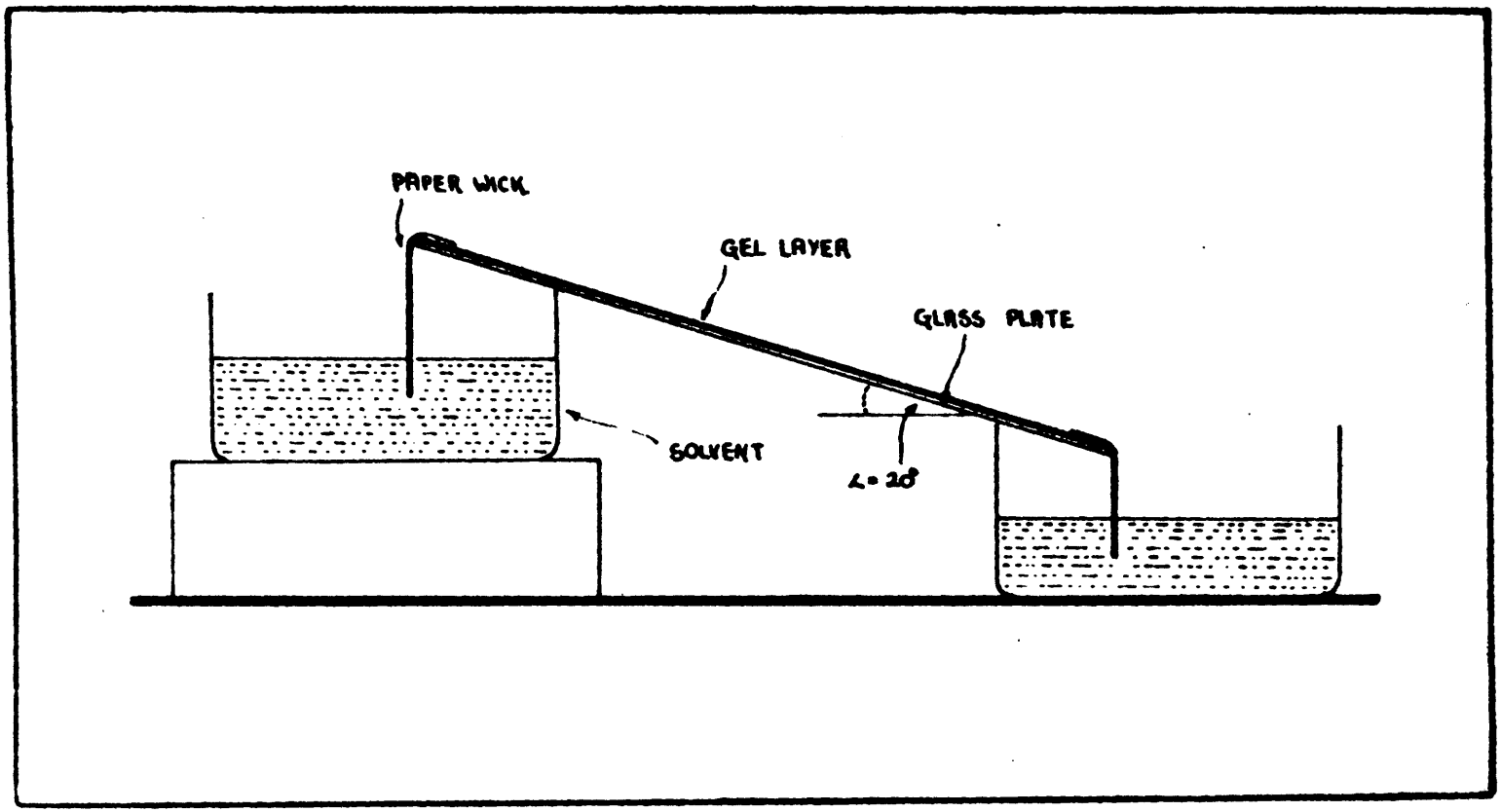


FIG. 3. Schematic representation of thin-layer gel filtration apparatus.

beginning an experiment, the plates were equilibrated by allowing the solvent to flow through the gel for about 15 h., a step which could be conveniently carried out over-night. After equilibration, the sample was applied with a fine-tipped micropipette, as a spot 1 cm. in diameter. The solvent system used was 0.05 M KCl pH 7.0.

After completion of a run the plate was removed from the apparatus and allowed to dry for about 10 min. It was then developed by a modification of the staining technique used in detecting the isoenzymes of adenosine deaminase separated by electrophoresis (Murphy, 1969). Instead of pouring the staining mixture (consisting in this case of 2.0 g. agar, 1.0 g. adenosine and 50 ml. satd. phenol violet) directly on to the thin-layer, it was found more satisfactory to pour it over a clean glass plate and allow it to solidify. A slab of solid gel was then cut from this and gently placed on the thin-layer plate, care being taken not to disrupt the layer. The sites of enzyme activity show up as blue spots on a yellow background.

Detection of protein zones after thin-layer gel filtration

Protein zones were located by a modification of the method used by Morris (Morris, 1964).

After chromatography the plate was supported horizontally and the wicks removed. A 10 x 20 cm. piece of cellulose acetate paper was then soaked in distilled water and partially dried. The moist paper was applied progressively to the gel surface starting near the origin and proceeding to the other end of the plate. Care had to be taken to avoid trapping air bubbles between the gel and the paper. The cellulose acetate was then removed and the proteins

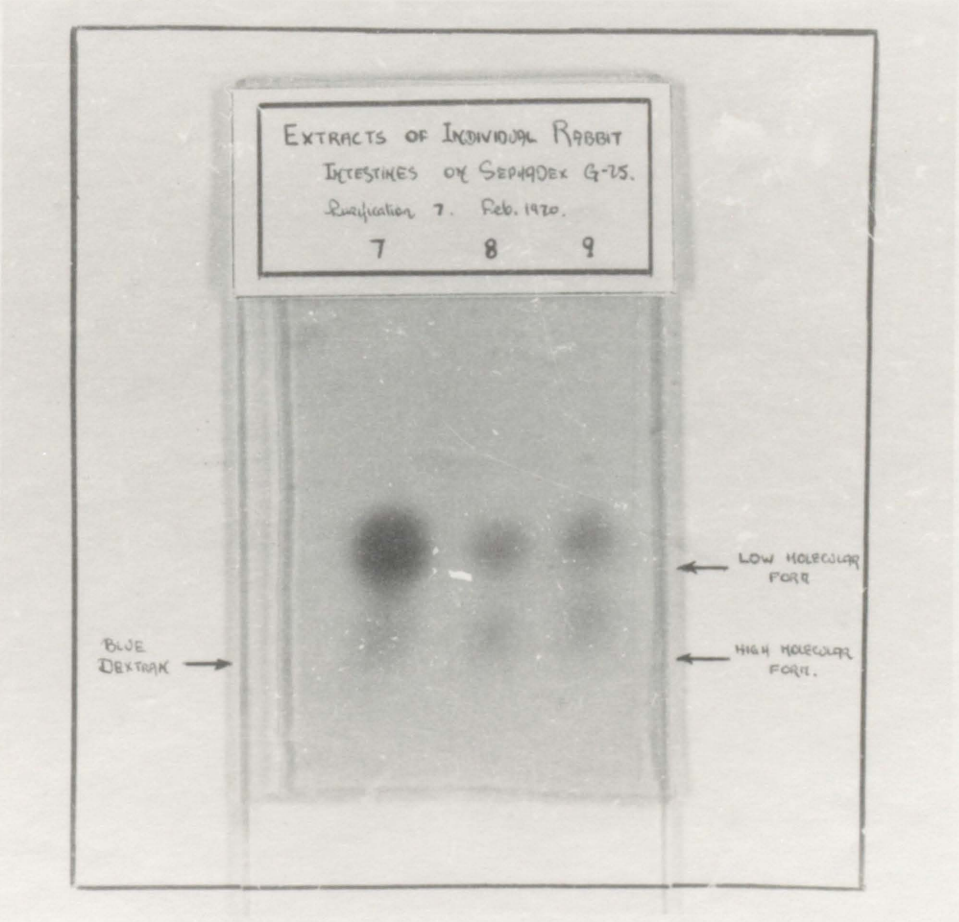


PLATE 1a SEPARATION OF THE HIGH AND LOW MOLECULAR WEIGHT FORMS OF RABBIT INTESTINAL ADENOSINE DEAMINASE BY THIN-LAYER GEL FILTRATION ON SEPHADEX G-75.

fixed by immediate immersion in 10% trichloroacetic acid. Staining was carried out as described on page 34 (using Method (iii)). A typical separation of the high and low molecular weight forms of the deaminase using the thin-layer gel filtration method is illustrated on Plate 1a. After chromatography the gel was stained using the zymogram staining method.

HIGH VOLTAGE ELECTROPHORESIS

Cellulose acetate paper (Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.) was supplied commercially in strips 40 cm x 5 cm. A half strip 20 cm. x 5 cm. was most suitable for separations. 30 mM phosphate buffer pH 7.0 was used in the buffer tanks and it was also used to moisten the paper connecting wicks, and the cellulose acetate strip. Care had to be taken to float the cellulose acetate strip on the buffer when wetting. If it was quickly submerged below the surface, air bubbles formed on portions of the paper, thus preventing it from becoming wetted, and unless the paper was dried thoroughly and re-wetted properly it could not be used.

After wetting, the cellulose acetate strip was placed between two sheets of absorbant paper to remove excess surface buffer, and transferred on to a perspex plate. Protein samples were applied to the strip by using a very small platinum wire loop or a narrow rectangular strip of cellulose acetate which had to be soaked in the protein solution and placed on the sheet. The samples were applied 4 cm. from one end of the strip, and 1 cm. in from the edge.

The wicks used were 60 cm. x 7 cm. strips of a very thin paper, having a low water absorbancy (Postlip filter paper, Technical Sales,

Ltd., London). These were folded over so that the dimensions were 30 cm. x 7 cm. The wicks were thoroughly wetted in the buffer and the excess moisture removed by blotting with absorbant filter paper. The cellulose acetate strip, to which the samples were applied, was then placed between the folds of the wicks, giving an overlap of about 1.5 cm. at the cathode end.

Both plates of the high voltage electrophoresis apparatus (Shandon Scientific Co. London) were covered with polythene sheets for insulation purposes. The cellulose acetate strip was placed on the plate of the electrophoresis apparatus. At pH 7.0 the enzyme ran towards the cathode. A polythene strip 6 cm. x 17 cm. was placed on top of the cellulose acetate, so that it covered the entire strip with the exception of the ends covered by the wicks. The top plate of the apparatus was clamped down to hold the cellulose acetate strip firmly.

A pressure of 14 lb./sq. in. was applied between the plates. The apparatus was then cooled by the passage of tap water through the plates. A potential of 4,000 V. was applied for 15 min. and the resultant current was usually between 10 and 15 mA. After the run (which normally took 15 min.) any moisture near the cellulose acetate was removed with absorbant paper and the strip transferred to a perspex plate. A strip of polythene 6 cm. x 22 cm. was placed over it, and any air bubbles formed between the cellulose acetate strip and the perspex plate were eased out by exerting slight pressure on the polythene strip. This was an essential step, because the pressure of air bubbles on the plate presented problems when testing for adenosine deaminase activity.

LOW VOLTAGE CELLULOSE ACETATE ELECTROPHORESIS

This technique is essentially the same as the high voltage method just described, except that the apparatus used is greatly simplified. The tank used (supplied by Shandon Scientific Co. Ltd.) had four compartments which were filled with buffer solution to a height of 2.5 cm. below the level of the cellulose acetate strip. Two buffer systems were employed depending on the type of experiment being carried out. 30 mM phosphate buffer pH 7.0 was used when isoenzyme patterns were being investigated, and when electropherogram and zymogram patterns were being compared. When staining for protein alone however, 50 mM barbitone buffer pH 8.6 was used, as this was found to give much sharper protein separations than the phosphate buffer system.

In this technique filter paper wicks were not used, the cellulose acetate strip being allowed to dip into the buffer at each end of the tank. The same procedure was adopted for wetting the strip and applying the protein as in the high voltage electrophoresis technique. In this method however, a voltage of only 300 V. was used, the running time being approximately 45 min.

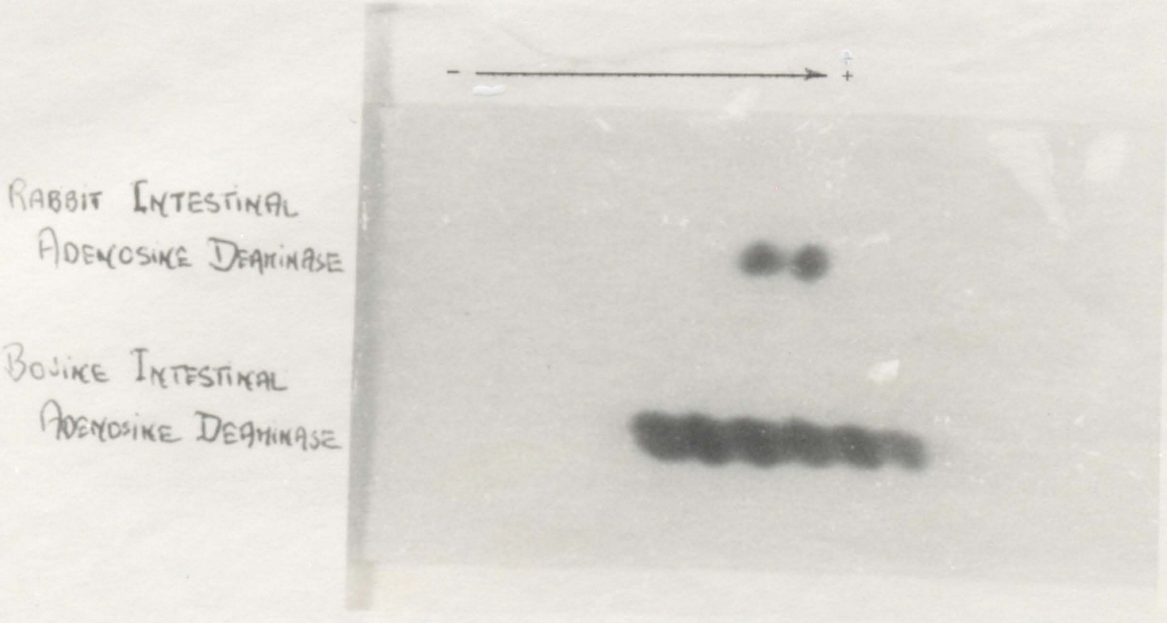


PLATE Ib SEPARATION OF THE ISOENZYMES OF ADENOSINE DEAMINASE BY HIGH VOLTAGE ELECTROPHORESIS ON CELLULOSE ACETATE PAPER. THE STAIN USED WAS THE ZYMOGRAM (PHENOL VIOLET) STAIN FOR ADENOSINE DEAMINASE.

LOCATION OF ADENOSINE DEAMINASE AFTER CELLULOSE ACETATE ELECTROPHORESIS

Adenosine deaminase was located on the paper using a modified form of the procedure used by Brady (Brady and O'Connell, 1962).

1.0 g. adenosine, 1.0 g. agar and 25 ml. saturated phenol violet were made up to 50 ml. with water and dissolved by heating to 90°C. It was cooled to 45°C and poured over the cellulose acetate strip. The reaction of the enzyme with adenosine, resulting in the production of ammonia changes the indicator from yellow to blue-black in those regions where the enzyme is located. A protein solution containing a minimum of 3 e.u./ml. was necessary to produce enough ammonia to change the colour of the gel. Plate 1b shows a separation (using the high voltage technique) of the isoenzymes of the low molecular weight form of rabbit intestinal adenosine deaminase as stained by this method.

PROTEIN STAINING TECHNIQUES FOR CELLULOSE ACETATE ELECTROPHORESIS

The following methods were used when staining for protein on the cellulose acetate strip.

(i) Ponceau S Stain : The cellulose acetate strip was immersed in a 0.02% solution of Ponceau S in 5% trichloroacetic acid

for 10 min. It was then removed, washed with 5% acetic acid and dried.

(ii) Nigrosine Stain : The cellulose acetate was placed in a 0.002% solution of Nigrosine in 2% acetic acid over-night. It was then removed, washed with running tap water and dried.

(iii) Ponceau S-Nigrosine Stain : This staining method is essentially a combination of the other two procedures. After staining the strip in the Ponceau S solution as above, it was removed and thoroughly washed with 5% acetic acid. It was then placed in the Nigrosine solution and the staining allowed to proceed overnight. This staining procedure used by Meighen (Meighen, 1970) gave much better results than Methods (i) and (ii).

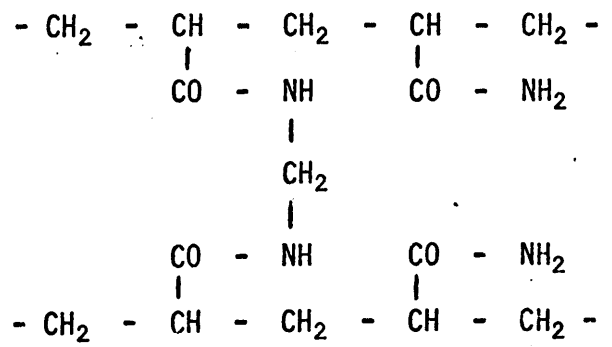
POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel as an electrophoretic medium was suggested by Raymond (1959) as an improvement over starch gel (Smithies, 1955), and is used either as horizontal or vertical slabs or in the "disc electrophoresis" microtechnique of Ornstein and Davies. Sharp separations of proteins and other mixtures have been reported. The following method is a modified form of the "disc" technique. In it both the "spacer" gel and the "large pore" gel of the Ornstein technique are omitted. Only microgram quantities of protein are required and electrophoretic separations are carried out vertically in homogenous

rod-shaped gels.

Procedure

Acrylamide gels were prepared by dissolving acrylamide monomer and cross-linking agent N, N'-methylene-bis acrylamide (BIS) in water. Properties are such that on addition of suitable catalyst-accelerator compounds, and exclusion of oxygen, strong gels are formed after 10-30 min. at room temperature. The gel consists of a three-dimensional network of long hydrocarbon chains, cross-linked at intervals by methylene groups:



The following working solutions were prepared.

(a) 30 g. acrylamide + 1 g. Bis + 123 ml. water
(b) 0.28% N, N, N', N'-tetramethylethylene diamine (V/V)
(c) 0.14% ammonium persulphate (W/V)
(d) 29 g. glycine + 6 g. Tris + 980 ml. water.

A 5% gel was obtained by mixing the working solutions in the following proportions:

2 vol. (a) + 1 vol. (b) + 4 vol. (c) + 1 vol. (d)

Before electrophoresis the tubes 5 mm x 35 mm were first of all cleaned with detergent, then rinsed with distilled water and finally rinsed in a 1 : 200 solution of "Photo-flo" solution and allowed to dry. The bottom end of each tube was then sealed with an inert polythene cap. The prepared acrylamide monomer solution

was quickly pipetted into each tube, filling each to the same mark (10 mm. from the top). Each tube was then overlayered with distilled water, this operation being completed within 10 minutes of adding the persulphate catalyst to the monomer solution. The function of the water layer is twofold: to exclude oxygen which would inhibit gelation of the top surface of the gel solution and also provide a flat surface (interface) with no meniscus so that the subsequent gel face is flat and the protein sample 'stacks' at a perfectly flat origin at right angles to the direction of electrophoretic migration. Polymerization in the tubes was indicated by the formation of a refractile boundary between the top of the gel and the water layer. The cap was then removed from the end of the gel tube, taking care to avoid disturbing the gel by suction. The overlayered water was poured off and the gel surface washed with a gentle stream of distilled water. The gel surface was never allowed to dry out.

Electrolyte used in the electrophoresis was 1:10 dilution of a stock solution prepared as follows:

29 g. glycine
6.0 g. Tris
5 ml. 1 N HCl
975 ml. distilled water

The pH of the diluted stock solution was then adjusted to 8.1. The lower tank of the electrophoretic apparatus was filled with electrolyte, and the gel tubes fitted into position. The protein solutions were mixed with an equal volume of 6% sucrose (containing a trace of bromophenol blue). After application to the gel surface, the protein solution was then carefully layered with the electrolyte, filling each tube to the top. The upper tank was next filled with

electrolyte, and the electrodes connected to the power supply.

A current of 2 ma/tube was used until the bromophenol blue had decended into the gel. It was then increased to 5 ma/tube and electrophoresis allowed to proceed until the dye had almost reached the end of the tube. This took approximately 20 min.

After electrophoresis, the gels were removed from the tubes by squirting water from a syringe between the gel and glass wall, and by using a pipette bulb to exert pressure.

Proteins were fixed and stained by immersion of the gels in 0.1% Amido Black in 7% acetic acid for 1 h. Stained gels were cleared of residual dye by leaching in 7% acetic acid.

Zymogram stain for adenosine deaminase

A modification of the zymogram stain developed by Harris for starch gel electrophoresis (Harris, 1968) was used.

0.2 g. agar and 10 mg. adenosine were added to 15 ml. phosphate buffer pH 7.5 and the mixture heated to 90^{o}C. On cooling to 40^{o}C, 8 µl nucleoside phosphorylase, 8 µl xanthine oxidase, 2 mg. tetrazolium salt (MTT) and 2 mg. phenazine metasulphate were dissolved in 5 ml. phosphate buffer pH 7.5 and added to it. The acrylamide gels were then placed in tubes containing this staining mixture, and the tubes kept in the dark for about 5 min. Sites of enzyme activity showed up as blue bands on a yellow background.

Satisfactory photographs of these gels could not be obtained as the yellow background quickly turned blue due to photo-oxidation of the dye. However, the number of bands present could be counted.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

Electrophoresis in polyacrylamide gels in the presence of the anionic detergent sodium dodecyl sulphate (SDS) has proved to be a useful tool for the separation and identification of polypeptide chains (Maizel, 1966; Shapiro, 1966; Vinvela, 1967). The following method is a modification of the technique developed by Weber (1969).

Preparation of protein solutions.

The proteins were incubated at 37°C for 2 hours in 0.01M sodium phosphate buffer pH 7.0, 1% in SDS and 1% in β-mercaptoethanol. After incubation the protein solutions were dialysed over-night against 500 ml. of 0.01M sodium phosphate buffer pH 7.0 containing 0.1% SDS and 0.1% β-mercaptoethanol.

Preparation of gels.

Gel buffer contained 8.8 g. NaH_2PO_4 $2H_2O$, 20.5 g. Na_2HPO_4 and 2g. SDS per litre of water. 11.1 g. of acrylamide and 0.3 g. of methylene-bis acrylamide (BIS) were dissolved in water to give 100 ml. of solution. The glass gel tubes were 10 cm. long with an inner diameter of 6 mm.

For a typical run the following mixture was prepared:

15 ml. gel buffer
13.5 ml. acrylamide solution
1.5 ml. ammonium persulphate (15 mg./ml.)
0.5 ml. N, N, N', N'-tetramethylethylenediamine(TEMED)

After mixing, each tube was filled with 2 ml. of this solution, which was then overlayed with water. After 10 to 20 min. an interface

could be seen indicating that the gel had solified.

Preparation of samples.

Prepared a solution containing the following reagents:

1 ml. 0.05% Bromophenol Blue
20 ml. glycerol
1.6 ml. mercaptoethanol
16 ml. dialysis buffer

The protein solution to be analysed was diluted 1:1 with this mixture, and applied to the gels. The two compartments of the electrophoresis apparatus were filled with gel buffer, diluted 1:1 with water. Electrophoresis was performed at a constant current of 8 ma per gel with the positive electrode in the lower chamber. Under these conditions the marker dye moved three-quarters through the gel in approximately 4 hours.

Staining and destaining.

After removing the gels from the glass tubes the position of the dye front was marked by inserting a piece of copper wire into the gel. The gels were placed in small tubes filled with staining solution prepared by dissolving 2.5 g. Amido Black in a mixture of 908 ml. of 50% methanol and 72 ml. of glacial acetic acid. Staining was at room temperature for a period of 1 h. The gels were removed from the staining solution, rinsed with distilled water and placed in destaining solution (908 ml. of 50% methanol and 72 ml. of acetic acid). After destaining, the gels were placed in 7% acetic acid for a few hours to enable them to swell to their original size(destaining caused shrinkage).

Measurement of protein mobilites

The mobilites of individual proteins after electrophoresis were calculated using the following equation:

$$\text{Mobility (cm.)} = \frac{6.5 \times \text{Distance travelled by protein (cm.)}}{\text{Distance travelled by dye (cm.)}}$$

6.5 (cm.) was an experimentally determined value of the distance normally travelled by the dye under the above experimental conditions. However, the value sometimes varied from one experiment to another and all mobilites were therefore re-calculated using the figure 6.5 as a standard dye mobility. Distance travelled (both for the dye and the proteins) was measured from the origin to the leading edge of the band (marked by the piece of copper wire).

UREA-POLYACRYLAMIDE GEL ELECTROPHORESIS

The following gel reagents were prepared:

(A)	Acrylamide 45g. N,N'-methylene bisacrylamide 5g. Water to 500 ml.
(B)	0.1 M ammonium persulphate
(C)	5% TEMED (v/v)
(D)	1.0 M Tris half neutralized with HCl (pH 8.1 approx.)

When preparing the gels 12 ml. (A), 0.5 ml. (C), 1.5 ml. (D), 1.0 ml. water and 15 g. urea were mixed, warmed to 20^{0}C and the solution then degassed. Polymerization was initiated by the addition of 0.1 ml. (B), and the mixture immediately pipetted into the gel

tubes. Water was layered onto the gel surface in the usual way. (Setting time was about 15 min.).

The samples (10-100µl per gel) were loaded in 8M urea containing 0.1M Tris half neutralized with HCl (pH 8.1). The cathode buffer contained 0.1M Tris base and 0.1M glycine pH 9.0 approximately, and the anode buffer contained 1.0 M Tris chloride pH 8.1. The current was 2 ma/tube initially and later 3 ma/tube. The running time was about 1h. during which time the bromophenol blue added with the sample migrated about 6 cm. The gels were ejected from the tubes in the usual manner, stained with 1% Amido Black in 7% acetic acid, and then destained using 7% acetic acid.

PHOTOGRAPHIC RECORDING OF ZYMOGRAMS AND ELECTROPHEROGRAMS

These were photographed with a 35 mm Durst model camera attached to its enlarger, loaded with 35 mm Ilford FPS film. The exposure time was 1/10 sec. at an aperture of F11. The patterns to be photographed were illuminated with four 150 watt enlarger lamps. When zymograms were being photographed, a yellow filter was used to increase contrast.

DENSITOMETRY

Densitometer tracings of both electrophoretic and chromatographic patterns were obtained using a Joyce Lobel Chromoscan.

RADIOACTIVITY MEASUREMENTS

Radioactivity was measured using a Nuclear Chigago Gas Flow Detector (Model 480) with a Basic Binary Scale (M 161 A) attachment. A voltage of 12,000 V and a gas pressure of 6 lb./ sq. inch were used. 0.5 ml. of each radioactive fraction was pipetted onto an aliminium planchet which was placed in the detecting chamber. The number of counts was read over a period of 10 min. from which the counts/min. could be determined.

HYDROLYSIS OF PROTEINS

Heavy walled Pyrex tubes (16 mm x 125 mm) were washed with chromic acid, rinsed in distilled water and finally rinsed in IN HCl. The residual HCl was removed in an air oven at 100^{o}C and the tubes stored inverted in a covered container. The weighed lyophilized protein sample was then placed in a tube and 1 ml. of constant boiling HCl (5.7N) added. The tube was evacuated using a mechanical pump, dearation being controlled by cooling the tube in an acetone-dry ice mixture. When bubble formation ceased the tube was sealed and heated at 110^{o}C in a thermostatically controlled oil-bath for a definite period of time. After hydrolysis the tube was removed, cooled to room temperature, and any liquid adhering to the walls was spun down by gentle centrifugation. The hydrolysate was then concentrated to dryness with a rotary evaporator.

AMINO ACID ANALYSIS

Amino acid analysis was performed according to Moore and Stein (1963) using a Lochart automatic amino acid analyser equipped with an automatic loading system. A complete amino acid analysis was performed on a single column using the following discontinuous buffer system: 0.2 N sodium citrate buffer pH 3.25 and 0.2N sodium citrate buffer pH 4.25 were used to elute the acidic and neutral amino acids and 0.35 N sodium citrate buffer pH 6.65 was used to elute the basic amino acids. The column was packed with a nominal 10% cross-linked resin (Permutit Zeo Carb 225). The eluted amino acids were detected by reaction with ninhydrin, the buffer and ninhydrin reagent being metered by Milton Roy Minipumps. The coloured complex was developed by passage through a coil of Teflon tubing submerged in a boiling water bath and estimated by passage through a flowthrough colorimeter, the results being presented on a Kent multipoint strip chart recorder. A complete analysis including column regeneration and equilibration took 8½h. The colour constants for each amino acid were determined using a solution containing 25 n moles of each amino acid (12.5 n moles of cystine were used).

RESULTS

SECTION 1

PRELIMINARY STUDIES

LEVEL OF ADENOSINE DEAMINASE ACTIVITY IN VARIOUS TISSUES

The level of enzyme activity in various rabbit tissues has already been investigated (Conway and Cooke, 1939; Clarke, 1952; Brady and O'Donovan, 1965). Spleen and duodenum were found by these workers to have highest concentrations of the enzyme. In the present investigation a small number of tissues, brain, spleen, liver and duodenum were selected for study, as possible starting material for purification of the enzyme. Both young and mature laboratory bred animals were examined.

1g. portions of each tissue were homogenised with 5 ml. glass distilled water, allowed to extract for 2 h. and centrifuged at 13,000 r.p.m. for 10 min. The supernatants were assayed for enzyme activity by the Conway Microdiffusion Method, and protein determined by the Biuret.

Table 1 shows the results obtained.

TABLE 1. Activity of Adenosine Deaminase in Rabbit Tissues.

Tissue	Activity (e.u./g. of Fresh Tissue.) Young Animal	Mature Animal
Duodenum	16.5 - 17.8(2)	15.0 - 18.0(3)
Spleen	6.6 - 10.4(4)	8.5 - 10.4(3)
Brain	0.6 - 1.3 (4)	2.1 - 2.5 (3)
Liver	0.5 - 1.1(4)	0.5 - 0.8 (3)

The figures in parentheses represent the number of animals studied.

The level of activity in the tissues studied shows a considerable fluctuation, the duodenum having the highest level and the liver the lowest. In table 2 the results obtained are compared with previously published data:

TABLE 2. Comparison of Adenosine Deaminase Activities in Rabbit Tissues with the Results of Previous Surveys.

Tissue	Clarke et al (1952)	Conway & Cooke (1939)	Brady & O'Donovan (1965)	Present Study	
				Mature	Young
Duodenum	-	4.6	10.7	16.2	17.2
Spleen	-	6.3	11.6	9.6	8.4
Brain	1.3	1.3	2.7	2.2	1.1
Liver	0.3	0.6	0.4	0.7	0.7

Values given are e.u./g. of fresh tissue

There is reasonable agreement between the results for spleen, brain and liver. However, the value obtained for the duodenum in the present study is considerably higher than that obtained in other surveys. An interesting point revealed in this investigation was the fact that the level of enzyme activity in the brain of the mature animal is twice that in the young. This may reflect an increase in enzyme activity during development of the animal. Another unusual feature of the rabbit brain is that the level of deaminase activity is almost tenfold the average activity of cat, dog, mouse, guinea-pig and rat brain (Brady and O'Donovan, 1965).

DISTRIBUTION OF ENZYME ACTIVITY IN THE INTESTINE

During preliminary studies carried out in this laboratory on the rabbit intestinal adenosine deaminase, the duodenum (approximately 20 cm. in length) was used as a source of the enzyme(Murphy, 1967; Meegan, 1969). However,the quantity of enzyme obtained from a single duodenum is so small that a considerable number of animals would be required for a purification from this source. It was therefore, with a view to obtaining more of the enzyme that a study of its distribution in the intestine was carried out.

The combined duodenum and small intestine were removed from a mature rabbit (as described), and cut into 12 sections (each 15 cm. in length). From each section extracted 0.5g. tissue with 4.5 ml. distilled water. After centrifugation the level of activity in the supernatants was estimated by the microdiffusion technique.

Fig. 4 shows the results obtained.

The plot shows that the upper small intestine contains a considerable amount of enzyme, the level of activity being almost as high as the duodenum in parts. The level falls off gradually towards the end of the ileum and the jejunum. The distribution of deaminase activity in porcine intestine shows a similar pattern (Noonan, unpublished data). These results however, differ from studies on the cat, rat and guinea-pig intestines, the level of activity in the jejuna of these animals being the same as in the duodena (Brady and O'Donovan, 1965).

Throughout the work on the purification of the rabbit enzyme therefore, the first 54 cm. (approx.) of intestine was used. This gave a much greater quantity of enzyme (Note: the duodenum is included

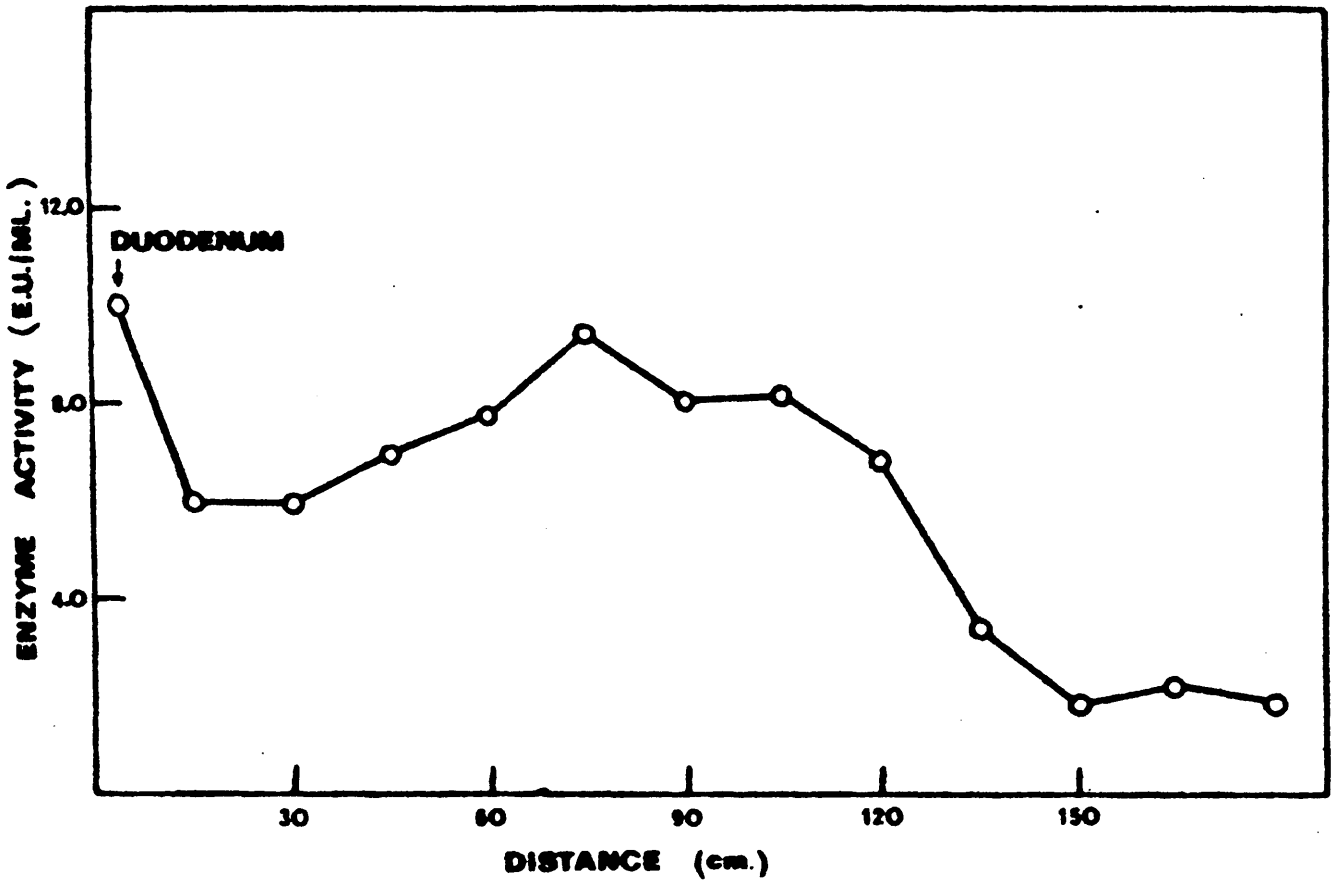


FIG. 4. Distribution of adenosine deaminase activity along the small intestine of the rabbit. Portions of tissue (0.5 g.) taken at 15 cm. intervals were extracted (dil. 1:10) and the activity of the supernatant fluid obtained after centrifugation estimated using the Microdiffusion Method of assay (pH 7.0).

in this length of intestine).

In the purification of adenosine deaminase from bovine intestine (Brady, 1962), the mucosa of the intestine was used as a source of the enzyme. This was not feasible with the rabbit intestine because of the small quantities of mucosa which can be obtained. A comparison was made however, of the level of activity in the intestinal wall and the mucosa.

Resuls are shown on Table 3.

TABLE 3. Level of Adenosine Deaminase Activity in Mucosa and Intestinal Wall Extracts of Rabbit.

Extract	e.u./ml.	Total e.u.	Sp. Activity(e.u./mg.)
Mucosal	0.95	4.75	0.11
Intestinal Wall	0.84	4.20	0.06

It can be seen that there is no significant difference in the level of activity between the mucosa and intestinal wall. However, the specific activity of the mucosal extract is almost double that of the intestinal wall extract, indicating that the mucosa would be a better source of the enzyme for purification providing enough of it could be obtained.

DISTRIBUTION OF THE TWO FORMS OF THE ENZYME IN THE SMALL INTESTINE, AND IN OTHER RABBIT TISSUES

Murphy and Brady (1969) have presented evidence that rabbit intestinal adenosine deaminase could be separated by gel filtration into two components: a high molecular weight one and a low molecular weight one. A typical Sephadex G-75 elution pattern is illustrated in Fig. 5. The high molecular weight form, being excluded from the gel, is eluted at the void volume of the column, and is followed by the low molecular weight peak of activity.

The distribution of the two molecular forms of the enzyme in different regions of the small intestine was investigated. Aqueous extracts (dilution 1:5) of duodenum, ileum and jejunum were made, and after centrifugation, samples of the supernatant applied to a previously equilibrated Sephadex G-75 coated thin-layer plate. 0.05 M KCl was used as solvent. After chromatography the enzyme zones were located by the zymogram stain for adenosine deaminase (p. 29)

Results are shown on Plate 2.

The ratio of the two molecular forms differs in the three regions of the intestine, the low molecular weight form being of highest concentration in the duodenum, and the high molecular weight form contributing to most of the activity in the jejunum. These results were obtained with four rabbits (two young and two mature) so that the pattern would appear to be a fairly general one.

The ratio of the molecular forms was also compared in the mucosal scrapings and the intestinal wall. However, no apparent difference could be detected between them.

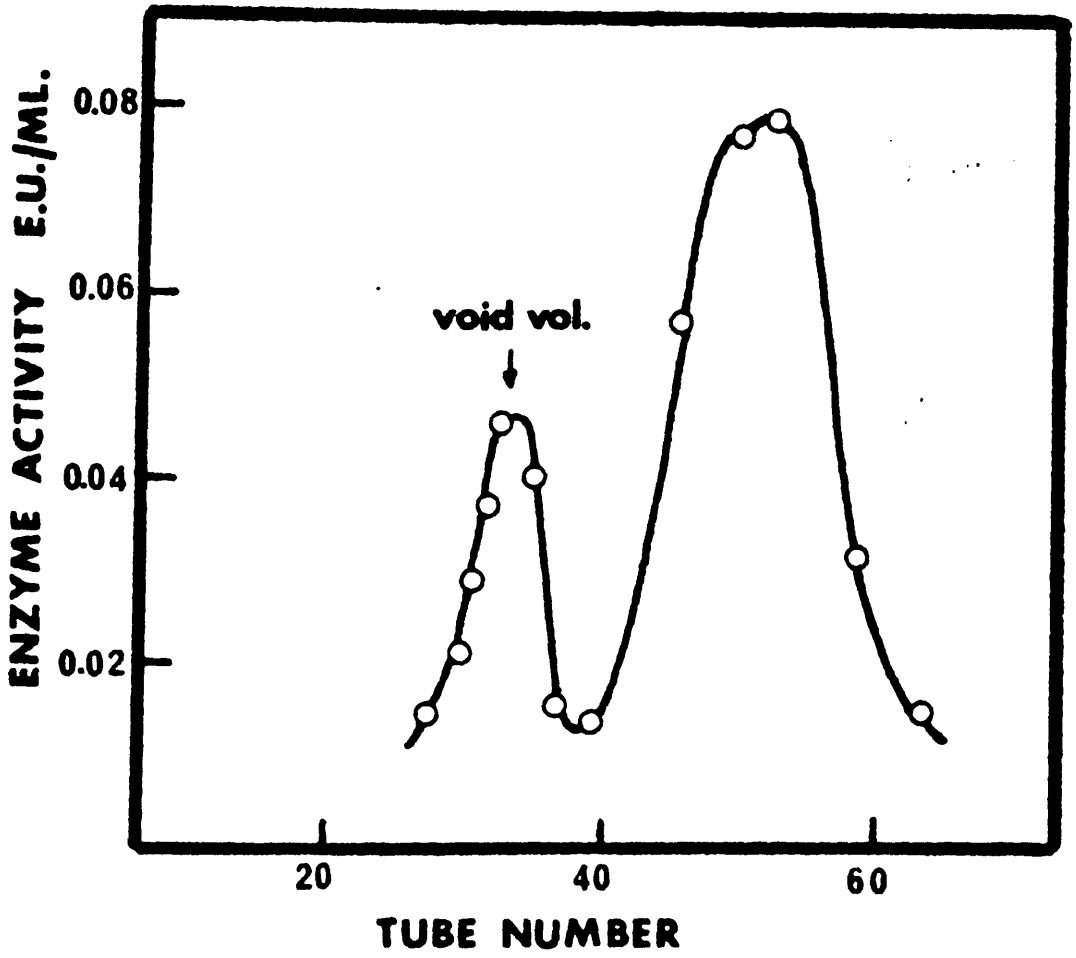


FIG. 5. Gel filtration of adenosine deaminase on Sephadex G-75(coarse). 3 ml. of an aqueous extract (dil. 1:5) of rabbit intestine was applied to the column which was equilibrated with 0.04 M Tris buffer pH 7.4 containing 0.24 M KCl. 3 ml. fractions were collected and enzyme activity estimated using the Microdiffusion Method of assay (pH 7.0).

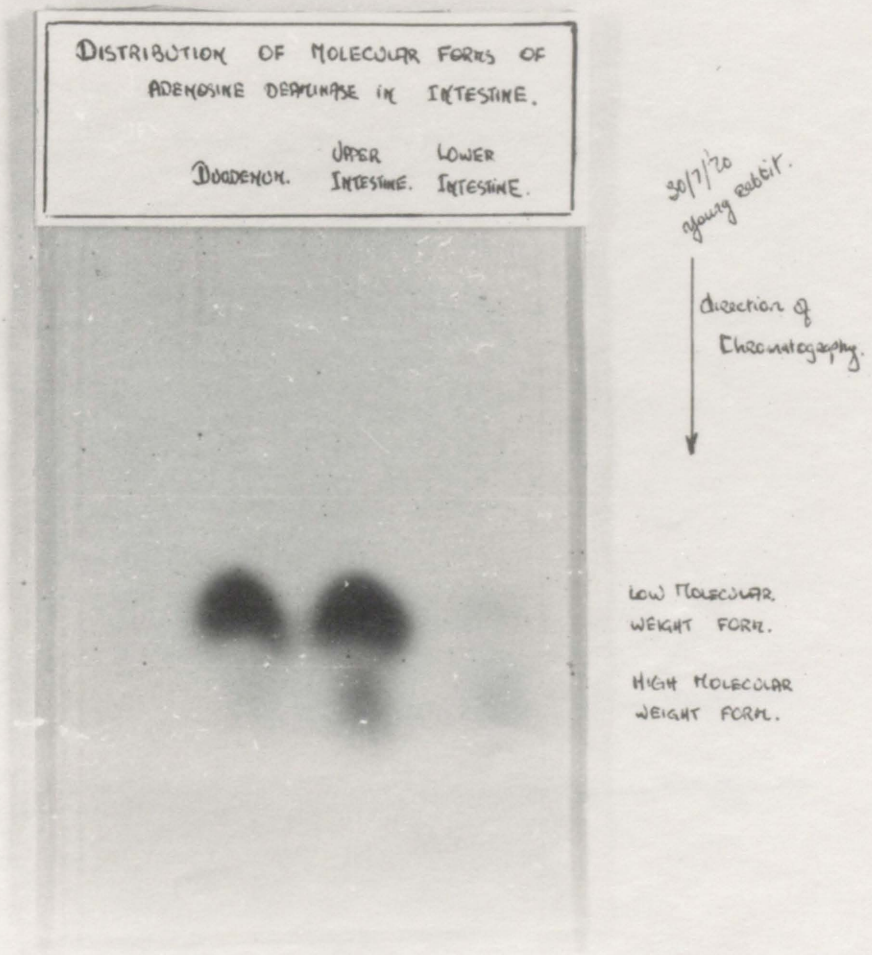


PLATE 2. THIN-LAYER GEL FILTRATION PATTERNS OF EXTRACTS FROM VARIOUS REGIONS OF THE RABBIT SMALL INTESTINE. THE STAIN USED WAS THE ZYMOGRAM (PHENOL VIOLET) STAIN FOR ADENOSINE DEAMINASE.

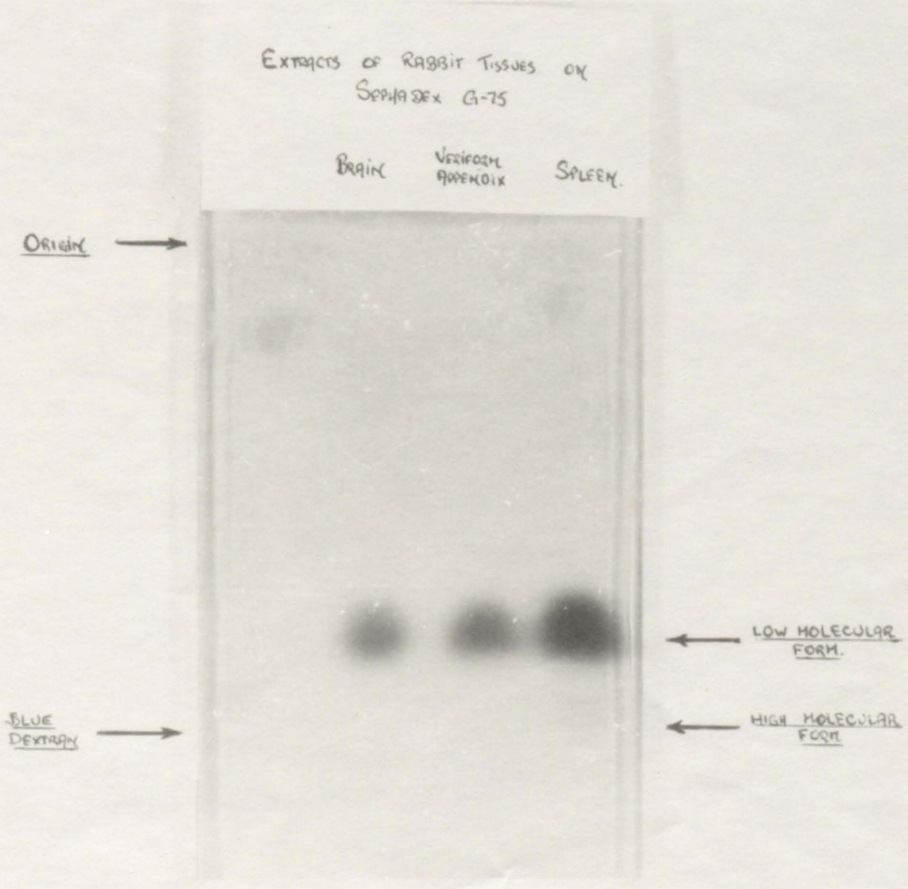


PLATE 3. THIN-LAYER GEL FILTRATION PATTERNS OF AQUEOUS EXTRACTS OF VARIOUS RABBIT TISSUES. THE STAIN USED WAS THE ZYMOGRAM (PHENOL VIOLET) STAIN FOR ADENOSINE DEAMINASE.

Distribution in other tissues.

Brain, veriform appendix and spleen were the only other rabbit tissues which were active enough to give results using the thin-layer gel filtration method. Aqueous extracts were prepared as before and applied to the plate. The patterns obtained are shown on Plate 3. No high molecular weight enzyme could be detected in either brain or veriform appendix, but a small amount was found to be present in the spleen preparation. This presumably is a similar enzyme to the high molecular weight adenosine deaminase found in the intestine as it moved to the same position as the Blue Dextran marker.

Rabbit liver, shown by Ma (Ma, 1969) to have almost all of its adenosine deaminase activity in the high molecular weight form, could not be analysed by this method as the activity in this tissue is extremely low (0.5 e.u./g. of fresh tissue). However, as will be seen later, preliminary steps in the purification of the rabbit liver enzyme have been worked out, and Ma's finding was verified.

EXTRACTION OF ADENOSINE DEAMINASE FROM RABBIT INTESTINE

A number of different extraction procedures were carried out in order to obtain an extract suitable for purification.

The purification scheme for the bovine enzyme developed by Brady (Brady, 1962) employed the use of acetone-dried powders of the intestinal mucosa as an enzyme source. These acetone-dried powders have the advantage that enzyme activity is retained indefinately when stored in this form. In the following experiment the whole intestine and an acetone-powder of the intestine were compared as sources of the rabbit enzyme.

Aqueous extracts of whole intestine and an acetone-powder of the intestine were prepared, and the activities of the extracts estimated. Results are shown on Table 4.

TABLE 4. Extraction of Adenosine Deaminase From Rabbit Intestine.

	Adenosine Deaminase Activity	
	Total e.u.	% Recovery
Aqueous extract	24.6	100
Acetone-dried intestine	7.6	31

Compared with aqueous extraction there is a marked reduction in activity after acetone treatment, only 31% recovery being obtained. This is very different from bovine mucosal deaminase which is not inactivated by acetone treatment (Hegarty, 1962).

Rabbit intestine differs from bovine intestine in that the former possesses a high molecular weight form of adenosine deaminase, not present in the bovine. The possibility that this form of the enzyme may be either compartmented or membrane bound was investigated by extracting intestinal homogenate with the following extractants:

Glass distilled water
1% Triton x-100
0.025 M sucrose
Trypsin (final concentration 0.5 mg:/ml.)

After allowing the extracts to stand at $4^{o}C$ overnight, they were centrifuged (10 min. at 15,000 r.p.m.) and the supernatants assayed for both adenosine deaminase and alkaline phosphatase activities.

Table 5 shows the results obtained.

TABLE 5. Extraction of Adenosine Deaminase and Alkaline Phosphatase from Rabbit Intestine.

	Adenosine Deaminase		Alkaline Phosphatase	
Extractant	ΔE/min.	% Recovery	ΔE/min.	% Recovery
Distilled water	0.28	100	0.16	100
1% Triton x-100	0.25	90	0.20	125
0.025 M Sucrose	0.30	107	0.20	125
Trypsin (solution)	0.30	107	0.19	120

It can be observed that there is little difference in the yields of adenosine deaminase obtained with the above extractants. Trypsin digestion increased the deaminase concentration by 7% and the alkaline phosphatase by 20%. These results contrast with those obtained by Brady (Brady and O'Connell, 1962) who found that digestion of bovine intestinal extracts with trypsin gave no increase in deaminase, but produced a 500% increase in alkaline phosphatase. The alkaline phosphatase in rabbit intestine is therefore apparently different from that found in bovine intestine.

The ratios of the molecular forms of adenosine deaminase in the above extracts were compared. After applying the extracts to a previously prepared thin-layer plate (Sephadex G-75 using 0.05 M KCl as solvent), development was allowed to proceed and the adenosine deaminase zones located by the zymogram stain. There was no apparent difference in the ratio of the two enzyme forms in each extract.

It would appear therefore from these results that the two forms

of the deaminase in rabbit intestine cannot be differentially extracted from this tissue using Triton x-100, sucrose or digestion with trypsin.

EXTRACTION USING ACID AND ALKALINE BUFFERS

In attempting to find a suitable extractant for large scale purification work the use of acid and alkaline buffers was investigated. Pancreatic enzymes have been extracted with dil. H_2SO_4 (Northrop, 1948), and a better yield of glyceraldehyde phosphate dehydrogenase was obtained by extracting muscle with dilute alkali than with water (Corri, 1948). In the present study the use of the following buffer systems was investigated and compared with aqueous extraction:

a) 0.2 M Acetate pH 5.5
b) Water
c) 0.2 M Tris pH 8.3

The intestine was homogenised with one volume of the buffers, A further four volumes were added and after centrifugation the enzyme and protein concentrations were estimated. Table 6 gives the Results obtained.

TABLE 6. Extraction of Adenosine Deaminase from Rabbit Intestine.

Extractant	Deaminase Activity e.u./ml	Protein Conc. mg/ml.	Sp. Activity e.u./mg
0.2 M Acetate pH 5.5	0.86	5.0	0.17
Water	0.82	5.8	0.14
0.2 M Tris pH 8.3	1.06	12.3	0.08

The specific activities show considerable variation, the value of the acetate extract being double that of the Tris extract. Although more enzyme was extracted with the pH 8.3 buffer, there was also a considerable amount of unwanted protein extracted. 0.2M acetate pH 5.5 was therefore chosen as the most suitable extractant because it gave a slightly higher specific activity than water alone.

The standard extraction procedure adopted for the purification of the enzyme was the following: the rabbit intestine was homogenised with 1 volume of 0.2 M acetate pH 5.5. Four volumes of the buffer was then added to the homogenate which was allowed to stand (with intermittent shaking) at $4^{o}C$ for approximately four hours. After centrifugation at 10,000 r.p.m. for 20 min. the supernatant was retained.

SECTION II

PURIFICATION OF THE HIGH AND LOW MOLECULAR WEIGHT FORMS OF THE DEAMINASE

PURIFICATION OF THE LOW MOLECULAR WEIGHT FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE

UNIVERSITY COLLEGE LIBRARY CORK

PURIFICATION 1

ACETONE FRACTIONATION OF THE CRUDE EXTRACT

One of the methods frequently used in the purification of enzymes is fractional precipitation using organic solvents. Since these reagents inactivate most enzymes at room temperature (acetone has already been shown to inactivate the rabbit intestinal adenosine deaminase. P. 53)special precautions were taken to keep the temperature as low as possible.

Pilot Acetone Fractionation

10 ml. of an acetate extract of the intestine was used. Ice-cold acetone was added gradually to the cold extract with intermittent shaking to prevent localized concentration of the solvent. The following fractions were collected:

50 volumes % acetone	Fraction 1
90 volumes % acetone	Fraction 11
170 volumes % acetone	Fraction 111
220 volumes % acetone	Fraction 1V

After addition of the 50 V.% acetone (5 ml.) the precipitate was quickly shaken and allowed to flocculate for 30 min. at $4^{o}C$ before centrifugation (20 min. at 10,000 r.p.m.) in a refrigerated centrifuge. The precipitate was dissolved in a minimum volume (3 ml.) of distilled water (Fraction 1). The supernatant was cooled in a freezing mixture with the minimum of delay and ice-cold acetone added to 90 V%. The precipitate was again allowed to flocculate for 30 min. in the cold and centrifuged for 20 min. at 10,000 r.p.m. in a refrig-

erated centrifuge. Having dissolved the precipitate in water (Fraction II) the same procedure was adopted for 170 volumes% and 220 volumes % as for the previous fractions.

The dissolved precipitates were dialysed overnight against distilled water to remove excess acetone before analysis. Results of the fractionation are shown in Table 7.

TABLE 7. Pilot Acetone Fractionation of Crude Extract.

	Vol. (ml)	Total e.u.	Sp. Act. e.u./mg.	Yield %	Purification factor	
Acetate Extract	10	13.2	0.20	100.0	1	
Fraction I	3	1.3	0.05	9.5	-	
Fraction II	5	4.0	0.59	33.2	2.9	4.0
Fraction III	3	4.6	1.15	30.8	5.8	
Fraction IV	3	0.0	0.00	0.0	0.0	

The bulk of the deaminase was precipitated in Fractions II and III, a recovery of 64% and a four-fold purification being obtained. It is interesting to note (in view of the fact that two enzyme forms are present) that approximately 50% of the recovered activity was in Fraction II and 50% in Fraction III.

Large Scale Acetone Fractionation

A small volume of the acetate extract was concentrated (using a DEAE cellulose column,(2.5 cm. x 1 cm.) and applied to a Sephadex G-75 (coarse) column. The elution pattern is shown in Fig. 6.

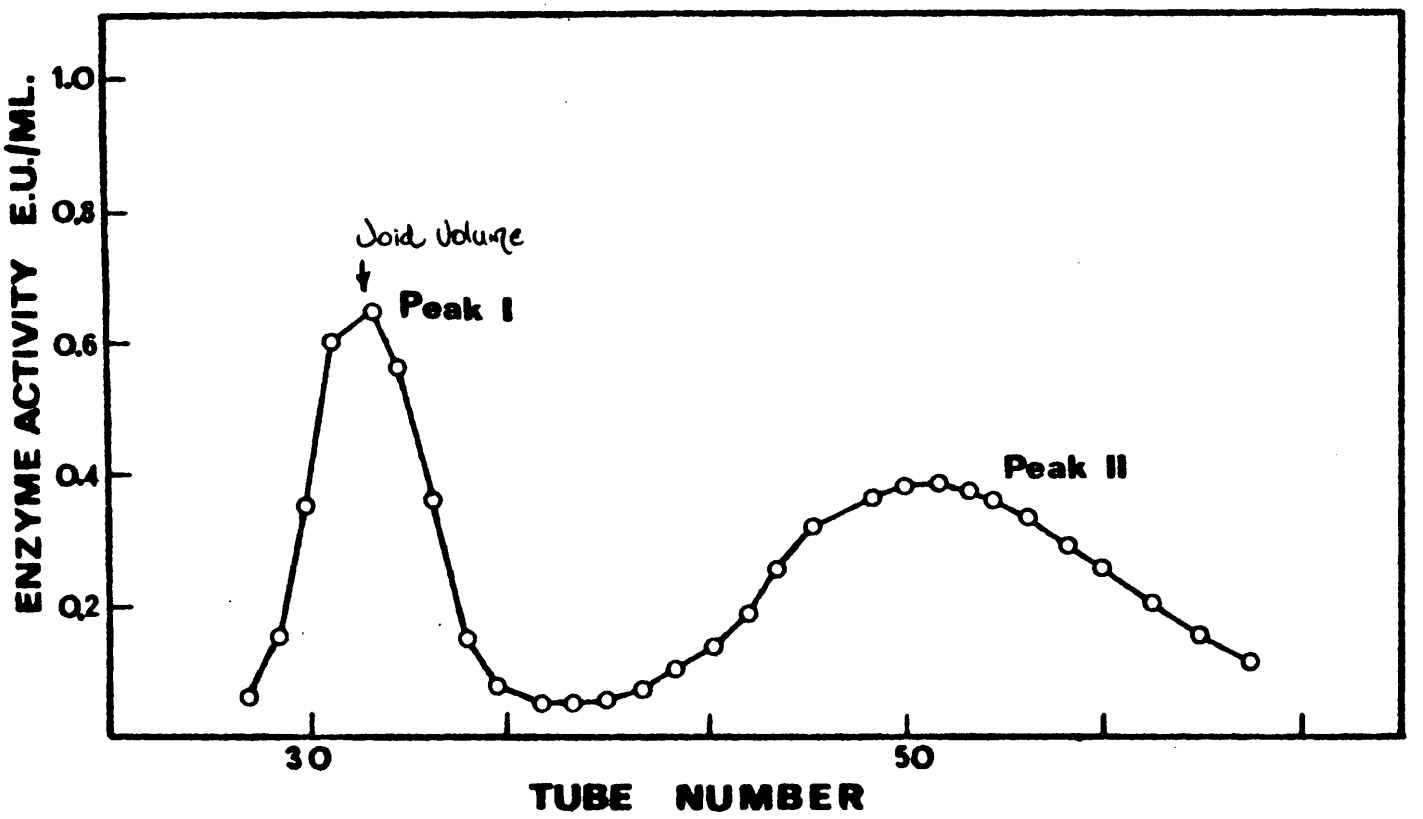


FIG. 6. Gel filtration of adenosine deaminase on Sephadex G-75 (coarse). 3 ml. of a concentrated acetate extract of rabbit intestine was applied to the column which was equilibrated with 0.04 M Tris buffer pH 7.4 containing 0.24 M KCl. 3 ml. fractions were collected and enzyme activity estimated using the Microdiffusion Method of assay (pH 7.0).

Measurement of the area under the peaks showed that 40% of the activity was present in Peak I (High molecular weight form) and 60% in Peak II (Low molecular weight form).

100 ml. of crude acetate extract (containing both forms of the enzyme as shown) was fractionated with acetone as already described. Results are shown in Table 8.

TABLE 8. Large Scale Acetone Fractionation of Crude Extract.

	Total e.u.	Sp. Act. e.u./mg.	Yield %	High Mol. Wt. form(%)	Low Mol. Wt.Form(%)
Acetate Extract	131.7	0.20	100	40	60
50-90% Acetone	28.6	0.29	21.8	88	12
90-170% Acetone	44.8	1.02	34.0	1	99

In this fractionation 21.8% of the activity was recovered in the 50-90% acetone fraction and 34% in the 90-170% acetone fraction, giving an overall recovery of only 56%.

3 ml. of each fraction was next applied to the Sephadex G-75 column. Fig. 7 shows the elution patterns obtained. It can be observed that separation of the two forms was achieved by acetone fractionation, the high mol. wt. form being almost exclusively present in the 50-90% acetone fraction, and the low mol. wt. form in the 90-170% acetone fraction.

Despite the fact that excellent separation of the two enzyme forms was achieved, the yield of enzyme after acetone fractionation was very low. In an effort to obtain better yields, further fractionations were carried out, taking extra precautions to keep the temp-

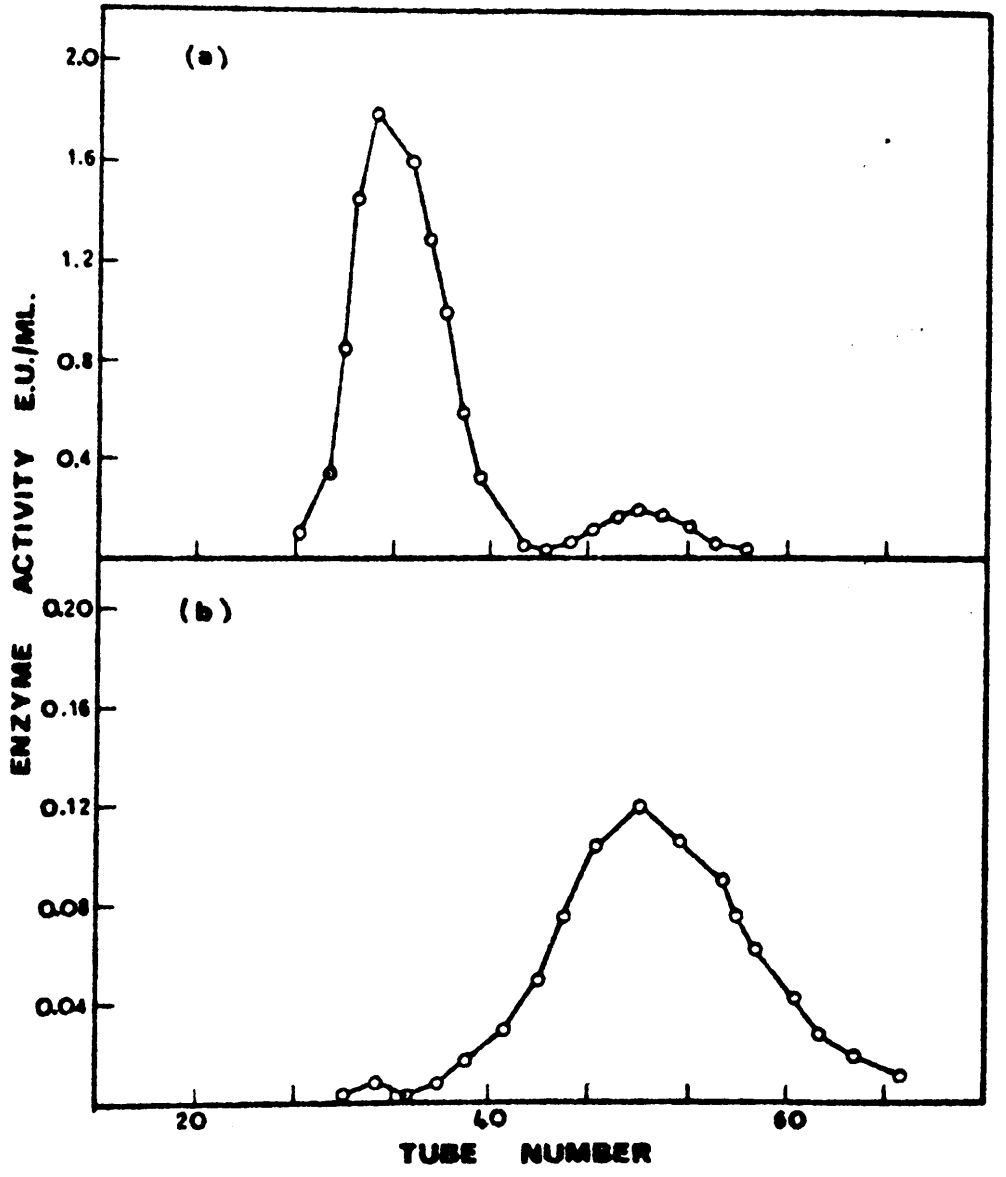


FIG. 7. Gel filtration of adenosine deaminase on Sephadex G-75 (coarse).
(a) 3 ml. of a 50-90% (v/v) acetone fraction (sp. act. 0.29 e.u.)
(b) 3 ml. of a 90-170% (v/v) acetone fraction (sp. act. 1.02 e.u.)
were applied to the column which was equilibrated with 0.04 M Tris buffer pH 7.4 containing 0.24 M KCl. 3 ml. fractions were collected and enzyme activity estimated using the Microdiffusion Method of assay (pH 7.0).

erature as low as possible (fractionations were carried out in the cold room at 4°C, keeping both the enzyme solution and acetone in a freezing ice mixture). In spite of this the yields were low and variable.

EXAMPLES OF TWO ACETONE FRACTIONATIONS. TABLE 9 AND TABLE 10

TABLE 9

	Total e.u.	Sp. Act. e.u/mg.	Yield %	High Mol. Wt.form(%)	Low Mol. Wt.form(%)
Acetate Extract	1870	0.31	100	33	66
50-90% Acetone	87	0.20	4	66	33
90-170% Acetone	563	1.44	31	2	98

TABLE 10

	Total e.u.	Sp. Act. e.u./mg.	Yield %
Acetate Extract	414	0.26	100
50-90% Acetone	51	0.18	12
90-170% Acetone	192	1.20	46

The results shown in Tables 9 and 10 were disappointing, the yield of enzyme in both cases being very low (35% and 58% respectively). The yield obtained in the 50-90% acetone fraction was considerably lower than previous results had indicated. However,

the fractionation procedure did separate the two enzyme forms (although not completely) as already observed.

DEAE CHROMATOGRAPHY AT pH 5.8

Although poor recoveries were obtained after acetone fractionation, it was decided to continue with the purification using DEAE chromatography as a further step. This anion exchanger has been successfully used (the columns being developed at pH 5.8) in the purification of the bovine intestinal deaminase (Murphy et al., 1969). Since the electrostatic binding properties of an enzyme depend on its isoelectric point, and the bovine intestinal and rabbit intestinal adenosine deaminases appear to have very similar isoelectric points (Brady and O'Donovan, 1965), chromatography was carried out at pH 5.8.

Previously prepared enzyme (See Table 9) was used. Because of the very low recovery (4%) in the 50-90% acetone fraction, it was decided to concentrate on the purification of the low molecular weight form only. However, both fractions were applied to the Sephadex G-75 column (Fig. 8). It is apparent that the bulk of the high molecular weight proteins were present in the 50-90% acetone fraction and emerge at the void volume of the column. The lower molecular weight proteins on the other hand were present in the 90-170% acetone fraction.

Tubes 33-71 (Fig. 8b), containing the bulk of the low molecular weight deaminase were pooled, dialysed against distilled water, and applied to a DEAE cellulose column 30 cm x 1 cm, previously equilibrated with 0.002M sodium citrate buffer pH 5.8. Unabsorbed, enzymatically inactive protein, was washed off using the equilibrating buffer before

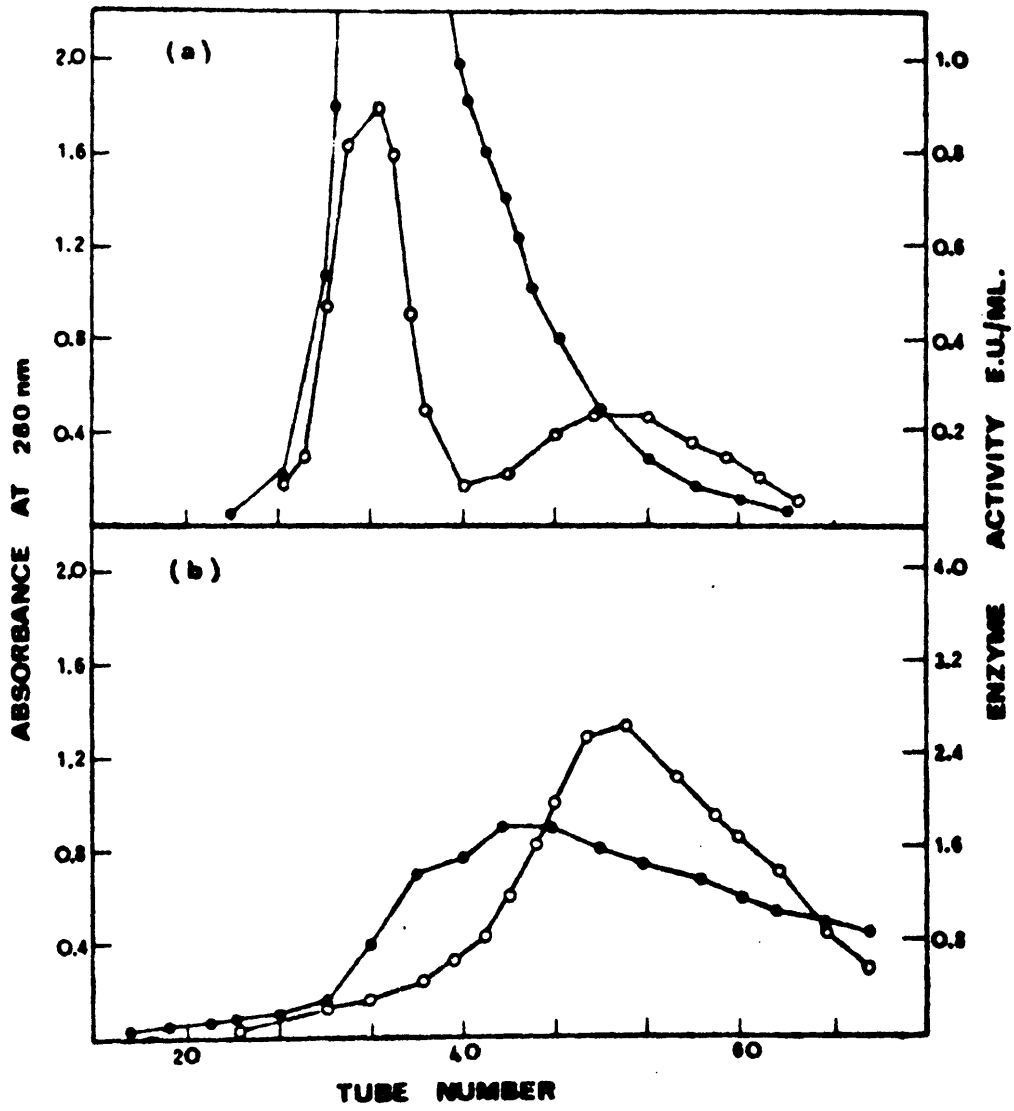


FIG. 8. Gel filtration of adenosine deaminase on Sephadex G-75 (coarse).
(a) 5 ml. of a 50 - 90% (v/v) acetone fraction (sp. act. 0.2 e.u.)
(b) 5 ml. of a 90 - 170% (v/v) acetone fraction (sp. act. 1.44 e.u.)
were applied to the column which was equilibrated with 0.04 M Tris buffer pH 7.4 containing 0.24 M KCl. 3 ml. fractions were collected. Protein concentration was measured by absorbtion at 280 nm and enzyme activity estimated using the Microdiffusion Method of assay (pH 7.0). ●——● , protein; o——o , adenosine deaminase activity.

a gradient from 0.002M - 0.03M citrate pH 5.8 was applied (See Methods section p. 24)

The pattern of separation is illustrated in Fig. 9. Most of the inactive protein was eluted before the deaminase peak which rose sharply and fell off gradually. It was calculated that 66% of the applied protein was recovered in the effluent during addition and subsequent washing of the column with the equilibrating buffer.

Tubes 86-130 were pooled, dialysed overnight against distilled water and concentrated using a small DEAE cellulose column.

	Vol. (ml.)	Total e.u.	Sp. Act. e.u./mg.	Yield %
DEAE pH 5.8	6.0	221	41	20

A summary of the purification procedure is shown in Table 11. This method of purification gave a 20% yield of enzyme having a specific activity of 41 e.u./mg. There was a 14-fold increase in specific activity after DEAE chromatography, indicating the usefulness of this step. However, only one form (low molecular weight form) of the enzyme can be prepared by this procedure. Because of this and also the fact that a very low yield (45%) was obtained at STEP 2, it was decided to consider using other fractionation methods as possible alternatives in the purification.

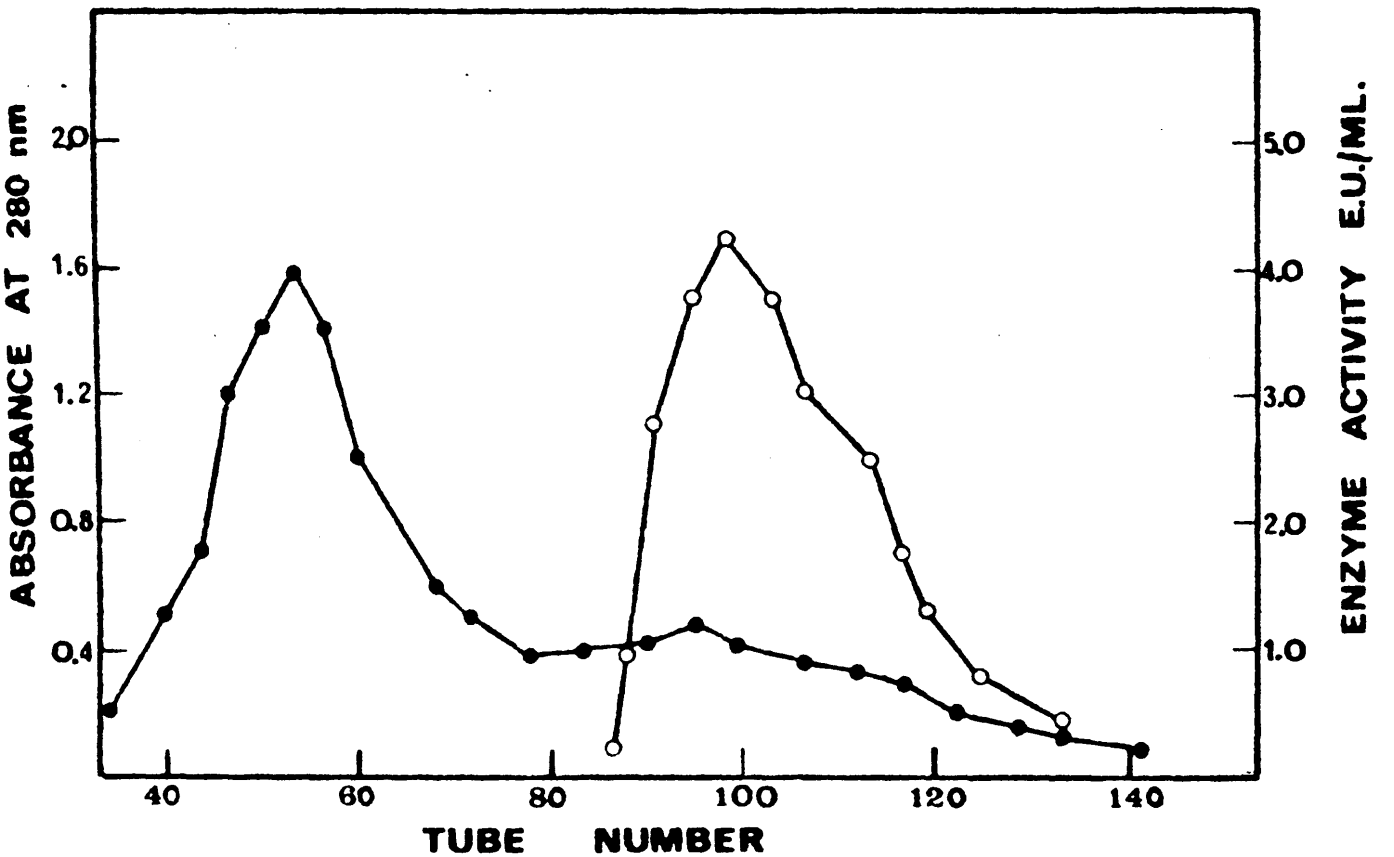


FIG. 9. Chromatography of adenosine deaminase on DEAE cellulose. 242 ml. of enzyme (after Sephadex G-75 chromatography : sp. act. 3.15 e.u.) was applied to the column and eluted using a sodium citrate gradient (pH 5.8) from 0.002 - 0.03 M. 3 ml. fractions were collected. Protein concentration was measured by absorption at 280 nm and enzyme activity estimated using the Microdiffusion Method of assay (pH 7.0). ●——● , protein; o——o , adenosine deaminase activity.

TABLE 11. Purification of the Low Molecular Weight Form of Adenosine Deaminase from Rabbit Intestine

Procedure		Vol. (ml.)	Activity (e.u./ml.)	Total e.u.	Protein (mg./ml.)	Total Protein(mg)	Sp. Act. e.u./mg.	Yield %	Purification Factor
Step. 1.	Acetate Extraction	950	1.31	1246*	6.3	5985	0.21	100	1
Step. 2.	90-170% Acetone	28	20.11	563	14.0	392	1.44	45	7
Step. 3.	Sephadex G-75	242	1.57	381	0.5	121	3.15	30	15.7
Step. 4.	DEAE pH 5.8	6	36.86	221	0.9	5.4	41	20	196

* The acetate extract as shown contained 33% High molecular weight form and 66% Low molecular weight form. Since the high molecular weight form was removed in the 50-90% acetone fraction, the value shown for total e.u(i.e. 1,246) is only 66% of the true experimental value. The % yields were calculated using this figure.

PURIFICATION 11

EFFECT OF HEAT AS A POSSIBLE MEANS OF PURIFICATION

The effect of temperature on rabbit intestinal extracts was examined. Heating on the aqueous.extract to 100°C surprisingly caused little or no precipitation of protein, whereas the deaminase was completely inactivated.

As it was decided to use acetate extraction at pH 5.5 as a preliminary step in the purification, the effect of heat on this extract was investigated. In contrast to aqueous extracts precipitation of protein did occur on heating the acetate extract. Samples were heated at 30°, 40°, 50°, 60° and 70°C for 10 min., immediately cooled in ice, and centrifuged at 10,000 r.p.m. for 20 min. The percentage activity and percentage protein remaining in the supernatants was determined (Fig. 10). It can be seen that the deaminase inactivation curve follows closely the protein precipitation curve so that little or no purification can be obtained by heating the acetate extract.

CALCIUM ACETATE FRACTIONATION

During an investigation of adenosine deaminase from Takadiastase powder (Minato, 1968) it was found that on addition of 1 M calcium acetate to extracts of the powder, considerable purification of the enzyme was obtained. However, addition of this reagent to extracts of rabbit intestine caused negligible precipitation of protein and

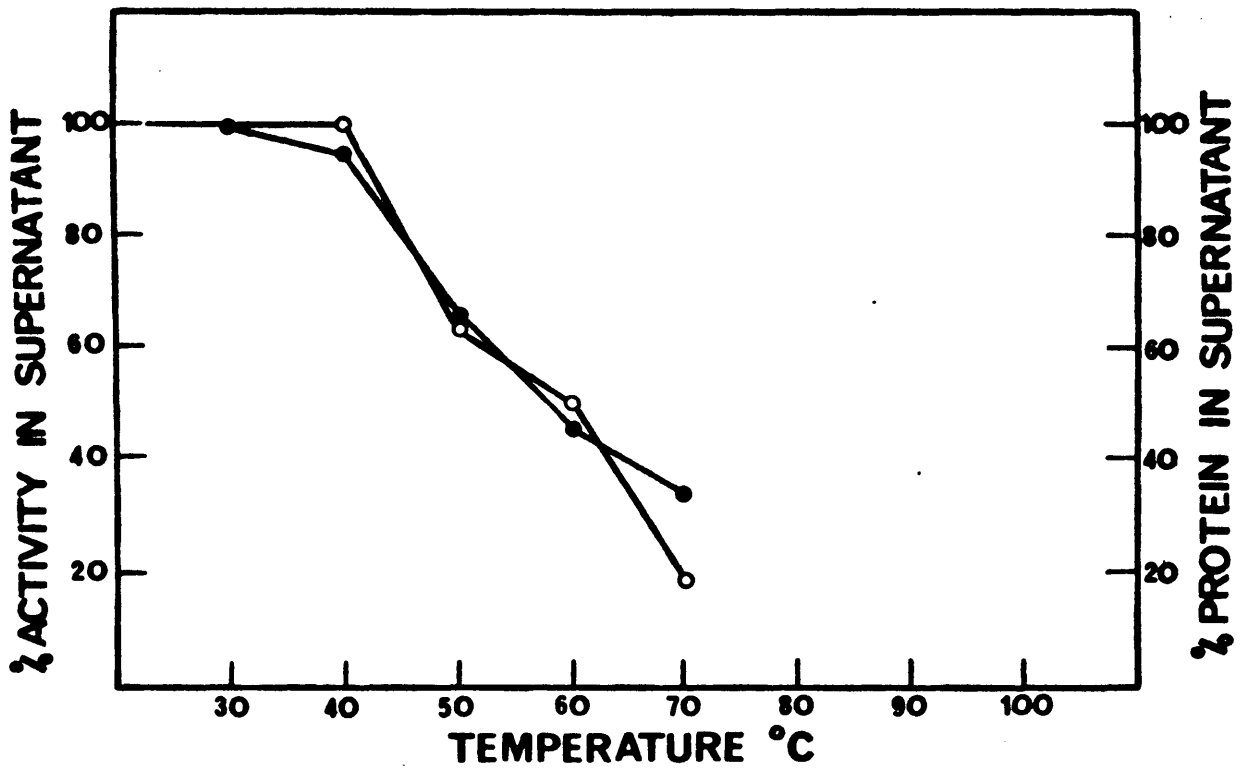


FIG. 10. Effect of temperature on an acetate exteact of rabbit intestine. Samples were heated at various temperatures for 10 min. and after centrifugation the protein concentration was measured using the Biuret Method, and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——●, protein; o——o , adenosine deaminase activity.

was therefore unsuitable for purification. As already mentioned, the Takadiastase enzyme is of similar molecular weight to the high molecular weight enzyme being studied in the present investigation.

AMMONIUM SULPHATE FRACTIONATION

Some preliminary experiments showed that adenosine deaminase was precipitated from the acetate extract by addition of ammonium sulphate to 60% saturation.

Procedure

The extract, after being poured through a fine nylon net to remove the floating layer of lipid material, was allowed to stand at room temperature for about 15 min. After addition of ammonium sulphate to the desired saturation, the precipitate was allowed to flocculate for 30 min. and centrifuged (20 min. at 10,000 r.p.m.). The bulk of the deaminase was precipitated in the 60% saturation fraction. Further addition of $(NH_4)_2SO_4$ to the centrifugate yielded only inactive protein.

After centrifugation the precipitate was dissolved in distilled water and checked for protein and enzyme activity (Table 12).

TABLE 12. Ammonium Sulphate Fractionation of Acetate Extract of Rabbit Intestine.

	Activity e.u./ml.	Protein mg./ml.	Sp. Act. e.u./mg.	Yield %	Purification factor
Acetate Extract	1.64	8.0	0.2	100	1
0-60% $(NH_4)_2SO_4$	12.05	10.0	1.5	96	5.8

Excellent recovery of the enzyme, and an increase in specific activity from 0.2 to 1.5 e.u/mg. was obtained by this procedure. In view of this enrichment it was decided to investigate the effect of refractionation with ammonium sulphate using acetone fractionation as an intermediate step. This pattern of fractionations has been found very useful in the purification of both bovine spleen and bovine lung adenosine deaminases (Noonan, 1969).

The 0-60% $(NH_4)_2SO_4$ fraction was therefore treated with 225 ml. ice cold acetone which brought the acetone saturation to 170% v/v. This precipitated all the deaminase activity which included both the high and low molecular weight forms. The precipitate obtained after centrifugation was dissolved in 120 ml. water, and refractionated with ammonium sulphate to give 60% saturation (12.8g. added). Results of the fractionation are shown in Table 13.

TABLE 13. $(NH_4)_2SO_4$ - Acetone - $(NH_4)_2SO_4$ Fractionation of Acetate Extract of Rabbit Intestine.

Procedure	Activity e.u./ml.	Protein mg./ml.	Sp. Act. e.u./mg.	Yield %
1. Acetate Extract	1.64	8.0	0.20	100
2. 0-60% $(NH_4)_2SO_4$	12.00	10.0	1.20	96
3. 0-170% Acetone	6.65	4.4	1.50	47
4. 0-60% $(NH_4)_2SO_4$	9.30	6.0	1.55	40

As previously observed there was a considerable loss of activity after acetone treatment. There is little difference in yield between Steps 3 and 4, indicating that during the second $(NH_4)_2SO_4$ fraction-

ation the bulk of the deaminase was again precipitated between 0 and 60% saturation.

DEAE Chromatography at pH 5.8

The material from STEP 4 was thoroughly dialysed against distilled water and applied to a previously equilibrated DEAE cellulose column. After washing off unadsorbed protein a salt gradient was applied as previously described. Fig. 11 illustrates the pattern obtained. A large inactive protein peak is seen to emerge before the adenosine deaminase peak. In this experiment the enzyme peak was largely symmetrical but showed skewing on the left hand side.

Tubes 43-68 were pooled, dialysed against distilled water, and concentrated to a volume of 3 ml. (approx.) using a small DEAE cellulose column.

Sephadex G-75 Chromatography at pH 7.4

The concentrated enzyme was then applied to a Sephadex G-75 column equilibrated with 0.05M Tris buffer pH 7.4 incorporating 0.24 M KCl. The elution pattern is shown in Fig. 12. Separation of the two enzyme forms was achieved, the high molecular weight peak being eluted with the bulk of the proteins at the void volume. Since there was no apparent protein peak corresponding to the low molecular weight form, it appeared likely that the preparation was still quite impure.

A summary of the purification procedure is shown in Table 14. Although a yield of 20% was obtained the specific activity of the

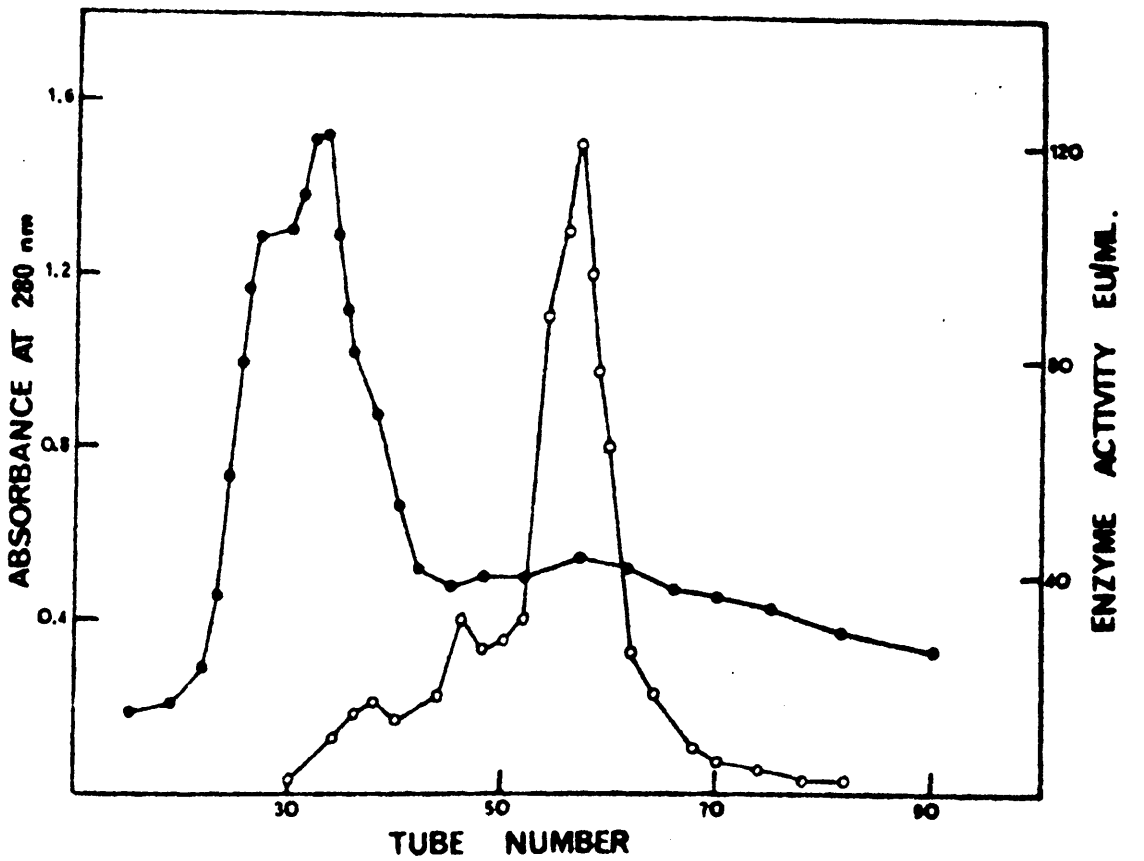


FIG. 11. Chromatography of adenosine deaminase on DEAE cellulose. The enzyme solution from STEP 4 (sp. act. 1.55) was applied to the column and eluted using a sodium citrate gradient (pH 5.8) from 0.002 - 0.03 M. 5 ml. fractions were collected. Protein concentration was measured by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (ph 7.0). ●——● , protein; o——o , adenosine deaminase activity.

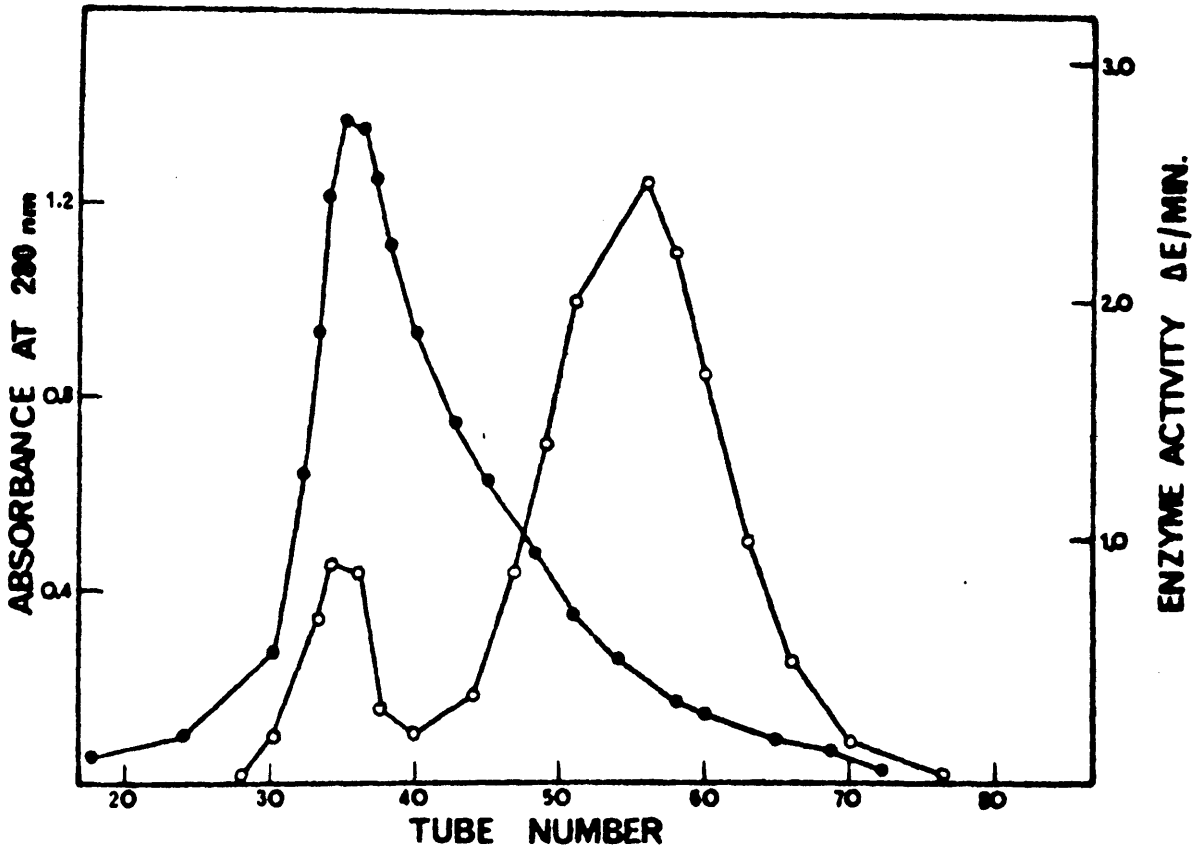


FIG. 12. Gel filtration of adenosine deaminase on Sephadex G-75(coarse). The enzyme solution after the DEAE chromatography step (sp. act. 8.5) was concentrated to a volume of 3 ml. and applied to the Sephadex column equilibrated with 0.04 M Tris buffer pH 7.4 containing 0.24 M KCl. 3 ml. fractions were collected. Protein concentration was measured by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——● , protein; o——o , adenosine deaminase activity.

TABLE 14. Purification of the Low Molecular Weight Form of Adenosine Deaminase from Rabbit Intestine

Procedure	Vol (ml.)	Activity (e.u./ml.)	Total e.u.	Protein (mg./ml.)	Total Protein	Sp. Act. (e.u./mg	Yield %	Purification factor
Step 1. Acetate Extraction	1059	1.6	1737	8.0	8472	0.20	100	1
Step 2. 0-60% $(NH_4)_2SO_4$	138	12.0	1663	10.0	1380	1.20	96	5.8
Step 3. 0-170% Acetone	122	6.6	811	4.4	537	1.50	47	7.3
Step 4. 0-60% $(NH_4)_2SO_4$	74	9.3	690	6.0	444	1.55	40	7.5
Step 5. DEAE pH 5.8	150	3.4	510	0.4	60	8.50	30	41.4
Step 6. Sephadex G-75	78	4.3	334	0.2	15.6	21.40	19.2	105

final preparation was only half that obtained in the previous purification. The series of fractionations carried out before the column stages would therefore appear to be unsuitable.

PURIFICATION III

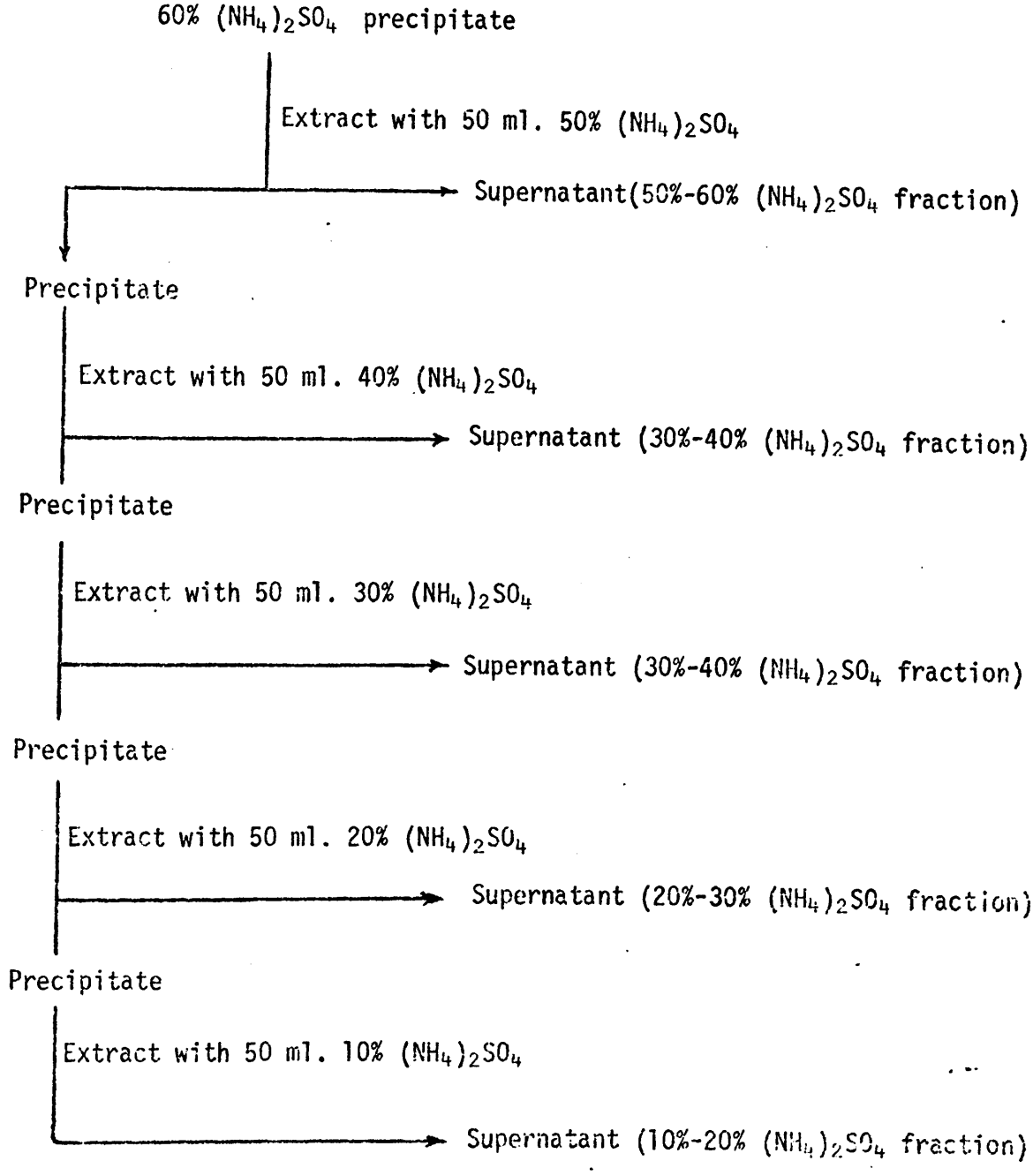
Although ammonium sulphate fractionation gave considerable purification, the concentration of inactive protein present at this stage was still too high for satisfactory application to the DEAE cellulose column. An alternative procedure, ammonium sulphate precipitation followed by re-extraction, used in the purification of human placental alkaline phosphatase (Ghosh, 1968) and rat mammary acetyl coenzyme A carboxylase (Miller, 1969) was therefore investigated. This method has been shown (at least in the case of the above enzymes) to be superior as a purification step to the conventional ammonium sulphate fractionation, and involves total precipitation of the enzyme with ammonium sulphate, followed by re-extraction using solutions of $(NH_4)_2SO_4$ of decreasing concentrations.

Procedure

An acetate extract was prepared from 90 g. of frozen rabbit intestine as previously described.

	Vol. (ml.)	Activity e.u./ml.	Protein mg/ml.	Sp. Act. e.u/mg.	Yield %
Acetate Extract	460	1.85	7.65	0.24	100

The extract was brought to 60% saturation by the addition of 180 g. of solid $(NH_4)_2SO_4$. After allowing to stand for 1 h., the precipitate was sedimented at 10,000 r.p.m. for 20 min. and the supernatant solution discarded. The precipitate was carefully suspended in 46 ml. of an aqueous solution of 50% $(NH_4)_2SO_4$, stirred for 30 min. and the suspension then centrifuged at 10,000 r.p.m.



SCHEME 1. Ammonium sulphate "re-extraction procedure" used in the purification of rabbit intestinal adenosine deaminase.

for 20 min. The supernatant solution was retained for assay. This procedure was repeated four times, each time using 50 ml. of aqueous ammonium sulphate solutions containing 40%, 30%, 20% and 10% $(NH_4)_2SO_4$ respectively. The fractionation procedure is outlined in Scheme I. The results obtained are shown in Table 15.

TABLE 15 Fractionation of Acetate Extract of Rabbit Intestine using $(NH_4)_2SO_4$ "Re-Extraction Method"

$(NH_4)_2SO_4$ (% Saturation)	Sp. Activity e.u./mg.	Yield %
50 - 60	0.20	4
40 - 50	0.30	18
30 - 40	0.50	65
20 - 30	0.13	13
10 - 20	-	0

Most of the activity (96%) was extracted between 20-50% $(NH_4)_2SO_4$. This material was therefore used for further purification.

Dialysis against Glass Distilled Water

The combined ammonium sulphate extracts were thoroughly dialysed against distilled water (4 changes), and centrifuged to remove insoluble material. The supernatant was retained for analysis.

TABLE 16

PROCEDURE	Vol. (ml)	Total e.u.	Protein mg.	Sp. Act. e.u./mg.	Yield %
20% -50% $(NH_4)_2SO_4$	162	826	2462	0.33	96
Dialysis/H_2O	236	826	991	0.90	96

It is apparent from Table 16 that the dialysis step resulted in the precipitation of a considerable quantity of protein with no loss in enzyme activity.

DEAE Chromatography at pH 5.8

The dialysed material was next applied to a DEAE cellulose column equilibrated at pH 5.8. After washing off unadsorbed protein, a salt gradient was applied as previously described. The elution pattern is shown on Fig. 13. Tubes 43-63 were pooled and analysed for enzyme activity and protein content. (Table 17).

TABLE 17

	Vol. (ml)	Activity e.u./ml.	Protein mg./ml.	Sp. Act. e.u./mg.	Yield %
DEAE pH 5.8	106	5.0	0.3	16.2	62

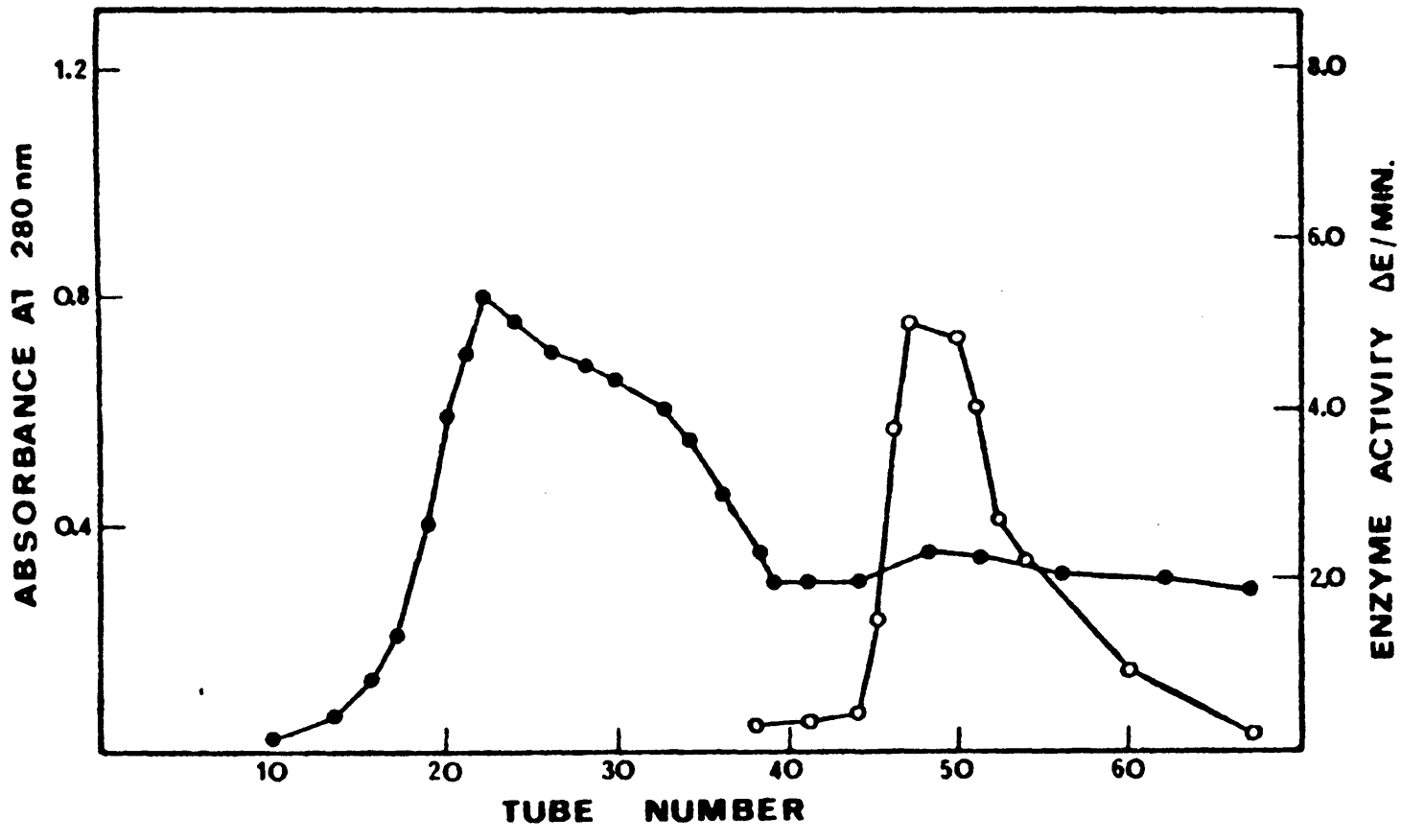


FIG. 13. Chromatography of adenosine deaminase on DEAE cellulose. 236 ml. of enzyme solution after the dialysis step (sp. act. 0.9 e.u.) was applied to the column and eluted using a sodium citrate gradient (pH 5.8) from 0.002 - 0.03 M. Protein concentration was measured by absorbtion at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●—●, protein; o—o , adenosine deaminase activity.

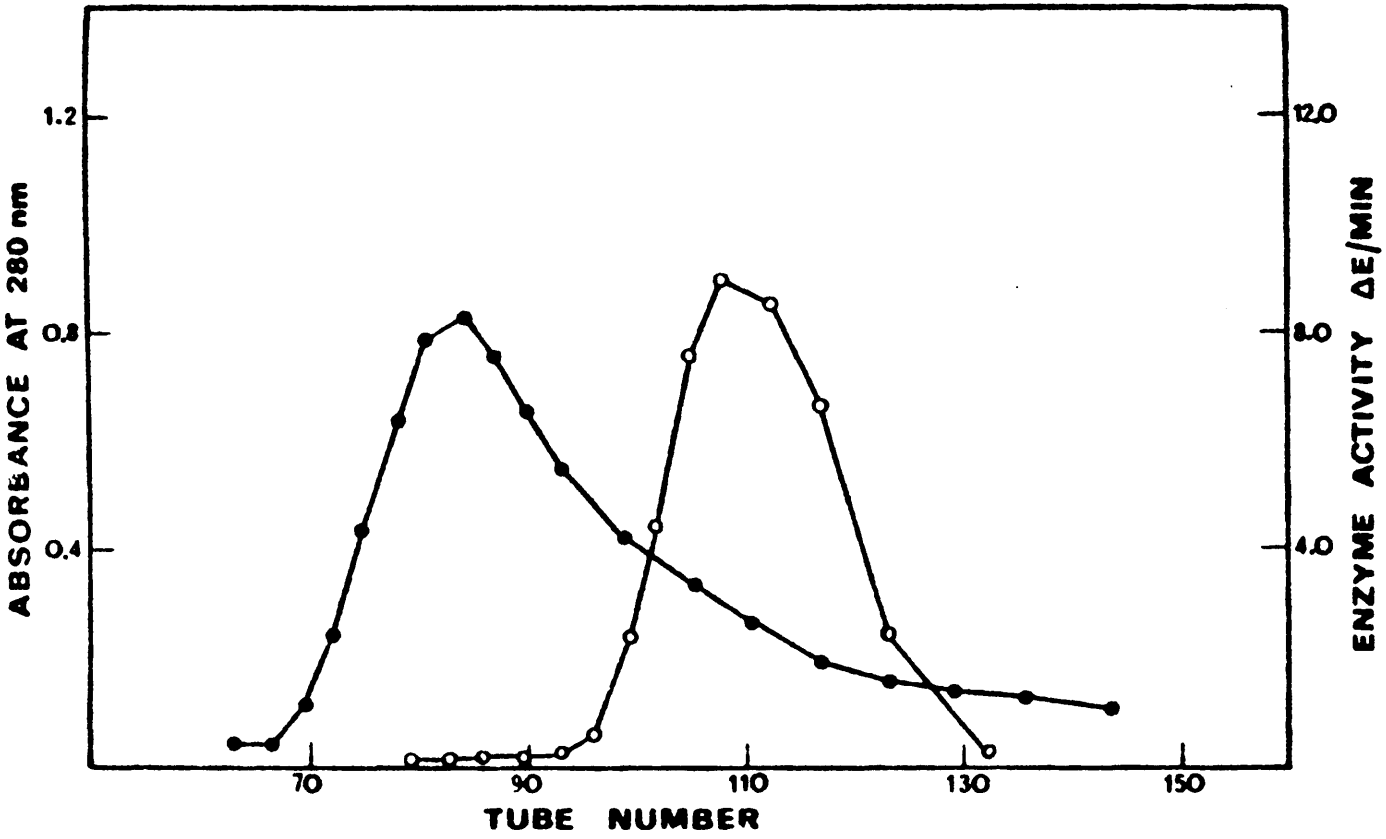


FIG. 14. Gel filtration of adenosine on Sephadex G-75 (coarse). 5 ml. of concentrated enzyme solution after the DEAE chromatography step (sp. act. 16.2 e.u.) was applied to the column which was equilibrated with 0.04 M Tris buffer pH 7.4 containing 0.24 M KCl. 3 ml. fractions were collected. Protein concentration was measured by absorbtion at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——●, protein; o——o , adenosine deaminase activity.

TABLE 18. Purification of the Low Molecular Weight Form of Adenosine Deaminase from Rabbit Intestine.

Procedure	Vol. (ml.)	Activity (e.u./ml.)	Total e.u.	Protein (mg./ml.)	Total Protein(mg)	Sp. Act. (e.u./mg.)	Yield %	Purification factor
Step 1. Acetate extraction	460	1.8	851	7.6	3519	0.24	100	1
Step 2. 20%-50% $(NH_4)_2SO_4$	236	3.5	826	4.2	991	0.90	97	4
Step 3. DEAE pH 5.8	106	5.0	530	0.3	32	16.60	62	70
Step 4. Sephadex G-75	42	8.4	353	0.2	8.4	42.00	41	175

Sephadex G-75 Chromatography at pH 7.4

The pooled material was dialysed overnight against distilled water and concentrated to a volume of 5 ml. (approx.) using a small DEAE cellulose column. The concentrated enzyme was then applied to a Sephadex G-75 column (Fig. 14). It can be seen that only one form of the enzyme (low molecular weight form) emerged from the column, having separated from a large peak of 280 nm-absorbing material which eluted at the void volume.

A summary of the complete purification scheme is outlined in Table 18. Although an excellent yield of 40% was obtained, the specific activity of the final preparation (41 e.u./mg.) was very low.

PURIFICATION IV

The following purification scheme was the final one adopted for the purification of the low molecular weight form of the enzyme. It has been repeated a number of times and shown to give consistent results. Essentially it is the same procedure as Purification III with the following modifications:

1). An alteration was made in the ammonium sulphate re-extraction procedure, so that the enzyme was re-extracted between 20% and 40% $(NH_4)_2SO_4$ instead of between 20% and 50% $(NH_4)_2 SO_4$.

2). Sephadex G-75(coarse grade) used in previous purifications was substituted by the finer grade Sephadex G-75 Superfine with more beneficial results. The volume of enzyme solution applied to the Sephadex column was also reduced (1 ml. approx. was used).

These modifications gave a substantial increase in the specific activity of the final preparation.

PURIFICATION PROCEDURE

Ten rabbits, from which 264 g. of intestine was obtained, were used for this purification. The acetate extract was prepared as already described.

TABLE 19

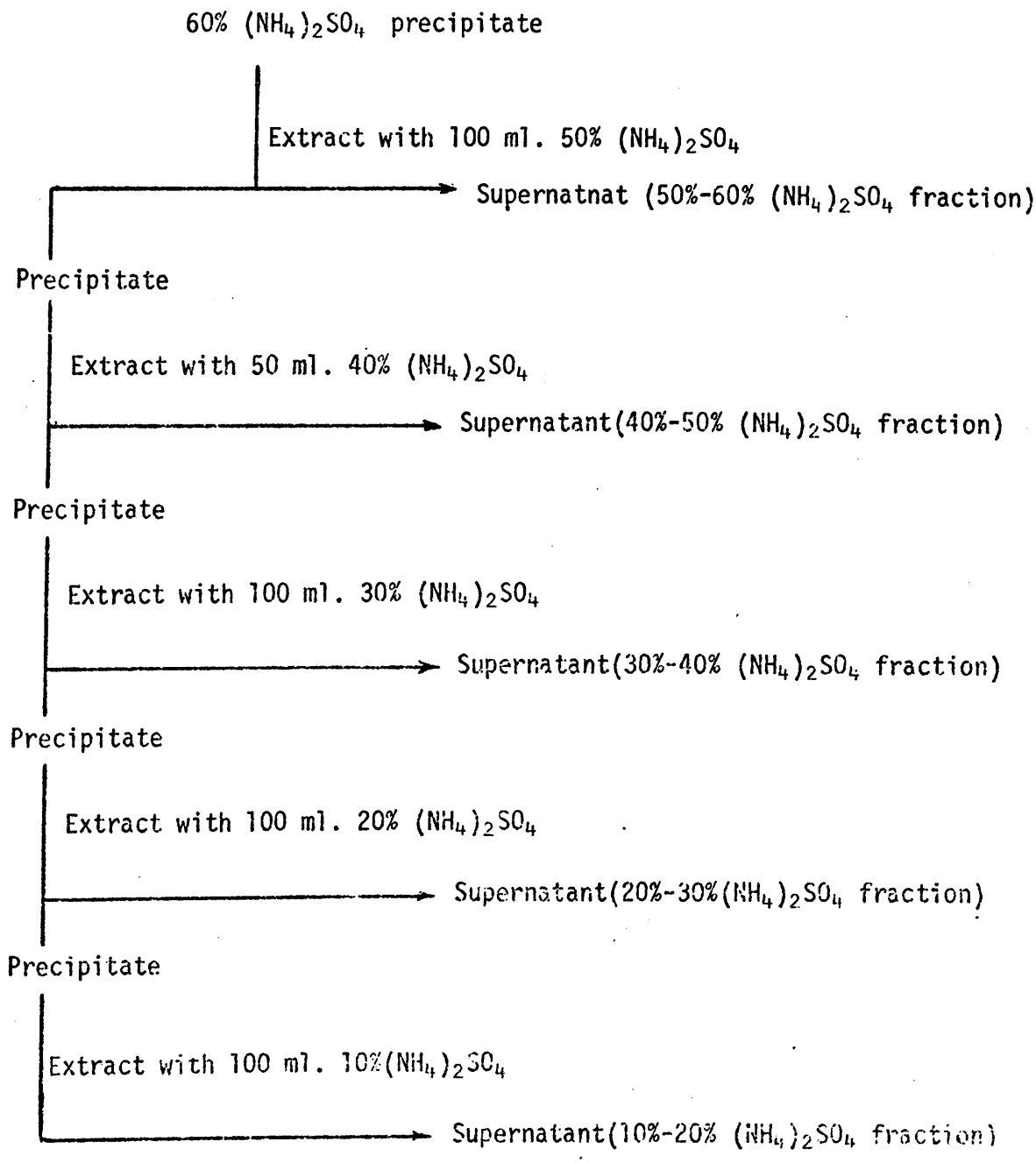
	Vol. (ml)	Activity (e.u./ml)	Protein (mg./ml)	Sp. Act. (e.u./mg.)	Yield %
Acetate Extract	1,340	1.1	6.4	0.17	100

Ammonium Sulphate Precipitation and Re-extraction

The enzyme was precipitated by the addition of 515 g. of $(NH_4)_2SO_4$ to 1,340 ml. of the acetate extract (60% saturation), and the precipitate re-extracted as before. In this purification (because of the larger quantity of starting material) 100 ml. volumes of ammonium sulphate were used for re-extraction. However, only half this volume i.e. 50 ml. was used in the second extraction (40% $(NH_4)_2SO_4$ solution). It can be seen from Table 20 that this results in the re-extraction of the bulk of the deaminase over a narrower range of $(NH_4)_2SO_4$ concentrations (between 20% and 40% $(NH_4)_2SO_4$).

TABLE 20 Fractionation of Acetate Extract of Rabbit Intestine Using $(NH_4)_2SO_4$ "Re-Extraction Method"

Concentration of $(NH_4)_2SO_4$ (% Saturation)	Vol of extracting Solution(ml)	Vol. of extract (ml.)	Total e.u.	Recovery %
50 - 60	100	125	11	0.8
40 - 50	50	65	85	6.0
30 - 40	100	115	700	50.0
20 - 30	100	115	356	25.0
10 - 20	100	100	90	6.0



SCHEME 11. Ammonium sulphate "re-extraction procedure" used in the purification of rabbit intestinal adenosine deaminase.

The method is diagrammatically represented in Scheme 11.

Dialysis against Distilled Water

The most active extracts 20% - 30% $(NH_4)_2SO_4$ and 30% - 40% $(NH_4)_2SO_4$ were combined and thoroughly dialysed against distilled water. The first three stages of the purification are summarized in Table 21.

TABLE 21

	Vol. (ml.)	Total e.u.	Protein mg.	Sp. Act. e.u./mg.	Yield %
Acetate Extract	1340	1474	8576	0.17	100
20-40% $(NH_4)_2SO_4$	230	1046	5106	0.20	75
Dialysis H_2O	323	1038	807	1.28	75

It is apparent that a large amount of non-enzymic protein was again removed after dialysis.

At this stage in the purification it was decided to measure the ratio of the two molecular weight forms at each step. This was done by applying individual samples to a previously prepared and equilibrated Sephadex G-75 thin-layer plate. After development and staining of the plate, densitometer tracings of the spots were obtained (Fig. 15).

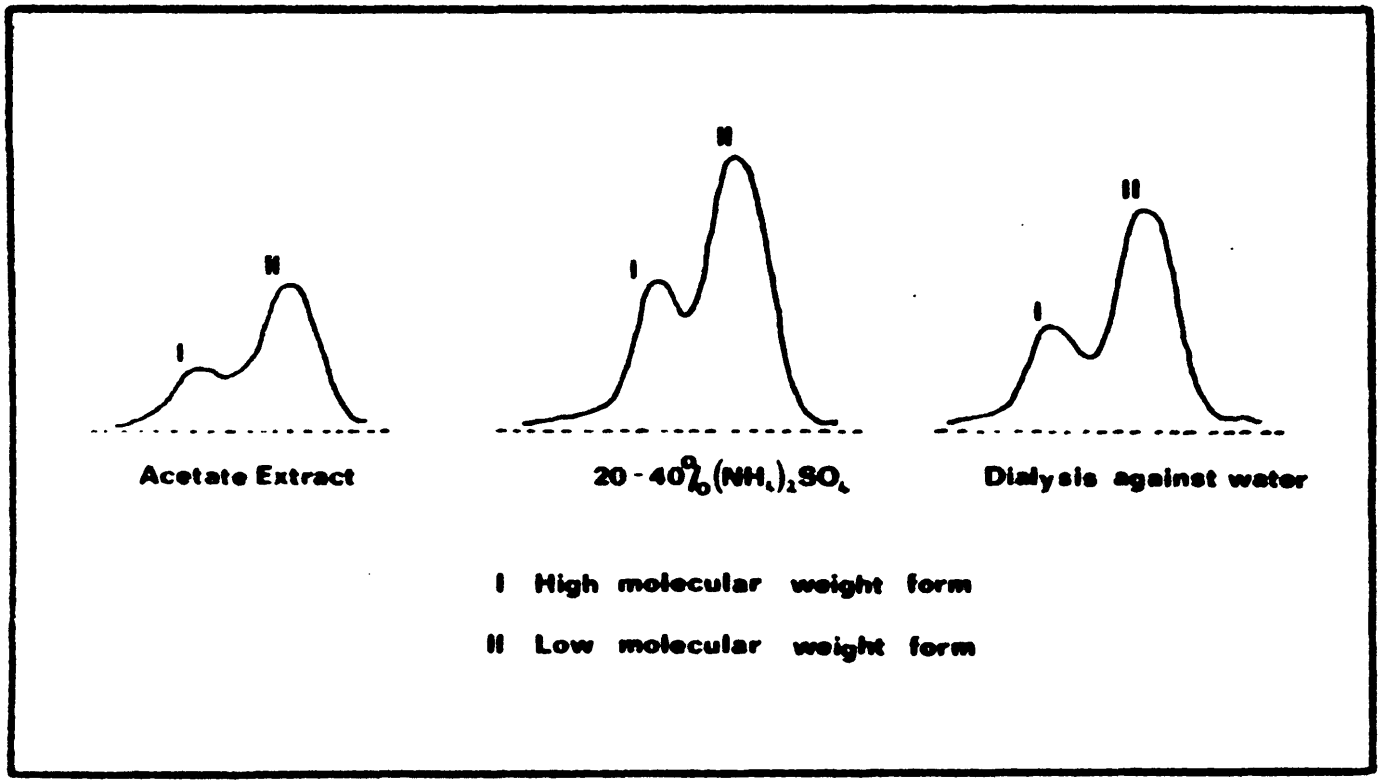


FIG.15. Densitometer tracings of thin-layer gel filtration patterns. Samples from the first three steps in the purification were applied to a Sephadex G-75 coated thin-layer plate and after development (using 0.05 M KCl as solvent) sites of enzyme activity were detected using the zymogram stain for adenosine deaminase.

TABLE 22

Purification Step	% High Molecular Weight form	% Low Molecular Weight form
Acetate Extract	26.3	73.7
20%-40% $(NH_4)_2SO_4$	29.4	70.6
Dialysis/H_2O	27.7	72.3

The relative percentages are given in Table 22. It is obvious that there is no change in the ratio during the first three stages of the purification.

DEAE Chromatography at pH 5.8

The dialysed enzyme was next applied to a previously equilibrated DEAE cellulose column. The elution pattern is illustrated in Fig. 16. Tubes 21 - 27 were pooled and dialysed before concentrating on a small DEAE cellulose column. Care was taken in this case to elute the enzyme in a very small volume (1 ml. approx.) with 2 M NaCl.

Chromatography on Sephadex G-75 (Superfine) at pH 5.8

A commercial Sephadex G-75 column (Superfine grade) supplied with plastic fittings was constructed, and equilibrated with 0.002M sodium citrate buffer pH 5.8. The pattern obtained when the concentrated enzyme preparation was applied to the column is illustrated in Fig. 17. It is evident that a slight [illegible] 280 nm-absorbing material coincided with the low molecular weight peak of enzyme activity.

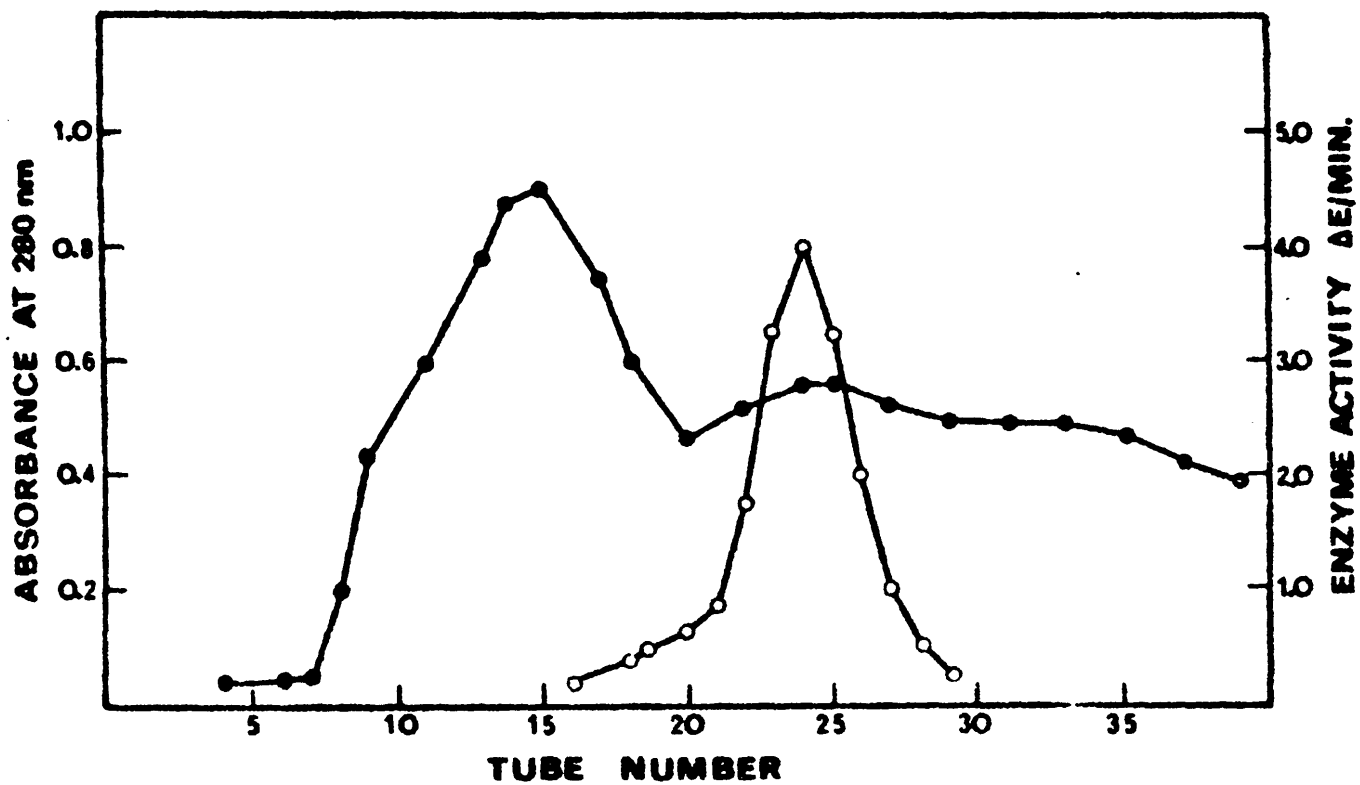


FIG. 16. Chromatography of adenosine deaminase on DEAE cellulose. 323 ml. of dialysed enzyme (sp. act. 1.28 e.u.) was applied to the column and eluted using a sodium citrate gradient (pH 5.8) from 0.002-0.03 M. 3 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——● , protein; o——o , adenosine deaminase activity.

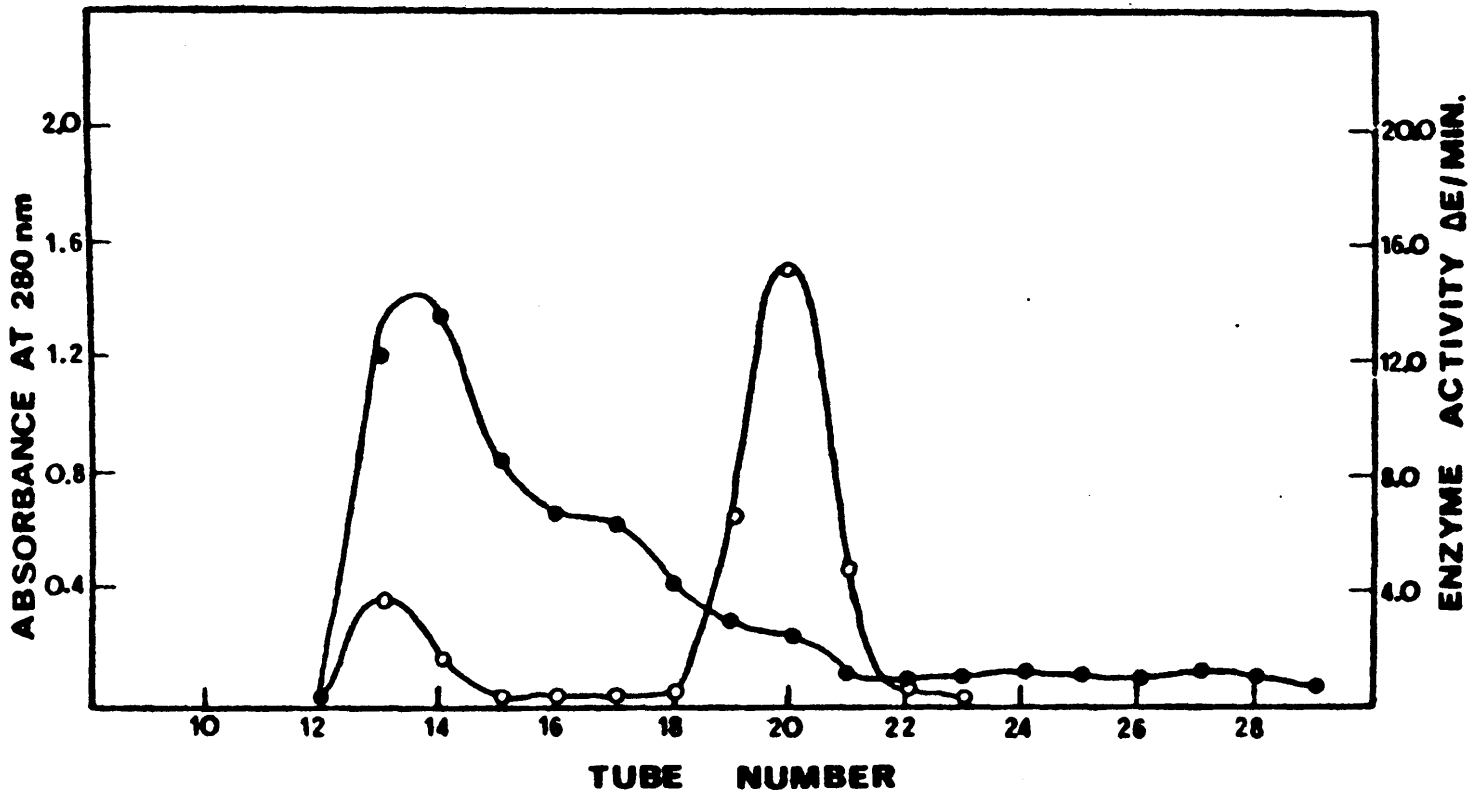


FIG. 17. Gel filtration of adenosine deaminase on Sephadex G-75 (Superfine). 1 ml. of concentrated enzyme solution after the DEAE chromatography step (sp. act. 16.8 e.u.) was applied to the column which was equilibrated with 0.002 M sodium citrate buffer pH 5.8. 5 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——● protein; o——o , adenosine deaminase activity.

TABLE 24. Purification of the Low Molecular Weight Form of Adenosine Deaminase from Rabbit Intestine

	Vol (ml.)	Activity (e.u./ml.)	Total e.u.	Protein (mg./ml.)	Total Protein(mg)	Sp. Act. (e.u./mg.)	Yield %	Purification factor
Step 1. Acetate Extraction	1340	1.1	1474	6.4	8576	0.17	100	1.0
Step 2. 20%-40% $(NH_4)_2SO_4$	230	4.5	1046	22.2	5106	0.20	75	1.2
Step 3. Dialysis against water	323	3.2	1038	2.5	807	1.28	75	7.5
Step 4. DEAE pH 5.8	80	8.4	672	0.5	40	16.80	46	94
Step 5. Sephadex G-75	5	57.5	287	0.24	1.2	240	20	1412

TABLE 23 Specific Activities of Adenosine Deaminase Solutions after Sephadex G-75 Chromatography.

Tube No.	Total Protein (Mg.)	Sp. Activity(e.u./mg)
19	1.50	81
20	1.25	240
21	0.50	126

A comparison of Table 23 and Table 18 (p. 84) shows that there is a great difference in specific activity between the DEAE and the Sephadex step in the present purification (16.8 e.u./mg. and 240 e.u./mg. respectively). This, therefore, illustrates the superiority of the Superfine Sephadex for purification purposes, over the coarser grade.

A summary of the purification scheme is shown in Table 24.

Preparations of the enzyme obtained from a number of purifications similar to the one just described were pooled and re-chromatographed on the Sephadex G-75 column (Fig. 18). A single protein peak emerged coincident with the deaminase peak, the specific activity of which was 300 e.u./mg.

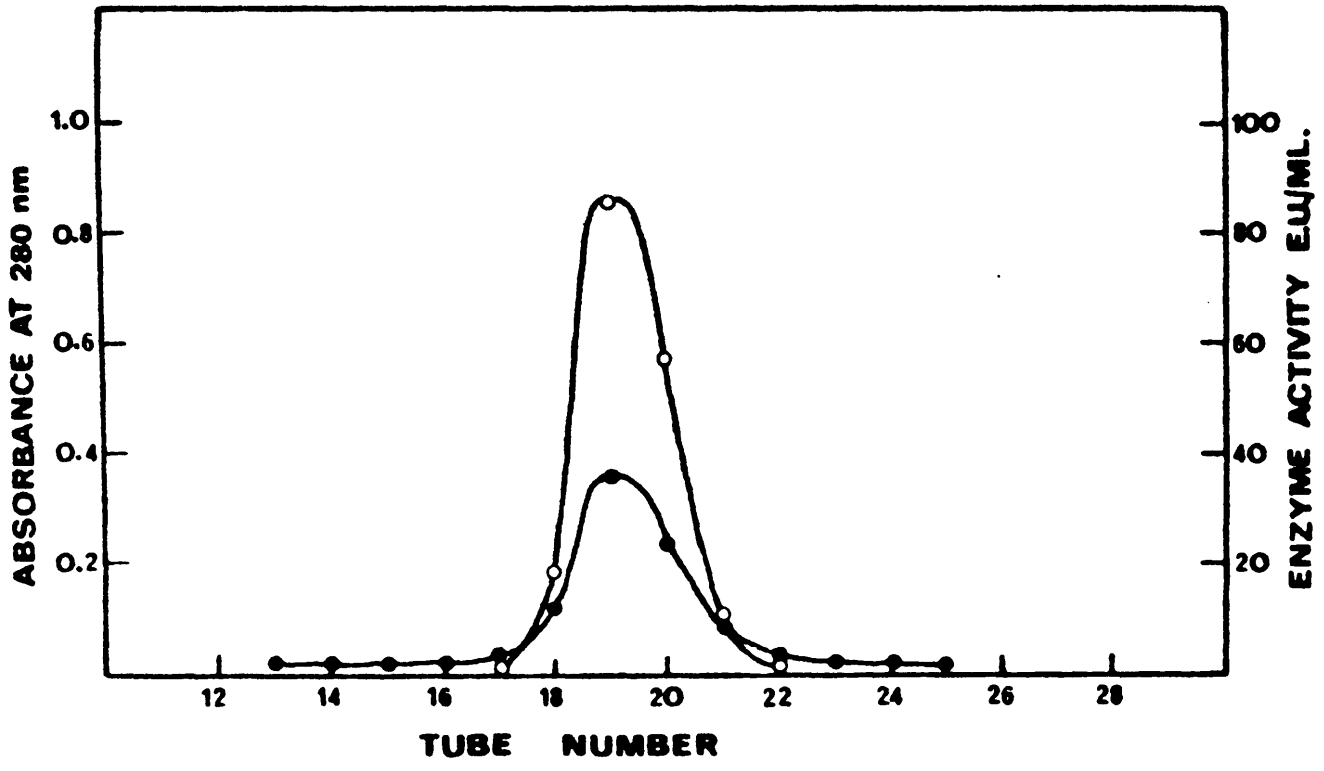


FIG. 18. Gel filtration of adenosine deaminase on Sephadex G-75 (Superfine). A number of low molecular weight enzyme preparations (prepared as described in Purification IV) were pooled, concentrated and applied to the column which was equilibrated with 0.002M sodium citrate buffer pH 5.8. 5 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——●, protein; o——o, adenosine deaminase activity.

COMPARISON OF THE AMMONIUM SULPHATE "RE-EXTRACTION METHOD" WITH THE "PRECIPITATION METHOD"

In the following experiment a direct comparison was made of the ammonium sulphate re-extraction method adopted in the purification, and the conventional ammonium sulphate fractionation procedure. This was done to justify the choice of the "re-extraction method" rather than the conventional "precipitation method".

Two separate 100 ml. samples of an acetate extract of rabbit intestine were subjected to fractionation by both procedures. The results obtained are shown in Table 25.

TABLE 25

	Precipitation Method		Re-extraction Method	
$(NH_4)_2SO_4$ conc. (% Saturation)	Sp. Act. e.u./mg.	Yield %	Sp. Act. e.u./mg.	Yield %
0 - 10	0.13	1.5	0.00	0.0
10 - 20	0.12	1.5	0.11	5.0
20 - 30	0.05	1.5	0.23	34.0
30 - 40	0.06	7.6	0.35	40.0
40 - 50	0.32	38.0	0.16	11.5
50 - 60	0.31	29.0	0.00	0.0
60 - 70	0.05	4.0	0.00	0.0

It is obvious that the enzyme was extracted over a narrower range of concentrations using the "re-extraction method". The more active fractions from both procedures were combined separately and

thoroughly dialysed against distilled water (4 changes). After centrifugation the supernatants were checked for enzyme activity. Table 26 compares the results obtained before and after dialysis.

TABLE 26

	Sp. Act. e.u./mg.	Yield %
Precipitation Method		
40%-60% $(NH_4)_2SO_4$	0.315	67
Post dialysis	0.90	56
Re-Extraction Method		
20%-40% $(NH_4)_2SO_4$	0.30	74
Post dialysis	1.80	56

Although the specific activity after the ammonium sulphate step was almost the same in both cases, the yield obtained using the re-extraction method was higher. Of greater significance is the fact that the specific activity in this fraction after dialysis was twice that of the preparation obtained using the precipitation method.

The extraction method was therefore the one of choice.

PURIFICATION OF THE HIGH MOLECULAR WEIGHT FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE

The intestines used as starting material in the purification procedures just described were taken mainly from the wild rabbit. It was observed when using this variety of rabbit that very little of the high molecular weight form of the enzyme was present in the intestine and in a number of cases, only the low molecular weight form was present. For example, during a survey of 23 individual rabbit intestines (wild variety) using the thin-layer gel filtration technique, 40% were found to have no high molecular weight form of the enzyme. Densitometer tracings of the thin-layer gel filtration patterns from a number of these intestines are illustrated in Fig. 19.

The level of the high molecular weight form in the intestine of the domestic (laboratory bred) variety of rabbit on the other hand, in general, appeared to be higher. Attempts at purifying this form of the enzyme were therefore made using the domestic variety.

PURIFICATION V

Intestines (total weight, 354 g.) were dissected from eight domestic rabbits, and the deaminase extracted with 5 volumes of 0.2 M acetate buffer pH 5.5. Because of the large quantity of starting material, the insoluble debri obtained after centrifugation of the extract was re-extracted with a smaller volume(approx.

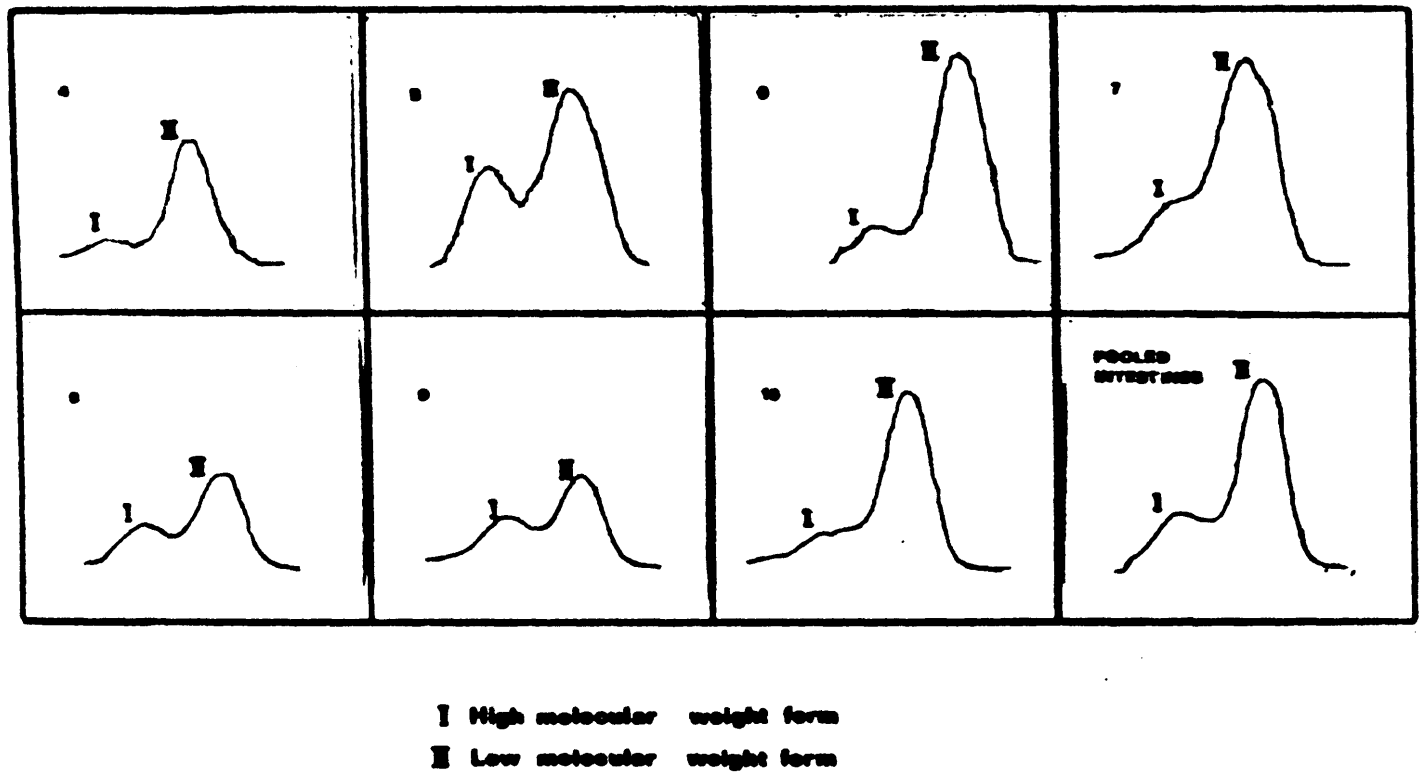


FIG. 19. Densitometer tracings of thin-layer gel filtration patterns. Samples of acetate extracts of individual rabbit intestines (wild variety) were applied to Sephadex G-75 coated thin-layer plates and after development (using 0.05 M KCl as solvent) sites of enzyme activity were detected using the zymogram stain for adenosine deaminase.

one-fifth of original volume) of acetate buffer (Table 27).

TABLE 27. Extraction of Adenosine Deaminase from Rabbit Intestine

	Vol. (ml.)	Activity (e.u./ml.)	Yield %
1st Extract	1,870	1.07	90
2nd Extract	400	0.70	10
Combined Extracts	2,270	1.30	100

The combined extracts were subjected to precipitation and fractional re-extraction with ammonium sulphate, followed by dialysis against distilled water as already described. The first three steps of the purification are summarized in Table 28.

TABLE 28.

		Protein (mg.)	Sp.Act. e.u./mg	Yield %	High Mol wt. form (%)	Low Mol wt. form (%)
Step 1.	Acetate extract	14,755	0.15	100	45	55
Step 2.	20-40% $(NH_4)_2SO_4$	13,050	0.17	88	49	51
Step 3.	Dialysis against H_2O	1,160	1.25	62	47	53

The ratio of the two forms of the enzyme was measured using the thin-layer gel filtration method followed by densitometry. The results obtained confirm the finding that the ratio does not alter during the

three stages of the purification.

It is apparent that the use of laboratory bred rabbits considerably increased the quantity of the high molecular weight form.

DEAE Chromatography at pH 5.8

The dialysate from above was passed through a column of DEAE cellulose, the deaminase being retained on the column. As in previous purifications, the enzyme was eluted using a salt gradient of sodium citrate pH 5.8. The eluate was collected in 10 ml. fractions, and the protein concentration and deaminase activity of each tube measured. The elution pattern is illustrated in Fig. 20. It is evident that the peak of enzymic activity is unsymmetrical, there being a tendancy towards skewing especially at the decending limb of the peak. It was decided therefore to measure the ratio of the two molecular forms of the enzyme in various tubes.

The tubes were pooled into three fractions (designated α, β and γ respectively) and the ratio of the enzyme forms in each fraction estimated using the thin-layer gel filtration method (Table 29).

TABLE 29

Tube Number	Fraction	High Mol. wt. form(%)	Low Mol. wt.form(%)
14, 15, 16, 17	α	100	0
18-25	β	40	60
26, 27, 28, 29	γ	75	25

Densitometer tracings of the thin-layer plates are shown in Fig. 21. It is evident that the high molecular weight form predominated in both the α and γ fractions, while the low molecular weight form eluted in the major part of the peak (the β fraction). This would appear to indicate that the low molecular weight form eluted as a rather sharp peak and the high molecular weight form as an overlapping, somewhat flattened peak of activity. (See theoretical drawing, Fig. 22 (b).

At this stage it was decided to use the β-fraction, which contained both the high molecular weight and low molecular weight forms, for further purification. The protein concentration and activity of this fraction are shown in Table 30.

TABLE 30.

	Activity e.u./ml.	Protein mg./ml.	Sp. Act. e.u./mg.	Yield %	Purification factor.
DEAE pH 5.8 (β fraction)	10.0	0.9	11.0	40	70

Sephadex G-75 I

The β-fraction was then dialysed against distilled water, concentrated to a volume of 1 ml. approx. on a small DEAE cellulose column, and applied to a Sephadex G-75 Superfine column. The elution pattern obtained is illustrated in Fig. 23. 40% of the total activity applied was found to be present in Peak A (high molecular

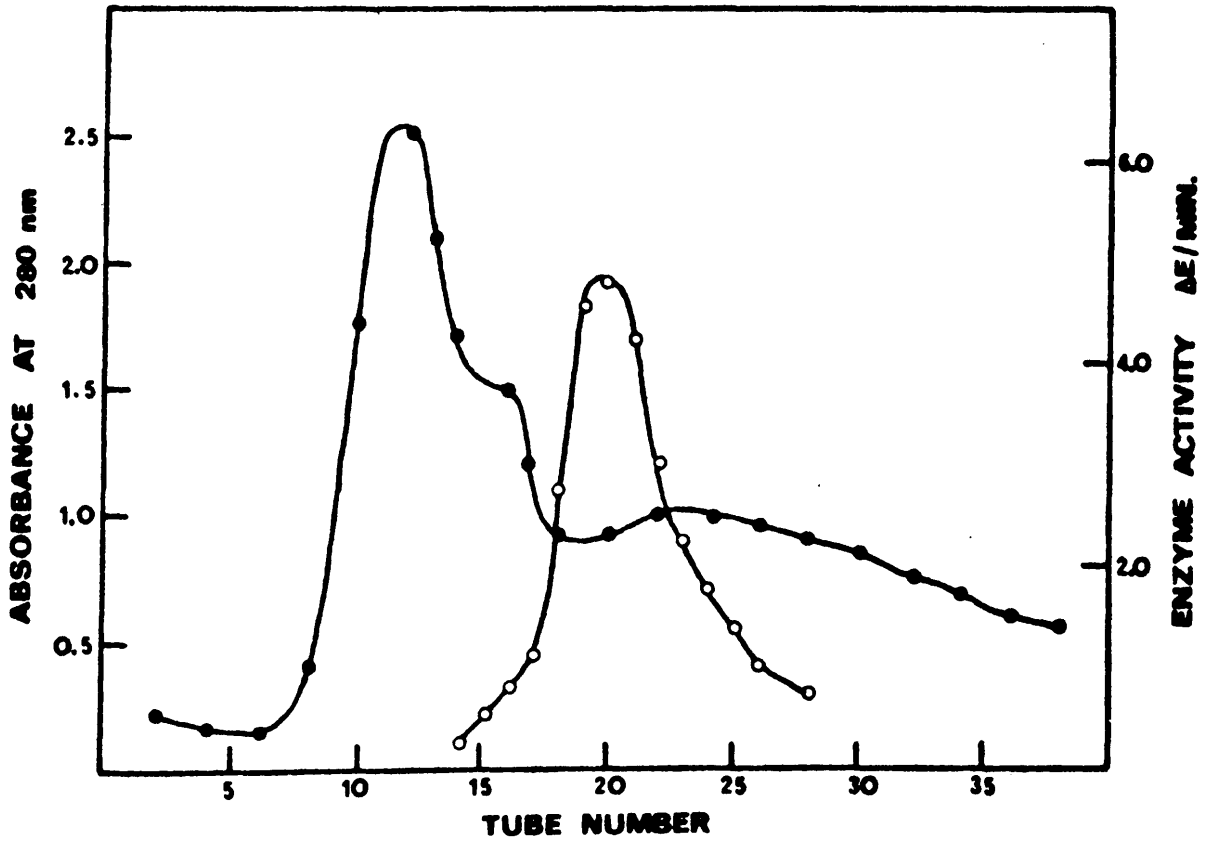


FIG. 20. Chromatography of adenosine deaminase on DEAE cellulose. The dialysed enzyme solution from Step 3 (sp. act. 1.25 e.u.) was applied to the column and eluted using a sodium citrate gradient (pH 5.8)from 0.002-0.03M. 10 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——●, protein; o——o adenosine deaminase activity.

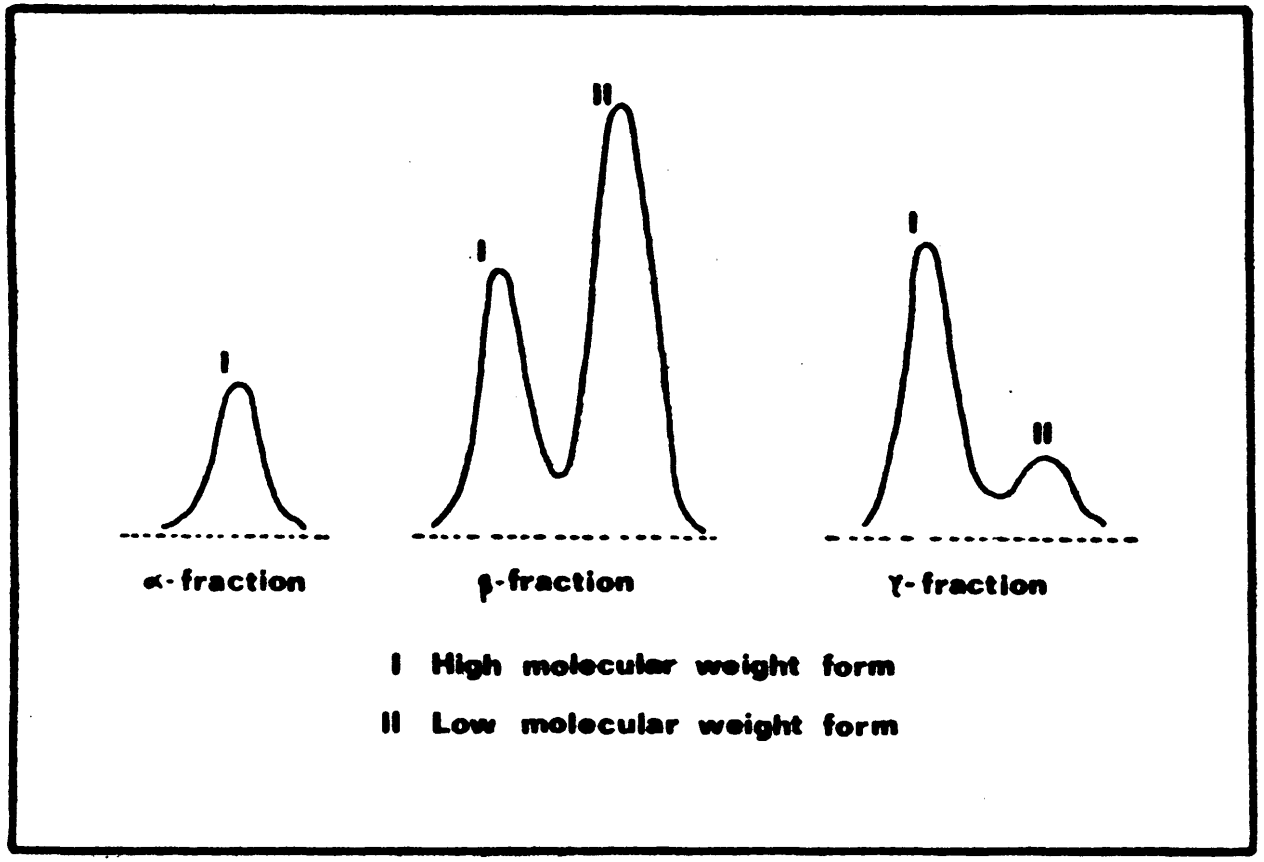


FIG. 21. Densitometer tracings of thin-layer gel filtration patterns. Samples of the α, β and γ fractions from the DEAE chromatography step were applied to a Sephadex G-75 coated thin-layer plate and after development (using 0.05 M KCl as solvent) sites of enzyme activity were detected using the zymogram stain for adenosine deaminase.

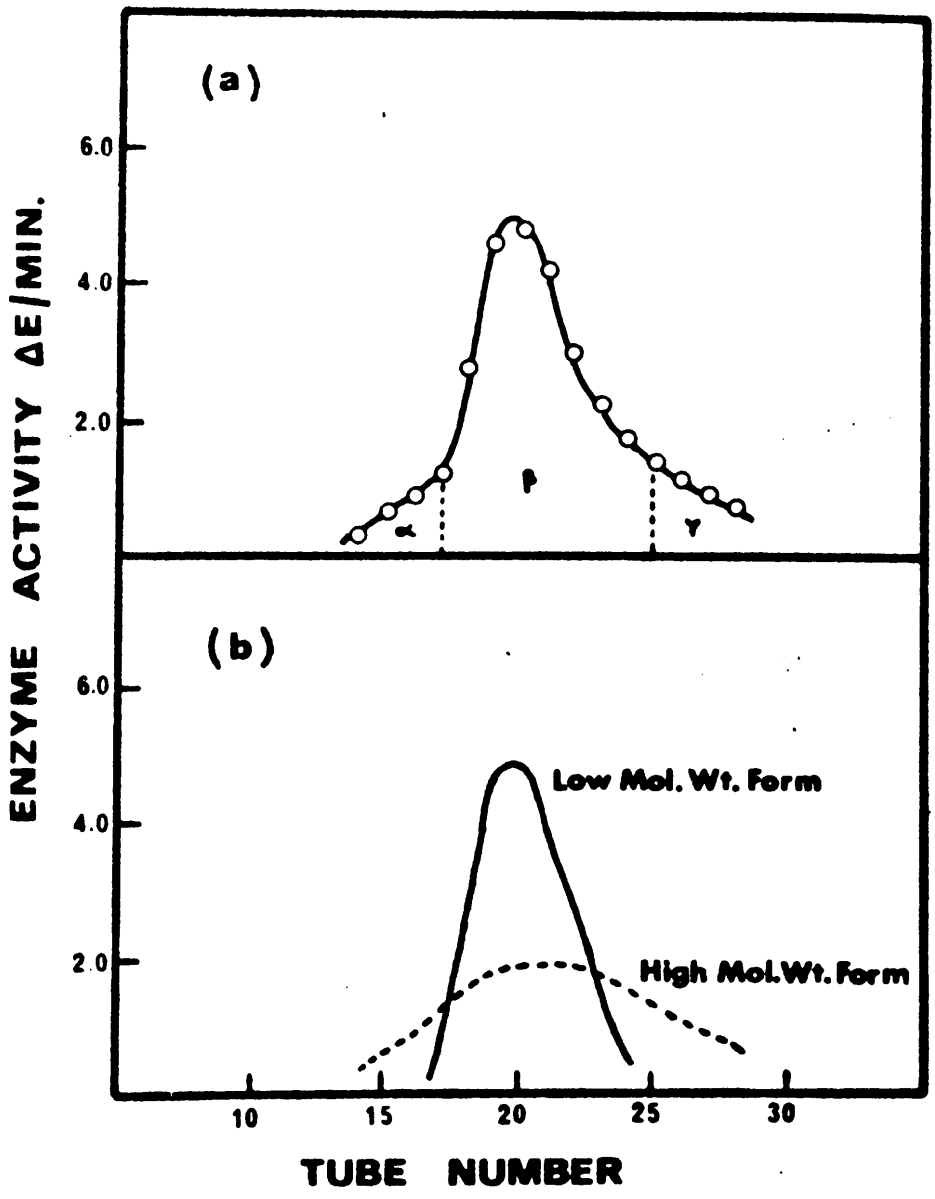


FIG. 22. (a) Chromatography of adenosine deaminase on DEAE cellulose (see FIG. 20). Tubes 14-17 (α-fraction), 18-24(β-fraction) and 25-28 (γ-fraction) were separately pooled.

(b) Theoretical drawing of the elution patterns of the high and low molecular weight forms of adenosine deaminase from the DEAE cellulose column based on the estimates made for the ratio of the two forms of the enzyme in the α-, β-and γ-fractions (see Table 29).

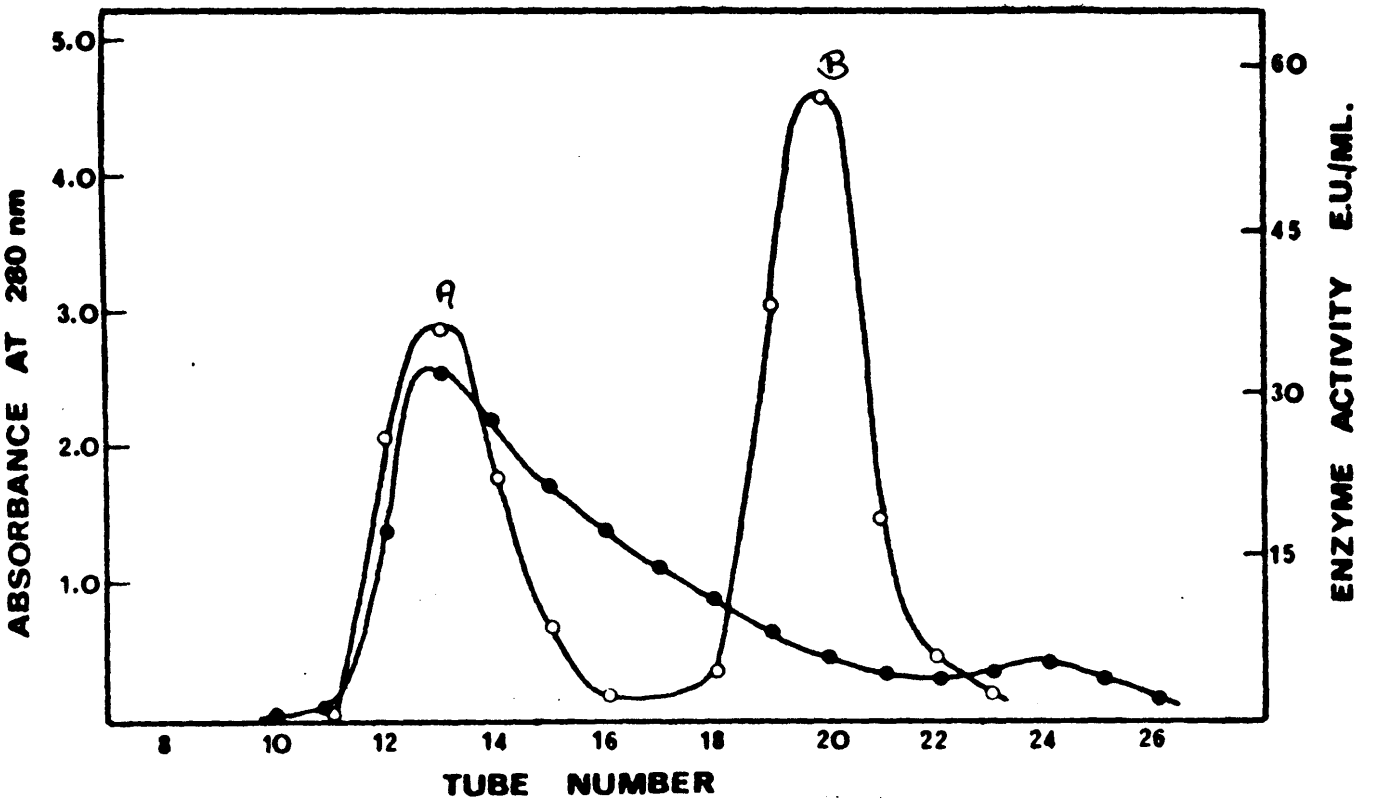


FIG. 23. Sephadex G-75 1. Gel filtration of adenosine deaminase on Sephadex G-75 (Superfine). The β-fraction from the DEAE chromatography step (sp. act. 11.0 e.u.) was concentrated and applied to the column which was equilibrated with 0.002M sodium citrate pH 5.8. 5 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0) ●——● , protein; o——o , adenosine deaminase activity.

weight form) and 60% in Peak B (low molecular weight form). These figures showed very good agreement with results obtained using the thin-layer gel filtration method (see Table 28).

Table 31 shows the specific activities of various tubes collected after chromatography.

TABLE 31. Specific Activities of Various Tubes after Sephadex G-75 I Chromatography.

Peak A	Tube No.	Total Protein(mg.)	Sp. Activity	
(High mol. wt. form)	12	7.0	18.9	15.5
	13	12.5	14.0	
Peak B	19	3.0	60	
(Low mol. wt. form)	20	2.25	120	
	21	2.0	50	

The combined tubes from Peak A (high molecular weight form) had a specific activity of 15.5 e.u./mg.

Further Purification of the Low Molecular Weight Form

Sephadex G-75 II.

The low molecular weight form was further purified by first re-chromatographing tubes 19 and 21 from Sephadex G-75 I. These were combined, concentrated to a volume of 1 ml. approx., and applied to the column. The resulting elution pattern is illustrated in Fig. 24.

TABLE 32. Specific Activities of Various Tubes after Sephadex G-75 II Chromatography.

Tube No.	Total Protein (mg.)	Sp. Activity (e.u./mg.)
18	0.75	22
19	0.88	120
20	0.75	125
21	0.50	76

Table 32 shows that tubes 19 and 20 had the highest specific activities.

Sephadex G-75 III

The most active tube from Sephadex G-75 I (tube 20) was combined with tubes 19 and 20 from Sephadex G-75 II and the resulting solution concentrated. This high active preparation was then re-applied to the Sephadex G-75 column. The pattern obtained after

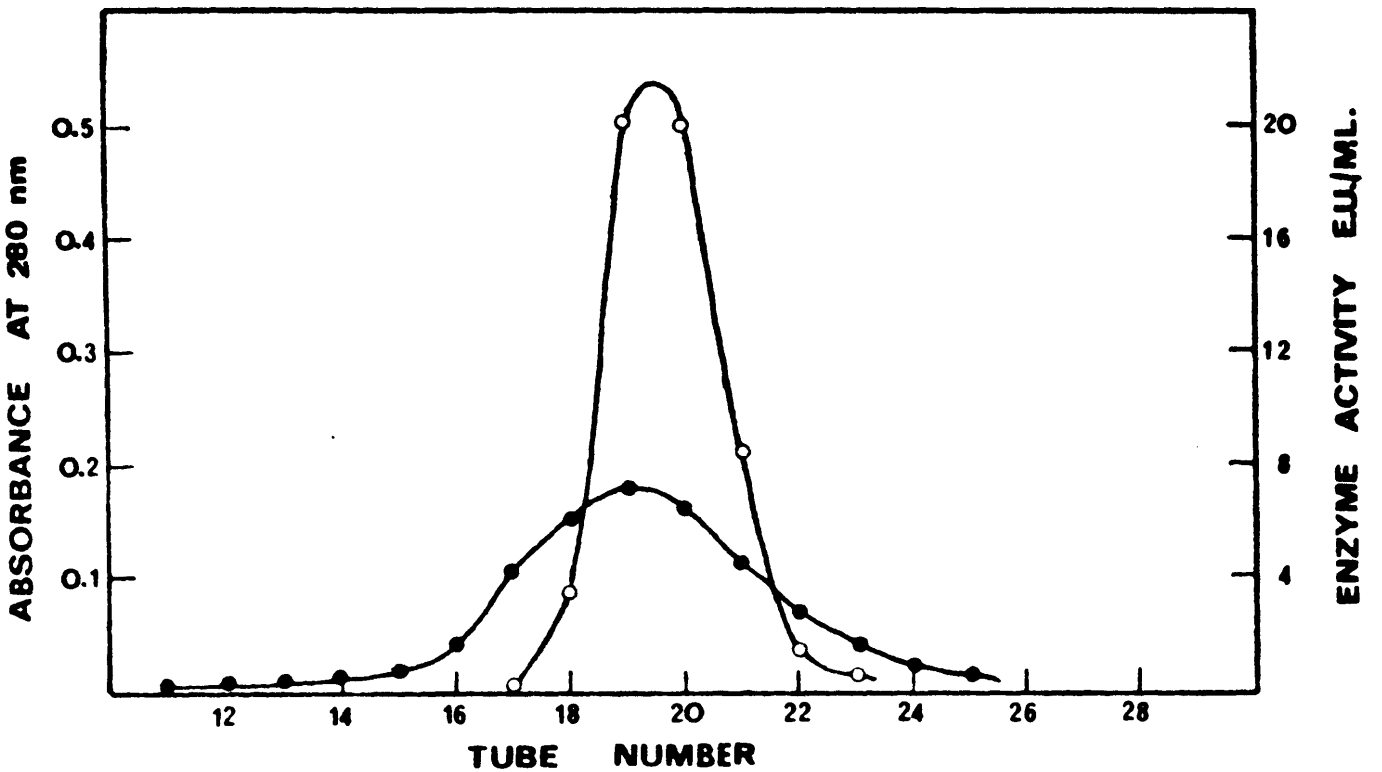


FIG. 24. Sephadex G-75 II. Gel filtration of adenosine deaminase on Sephadex G-75 (Superfine). Tubes 19 and 21 from Sephadex G-75 I (FIG. 23) were pooled, concentrated and applied to the column equilibrated with 0.002 M sodium citrate pH 5.8. 5 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●—●, protein; o—o, adenosine deaminase activity.

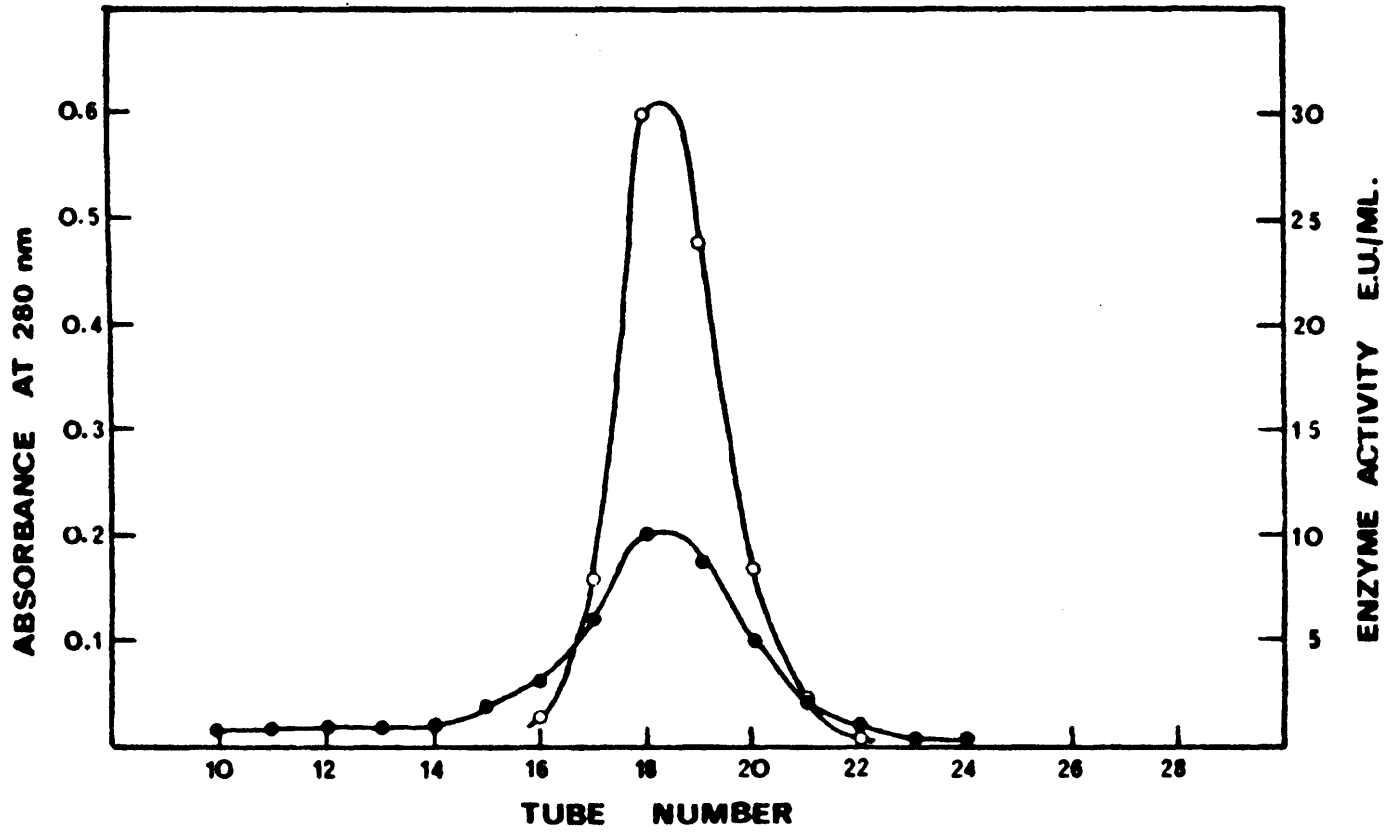


FIG. 25. Sephadex G-75 III. Gel filtration of adenosine deaminase on Sephadex G-75 (Superfine). Tube 20 from Sephadex G-75 I (FIG. 23) and tubes 19 and 20 from Sephadex G-75 II (FIG. 24) were pooled, concentrated and applied to the column equilibrated with 0.002 M sodium citrate buffer pH 5.8. 5 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity was estimated spectrophotometrically at 265 nm (pH 7.0). ●——● , protein; o——o , adenosine deaminase activity.

elution is shown in Fig. 25. Only a single peak of 280 nm-absorbing material emerged, which coincided with the peak of enzymic activity.

TABLE 33. Specific Activities of Various Tubes after Sephadex G-75 III Chromatography

Tube No.	Total Protein (mg.)	Sp. Activity (e.u./mg.)	Yield %
17	0.625	73	3.0
18	1.000	150	7.0
19	0.875	133	6.0
20	0.500	84	3.3

Although the specific activity of the enzyme in this case (Table 33) was much lower than previously obtained, the symmetry of the two curves indicated that the preparation was nearly homogenous.

Further Purification of the High Molecular Weight Form

Tubes 12 and 13 (Sp. activity: 15.5 e.u./mg.) from Sephadex G-75 I were used for purification of the high molecular weight form. Because of the very large size of the proteins present in this preparation (they eluted at the void volume of the Sephadex G-75 column) it was decided to replace the Sephadex G-75 with a

Sephadex G-200 chromatography step.

Sephadex G-200 Chromatography

A Sephadex G-200 column (40 cm. x 2.5 cm.) was constructed as previously described (p. 21), and equilibrated for several days with 0.002M sodium citrate buffer pH 5.8. The flow rate through the column was 0.04 ml./min. (approx.). The high molecular weight enzyme solution from above was concentrated on a small DEAE cellulose column and applied to the Sephadex G-200 column. The resulting elution pattern is illustrated in Fig. 26. It is evident that the first major protein peak coincided with the peak of enzyme activity. Tubes 19 and 20 were combined and concentrated.

TABLE 34.

Purification Step	Total e.u.	Protein (mg.)	Sp. Act. e.u./mg.	Yield %
Sephadex G-75 I (Tubes 12 and 13)	310	19.7	15.5	13
Sephadex G-200 (Tubes 19 and 20)	150	5.8	26.0	7

Table 34 shows the results obtained before and after chromatography. The specific activity of the preparation was increased from 15.5 e.u./mg. to 26.0 e.u./mg. which indicates the usefulness of the Sephadex G-200 chromatography step. The protein removed by

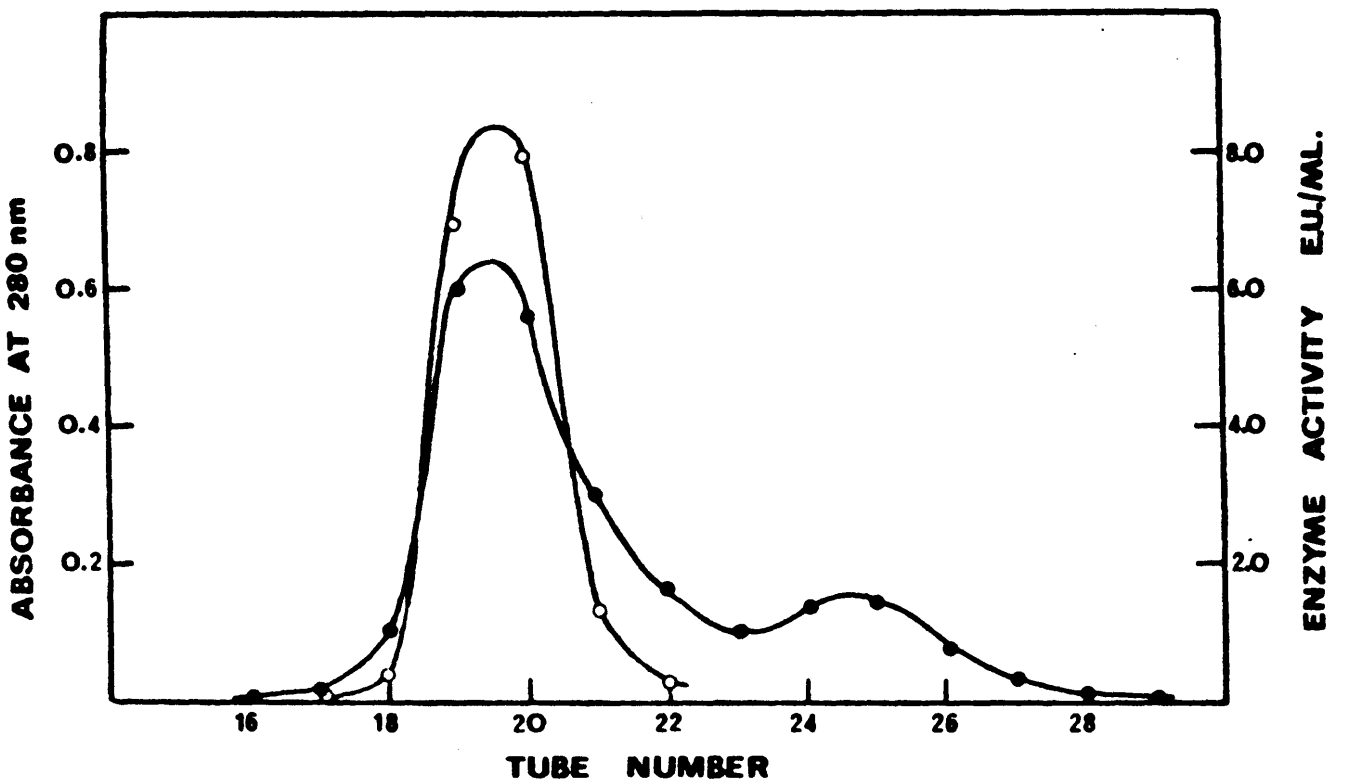


FIG. 26. Gel filtration of adenosine deaminase on Sephadex G-200 (Superfine). Tubes 12 and 13 from Sephadex G-75 1 (FIG. 23) were pooled, concentrated and applied to the column which was equilibrated with 0.002M sodium citrate buffer pH 5.8. 5 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity was estimated spectrophotometrically at 265 nm (pH 7.0). ●——● , protein; o——o , adenosine deaminase activity.

this step is illustrated on Plate 4 which shows the patterns obtained when both samples (i.e before the Sephadex G-200 and after) were analysed by low voltage electrophoresis using cellulose acetate paper. It is evident that two proteins of different electrophoretic mobilites were present in the final preparation.

A summary of the entire purification procedure is illustrated in Table 35. Although the final yields of enzyme (13% for the low molecular weight from and 7% for the high molecular weight form) were very low, the values are only apparent, since the starting crude extract contained both enzyme forms. Table 36 shows the true enzyme yields calculated on the basis that the original acetate extract possessed 45% of its deaminase activity as high molecular weight enzyme and 55% as low molecular weight enzyme, these estimates having been made using the thin-layer gel filtration technique (p. 27).

TABLE 36.

	Percentage Yield	
	Low Molecular weight form	High Molecular weight form
Apaprent Yield	13.0	7.0
True Yield	23.0	15.5

TABLE 35. Purification of the High Molecular Weight and Low Molecular Weight Forms of Adenosine Deaminase from Rabbit Intestine.

Purification Step	Vol. (ml.)	Activity (e.u./ml.)	Total e.u.	Protein (mg/ml.)	Total Protein	Sp. Act.	Yield %	Purification factor
Step 1. Extraction with acetate	2270	1.03	2338	6.5	14755	0.16	100	1.0
Step 2. 20%-40% $(NH_4)_2 SO_4$	290	7.6	2204	45.0	13050	0.17	94	1.06
Step 3. Dialysis against H_2O	365	4.0	1460	3.18	1160	1.25	62	7.7
Step 4. DEAE pH 5.8	92	10.0	920	0.90	83	11.0	40	70

Low Molecular Weight Form.

Purification Step	Protein(mg.)	Sp. Act.	Yield(%)
Sephadex G-75 I	2.25	120	24
Sephadex G-75 III	1.87	140	13

High Molecular Weight Form.

Purification Step	Protein(mg.)	Sp. Act.	Yield(%)
Sephadex G-75 I	19.75	15.5	13
Sephadex G-200	5.8	26.0	7

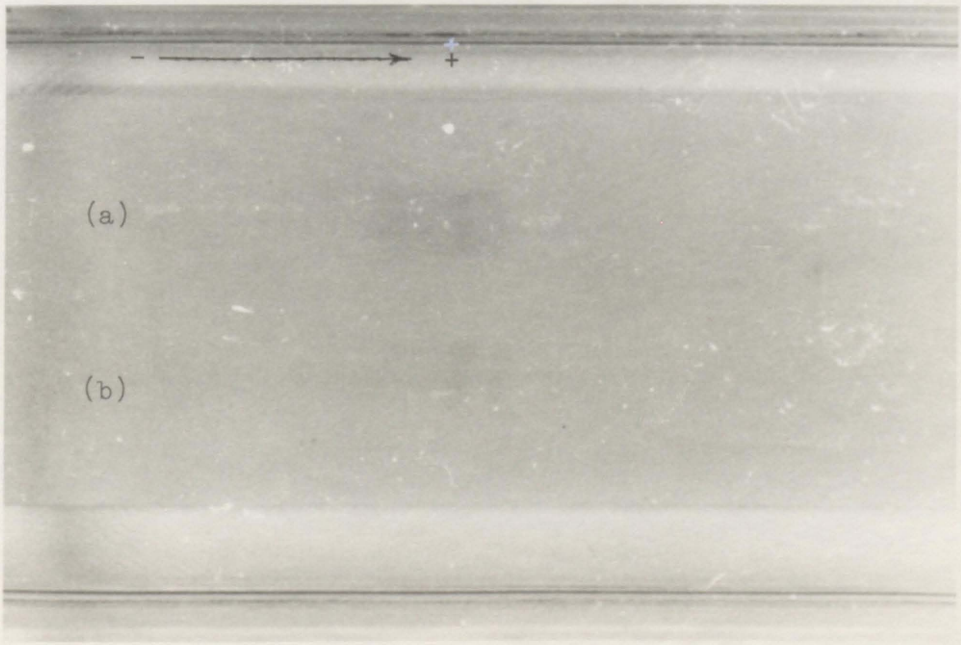


PLATE 4. LOW VOLTAGE CELLULOSE ACETATE ELECTROPHORESIS OF THE HIGH MOLECULAR WEIGHT FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE.

(a) POST SEPHADEX G-75 I
(b) POST SEPHADEX G-200

STAINING WAS CARRIED OUT USING THE PONCEAU S - NIGROSINE PROTEIN STAIN.

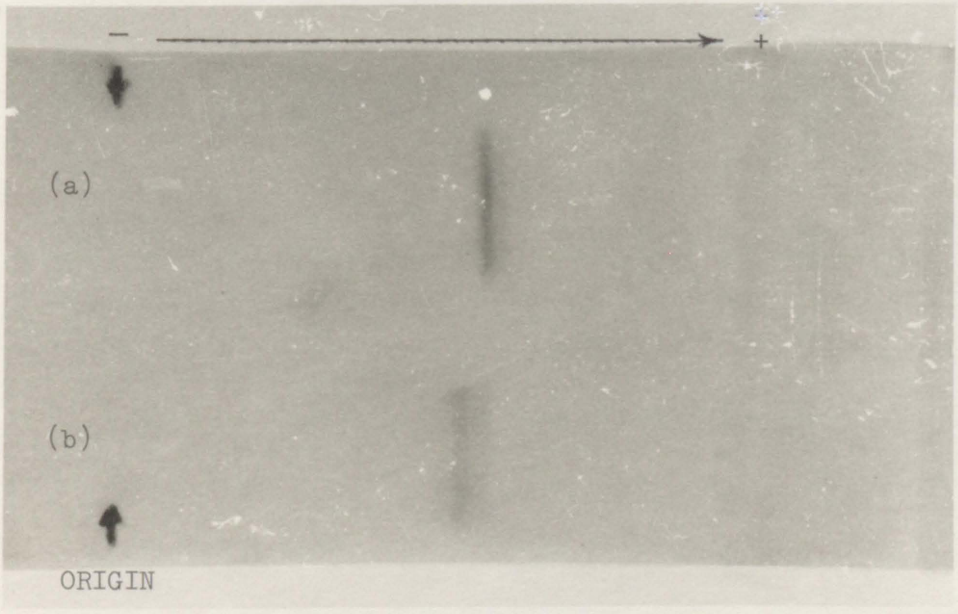


PLATE 5. LOW VOLTAGE CELLULOSE ACETATE ELECTROPHORESIS OF RABBIT INTESTINAL ADENOSINE DEAMINASE

(a) LOW MOLECULAR WEIGHT FORM
(b) HIGH MOLECULAR WEIGHT FORM

STAINING WAS CARRIED OUT USING THE PONCEAU S - NIGROSINE PROTEIN STAIN.

Electrophoretic Analysis.

Both forms of the enzyme were analysed by low voltage electrophoresis on cellulose acetate. Samples were applied to the strip and electrophoresis was carried out using 300 volts for 45 mins. The paper was fixed and stained for protein as described in the Methods section (p.33). The patterns obtained are illustrated in Plate 5. Two distinct protein bands were evident in the case of both forms, each enzyme consisting of a fast moving minor band followed closely by a slow moving major band. As will be seen later both protein bands in each case possess enzymatic activity.

PURIFICATION VI

In Purification V the ∝ and γ fractions, obtained after DEAE cellulose chromatography, were not used for further purification as the total number of enzyme units was very low in these fractions. It was later realised however, that the high molecular weight form was an enzyme of very low specific activity. Because this form contributed to most of the deaminase activity in both the ∝ and γ fractions, the purification scheme was revised, the ∝ and γ fractions being retained and used for further purification of the high molecular weight form.

293 g. of frozen intestine, taken from both wild and domestic rabbits, was used for this purification. The deaminase was extracted from the tissue with 0.2M acetate buffer pH 5.5 and purified to the dialysis stage as already described. The results are summarized in Table 37.

TABLE 37.

Procedure		Protein (mg.)	Sp.Act. e.u./mg	Yield %	High Mol. wt. form (%)	Low Mol. wt. form (%)
Step 1.	Acetate extract	11375	0.16	100	22	78
Step 2.	20-40%$(NH_4)_2SO_4$	8600	0.19	86	-	-
Step 3.	Dialysis against H_2O	887	1.33	62	30	70

DEAE Chromatography at pH 5.8

The dialysed material was then applied to a DEAE cellulose column. After washing off unabsorbed protein using 0.002M sodium citrate pH 5.8, a salt gradient was applied as described in previous purifications. The elution pattern obtained is illustrated in Fig. 27. In this case the enzyme peak showed considerable skewing on both sides. Tubes which showed adenosine deaminase activity were pooled as before into three fractions, the α fraction consisting of tubes 14 through 17, the β fraction of tubes 18 through 24 and the γ fraction of tubes 25 through 28. Densitometer tracings of thin-layer gel filtration patterns of the three fractions are shown in Fig. 28. It is evident that both the α and γ fractions possessed a considerable quantity of the high molecular weight form, whereas the main peak (β fraction) contained most of the low molecular weight form. From a knowledge of the total enzyme units and the ratio of the two molecular weight forms in each fraction, it was calculated that approximately 50% of the high molecular weight form applied to the DEAE cellulose column eluted in the β fraction, and the remaining 50% eluted in the combined α and γ fractions.

The β fraction was therefore used for the purification of both the low molecular weight and high molecular weight forms, and the combined α and γ fractions also used for further purification of the high molecular weight form.

The results obtained after DEAE chromatography are summarized in Table 38.

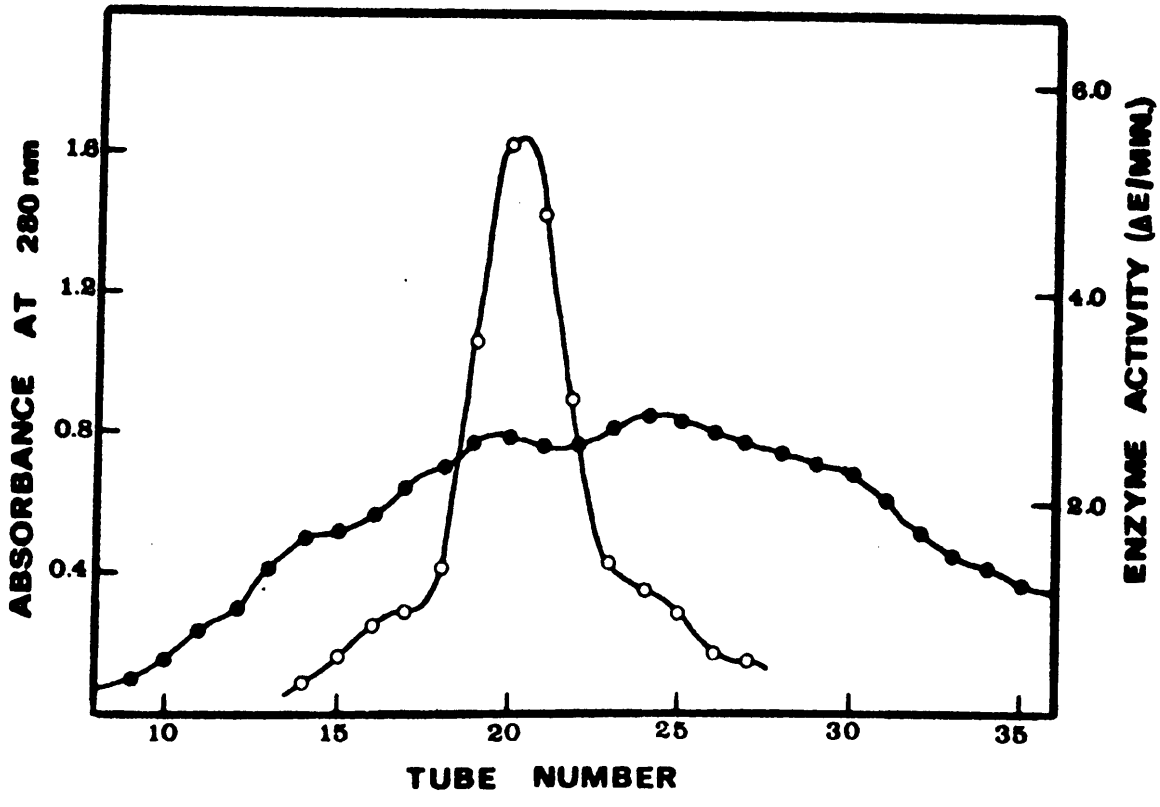


FIG. 27. Chromatography of adenosine deaminase on DEAE cellulose. Dialysed enzyme solution from Step 3 (sp. act. 1.33 e.u.) was applied to the column and eluted using a sodium citrate gradient (pH 5.8) from 0.002-0.03 M. 10 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——● , protein; o——o , adenosine deaminase activity.

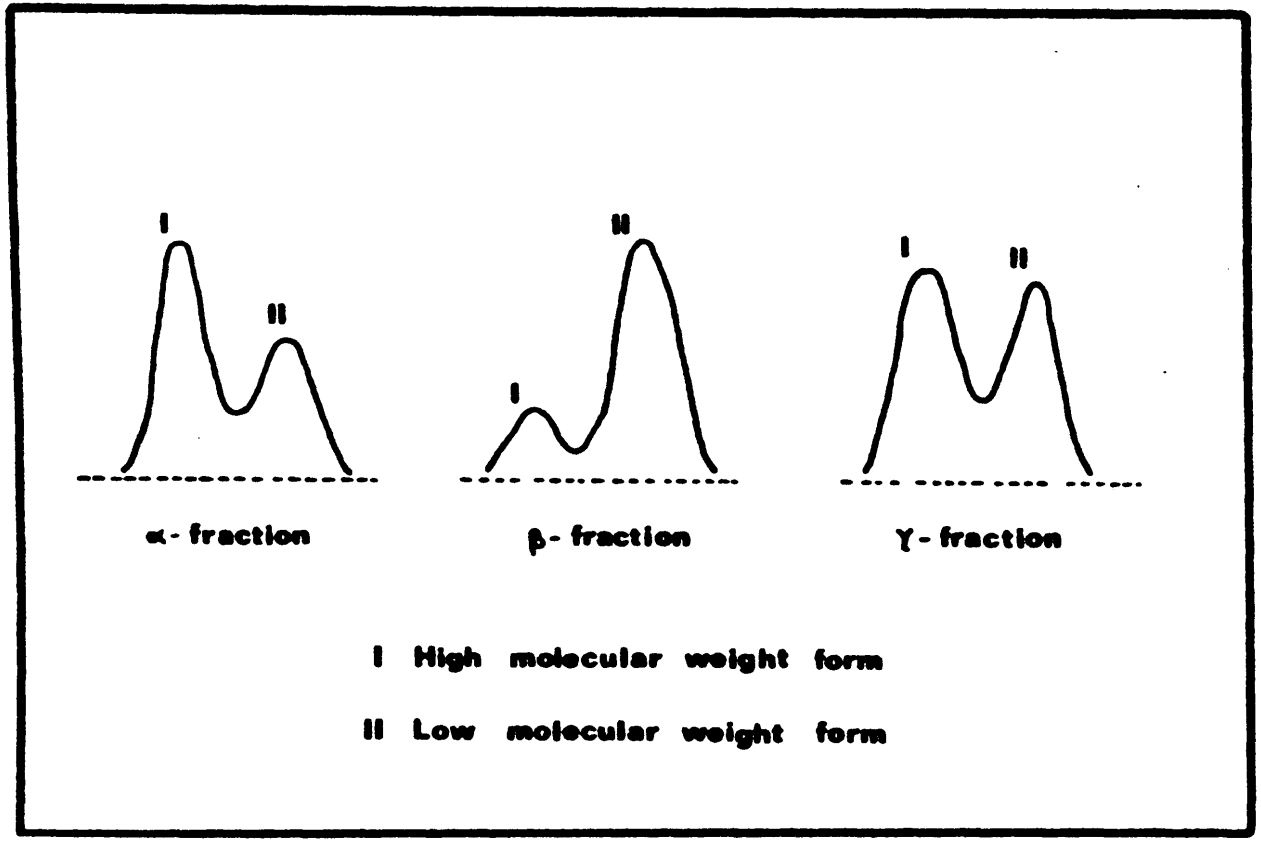


FIG. 28. Densitometer tracings of thin-layer gel filtration patterns. Samples of the α-, β-and γ-fractions from the DEAE chromatography step were applied to a Sephadex G-75 coated thin-layer plate and after development (using 0.05 M KCl as solvent) sites of enzyme activity were detected using the zymogram stain for adenosine deaminase.

TABLE 38.

	Protein (mg.)	Sp.Act. e.u./mg	Yield %	High Mol. wt.form (%)	Low Mol. wt. form (%)
DEAE (β fraction)	51.6	15.2	42	17	83
DEAE (αγ fraction)	67.2	3.2	12	60	40

PURIFICATION OF THE LOW MOLECULAR WEIGHT AND HIGH MOLECULAR WEIGHT FORMS FROM THE β FRACTION

The β fraction was dialysed against distilled water, concentrated and applied to a Sephadex G-75 Superfine column. The pattern obtained (designated Sephadex G-75 Iβ) is shown in Fig. 29. It is evident that complete separation of the two forms was obtained.

Purification of the low Molecular Weight Form

The low molecular weight form was further purified by first re-chromatographing tubes 15 and 18 from Sephadex G-75 Iβ. The pattern obtained (designated Sephadex G-75 IIβ) is illustrated in Fig. 30. Tube 19 (Sephadex G-75 IIβ) which had the highest specific activity was then pooled with tubes 16 and 17 from Sephadex G-75 Iβ and the resulting solution concentrated and applied to the Sephadex G-75 column. Fig. 31 shows the elution pattern from which it is

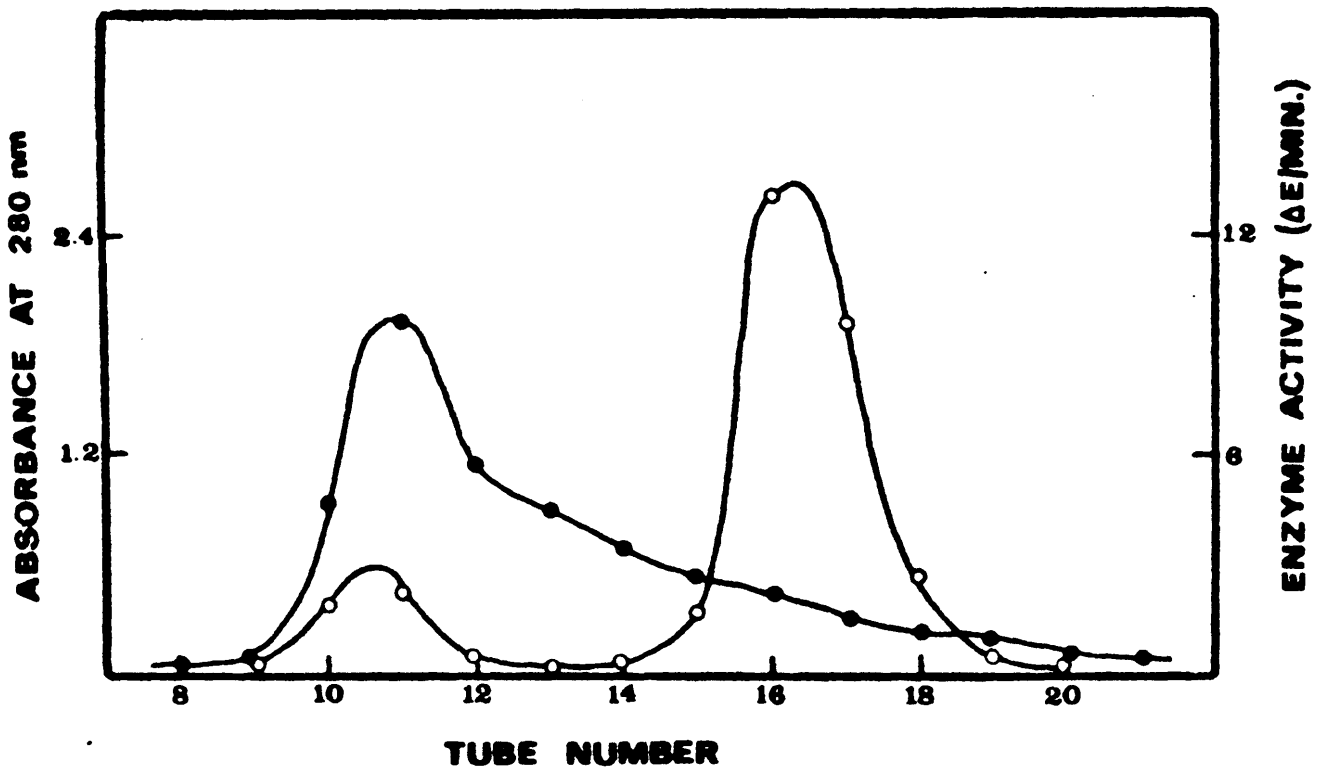


FIG. 29. Sephadex G-75 1β. Gel filtration of adenosine deaminase on Sephadex G-75 (superfine). The β-fraction from the DEAE chromatography step (sp. act. 15.2 e.u.) was concentrated and applied to the column which was equilibrated with 0.002 M sodium citrate buffer pH 5.8. 5 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——● , protein; o——o , adenosine deaminase activity.

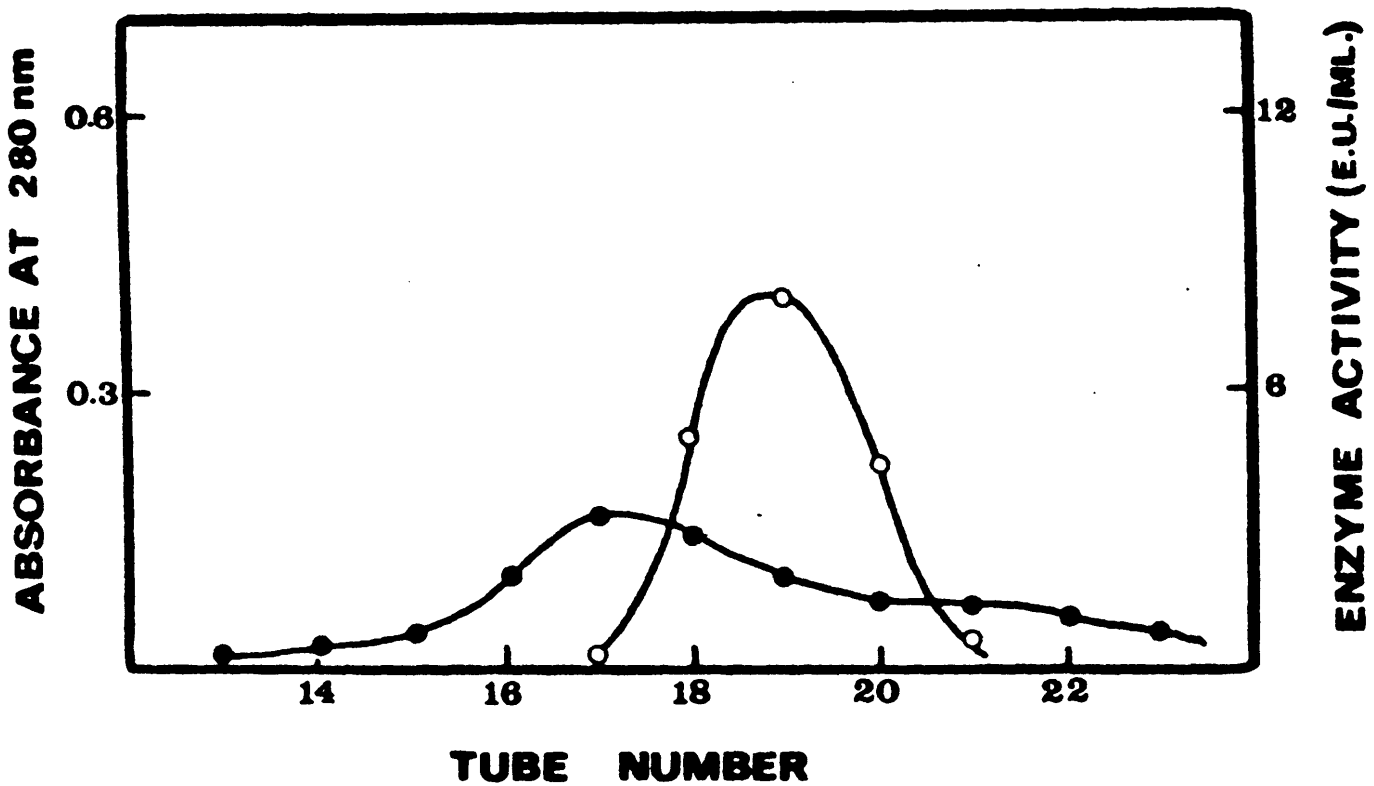


FIG. 30. Sephadex G-75 11β. Gel filtration of adenosine deaminase on Sephadex G-75 (Superfine). Tubes 15 and 18 from Sephadex G-75 1β (FIG. 29) were pooled, concentrated and applied to the column which was equilibrated with 0.002 M sodium citrate buffer pH 5.8. 5 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——● protein; o——o , adenosine deaminase activity.

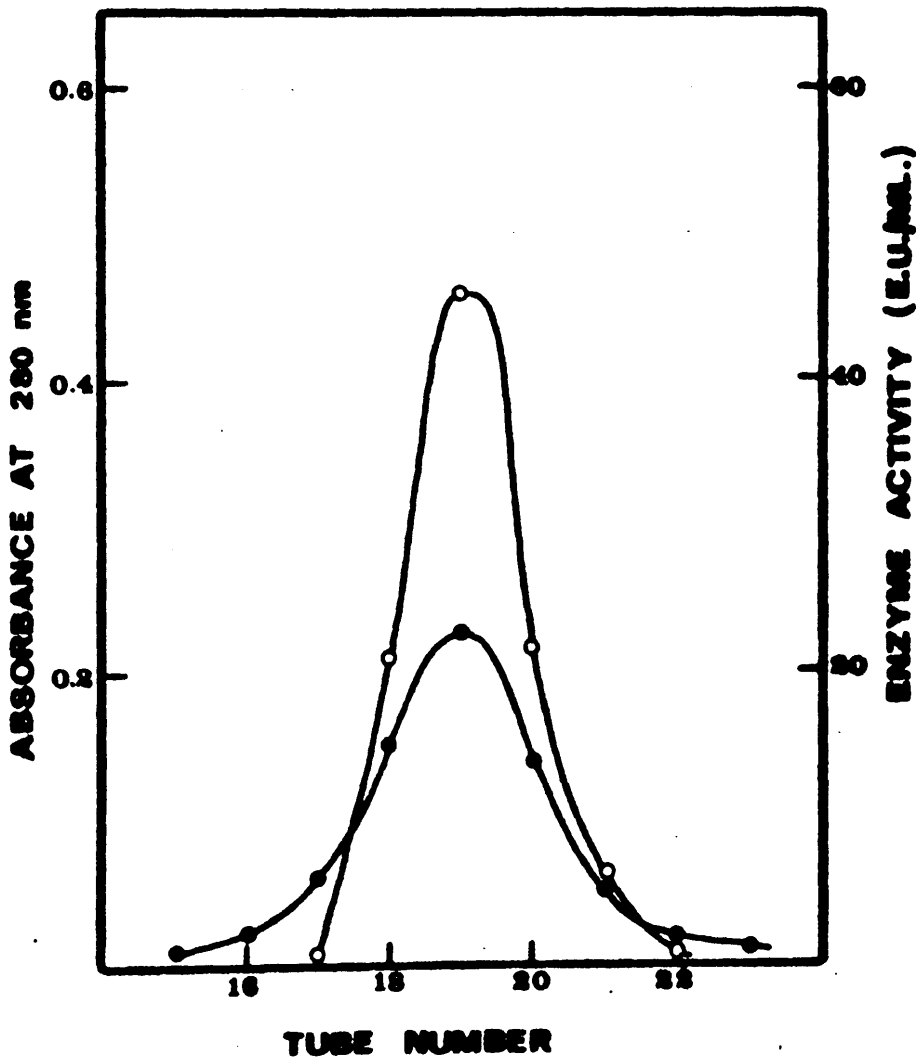


FIG. 31. Sephadex G-75 111β. Gel filtration of adenosine deaminase on Sephadex G-75 (Superfine). Tubes 16 and 17 from Sephadex G-75 1β (FIG. 29) and tube 19 from Sephadex G-75 11β (FIG. 30) were pooled, concentrated and applied to the column equilibrated with 0.002 M sodium citrate buffer pH 5.8. 5 ml. fractions were collected. Protein concentration was determined absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——● , protein; o——o , adenosine deaminase activity.

UNIVERSITY COLLEGE LIBRARY CORK

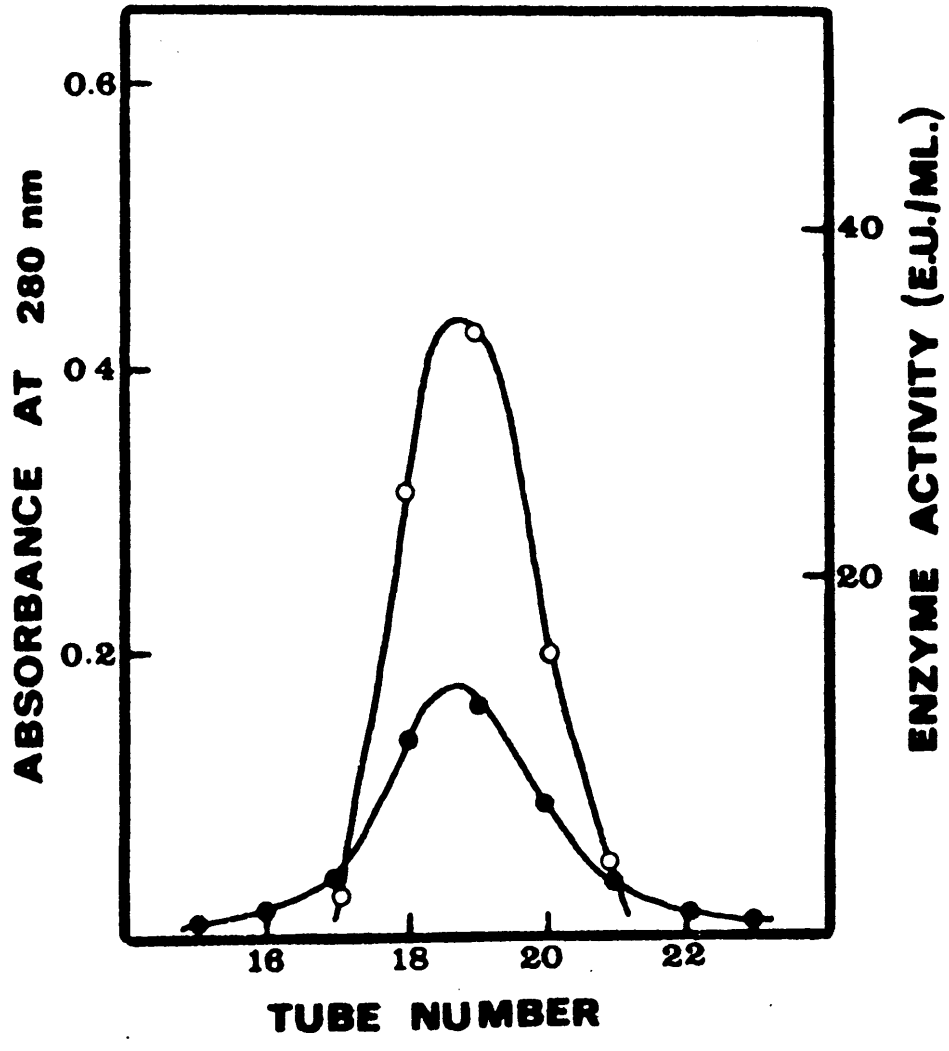


FIG. 32. Sephadex G-75 IVβ. Gel filtration of adenosine deaminase on Sephadex G-75 (Superfine). Tubes 18, 19 and 20 from Sephadex G-75 IIIβ (FIG. 31) were pooled, concentrated and applied to the column which was equilibrated 0.002 M sodium citrate buffer pH 5.8. 5 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——● , protein; o——o , adenosine deaminase activity.

evident that only a single protein peak emerged coincident with the peak of enzymic activity. The specific activities of various fractions are given in Table 39.

TABLE 39. Specific Activities of Adenosine Deaminase Solutions after Sephadex G-75 Chromatography (Sephadex G-75 IIIβ)

Tube No.	Total Protein (mg)	Sp. Act. (e.u./mg.)
17	0.30	12
18	0.75	140
19	1.15	210
20	0.70	156
21	0.25	110

Although the protein and enzyme peaks appeared to be symmetrical, the specific activity of the preparation was low. Tubes 18, 19 and 20 were therefore pooled, concentrated and re-applied to the Sephadex G-75 column. Fig. 32 illustrates the elution pattern obtained.

The final quantity of purified enzyme was somewhat increased (1.8 mg.), but the specific activity of 210 e.u. had not increased.

Purification of the High Molecular Weight Form

Tubes 10 and 11 from Sephadex G-75 Iβ (which contained the high molecular weight form enzyme) were pooled, concentrated and applied to a Sephadex G-200 Superfine column. Fig. 33 shows the

elution pattern. Three peaks of 280 nm-absorbing material emerged, the first of which coincided with the peak of enzyme activity. The specific activities of various fractions are given in Table 40.

TABLE 40. Specific Activities of Adenosine Deaminase Solutions after Sephadex G-200 Chromatography (Sephadex G-200β)

Tube No.	Total Protein(mg.)	Sp. Act. (e.u./mg.)
31	1.4	5.0
32	1.8	12.2
33	1.5	19.0
34	1.2	10.5

Most of the enzyme was present in tubes 32, 33 and 34 which were pooled and retained.

Purification of the High Molecular Weight Form From the αγ Fraction

The combined α and γ fractions from the DEAE cellulose chromatography step (p.) were dialysed against distilled water and concentrated using a small DEAE column. The resulting solution was applied to a Sephadex G-75 column. The pattern obtained (designated Sephadex G-75-αγ) is illustrated in Fig. 34. Tubes 12 and 13 which contained the bulk of the high molecular

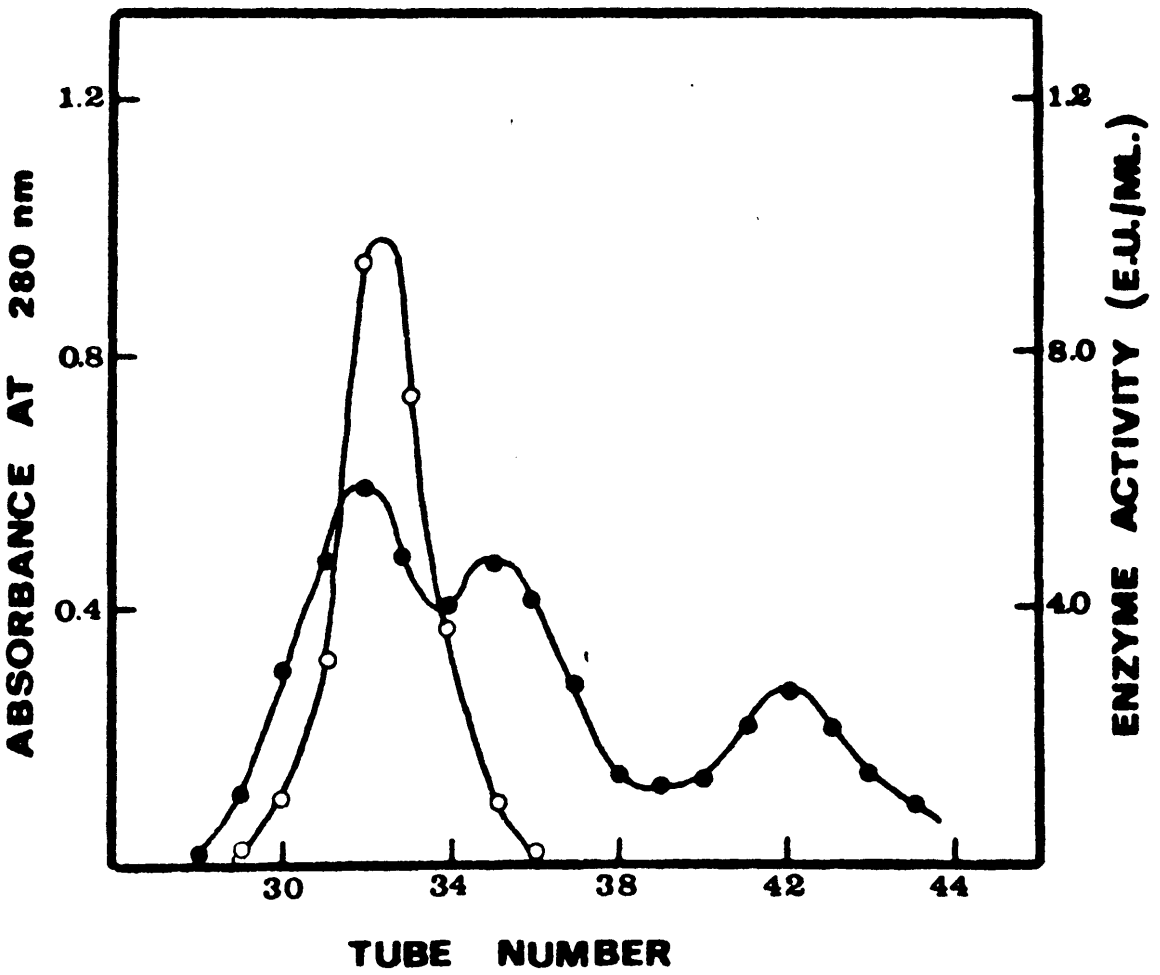


FIG. 33. Sephadex G-200β. Gel filtration of adenosine deaminase on Sephadex G-200 (Superfine). Tubes 10 and 11 from Sephadex G-75 1β (FIG. 29) were pooled, concentrated and applied to the column equilibrated with 0.002 M sodium citrate buffer pH 5.8. 3 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——● , protein; o——o , adenosine deaminase activity.

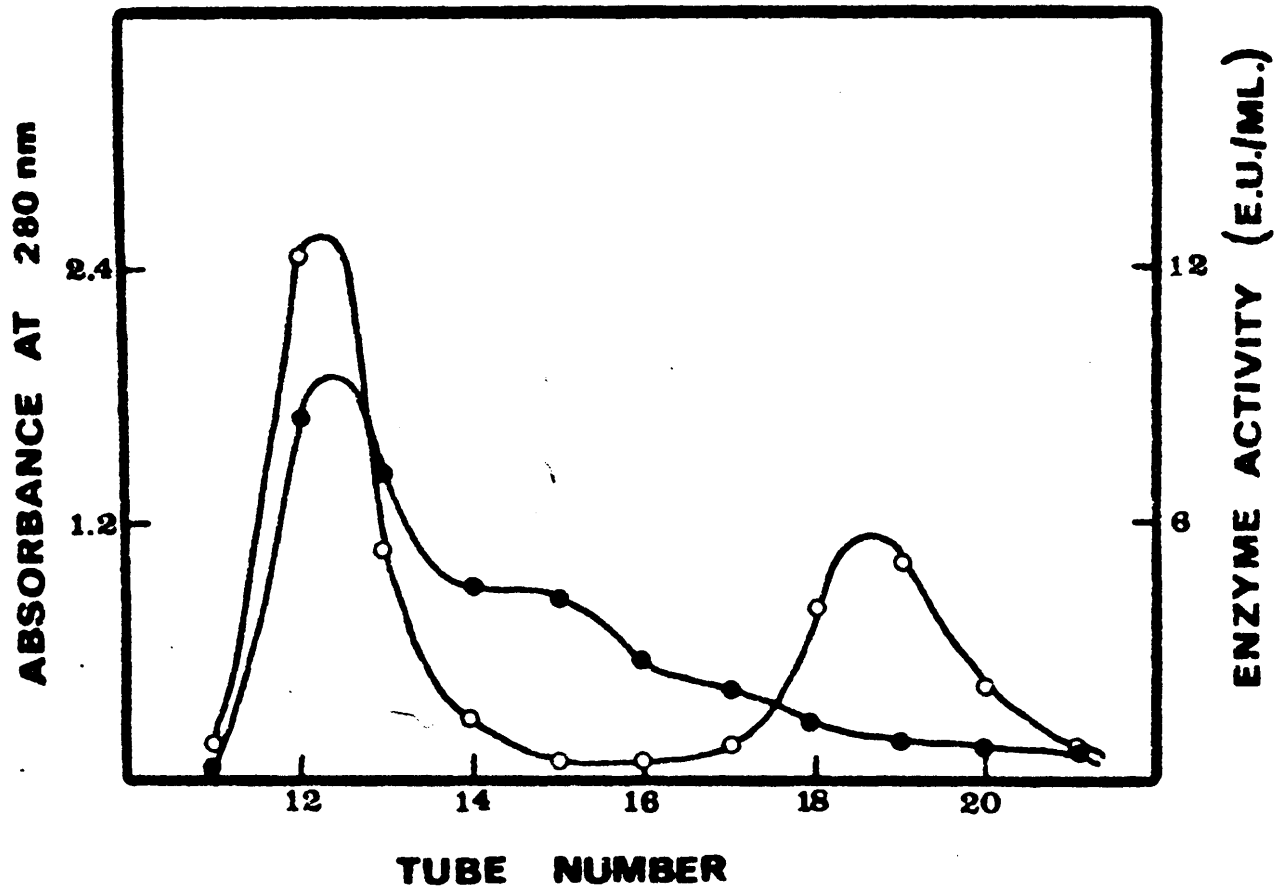


FIG. 34. Sephadex G-75 $\alpha\gamma$. Gel filtration of adenosine deaminase on Sephadex G-75 (Superfine). The α-and γ-fractions from the DEAE chromatography step (see Table 38 were combined (sp. act. 3.2 e.u.), concentrated and applied to the column which was equilibrated with 0.002 M sodium citrate buffer pH 5.8. 5 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (ph 7.0). ●——● ,protein; o——o , adenosine deaminase activity.

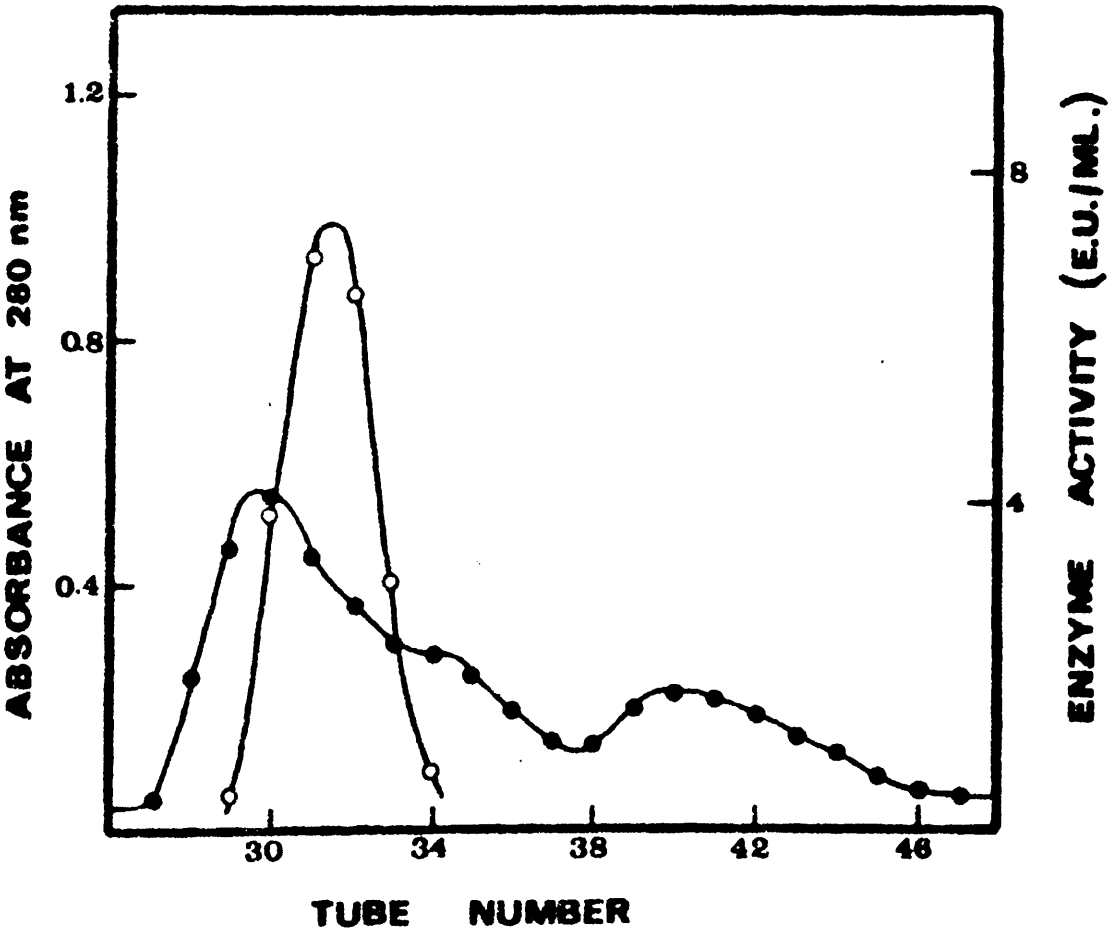


FIG. 35. Sephadex G-200$\alpha\gamma$. Gel filtration of adenosine deaminase on Sephadex G-200 (Superfine). Tubes 12 and 13 from Sephadex G-75$\alpha\gamma$ (FIG. 34) were pooled, concentrated and applied to the column which was equilibrated with 0.002 M sodium citrate buffer pH 5.8. 3 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——● , protein; o——o adenosine deaminase activity.

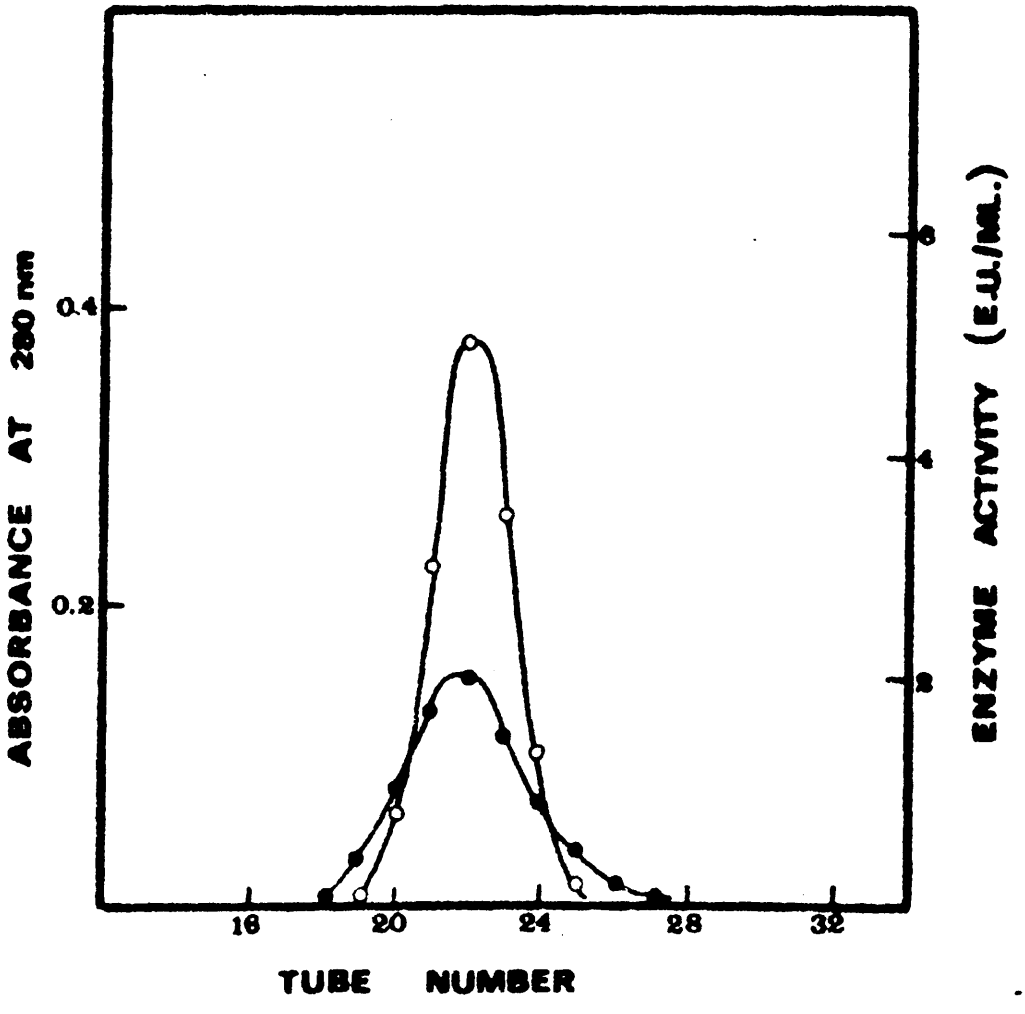


FIG. 36. Final Sephadex G-200. Gel filtration of adenosine deaminase on Sephadex G-200 (Superfine). Tubes 32, 33 and 34 from Sephadex G-200β (FIG. 33) were combined with tubes 30, 31, 32 and 33 from Sephadex G-200α-γ (FIG. 35), concentrated and applied to the column equilibrated with 0.002 M sodium citrate buffer pH 5.8. 5 ml. fractions were collected. Protein concentration was determined by absorption at 280 nm and enzyme activity estimated spectrophotometrically at 265 nm (pH 7.0). ●——● protein; ○——○ , adenosine deaminase activity.

weight form were pooled, concentrated as before and applied to a Sephadex G-200 Superfine column. Fig. 35 shows the elution pattern (designated Sephadex G-200$\alpha\gamma$). The specific activities of various fractions are given in Table 41.

TABLE 41. Specific Activities of Adenosine Deaminase Solutions after Sephadex G-200 Chromatography (Sephadex G-200$\alpha\gamma$)

Tube No.	Total Protein (mg)	Sp. Act. (e.u./mg.)
30	1.56	9.0
31	1.32	16.0
32	1.14	17.2
33	0.99	9.0

The final stage in the purification of the high molecular weight form involved pooling the active tubes from Sephadex G-200β (i.e. tubes 32, 33 and 34) with the active tubes from Sephadex G-200$\alpha\gamma$ (tubes 30, 31, 32 and 33). The resulting solution was concentrated and applied to the Sephadex G-200 column. A single protein peak emerged coincident with the peak of enzyme activity (Fig. 36).

Table 42 shows the specific activities of the various fractions obtained.

TABLE 42. Specific Activities of Adenosine Deaminase Solutions After Final Sephadex G-200 Chromatography Step

Tube No.	Total Protein (mg.)	Sp. Act. (e.u./mg.)
20	0.4	9.0
21	0.7	24.0
22	0.8	33.0
23	0.6	31.0
24	0.3	16.0

Tubes 21, 22 and 23 were combined. This purified preparation (2.1 mg.) had a specific activity of 30 e.u./mg.

A summary of the steps involved in the purification of both forms is outlined in Tables 43 and 44. Because of the complexity of the procedure (particularly at the chromatographic stages) schematic representations of the individual steps are illustrated in Schemes 3 and 4.

TABLE 43. Purification of the Low Molecular Weight Form of Adenosine Deaminase from Rabbit Intestine

Procedure	Vol. (ml.)	Activity (e.u./ml.)	Total e.u.	Protein (mg./ml.)	Total Protein(mg)	Sp. Act. (e.u./mg.)	Yield %	Purification factor
Step 1. Acetate Extract	1820	1.03	1874	6.25	11375	0.16	100	1
Step 2. 20%-40% $(NH_4)_2$ SO_4	248	6.50	1609	34.8	8600	0.19	86	1.2
Step 3. Dialysis against H_2O	306	3.85	1178	2.90	887	1.33	62	8.3
Step 4. DEAE pH 5.8 (β fraction)	86	9.20	800	0.60	51.6	15.2	42	95
Step 5. Sephadex G-75 I_β	20	25.5	510	0.38	7.6	70	30	438
Step 6. Sephadex G-75 $\underline{III}_\beta$	15	28.0	420	0.17	2.6	180	22	1125
Step 7. Sephadex G-75 $\underline{IV}_\beta$	15	25.2	368	0.12	1.8	210	19	1313

TABLE 44. Purification of the High Molecular Weight Form of Adenosine Deaminase from Rabbit Intestine

Procedure	Vol. (ml.)	Activity (e.u./ml.)	Total e.u.	Protein (mg./ml.)	Total Protein (mg).	Sp. Act. (e.u./mg.)	Yield %	Purification factor
Step 1. Acetate Extract	1820	1.03	1874	6.25	11375	0.16	100	1
Step 2. 20%-40% $(NH_4)_2SO_4$	248	6.50	1609	34.8	8600	0.19	86	1.2
Step 3. Dialysis against H_2O	306	3.85	1178	2.9	887	1.33	62.2	8.3
* Step 4. DEAE pH 5.8 (β fraction)	86	9.20	800	0.60	51.6	15.2	42.0	95.0
($\alpha\gamma$ fractions)	80	2.65	212	0.84	67.2	3.2	12.0	20.0
Step 5. Sephadex G-75 Iβ	10	7.80	78	1.06	10.6	7.0	4.2	44.0
Step 6. Sephadex G-200β	9	7.00	63	0.50	4.5	14.0	3.4	87.0
Step 7. Sephadex G-75$\alpha\gamma$	10	8.80	88	1.50	15.0	5.8	4.8	35.0
Step 8. Sephadex G-200$\alpha\gamma$	12	5.10	61	0.42	5.0	12.5	3.3	78.0
**Step 9. Final Sephadex G-200	15	4.20	63	0.14	2.1	30.0	3.2	200

* The high specific activity and high yield in the β fraction were due to the presence of the low molecular weight form, which was removed in the next step (Step 5.).

** The final purification step involved the application of the active fractions from Steps 6 and 8 to the Sephadex G-200 column.

Acetate Extract

|

20% - 40% $(NH_4)_2SO_4$

|

Dialysis against water

|

DEAE cellulose pH 5.8
(β-fraction)

|

Sephadex G-75 Iβ

|

Sephadex G-75 IIβ

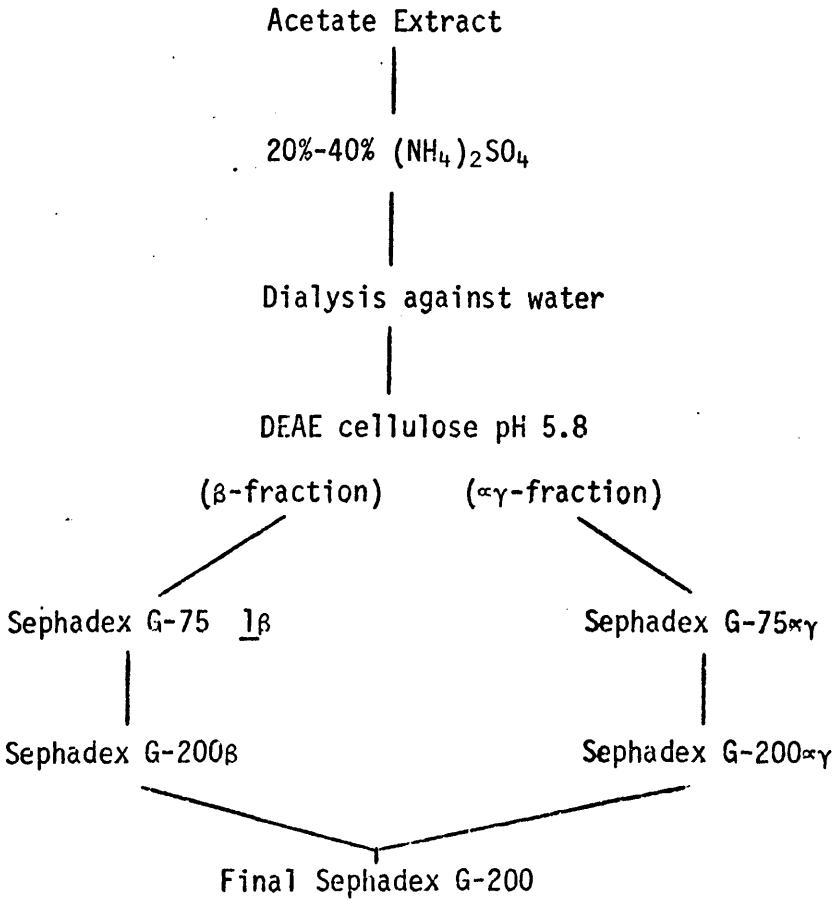
|

Sephadex G-75 IIIβ

|

Sephadex G-75 IVβ

SCHEME III. Purification scheme for the low molecular weight form of rabbit intestinal adenosine deaminase.



SCHEME IV. Purification scheme for the high molecular weight form of rabbit intestinal adenosine deaminase.

Evidence for the Purity of the Molecular Forms

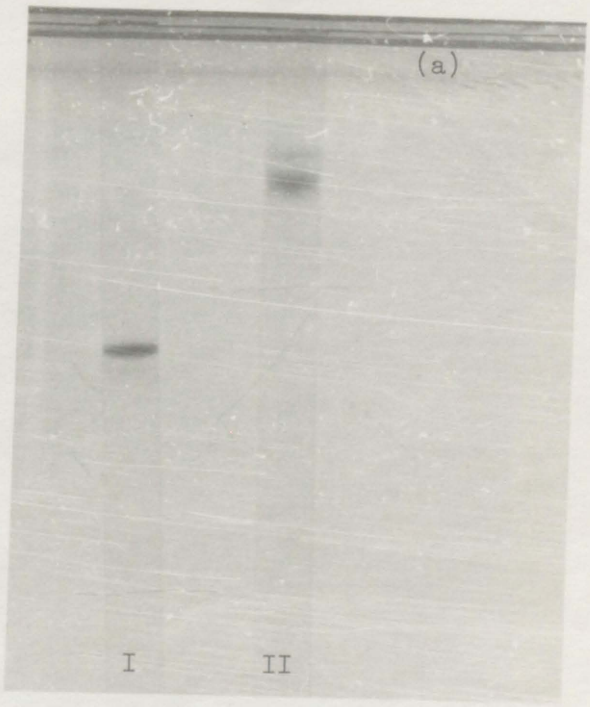
Homogenity by Polyacrylamide Gel Electrophoresis

The purified preparations obtained after the final gel filtration steps were subjected to polyacrylamide gel electrophoresis. Samples of the enzymes containing 10-50 µg protein were applied to the gels and electrophoresis carried out at a current of 5 ma/tube for 30 min. at room temperature. Some of the gels were then fixed and stained for protein by immersion in 1% (w/v) Amido Black in 7% (v/v) acetic acid and the rest were stained for enzyme activity. The patterns obtained in a typical run are illustrated in Plate 6. In each case two bands of enzyme activity were detected which corresponded with the observed protein bands. As already mentioned (Methods section p. 37) the procedure developed for the detection of adenosine deaminase activity in polyacrylamide gels was not entirely satisfactory, since the gels could not be photographed due to rapid background coloration. The bands shown in Plate 6 (b) are therefore diagramatic representations of what was observed immediately after staining.

Chromatographic Evidence of Purity

The fact that both forms of the enzyme at the final stage of purification emerged from the Sephadex columns as symmetrical peaks coincident with their respective protein peaks, was a strong indication of homogenity.

ELECTROPHEROGRAM



ZYMOGRAM

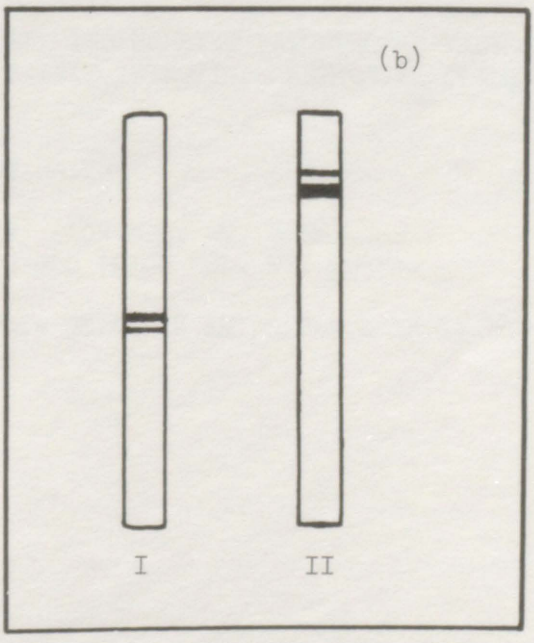
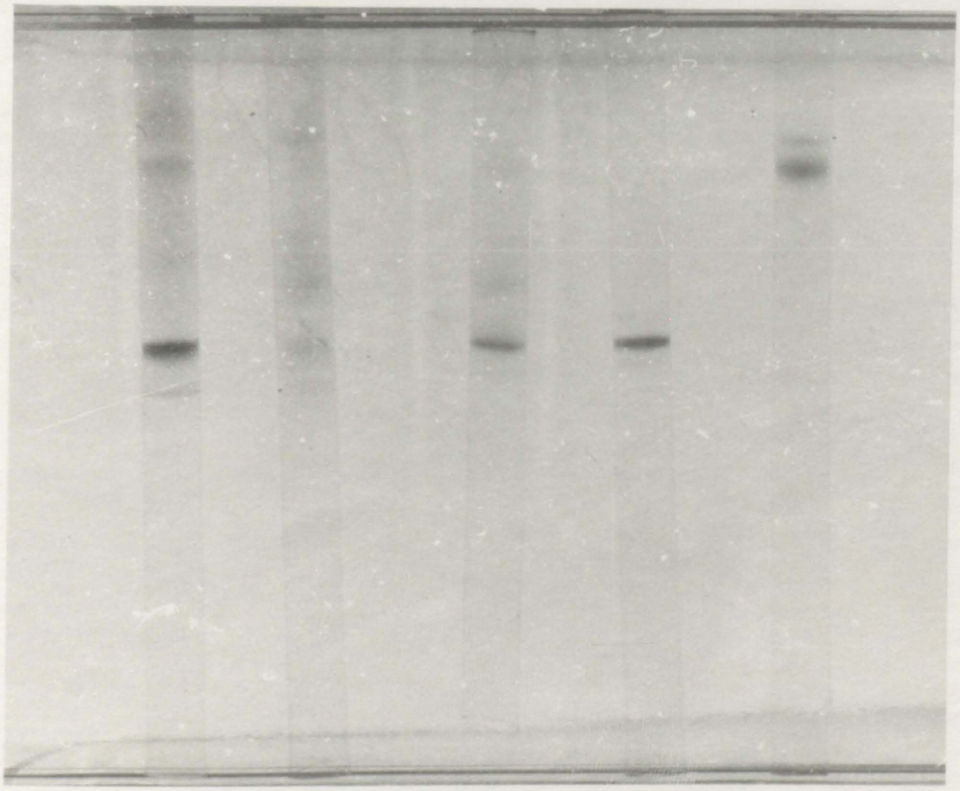


PLATE 6. POLYACRYLAMIDE GEL ELECTROPHORETIC PATTERNS OF PURIFIED RABBIT INTESTINAL ADENOSINE DEAMINASE.

I LOW MOLECULAR WEIGHT FORM
II HIGH MOLECULAR WEIGHT FORM

THE ELECTROPHEROGRAM (a) WAS STAINED FOR PROTEIN USING 1% AMIDO BLACK IN 7% ACETIC ACID. THE ZYMOGRAM (b) WAS STAINED FOR ENZYME ACTIVITY USING THE ZYMOGRAM STAIN FOR ADENOSINE DEAMINASE.



(a) (b) (c) (d) (e)

PLATE 7. POLYACRYLAMIDE GEL ELECTROPHORETIC PATTERNS AT VARIOUS STAGES IN THE PURIFICATION OF RABBIT INTESTINAL ADENOSINE DEAMINASE.

(a) ACETATE EXTRACT
(b) POST DIALYSIS AGAINST WATER
(c) POST DEAE pH 5.8
(d) POST SEPHADEX G-75 IV_β (LOW MOL. WT. FORM).
(e) POST FINAL SEPHADEX G-200 (HIGH MOL. WT. FORM).

THE GELS WERE STAINED FOR PROTEIN USING 1% AMIDO BLACK IN 7% ACETIC ACID.

Electrophoretic Patterns Throughout the Purification

The progress in the purification of both forms was followed by subjecting samples from the active fractions of individual purification steps to electrophoresis in 5% polyacrylamide gels. The protein was located by staining the gels with Amido Black in 7% acetic acid. Results are illustrated on Plate 7. The material from Step 2 (20%-40% $(NH_4)_2SO_4$) was not analysed because of the high concentration of $(NH_4)_2SO_4$ present in the solution.

Removal of Alkaline Phosphatase Activity During the Purification

In the purification of adenosine deaminase from bovine intestine (Brady and O'Connell, 1962) the elimination of alkaline phosphatase was a constant objective. The level of phosphatase activity at various steps in the present purification procedure was estimated.

TABLE 45.

Procedure	Adenosine Deaminase Yield %	Alkaline Phosphatase Yield %
Step 1. Acetate Extract	100	100
Step 2. 20%-40% $(NH_4)_2SO_4$	86	55
Step 3. Dialysis against H_2O	62	3.6
Step 4. DEAE pH 5.8	42	-

The results summarized in Table 45 show that only 3.6% of the alkaline phosphatase activity remained after the dialysis step, whereas 62% of the original adenosine deaminase was retained.

The A_{280} : A_{260} Ratio at Each Step in the Purification

Table 46 shows the absorbancy ratio (A_{280} : A_{260}) measured at different stages in the purification. The removal of nucleic acid and other compounds which absorb at 260 nm, is illustrated by the gradual increase in the ratio.

TABLE 46.

Procedure	Absorbancy Ratio
Step 1. Acetate Extract	0.98
Step 2. 20%-40% $(NH_4)_2SO_4$	1.00
Step 3. Dialysis against H_2O	1.70
Step 4. DEAE pH 5.8	1.60

Stability of Adenosine Deaminase

The stability of the enzyme during the early stages of the purification procedure was investigated. This was done to test the possibility of storing the crude or partially purified enzyme. Samples from the first three steps were kept for two months under the following conditions:

a) $4^{o}C$

b) $4^{o}C$ incorporating 50 mM NaF

c) Deep freeze ($-10^{o}C$)

d) Deep freeze incorporating 50 mM NaF

Assays were carried out at various intervals up to a period of two months, and the results showed that there was no loss in activity. It is therefore possible to store the enzyme under any of the above conditions for two months without loss in activity.

SECTION III

PROPERTIES OF THE HIGH AND LOW MOLECULAR WEIGHT FORMS OF RABBIT INTESTINAL ADENOSINE DEAMINASE

Having purified both forms of the enzyme it was considered of interest to characterize them with a view to establishing whether any differences existed between them. The first part of the following section constitutes a survey of some of the physical, chemical and catalytic properties of the two forms. These include pH optima, pH stabilites, isoelectric points, activation energies, Kms and effects of chemical reagents.

In the latter half of the section studies on the high molecular weight form are described with emphasis on attempts to convert the high molecular weight form into the low molecular weight form using urea, guanidine hydrochloride, sodium dodecyl sulphate and other dissociating agents.

The Effect of pH on Enzyme Activity

The pH optima for both forms of the enzyme were estimated using adenosine and deoxyadenosine as substrates. Activity determinations were carried out over the pH range 3.0 - 10.6 using purified enzyme preparations. Phosphate buffer was used over the pH range 3.0 - 8.0 and glycine buffer from pH 9.0-10.6. Assays were performed spectrophotometrically at 37^{o}C by adding 0.1 ml. enzyme to 3 ml. of substrate buffered at the appropriate pH. The results obtained when pH was plotted against % activity are shown in Figs. 1 and 2.

The observed pH optima are given in Table 1.

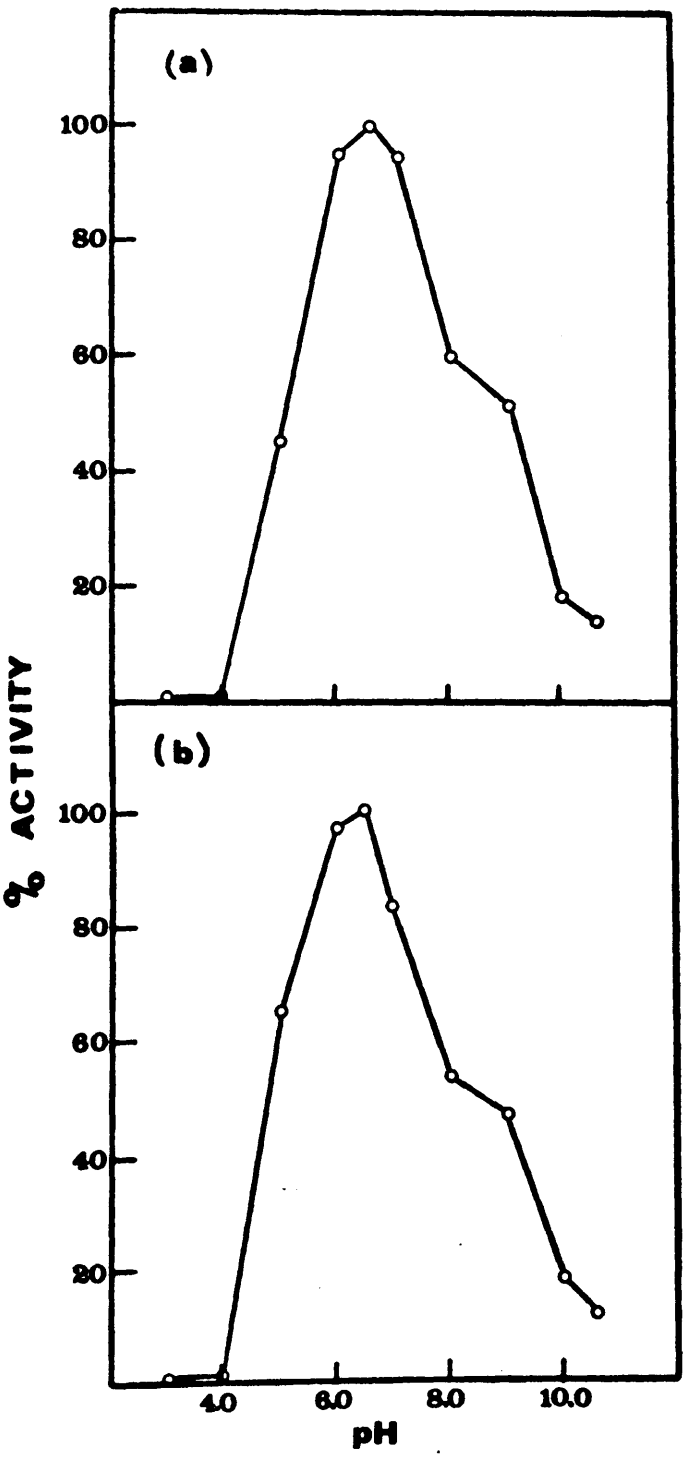


FIG. 1. pH activity profiles of the low molecular weight form of rabbit intestinal adenosine deaminase (a) using adenosine and (b) using deoxyadenosine as substrates. Phosphate buffer (pH 3.0-8.0) and glycine buffer (pH 9.0 - 10.6) were used, and assays performed spectrophotometrically at 265 nm.

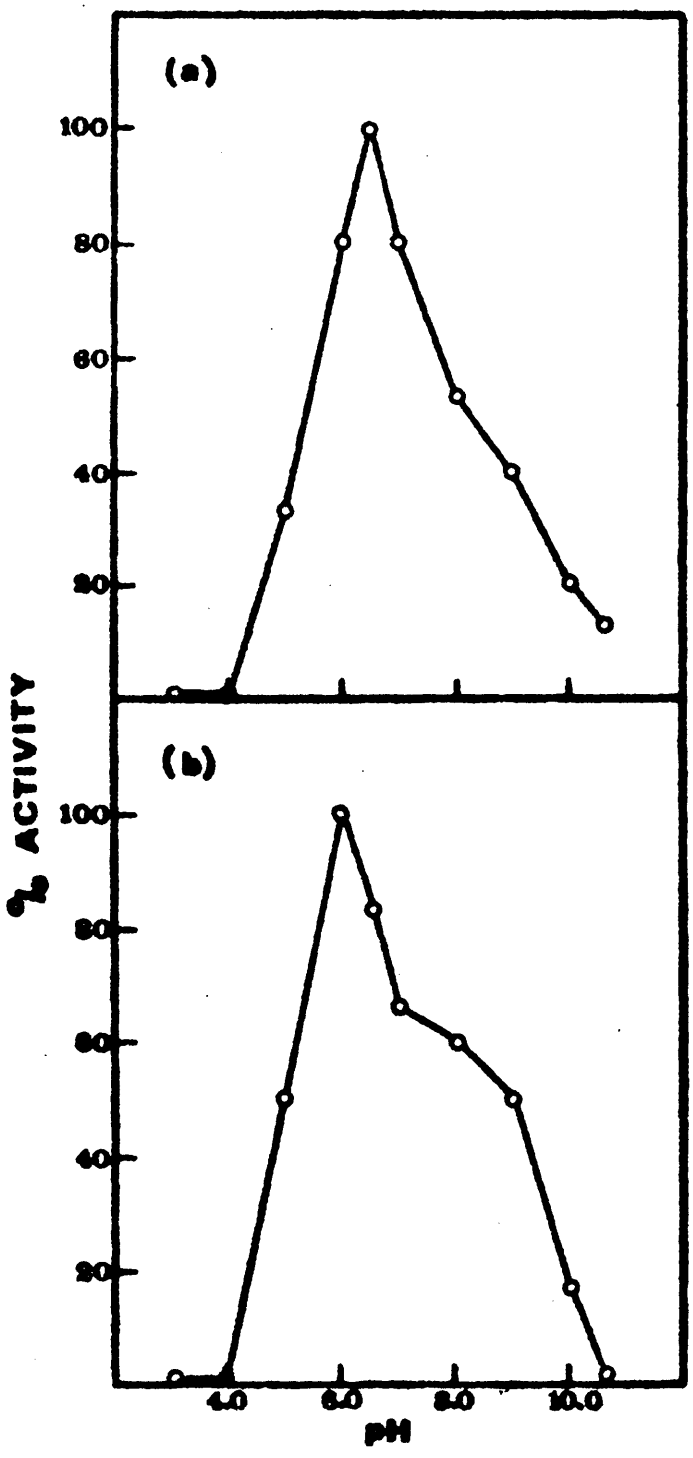


FIG. 2. pH activity profiles of the high molecular weight form of rabbit intestinal adenosine deaminase, (a) using adenosine and (b) using deoxyadenosine as substrates. Phosphate buffer (pH 3.0-8.0) and glycine buffer (pH 9.0-10.6) were used, and assays performed spectrophotometrically at 265 nm.

TABLE 1 pH Optima of the High and Low Molecular Weight Forms of Adenosine Deaminase from Rabbit Intestine

Enzyme Form	Substrate used	pH Optima
High molecular weight	Adenosine	6.5
High molecular weight	Deoxyadenosine	6.0
Low molecular weight	Adenosine	6.5
Low molecular weight	Deoxyadenosine	6.5

It can be seen that for both enzyme forms the pH optimum with adenosine was at pH 6.5, but with deoxyadenosine the high molecular weight form had a pH optimum at pH 6.0. It is also apparent that the fall on the acid side of each pH curve is steeper than on the alkaline side, there being no detectable enzyme activity below pH 4.0.

pH Stability of the High and Low Molecular Weight Forms

The pH stability of both forms was determined over the pH range 3.0-10.6. Buffers were the same as those used in the pH optima studies. The enzymes were maintained at the various pHs for a period of 24 h. and were then assayed spectrophotometrically at pH 6.5 using adenosine and deoxyadenosine as substrates. In the case of the high molecular weight form (using deoxyadenosine as substrate) the assays were performed at pH 6.0. The results

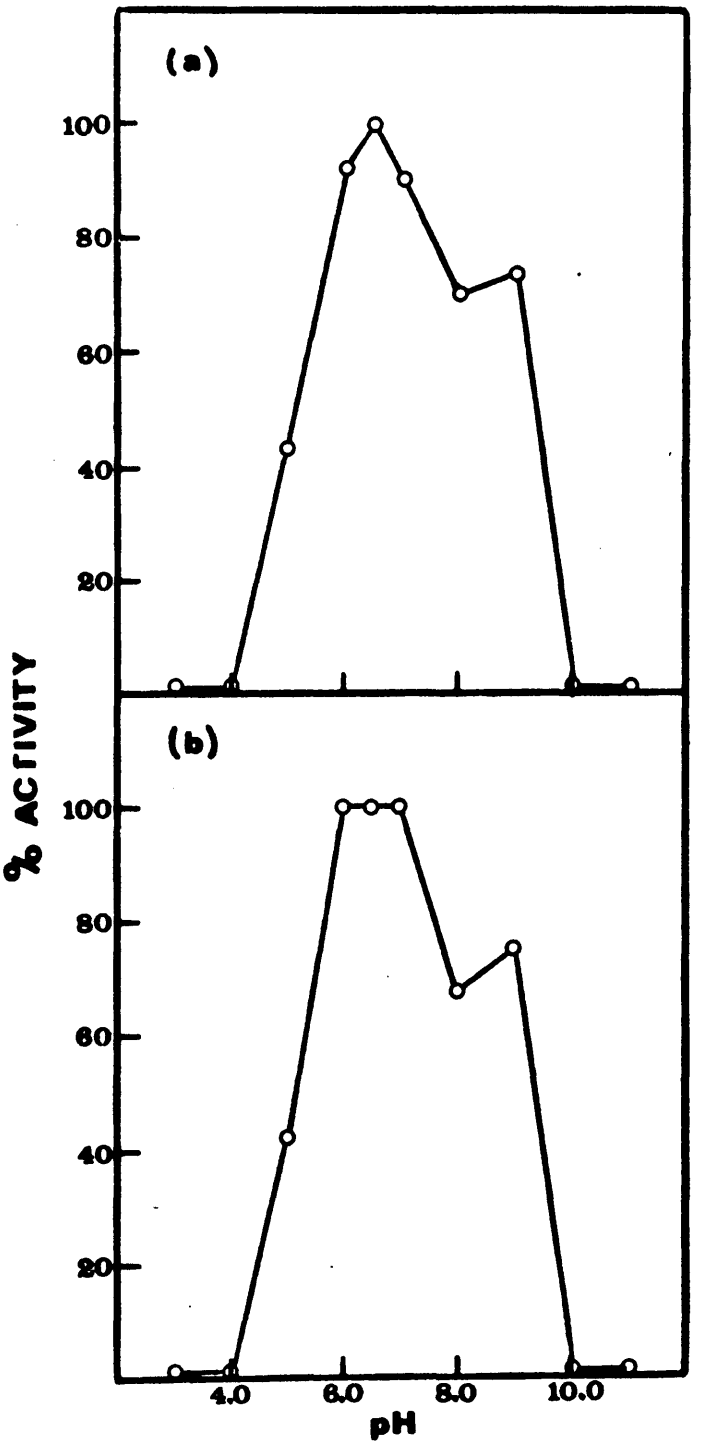


FIG. 3. pH stability profiles of the low molecular weight form of rabbit intestinal adenosine deaminase (a) using adenosine and (b) using deoxyadenosine as substrates. Phosphate buffer (pH 3.0-8.0) and glycine buffer (pH 9.0-10.6) were used, and assays performed spectrophotometrically at 265 nm.

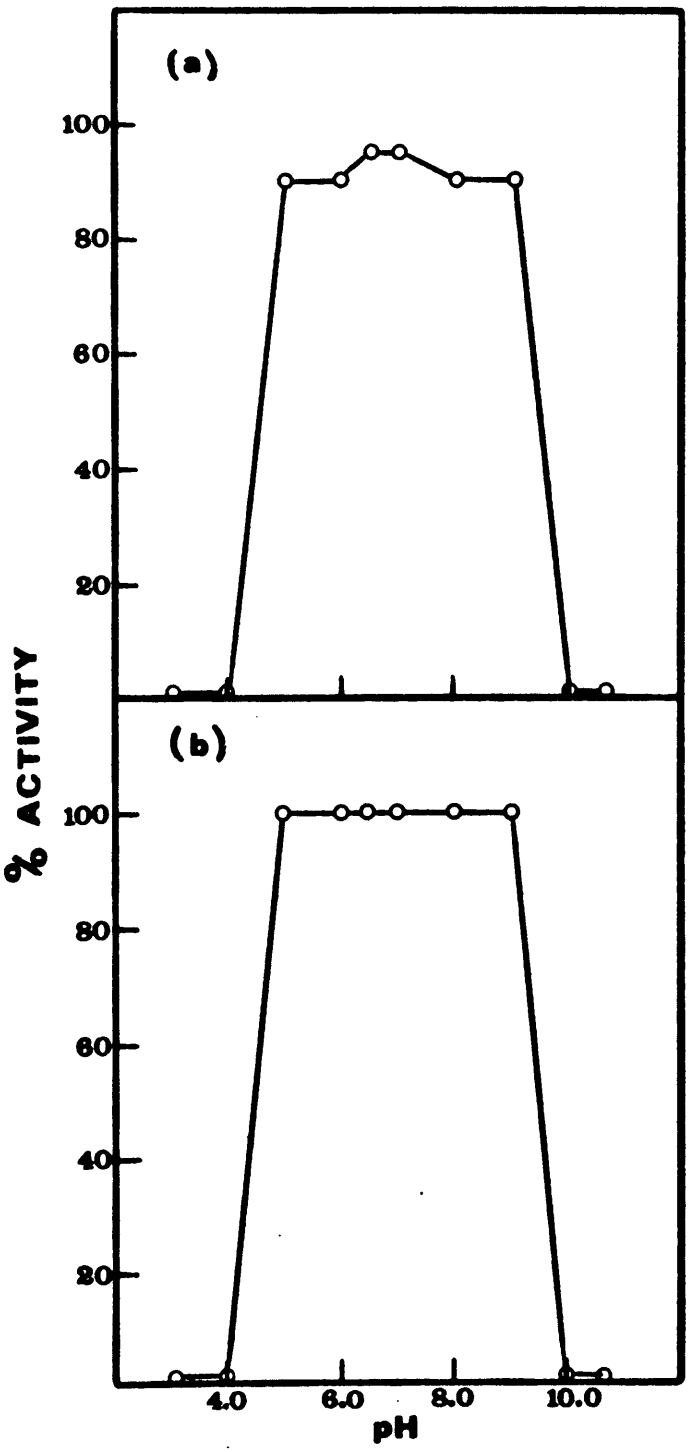


FIG. 4. pH stability profiles of the high molecular weight form of rabbit intestinal adenosine deaminase (a) using adenosine and (b) using deoxyadenosine as substrates. Phosphate buffer (pH 3.0-8.0) and glycine buffer (pH 9.0-10.6) were used, and assays performed spectrophotometrically at 265 nm.

obtained are graphically illustrated in Figs. 3 and 4.

It can be observed that the low molecular weight enzyme is less stable towards pH than the high molecular weight form. The latter is stable from pH 5.0 to pH 9.0, the enzyme regaining 100% of its activity on assay after being maintained in that pH range for 24 h. However, the low molecular weight form did not regain full activity on the alkaline side of the curve making its stability range narrower than that of the high molecular weight enzyme.

The Effect of Heating on Enzyme Activity

The rate of inactivation of enzymes in solution increases rapidly with temperature. In nearly all cases inactivation becomes virtually instantaneous at temperatures well below 100°C. In the following experiment the stability of the two forms of the rabbit intestinal deaminase towards heat was investigated. Purified preparations of the enzymes were used in the study.

The suitably diluted enzymes (in 0.1M phosphate buffer pH 6.5) were incubated for 15 min. at 20°, 37°, 50°, 60°, 70° and 80°C. 0.1 ml. samples were then removed and assayed spectrophotometrically at pH 6.5 using adenosine and deoxyadenosine as substrates. % activity was plotted against temperature (°C). The inactivation curves obtained are illustrated in Figs. 5 and 6 which indicate that there is practically no difference between the two forms of the enzyme in their stability towards heating.

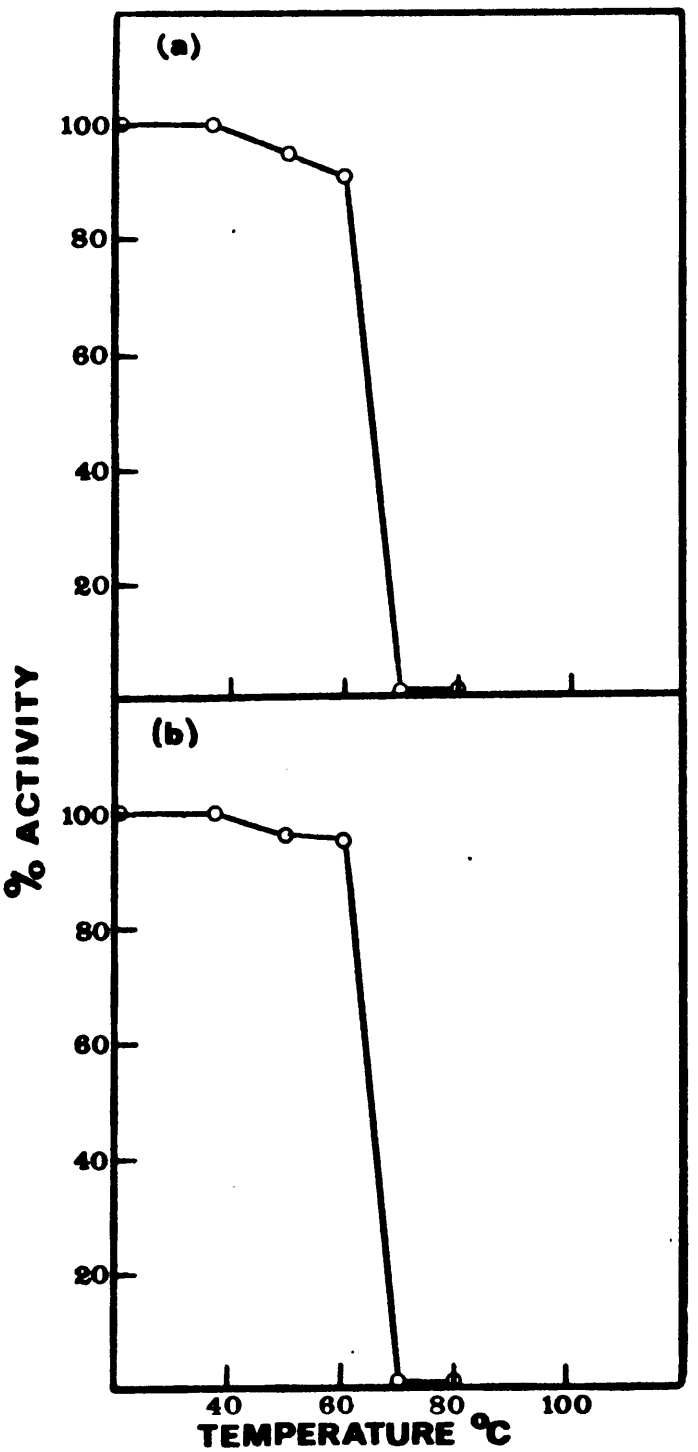


FIG. 5. Heat stability of the low molecular weight form of rabbit intestinal adenosine deaminase (a) using adenosine and (b) using deoxyadenosine as substrates. The suitably diluted enzyme (in 0.1 M phosphate pH 7.0) was heated for 15 min. at various temperatures and assays performed spectrophotometrically at 265 nm (pH 6.5).

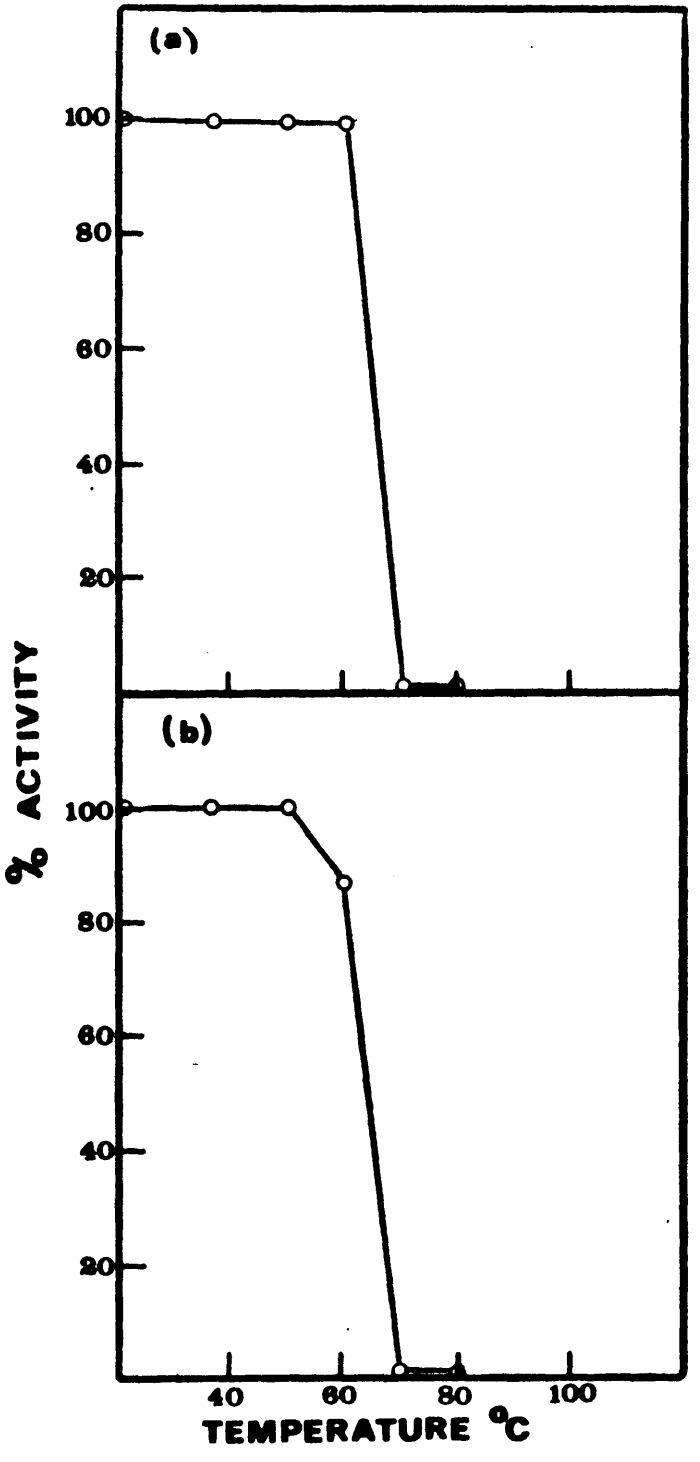


FIG. 6. Heat stability of the high molecular weight form of rabbit intestinal adenosine deaminase (a) using adenosine and (b) using deoxyadenosine as substrates. The suitably diluted enzyme (in 0.1 M phosphate pH 7.0) was heated for 15 min. at various temperatures, and assays performed spectrophotometrically at 265 nm.

In all cases the enzymes were stable up to 60°C, but were completely inactivated on heating at 70°C for 15 min.

Determination of the Activation Energies of the High and Low Molecular Weight Forms

When fixed concentrations of reactants are maintained, a reaction proceeds at different rates at different temperatures. This means that the magnitude of the rate constant must be temperature dependant. The relationship between the value of the rate constant and the absolute temperature is defined by the Arrhenius equation:

$$k = Ae^{\frac{-E}{RT}}$$

where k = rate constant, T = temperature °K and R = gas constant. A is a constant and E is a second constant called the activation energy of the reaction. Whatever is its theoretical basis, the activation energy evidences itself as an energetic obstacle to reaction, whose magnitude to a large extent determines the rate of the reaction. The Arrhenius equation provides a means whereby the value of the activation energy can be determined.

$K = Ae^{\frac{-E}{RT}}$ can be written in logarithmic form

$$\ln k = \ln A - \frac{E}{RT}$$

$$\text{i.e. } \log k = \log A - \frac{E}{2.303 \times R} \cdot \frac{I}{T}$$

This is the equation of a straight line obtained by plotting log k against $\frac{1}{T}$. The slope of the line equals - E/2.303 x R: as 2.303 x R equals 4.576 cal. mole^{-1} degree^{-1}, the value of the activation energy can be determined from the "Arrhenius Plot" as being equal to -(slope x 4.576) cal./mole.

Experimental Procedure:

Adenosine and deoxyadenosine (0.1 mM) were both used as substrates. Assays were performed spectrophotometrically at 25°, 30°, 35°, 40° and 45°C. The pH of the assay mixture was 6.5 except in the case of the high molecular weight form using deoxyadenosine as substrate where the pH was 6.0. Both the enzymes and substrates were separately equilibrated at each temperature before assay. The results obtained are graphically illustrated in Fig. 7 and the calculated activation energies given in Table 2.

TABLE 2. Activation Energies of the High and Low Molecular Weight Forms of Rabbit Intestinal Adenosine Deaminase

Form of the enzyme	Substrate	Activation energy (k cal.)
High mol. wt.	Adenosine	1.15
High mol. wt.	Deoxyadenosine	1.25
Low mol wt.	Adenosine	7.8
Low mol wt.	Deoxyadenosine	7.6

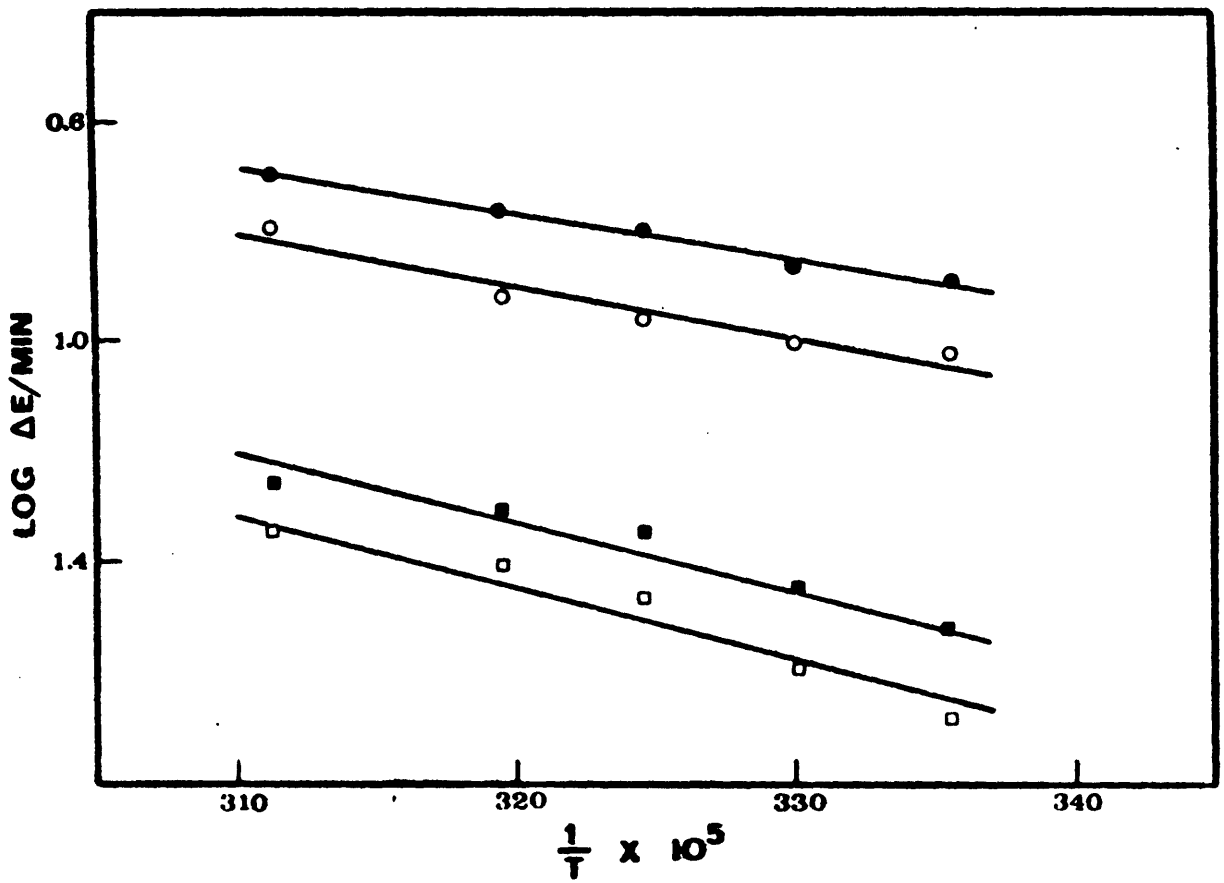


FIG. 7. An Arrhenius plot of log v (ΔE/min.) versus the reciprocal of temperature in ^{O}K for the low molecular weight form (●—● , using adenosine and ○—○ , using deoxyadenosine as substrates) and the high molecular weight form (■—■ , using adenosine and □—□ , using deoxyadenosine as substrates) of rabbit intestinal adenosine deaminase. Temperatures ranging from 25^{O} to $45^{O}C$ were used, and assays performed spectrophotometrically at 265 nm.

It is apparent that there is approximately a 6-fold difference in the activation energies of the two forms.

Determination of the Isoelectric Points

The Suramin inhibition method of Wills (1952) was used to measure the isoelectric points of both forms of the deaminase. Suramin (mol. wt. 1429.2) is a trypanocidal drug and was obtained from Imperial Chemical Industries Ltd. (Pharmaceutical Division), Wilmslow, Cheshire, England. The method is based on the fact that a sharp drop is observed in the inhibition pH curve at or near the isoelectric point of the enzyme when it is inhibited by suramin.

Experimental conditions:

The microdiffusion method of assay rather than the spectrophotometric method was used because the suramin was found to absorb strongly at 265 nm. Activity was determined over a pH range of 4.2 to 5.4 in 0.1M citrate-phosphate buffer in the presence and absence of suramin. Enzyme solutions were incubated at 37^{o}C with 1.0 mM suramin for 20 min. prior to addition of the substrate. The isoelectric point is taken as the pH at which 50% inhibition occurs. The results obtained with both forms of the enzyme are shown in Fig. 8.

Enzyme form	Isoelectric point
High Molecular Weight	4.94
Low Molecular Weight	5.16

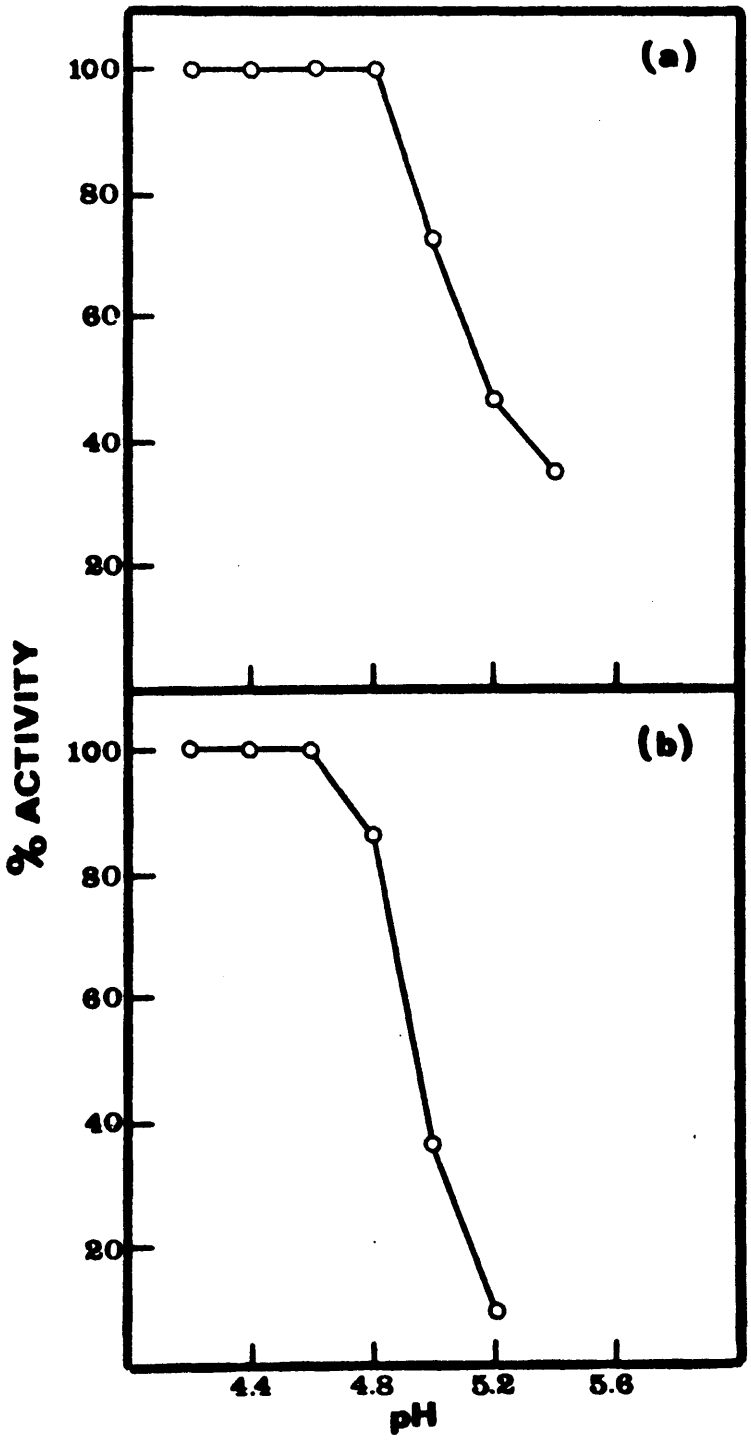


FIG. 8. The effect of Suramin on (a) the low molecular weight form and (b) the high molecular weight form of rabbit intestinal adenosine deaminase at various pH values. 0.1 M citrate-phosphate buffer (pH 4.2-5.4) was used and assays performed spectrophotometrically at 265 nm (pH 6.5).

A slightly higher value was obtained for the low molecular weight enzyme. However as there is no definite theoretical basis for the method, the isoelectric points of the two forms cannot be said to differ. A value of 4.7 was obtained by Brady and O'Donovan (1965) using a crude extract of rabbit duodenum.

Determination of the Michaelis Constants (Km) for the High and Low Molecular Weight Forms of Adenosine Deaminase

Experimental conditions:

Stock solutions of adenosine and deoxyadenosine (0.2 mM) were prepared in 0.1M phosphate buffer pH 7.0. For enzyme assay dilutions of the stock solutions were made to give substrate concentrations ranging from 0.01 mM to 0.15 mM. The assay procedure involved measuring the change in absorbance (ΔE) at 265 nm with time at 37°C, when 0.1 ml. of enzyme was added to 3 ml. of substrate. Km values were calculated according to the Lineweaver-Burke method (1934). Results obtained for both forms of the enzyme are shown in Figs. 9 and 10.

Table 3 shows the various Km values obtained using adenosine and deoxyadenosine as substrates.

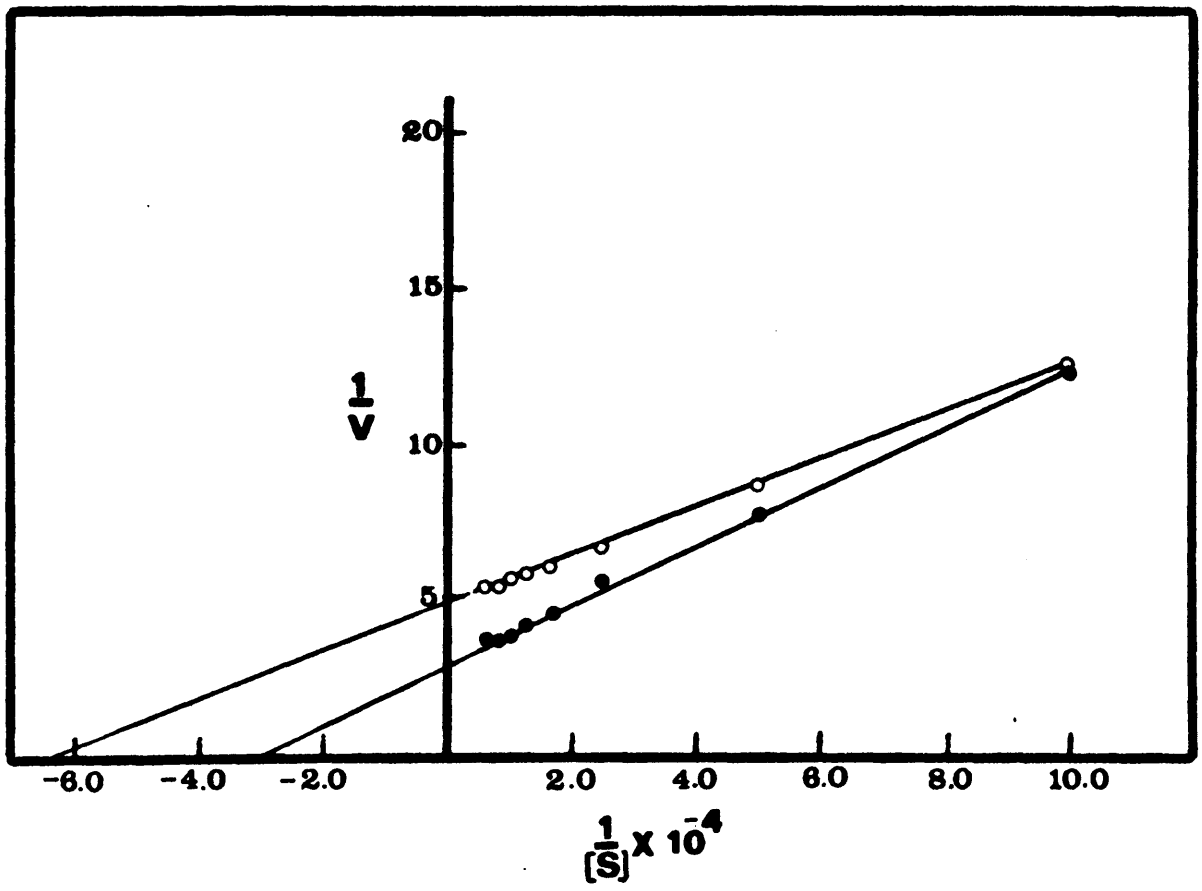


FIG. 10. Lineweaver-Burke plots of the high molecular weight form(●——●) and the low molecular weight form (o——o) of rabbit intestinal adenosine deaminase using deoxyadenosine as substrate. V represents ΔE/min. and the concentration of substrate is in moles /l.

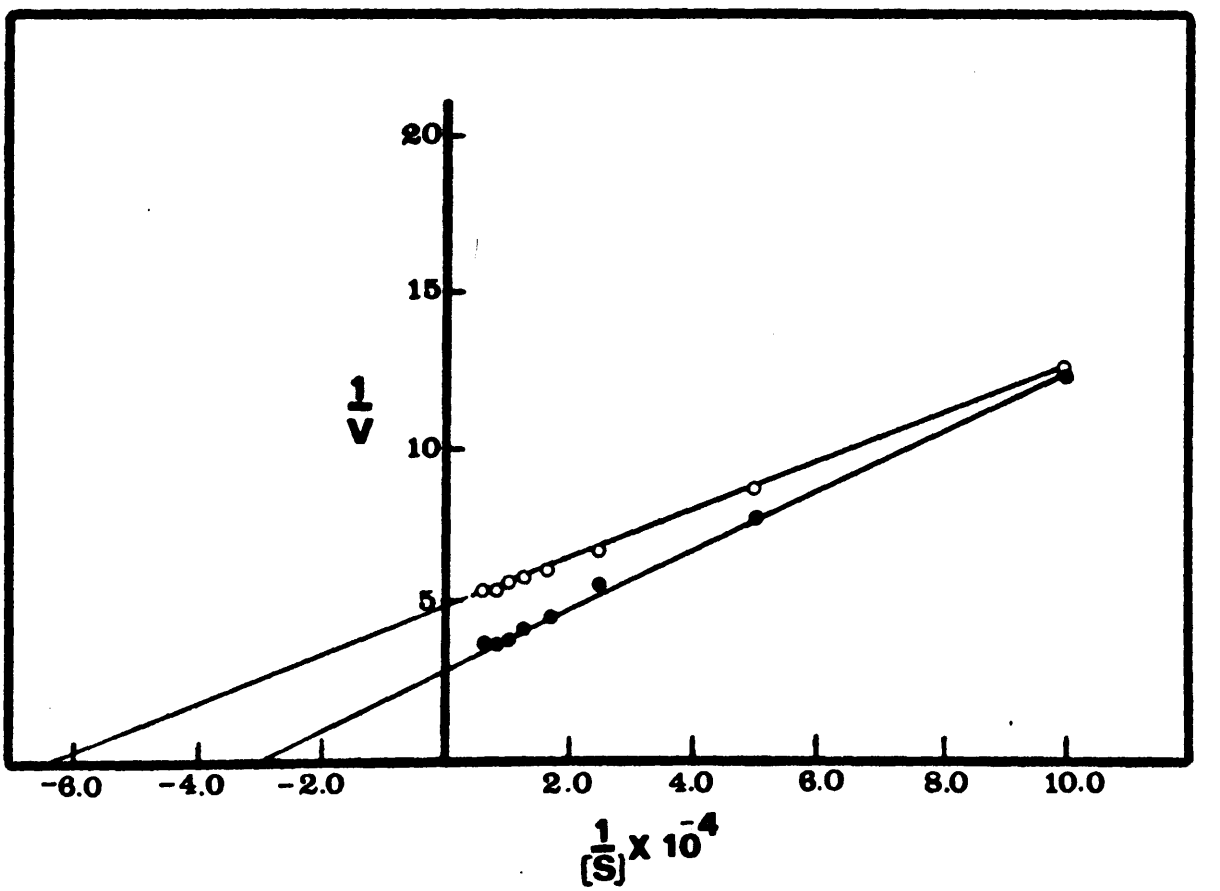


FIG. 10. Lineweaver-Burke plots of the high molecular weight form(●——●) and the low molecular weight form (o——o) of rabbit intestinal adenosine deaminase using deoxyadenosine as substrate. V represents ΔE/min. and the concentration of substrate is in moles /l.

TABLE 3. Km Values for the High and Low Molecular Weight Forms using Adenosine and Deoxyadenosine as Substrates

Enzyme form	Substrate	Km
High molecular weight	Adenosine	33.3×10^{-5}
High molecular weight	Deoxyadenosine	20×10^{-5}
Low molecular weight	Adenosine	3.3×10^{-5}
Low molecular weight	Deoxyadenosine	1.6×10^{-5}

A higher value was obtained (using both substrates) for the high molecular weight enzyme, the Km being approximately ten times greater than that of the low molecular weight form.

Deamination of Adenosine, 2'-Deoxyadenosine, and 3'-Deoxyadenosine (Cordecypin) by the High and Low Molecular Weight Forms of Adenosine Deaminase.

The spectrophotometric method of assay was used in this study.

Experimental conditions:

0.1 mM substrates (pH 7.0) were used in all cases. 0.1 ml. of enzyme was added to 3 ml. of each substrate and the fall in absorbance at 265 nm measured at 37°C.

The results are given in Table 4. These are expressed as a ratio of deoxyadenosine to adenosine from values obtained (ΔE) at 265 nm after 2 minutes.

TABLE 4. Comparison of the Rates of Deamination of Adenosine, 2'-Deoxyadenosine and 3'-Deoxyadenosine by the High and Low Molecular Weight forms of Adenosine Deaminase

	2'-Deoxyadenosine/ Adenosine	3'-Dexoyadenosine Adenosine
High mol. wt. form	0.75	0.77
Low mol. wt. form	0.75	1.00

Both forms of the enzyme deaminated 2'-deoxyadenosine at a similar rate. However, the low molecular weight form deaminated 3'deoxyadenosine at a faster rate.

THE EFFECT OF UREA ON ENZYME ACTIVITY

Ultrapure urea (obtained from Mann Research Laboratories, U.S.A.) which did not require re-crystallization was used in this investigation. Urea has already been shown to markedly reduce the pH stability of adenosine deaminase isolated from bovine intestinal mucosa (Brady and O'Sullivan, 1967).

Experimental conditions:

A stock 10M urea solution was prepared in 0.1M phosphate buffer pH 7.0. This was suitably diluted with buffer and the enzyme to give final urea concentrations ranging from 1-9M. The urea-enzyme solutions were incubated at 37°C and 0.1 ml. samples

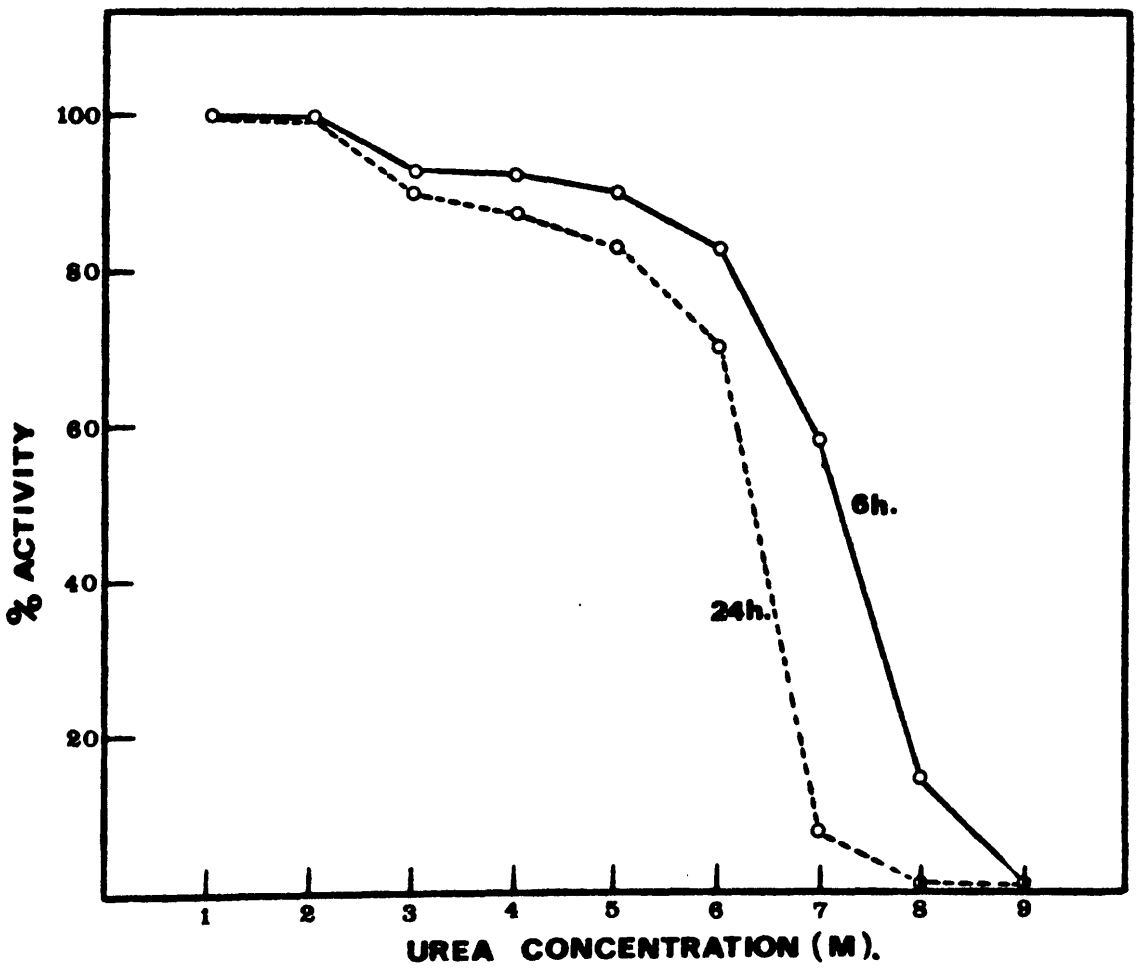


FIG. 11. The effect of urea on the activity of the high molecular weight form of rabbit intestinal adenosine deaminase. The diluted enzyme (in 0.1 M phosphate pH 7.0) was incubated at 37°C in urea solutions (1-9 M) for 6 h. and 24 h. and assays performed spectrophotometrically at 265 nm (pH 7.0).

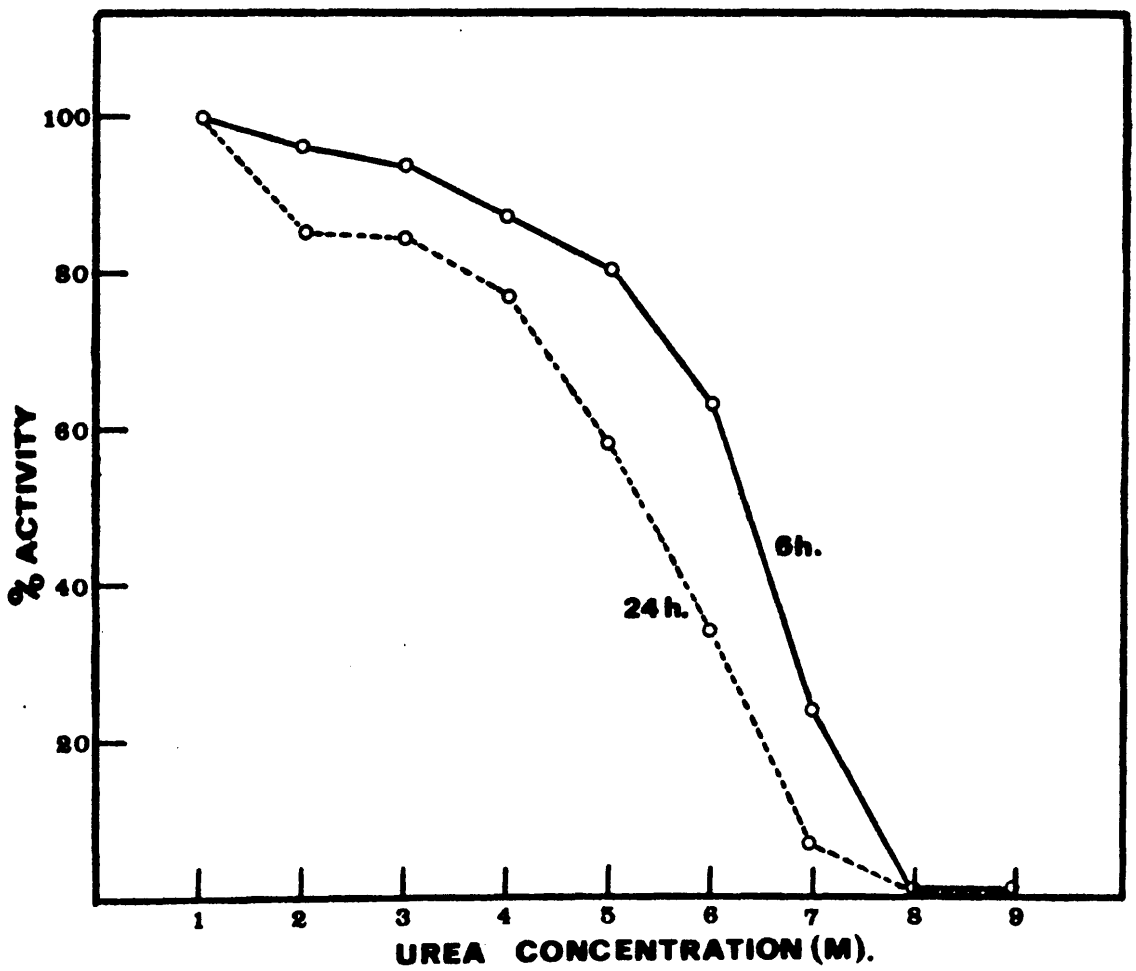


FIG. 12. The effect of urea on the activity of the low molecular weight form of rabbit intestinal adenosine deaminase. The diluted enzyme (in 0.1 M phosphate pH 7.0) was incubated at 37°C in urea solutions (1-9 M) for 6 h. and 24 h. and assays performed spectrophotometrically at 265 nm (pH 7.0).

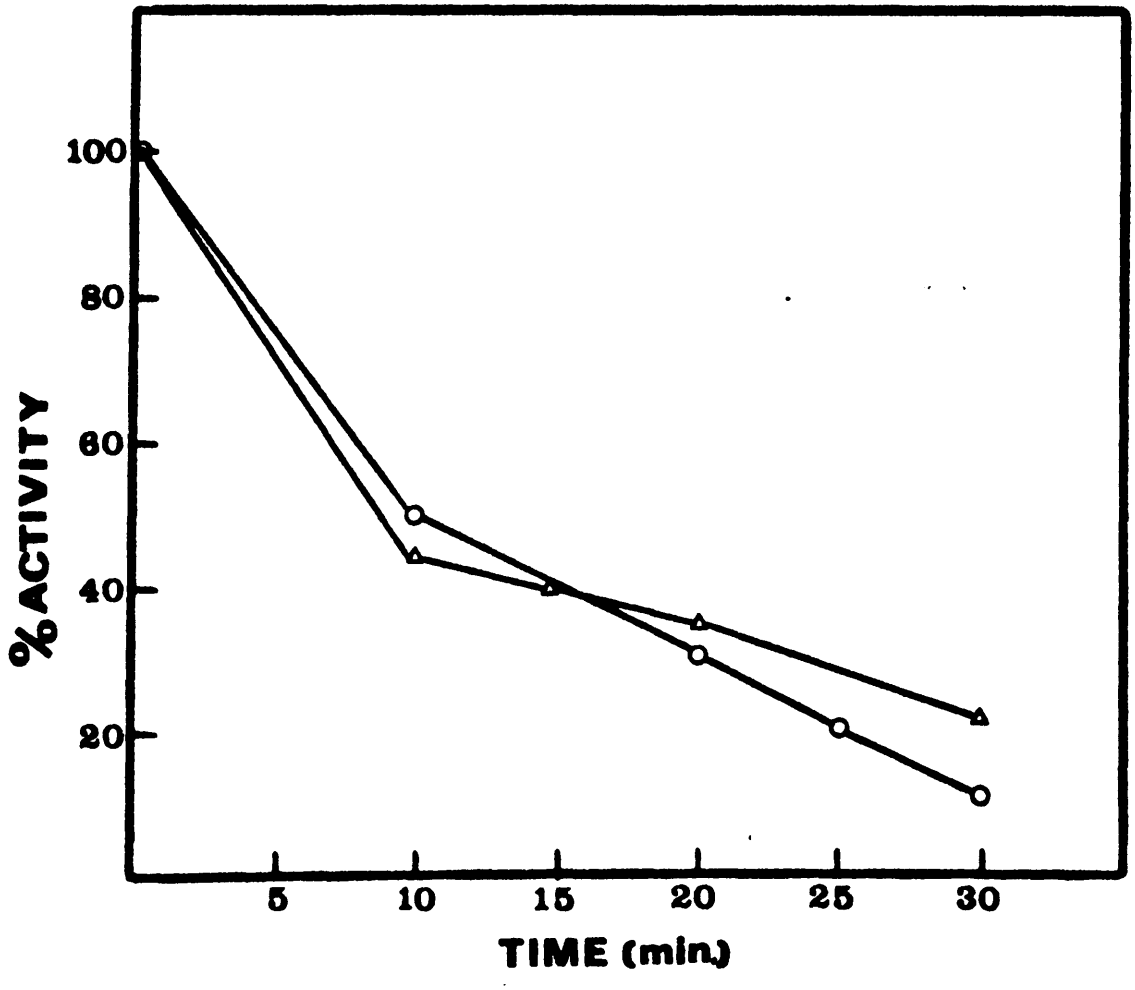


FIG. 13. Effect of 8M urea on the activities of the high molecular weight form (o——o) and the low molecular weight form (Δ——Δ) of rabbit intestinal adenosine deaminase. The enzymes were incubated for 30 min. at 37°C in 8M urea (in 0.1 M phosphate pH 7.0) and assays performed spectrophotometrically at 265 nm (pH 7.0).

removed for spectrophotometric assay after 6 h. and 24h. respectively. Results obtained for the high and low molecular weight forms are illustrated in Figs. 11 and 12.

It can be observed that the inactivation patterns are very similar, both forms of the enzyme being completely inactivated by incubation in 8M urea for 24h.

Another experiment was performed to determine if any differences could be detected between the two forms in the denaturation process. Both forms were suitably diluted and incubated with 8M urea in 0.1M phosphate buffer pH 7.0. The loss of enzyme activity was measured over a period of 30 min. by removing 0.1 ml. volumes for assay at various times. Fig. 13 shows a plot of the results obtained. It is evident that the rate of loss in activity in both cases was almost the same, demonstrating the similarity of the denaturation of the high and low molecular weight forms by urea.

THE EFFECT OF GUANIDINE HYDROCHLORIDE ON ENZYME ACTIVITY

Guanidine hydrochloride is also known to denature many enzymes but at a much lower concentration than urea. In the following experiment its effect on the activity of the high and low molecular weight forms of the deaminase was investigated.

The enzymes were incubated at 37^{o}C with guanidine hydrochloride in 0.1M phosphate pH 7.0. The final concentrations of the denaturant were 0.5M, 1M and 2M respectively. Deaminase activity

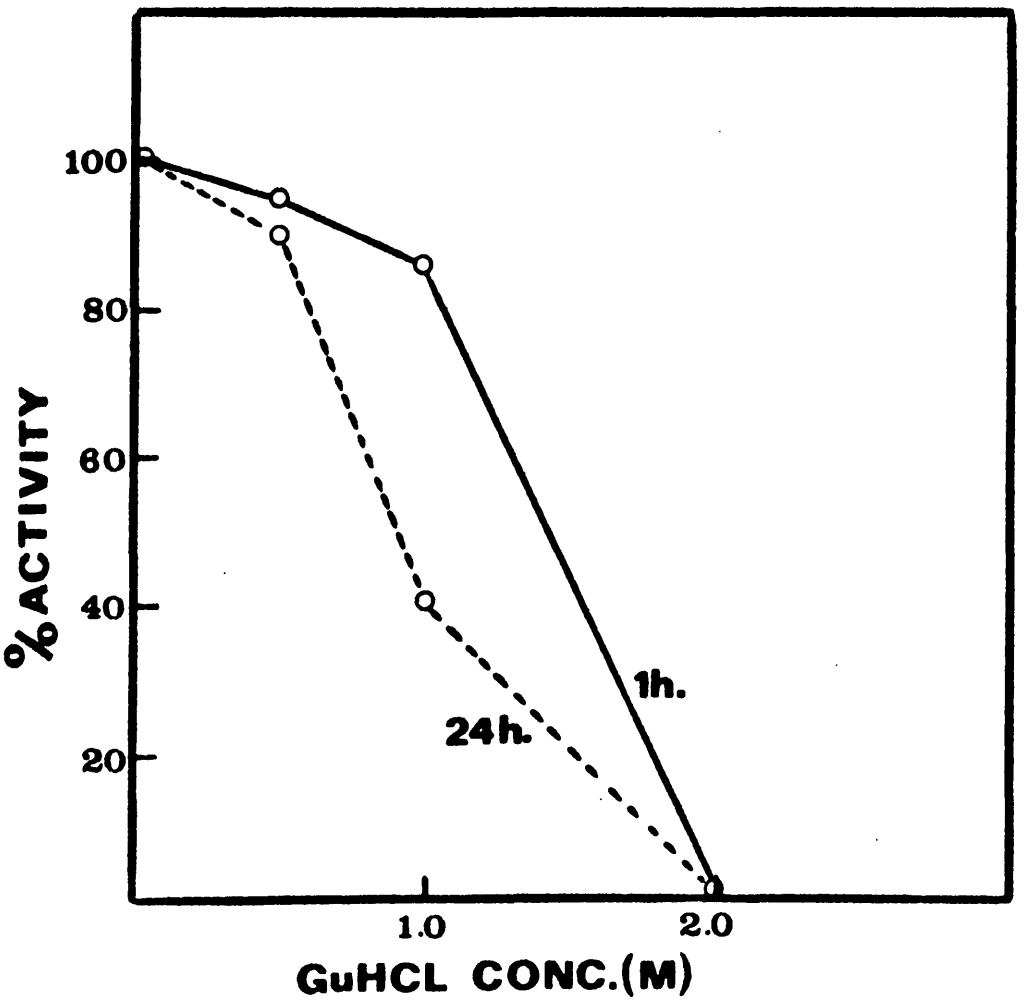


FIG. 14. Effect of guanidine hydrochloride on the activity of the low molecular weight form of rabbit intestinal adenosine deaminase. The diluted enzyme (in 0.1 M phosphate pH 7.0) was incubated at 37°C for 1 h. and 24 h. with the GuHCl solutions (1-2 M) and assays performed spectrophotometrically at 265 nm (pH 7.0).

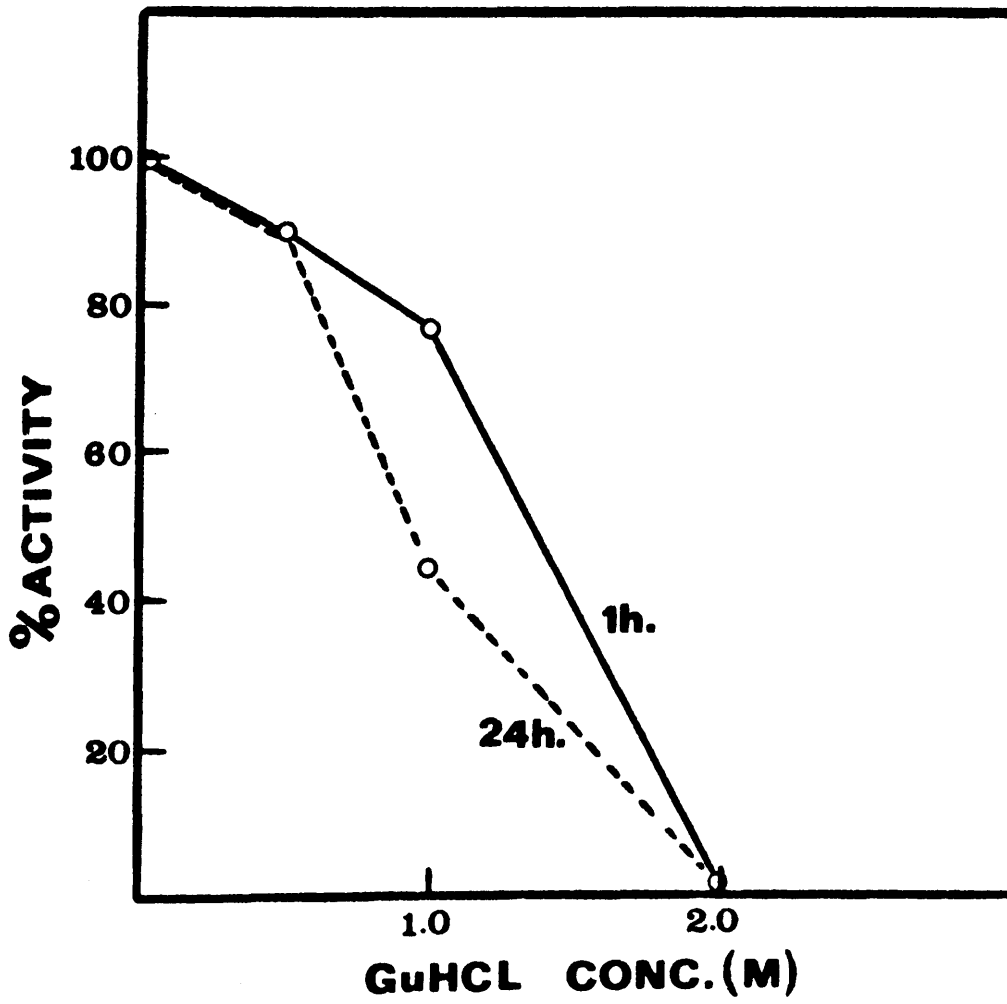


FIG. 15. Effect of guanidine hydrochloride on the activity of the high molecular weight form of rabbit intestinal adenosine deaminase. The diluted enzyme (in 0.1 M phosphate pH 7.0) was incubated at 37°C for 1 h. and 24 h. with the GuHCl solutions (1-2 M) and assays performed spectrophotometrically at 265 nm (pH 7.0).

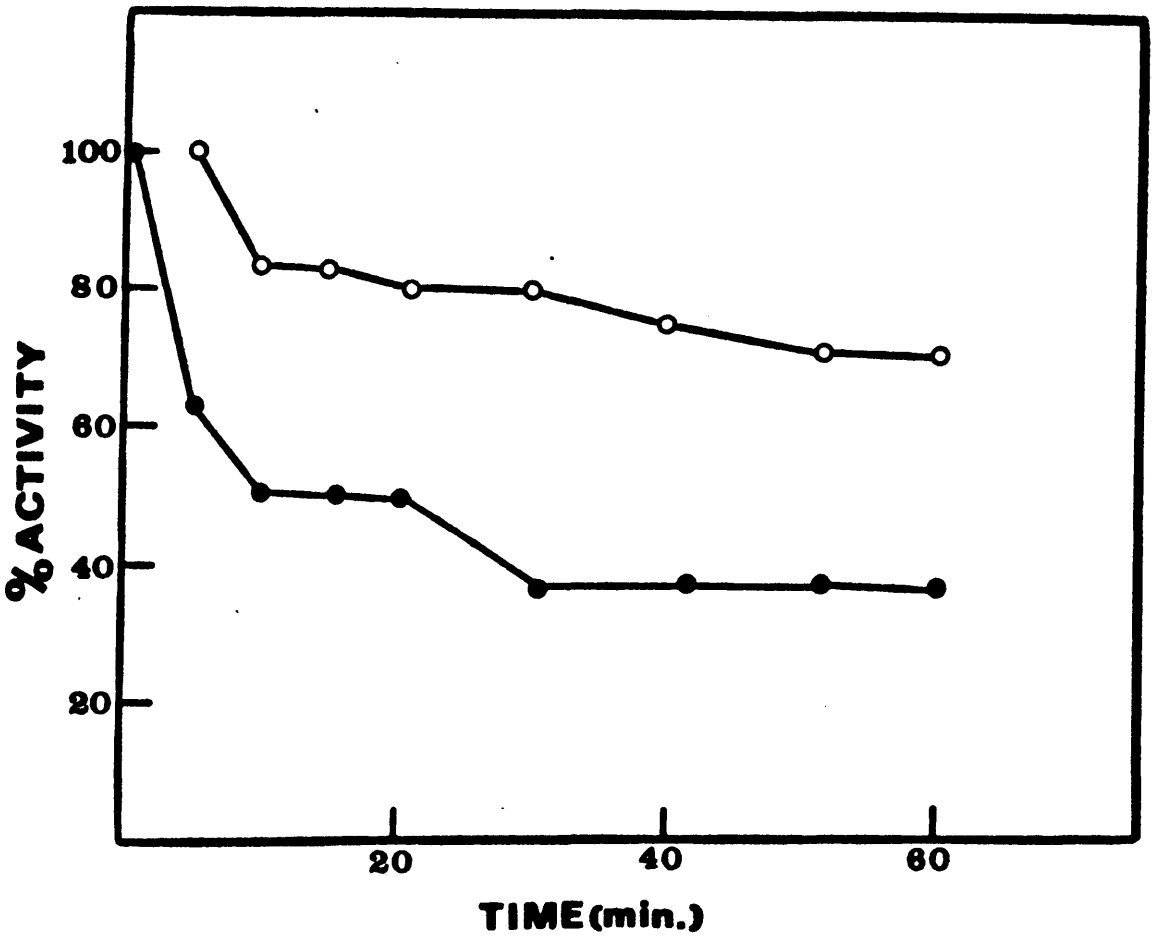


FIG. 16. The effect of 1.5 M guanidine hydrochloride on the activity of the low molecular weight form (o—o) and the high molecular weight form (●—●) of rabbit intestinal adenosine deaminase. The enzymes were incubated for 60 min. at 27°C in 1.5 M GuHCl (in 0.1M phosphate pH 7.0) and assays performed spectrophotometrically at 265 nm (pH 7.0).

was measured spectrophotometrically at pH 7.0 after 1 h. and 24 h. The results which are depicted in Figs. 14 and 15 illustrate that the high and low molecular weight forms show very similar inactivation patterns with this reagent.

As in the case of the urea experiments the loss in activity with time was investigated using a single concentration of guanidine hydrochloride. The enzymes were incubated at 37°C with 1.5 M guanidine hydrochloride in 0.1M phosphate pH 7.0 and assays performed at various times. The results obtained are graphically illustrated in Fig. 16. This shows that the low molecular weight form is more stable towards guanidine hydrochloride than the high molecular weight enzyme, 70% of its activity remaining after 60 min. compared to 37% for the high molecular weight form.

THE EFFECT OF DIMETHYLFORMAMIDE ON ENZYME ACTIVITY

Dimethylformamide (DMF) or formdimethylamide is an organic solvent which causes denaturation and often dissociation of certain proteins (Cooper, 1971). In the following experiment its effect on the activity of the high and low molecular weight forms of the deaminase is studied.

Experimental conditions :

The enzymes were incubated at 37°C for 30 min. with various concentrations of dimethylformamide which ranged from 10% (v/v) to 50% (v/v) in 0.1M phosphate pH 7.0. Controls were included without the denaturant. After incubation assays were performed

spectrophotometrically at pH 7.0. The results obtained are summarized in Table 5.

TABLE 5. Effect of DMF on the Activity of the High and Low Molecular Weight Forms of Adenosine Deaminase

	% Activity remaining after 30 min. incubation	
% DMF (v/v)	High mol.wt. form	Low mol. wt. form
10	100	100
20	85	90
30	55	66
40	0	0
50	0	0

It is evident that the reagent inactivated the enzymes to the same extent, both forms loosing all activity when incubated with 40% DMF (v/v).

METAL-ION INHIBITION OF THE HIGH AND LOW MOLECULAR WEIGHT FORMS OF ADENOSINE DEAMINASE

Various metal ions have already been shown to effect the activity of adenosine deaminase isolated from bovine mucosa(Brady, 1942; Zittle, 1946). In the following experiment a systematic study of the effect of a number of metal ions on the activity of

the rabbit enzymes was carried out.

Experimental conditions:

10 mM solutions of the following salts were made up in 0.1M Tris pH 7.0:

$ZnSO_4$, $CuCL$, $CuCL_2$, $MgCL_2$ $MnCL_2$, $CdCL_2$, $CoCL_2$
$SrCL_6$, $FeCL_3$, $BaCL_2$, $CrCL_3$, $CaCL_2$, $NiCL_2$ and EDTA.

Equal volumes of both forms of the deaminase were incubated at 37°C for 30 min. with an equal volume of each salt so that the final metal ion concentration was 5 mM. Assays were performed spectrophotometrically at pH 7.0. The results obtained are listed in Table 6.

TABLE 6. Effect of Metal Ions on the activity of the High and Low Molecular Weight Forms of Adenosine Deaminase

	% inhibition after 30 min. incubation	
Metal ion	High mol. wt. form	Low mol. wt. form
Zn^{++}	0	0
Cu^{+}	100	100
Cu^{++}	100	100
Mg^{++}	0	0
Mn^{++}	0	0
Cd^{++}	33	0
Co^{++}	20	0
Ba^{++}	0	0
Ca^{++}	0	0
Ni^{++}	0	0
Fe^{+++}	100	7
Cr^{+++}	67	12
Sr^{++}	0	0
EDTA	0	0

Zn^{++}, Mg^{++}, Mn^{++}, Ba^{++}, Ca^{++}, Ni^{++} and Sr^{+6} had no effect on the activity of the enzymes, whereas both Cu^{+} and Cu^{++} completely inactivated the two forms. Cd^{++} and Co^{++} inhibited the high molecular weight form by 33% and 20% respectively with neither metal ion affecting the activity of the low molecular weight form. Fe^{+++} and Cr^{+++} inhibited the high molecular weight enzyme by 100% and 67% respectively, but inhibited the low molecular weight form by only 7% and 12%.

STUDIES ON THE ACTIVE SITE OF ADENOSINE DEAMINASE FROM RABBIT INTESTINE

As will be seen later in this section, various suggestions have been made as regards the nature of the high molecular weight form and its relationship to the low molecular weight form. As both forms possess adenosine deaminase activity, it was considered of interest to investigate , to some extent, the nature of their active sites with a view to establishing whether or not there were similarities between them. It has already been shown that a sulphydryl group is necessary for the activity of adenosine deaminase isolated from bovine intestinal mucosa (Ronca et al.1967; Murphy, 1967) chicken duodenum (Hoagland and Fisher, 1967) and bovine placenta (Maguire and Sim, 1971). Because of the similarity between these enzymes and the low molecular weight adenosine deaminase from rabbit intestine, it was considered that a sulphydryl group might be necessary for the activity of the rabbit enzyme. The effect of a number of reagents known to react with sulphydryl groups was therefore studied using both forms of the enzyme.

Evidence has been presented (Maguire and Sim, 1971) which indicates that histidine and possibly tryptophan may play a role in the deamination reaction catalysed by the bovine placental adenosine deaminase. Reagents which react with these amino-acids were also used in the study of the rabbit enzymes.

THE EFFECT OF THIOL REAGENTS ON DEAMINASE ACTIVITY

Preliminary experiments using thiol reagents showed that iodoacetic acid and its amide iodoacetamide had no effect on enzyme activity when used at a concentration of 2.5 mM in 0.1M phosphate pH 7.0. Both the high and low molecular weight forms retained 100% of their activity even after 15 h. incubation at 37°C with the reagents. The fact that these alkylating compounds did not inhibit adenosine deaminase activity does not necessarily exclude the possibility of the presence of sulphydryl groups in the enzymes, as iodoacetic acid and iodoacetamide are not very reactive.

Iodosobenzoic acid (0.25 mM in 0.1M phosphate pH 7.0) however completely inactivated both forms of the enzyme after 5 min. incubation at 37°C. In an effort to determine if this reagent had a differential effect on the two enzyme forms a lower concentration was used. Fig. 17 shows the effect of 10 µM iodosobenzoic acid (pH 7.0) with time. Only a small difference was observed, the high molecular weight form being slightly more stable than the low molecular weight form after 16 h. incubation with the oxidizing agent.

The effect of 5'-5'-dithiobis-(2-nitrobenzoate), a thiol specific reagent developed by Ellman (1959), was also investigated However, the reagent (0.1 mM in 0.1M phosphate pH 8.0) had no effect on the activity of the high and low molecular weight forms after 3 h. incubation at 37°C.

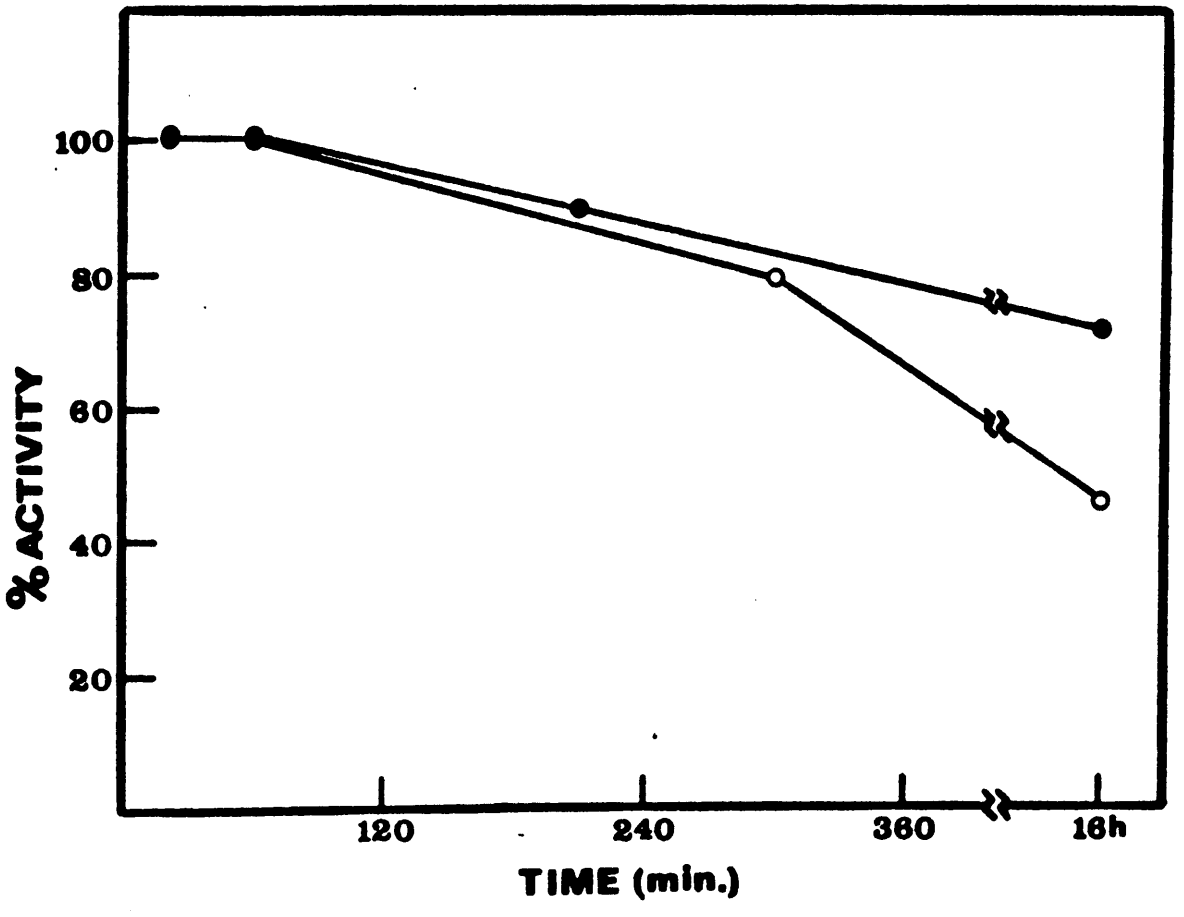


FIG. 17. The effect of iodozobenzoic acid on the activities of the high molecular weight form (●——●) and the low molecular weight form (○——○) of rabbit intestinal adenosine deaminase. The enzymes were incubated for 5 min. with 10μM iodozobenzoic acid (in 0.1 M phosphate pH 7.0) and assays performed spectrophotometrically at 265 nm (pH 7.0).

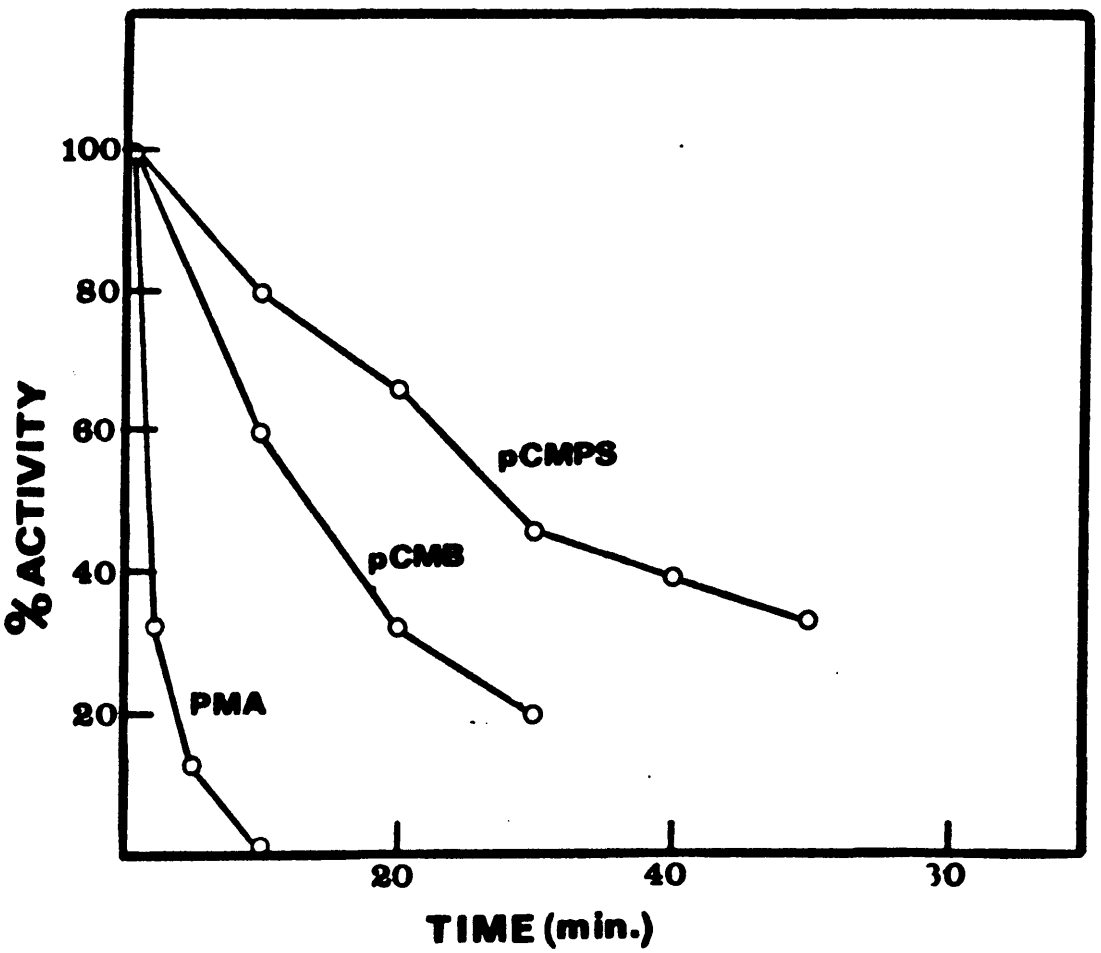


FIG. 18. The effect of organomercurials on the activity of the high molecular weight form of rabbit intestinal adenosine deaminase. The enzyme was incubated at 37°C with each reagent (0.2 M in 0.1M phosphate pH 7.0) and assays performed spectrophotometrically at 265 nm (pH 7.0).

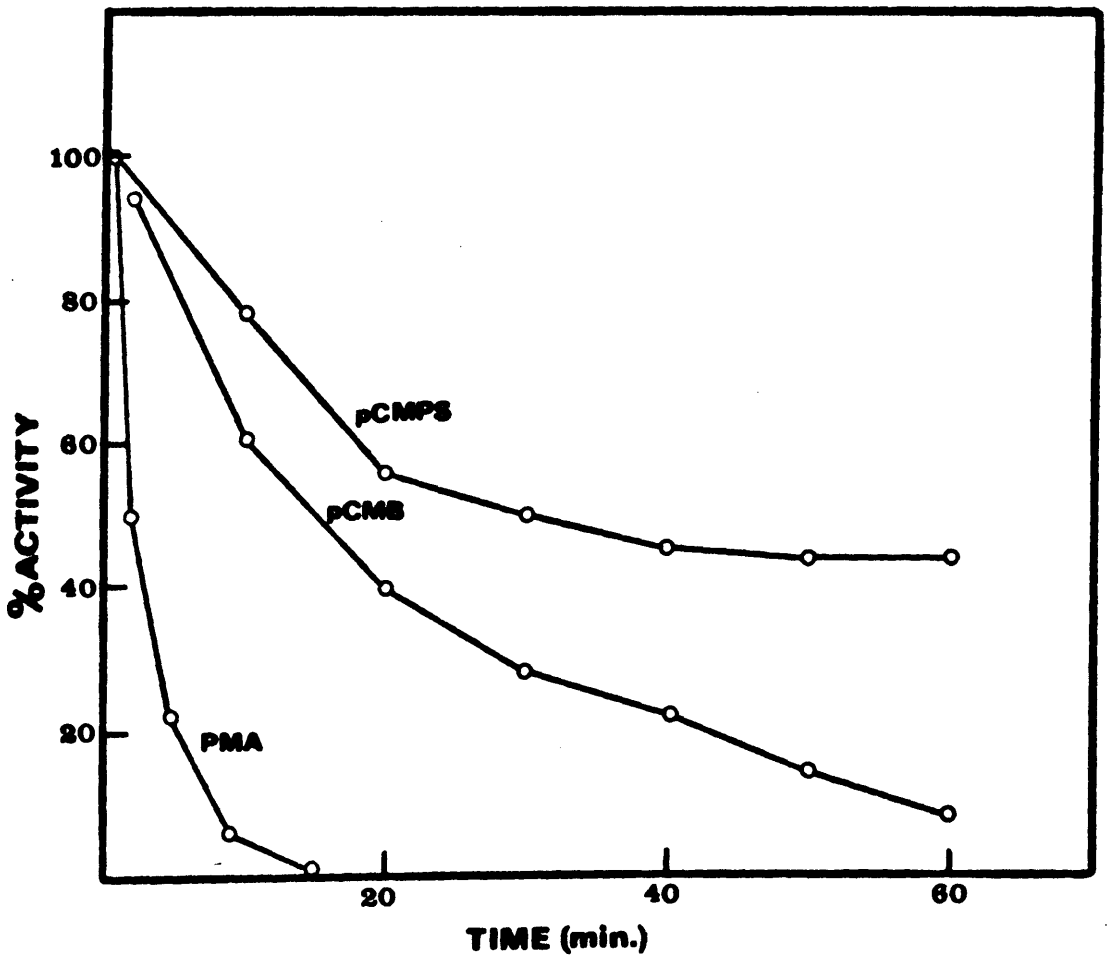


FIG. 19. The effect of organomercurials on the activity of the low molecular weight form of rabbit intestinal adenosine deaminase. The enzyme was incubated at 37°C with each reagent (0.2 mM in 0.1 M phosphate pH 7.0) and assays performed spectrophotometrically at 265 nm (pH 7.0).

THE EFFECT OF ORGANOMERCURIALS ON ADENOSINE DEAMINASE ACTIVITY

Murphy (1967) found that the bovine intestinal adenosine deaminase was not inhibited by iodoacetic acid or iodoacetamide, but did, however, react with organomercurials. The effect of these thiol reagents on the activity of the rabbit enzymes was therefore investigated.

The following mercurials were used in the study:

p-chloromercuriphenylsulphonic acid (PCMPS)

p-chloromercuribenzoate (PMB)

phenyl-mercury acetate (PMA)

The enzymes were incubated at 37°C with the mercurials (Final concentration, 0.2 mM in 0.1 phosphate pH 7.0). The spectrophotometric method of assay was used. Figs. 18 and 19 show the loss in activity with time for both forms of the enzyme. It is evident that the inhibition by the mercurials is time dependent for the high and low molecular weight forms. The order of reactivity of the mercurials was PMA > PMB > PCMPS.

REACTIVATION OF THE MERCURIAL INHIBITED DEAMINASES

The above results indicate the presence of sulphydryl groups in both forms of the enzyme. A criterion of mercaptide formation is that the reaction is readily reversible. The following experiment was designed so as to determine if β-mercaptoethanol,

dithiothreitol (Clelands reagent) or dithioerythritol (DTE) would reactivate the enzymes inhibited by the mercurials.

The high and low molecular weight forms were treated with p-chloromercuribenzoate (0.2 mM) at pH 7.0 at 37°C for 30 min. This gave approximately 90% inhibition in each case. After this time β-mercaptoethanol (33 mM), dithiothreitol (3.3 mM) and dithioerythritol (1.66 mM) were added in separate tubes to the treated enzymes, and assays performed spectrophotometrically at various time intervals. The results obtained are plotted in Figs. 20 and 21. As may be seen, reactivation was achieved in all cases, the patterns being very similar for the high and low molecular weight forms. The degree of reactivation also increased with increasing molarity of the reactivating agents. However the reactivation was only partial. Murphy (1967) also found that the bovine intestinal adenosine deaminase could be only partially reactivated after treatment with p-chloromercuribenzoate and he showed that this partial reactivation obtained at longer incubation periods was due to slow precipitation of the PMB-enzyme complex. Precipitation of the PMB-enzyme complex could also account for the partial reactivation of the mercurial treated rabbit intestinal deaminases

The inhibition of deaminase by the organomercurials and the subsequent reactivation by β-mercaptoethanol, dithiothreitol and dithioerythritol indicates that sulphydryl groups are necessary for the enzymatic activity of the high and low molecular weight forms of rabbit intestinal adenosine deaminase.

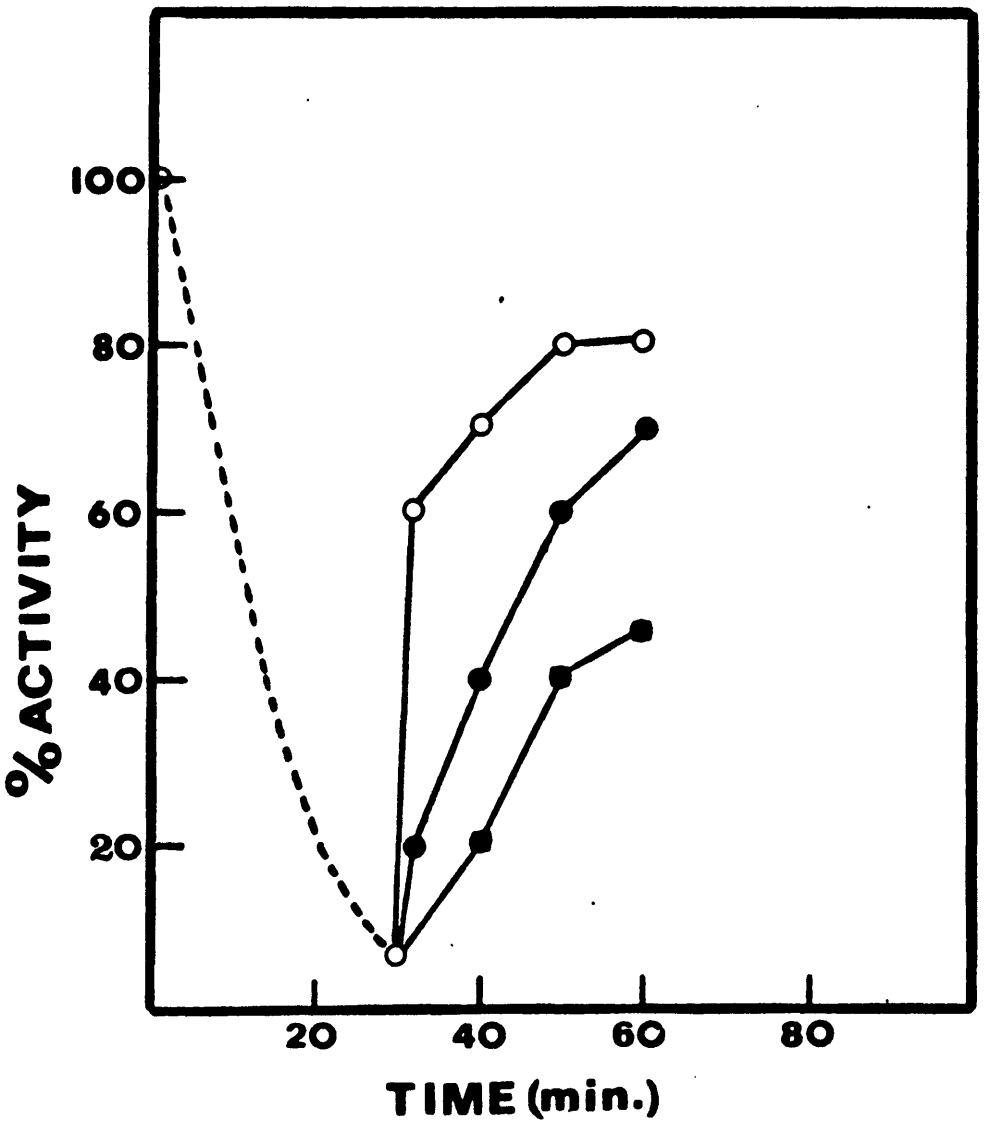


FIG. 20. Reactivation of the PMB-inhibited high molecular weight form of rabbit intestinal adenosine deaminase using 1.66 mM DTE (■——■), 3.3 mM Clelands reagent (●——●), and 33 mM β-mercaptoethanol (○——○). The dotted line represents inactivation of the enzyme with 0.2 mM PMB (in 0.1 M phosphate pH 7.0).

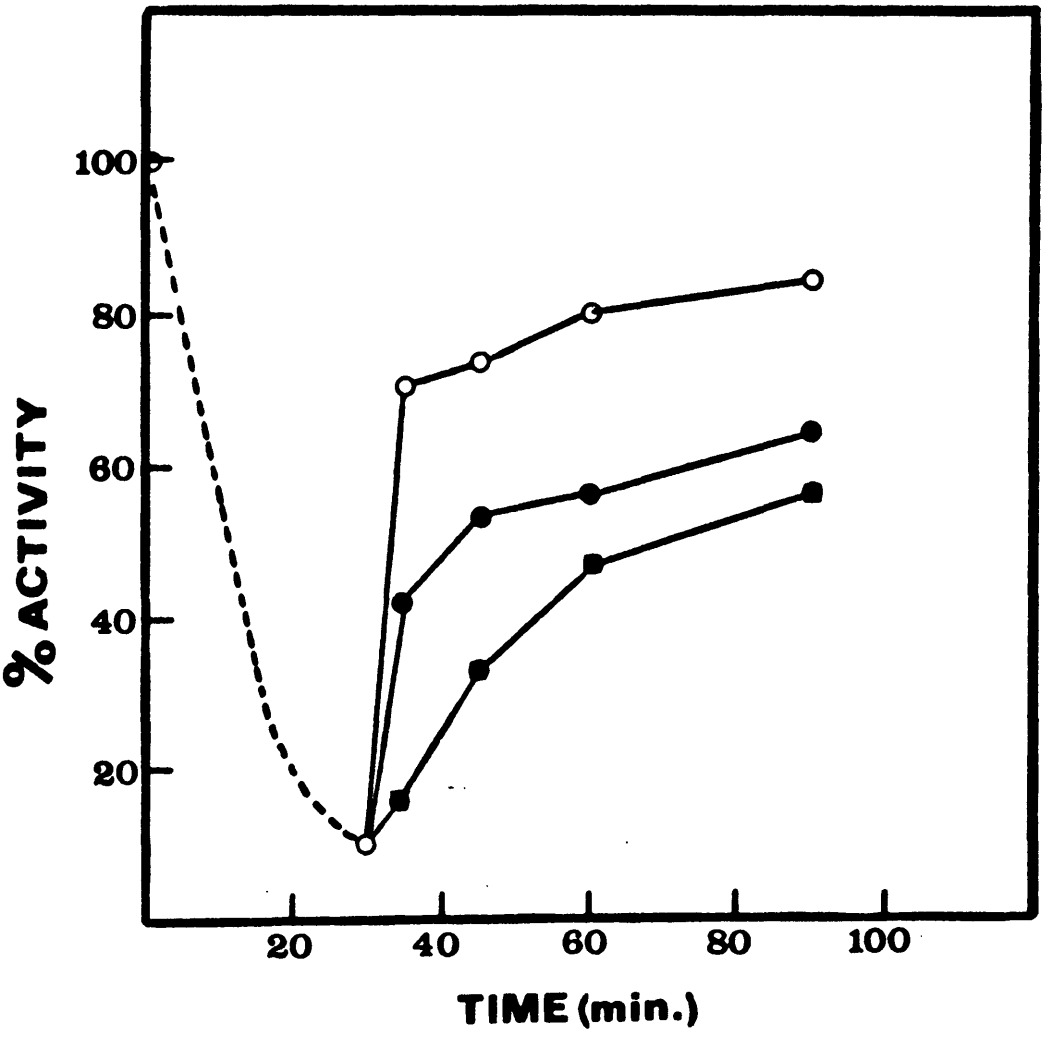


FIG. 21. Reactivation of the PMB-inhibited high molecular weight form of rabbit intestinal adenosine deaminase using 1.66 mM DTE (■——■), 3.3 mM Clelands reagent (●——●) and 33 mM β-mercapto-ethanol (○——○). The dotted line represents inactivation of the enzyme with 0.2 mM PMB (in 0.1M phosphate pH 7.0).

PHOTOOXIDATION STUDIES

Following the use of thiol reagents, it was decided to study the effect of photooxidation on the activity of the high and low molecular weight forms of the deaminase.

Molecular oxygen, which is generally ineffective towards amino acids and proteins, can be photoactivated to promote the oxidation of polypeptide molecules by irradiation with visible light in the presence of sensitizers (Spikes and Livingston, 1969). Although the mechanism of the photooxidation process has not yet been completely established, the technique has been used for the chemical modification of proteins in the study of structure-function relationships (Westhead, 1965; Martinez-Carrion et al., 1967; Barker et al., 1971). The amino acid moities, which are normally considered to be susceptible to modification by this technique, are the thiol group of cysteine, the thioether function of methionine, the imidazole ring of histidine, the indole moiety of tryptophan and the phenolic side chain of tyrosine. The most commonly used sensitizers are methylene blue, rose bengal and crystal violet.

Experimental Procedure:

The experimental procedure employed was the same in all experiments.

The purified deaminase was diluted with the appropriate buffer to give a reasonable fall in absorbance on assay. To 0.5 ml. of the deaminase solution was added 0.5 ml. of sensitizer (0.02%

solution in the same buffer) and the mixture irradiated at room temperature by four 150 W spotlight lamps, each placed at a distance of 20 cm. After appropriate intervals, 0.1 ml. of the mixture was withdrawn to measure the enzyme activity. Control tubes were included without the sensitizer.

PHOTOOXIDATION OF ADENOSINE DEAMINASE IN THE PRESENCE OF METHYLENE BLUE

In the first experiment, methylene blue was incubated with solutions of the high and low molecular weight forms of rabbit intestinal adenosine deaminase. The bovine intestinal deaminase was also included in the experiment, the pH of the incubation mixtures being 6.5. The loss of activity with time is shown in Fig. 22. It is evident that photooxidation was complete after 1h. incubation, the bovine enzyme being less susceptible than the high and low molecular weight rabbit deaminases. This experiment indicated that a photooxidizable amino acid is necessary for the activity of adenosine deaminase.

Since the rate of photooxidation is strongly dependent on the pH of the reaction medium (McLaren, 1964) the process was investigated at different pH values. The following buffers were used:

0.1 M acetate pH 5.0
0.1 M phosphate pH 6.5
0.1 M Tris pH 8.6

Methylene blue was used as sensitizer. The effect of photo-

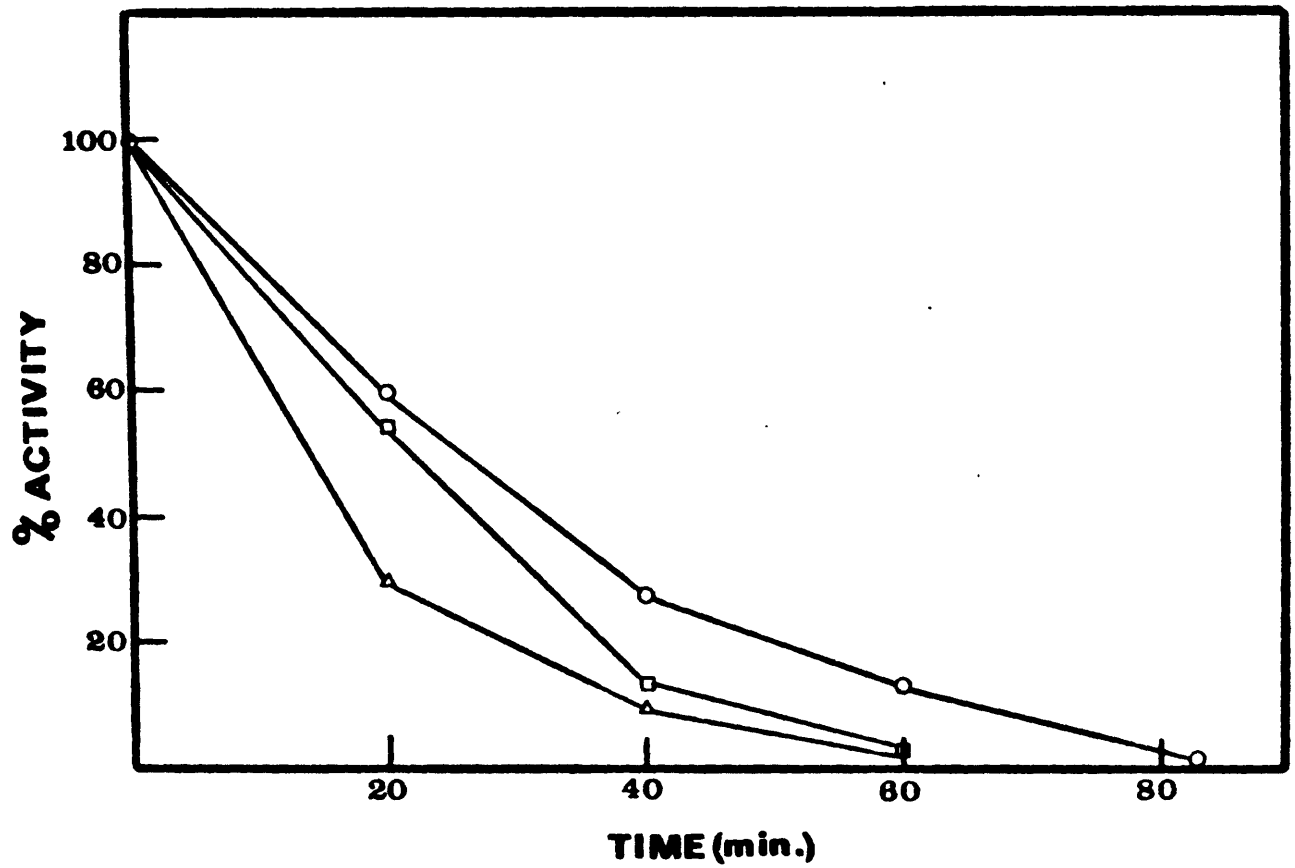


FIG. 22. The effect of photooxidation (using 0.01% methylene blue as sensitizer) on the activity of bovine intestinal adenosine deaminase (o——o) and the high (□——□) and low (△——△) molecular weight forms of rabbit intestinal adenosine deaminase. The pH of the reaction mixture was 6.5.

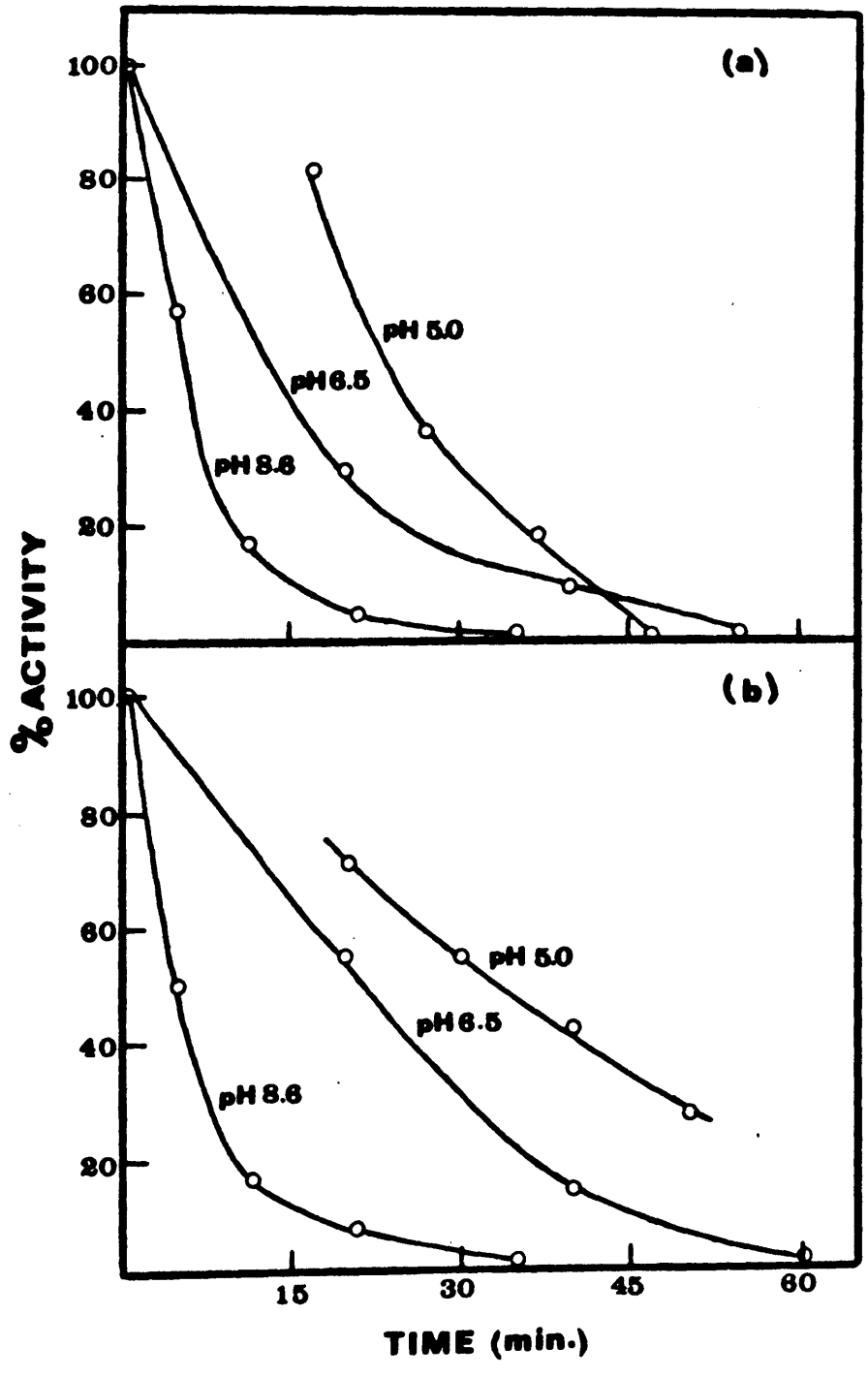


FIG. 23. The effect of photooxidation (using 0.01% methylene blue as sensitizer) on the activity of (a) the low molecular weight form and (b) the high molecular weight form of rabbit intestinal adenosine deaminase at various pH values.

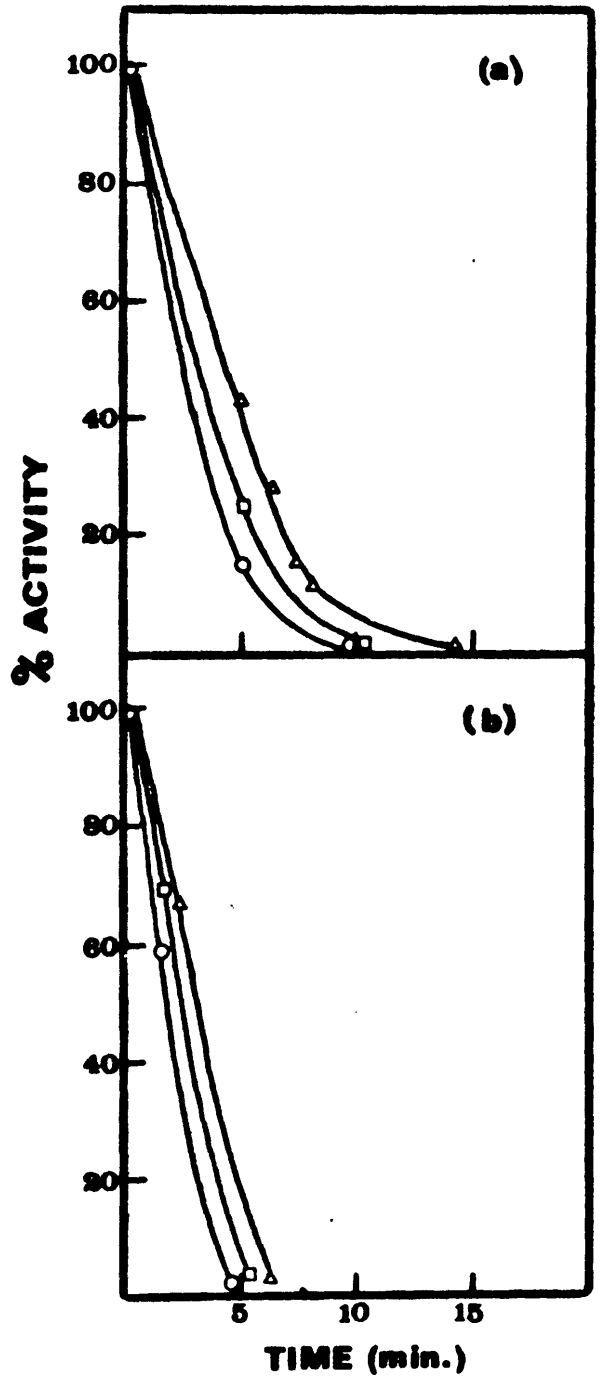


FIG. 24. The effect of photooxidation (using 0.01% rose bengal as sensitizer) on the activity of (a) the low molecular weight form and (b) the high molecular weight form of rabbit intestinal adenosine deaminase. ○—○, pH 6.5; □—□, pH 5.0; △—△ pH 8.6.

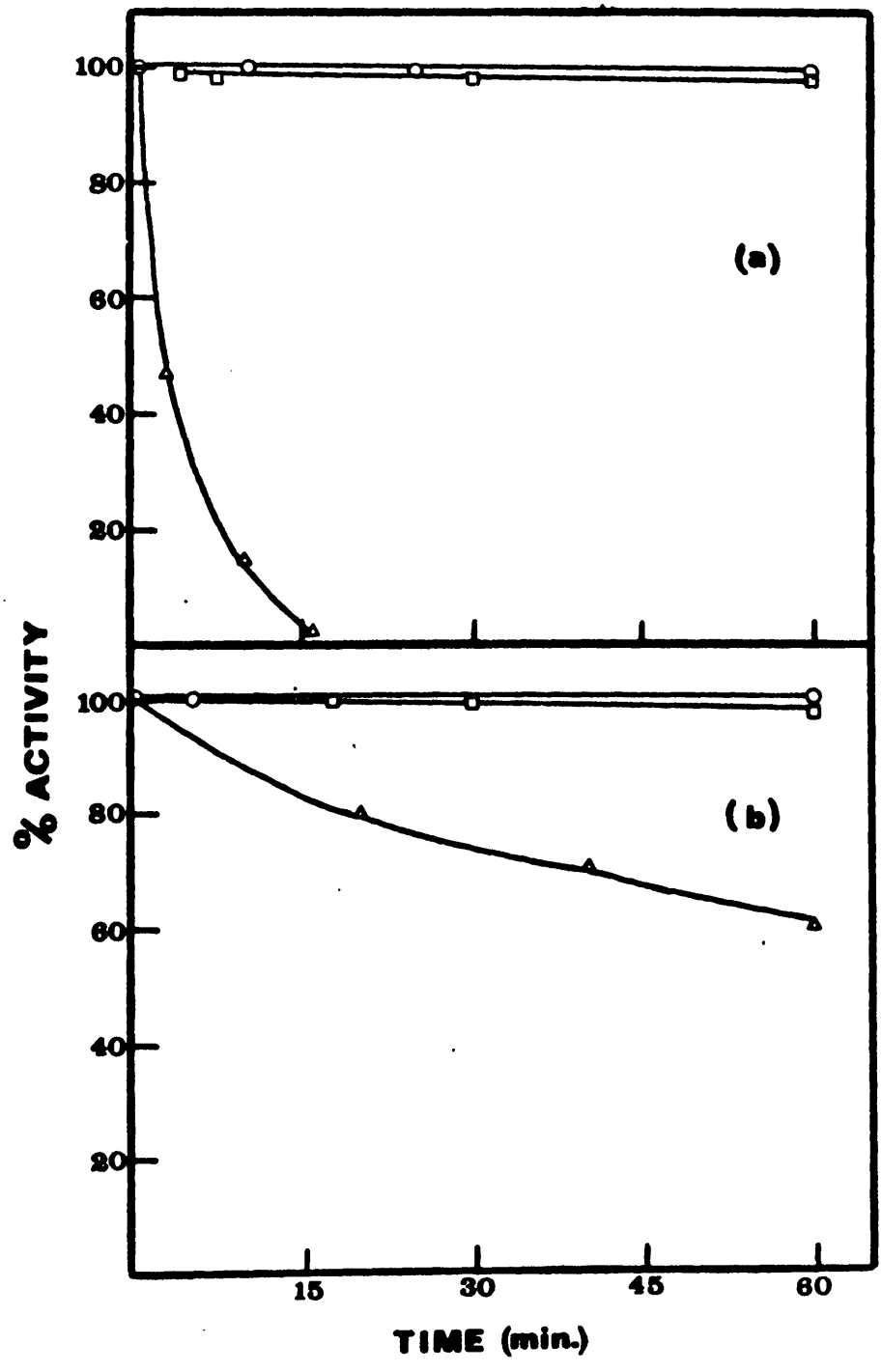


FIG. 25. The effect of photooxidation (using 0.01% crystal violet as sensitizer) on the activity of (a) the low molecular weight form and (b) the high molecular weight form of rabbit intestinal adenosine deaminase. O——O , 5.0; □——□ pH 6.5; Δ——Δ pH 8.6.

oxidation with time at these pH values is illustrated in Fig. 23. The inactivation patterns observed appear to be somewhat similar for both forms of the enzyme, the rate of photooxidation being greatest at the alkaline pH and least at the acid pH value.

PHOTOOXIDATION OF ADENOSINE DEAMINASE IN THE PRESENCE OF ROSE BENGAL

Rose Bengal has also been widely used as a sensitizer in photooxidation studies. It has been found to be more effective than methylene blue (Cohen, 1968) and also more specific, histidine residues being primarily oxidized (Glazer, 1970).

The inactivation patterns obtained for the high and low molecular weight forms of the deaminase are shown in Fig 24. It is evident that the rate of photooxidation was much faster here than in the case of the methylene blue-sensitized photooxidations. In this experiment the low molecular weight enzyme was photooxidized at a somewhat slower rate than was the high molecular weight form.

PHOTOOXIDATION OF ADENOSINE DEAMINASE IN THE PRESENCE OF CRYSTAL VIOLET

Because methylene blue and rose bengal are not very specific

as sensitizers, only tentative conclusions can be drawn from the results of the above experiments. An example of absolute specificity of a sensitizer for a given amino acid is offered by the crystal violet-sensitized photooxidation of cysteine. Following the first experiments of Bellin and Yankus (1968), Jori, et al. (1969) showed that this dye acts selectively on cysteine in aqueous solution over the pH range 2.5 - 9.0, and suggested that the inactivation of the sulphydryl enzyme papain was due to a specific binding of the crystal violet with the thiol group of cysteine.

Photooxidation of the high and low molecular weight forms of rabbit intestinal adenosine deaminase was investigated using crystal violet as sensitizer. The conditions were the same as those used in previous oxidation studies. The inactivation patterns obtained are illustrated in Fig. 25. It is apparent that the enzymes were photooxidized, although only at the alkaline pH, indicating the presence of a sulphydryl group necessary for activity in both forms of the enzyme. The rate of inactivation of the low molecular weight form was however faster than that of the high molecular weight enzyme.

In summary, therefore, a photooxidizable amino acid appears to be necessary for the activity of both forms of the deaminase. From the results obtained using crystal violet as sensitizer, this would appear to be cysteine. However, as the oxidation rates in the presence of methylene blue and rose bengal were much faster, it is possible that histidine or(and) tryptophan may be also involved.

EFFECT OF n-BROMOSUCCINIMIDE ON ENZYME ACTIVITY

Following the photooxidation studies, it appeared that a tryptophan residue may be necessary for enzyme activity. A reagent which has been found to react specifically with this amino acid is n-bromosuccinimide. This compound, when used at low concentrations and at acid pH, oxidizes tryptophan residues in many proteins and enzymes (Rao and Ramachandran, 1962; Spande and Witkop, 1967).

The effect of n-bromosuccinimide on the activity of the rabbit deaminases and also the bovine intestinal adenosine deaminase was investigated. The enzymes were incubated at 37^{o}C in 0.1M acetate buffer pH 5.0 with 10 mM and 10 µM n-bromosuccinimide. At the higher concentration (10 mM) almost 100% inactivation of the enzymes was obtained after 5 min. However, using 10 µM n-bromosuccinimide the low molecular weight form was almost completely inactivated after 10 min., whereas the reagent had no effect on the activity of the high molecular weight enzyme (Fig. 26). The bovine intestinal deaminase gave the same pattern of inhibition as the low molecular weight rabbit enzyme.

n-bromosuccinimide, therefore, does appear to have a differential effect on the activity of the low and high molecular weight forms of the deaminase. The fact that inactivation was obtained however, indicates that a tryptophan residue may be necessary for enzyme activity.

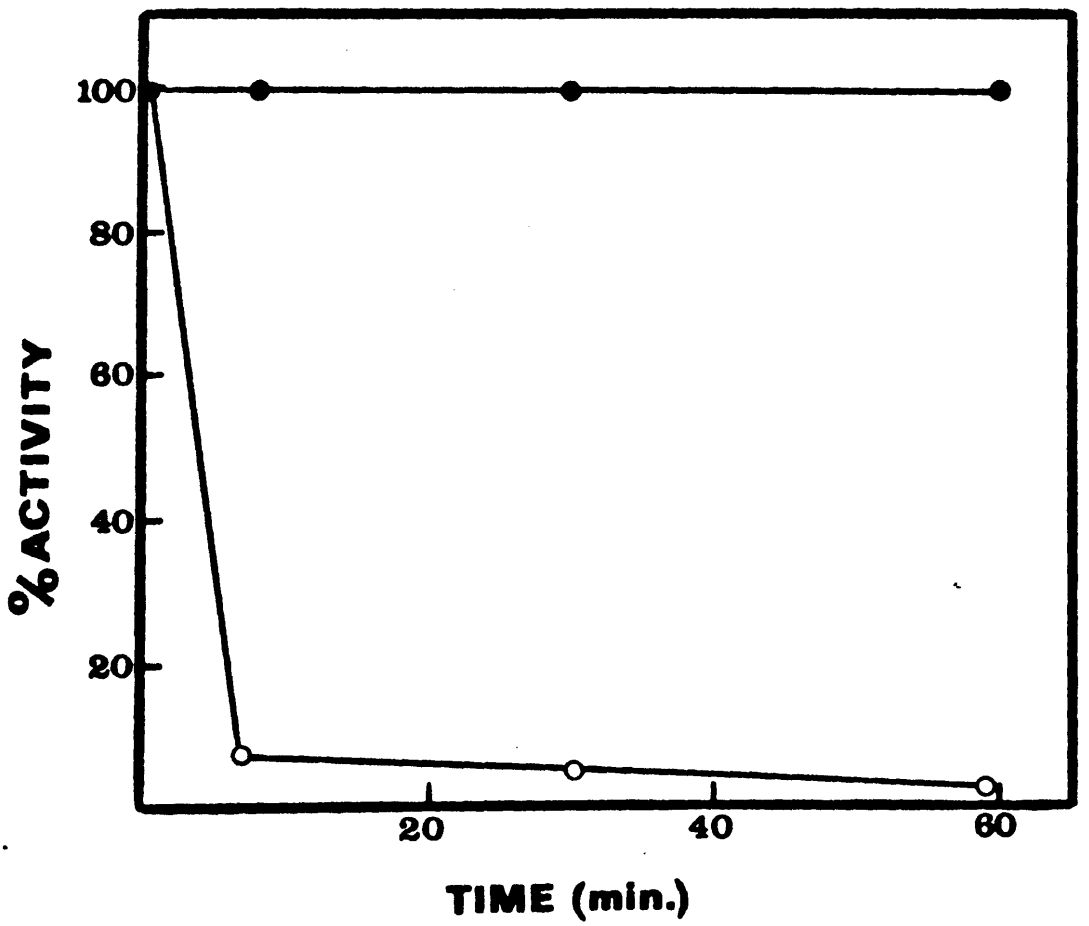


FIG. 26. The effect of n-bromosuccinimide on the activity of the high molecular weight form (●——●) and the low molecular weight form (○——○) of rabbit intestinal adenosine deaminase. The n-bromosuccinimide (10μM in 0.1M acetate pH 5.0) was incubated at 37°C with the enzymes and activity estimated spectrophotometrically at 265 nm (pH 7.0) at various time intervals.

MOLECULAR WEIGHT DETERMINATIONS

The molecular weights of the high and low molecular weight forms of rabbit intestinal adenosine deaminase have been determined (Murphy et al., 1969b) and were estimated to be in the order of 260,000 and 34,000 respectively. However, since these values were obtained using crude rabbit duodenal extracts it was decided to determine the molecular weights of the purified enzymes.

DETERMINATION OF THE MOLECULAR WEIGHT OF THE PURIFIED LOW MOLECULAR WEIGHT FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE BY GEL FILTRATION

A sephadex G-75 Superfine column (40 cm. x 2.5 cm.) was prepared as described in the Methods section (p.21) and equilibrated with 0.002M sodium citrate pH 5.8 incorporating 0.1 M KCl. Pure proteins of known molecular weights were applied to the column using the "Layering technique" (p.22) and 3 ml. fractions collected after elution. Table 7. lists the proteins, their molecular weights and the quantities used in calibrating the column. In all cases the proteins were determined by their ultraviolet absorption at the appropriate wavelength.

Fig. 27 shows the elution pattern of some of the pure proteins listed in Table 7 which were used for calibrating the column. Blue Dextran was used to determine the void volume.

Fig. 28 shows the relationship which exists between the molecular weights of these proteins (when plotted on a logarithmic scale) and the peak elution volumes. The relationship was linear between

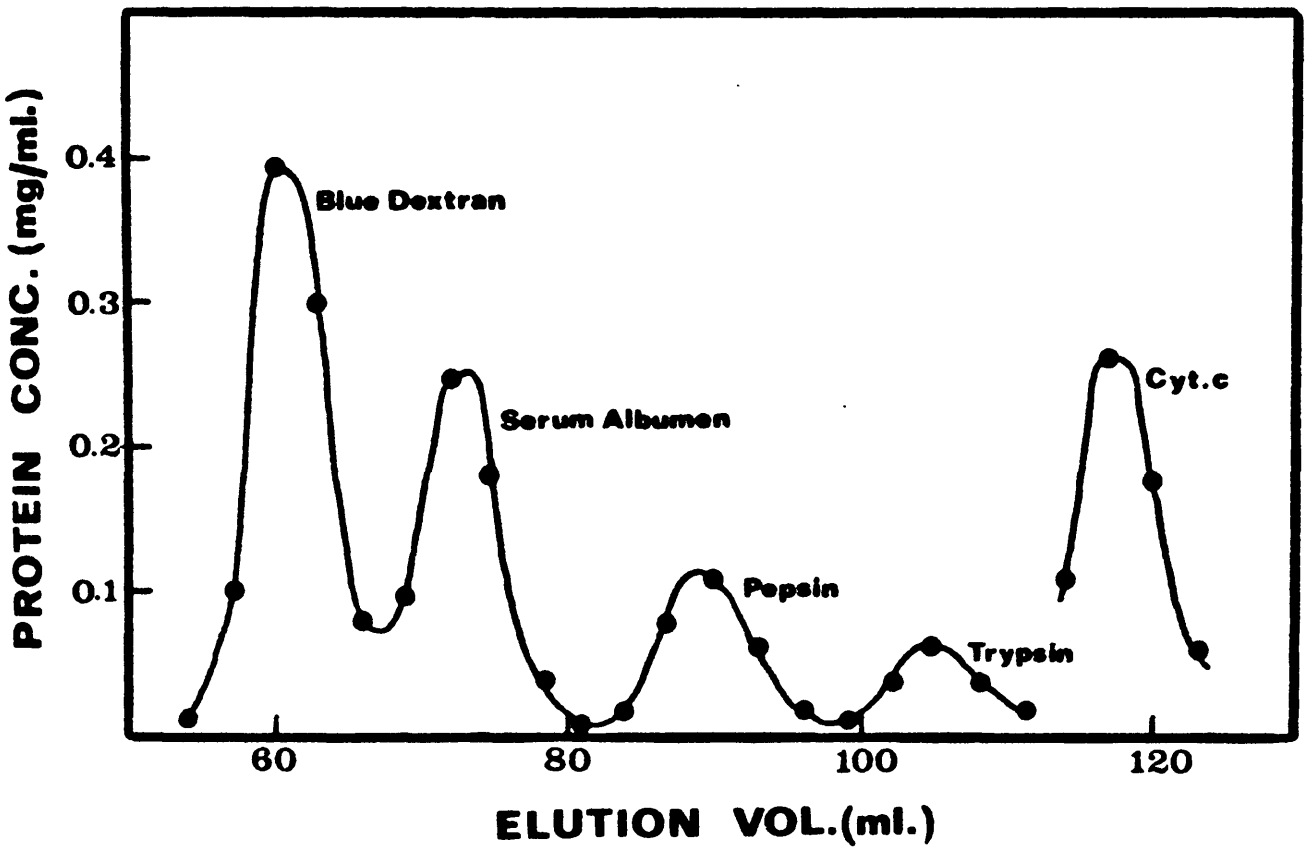


FIG. 27. Elution pattern of a number of purified proteins of known molecular weight from a Sephadex G-75 (Superfine) column equilibrated with 0.002 M sodium citrate pH 5.8 containing 0.1 M KCl. 2 ml. of each protein was applied to the column and 3 ml. fractions collected. Protein was determined by ultraviolet absorption at the appropriate wavelength.

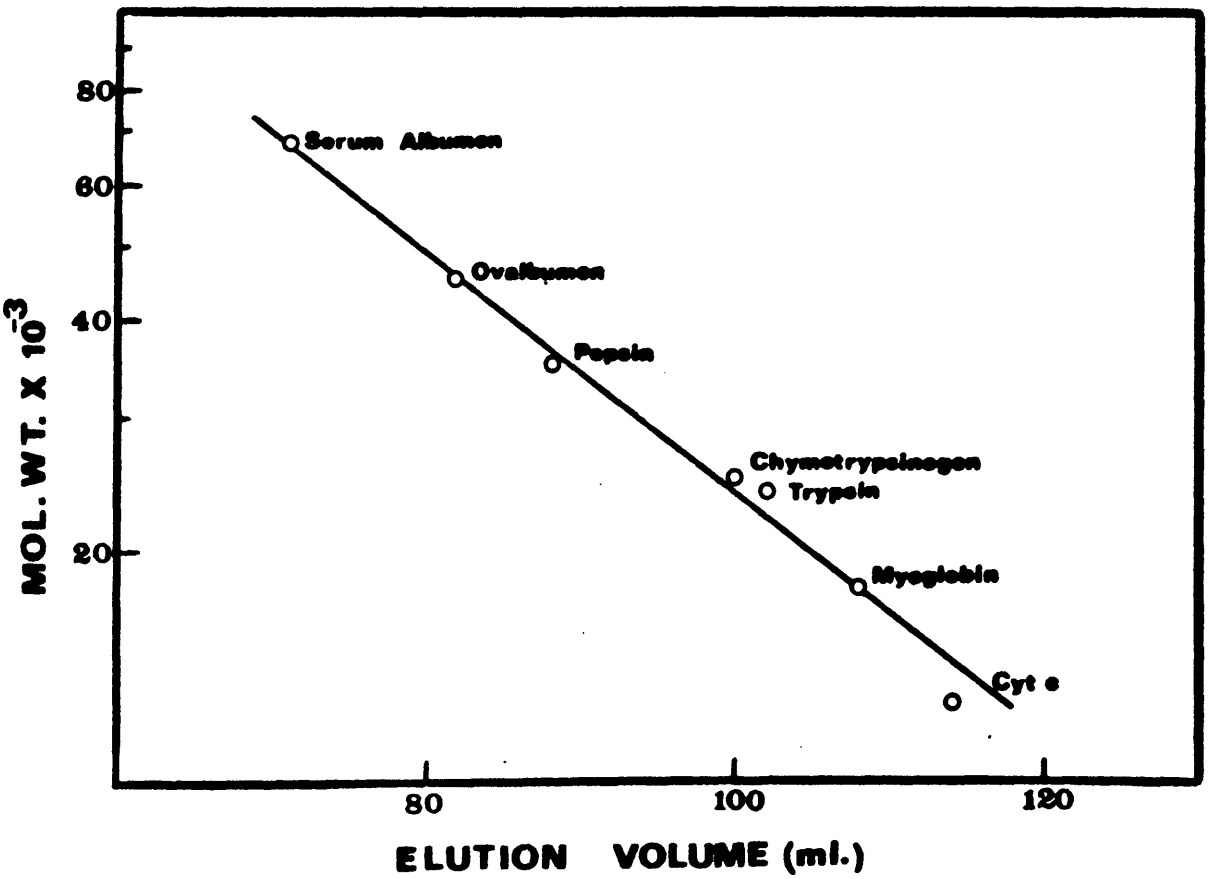


FIG. 28. Standard graph for molecular weight determination on Sephadex G-75 (Superfine). Purified proteins (2 ml. volumes) were applied to a Sephadex G-75 column equilibrated with 0.002 M sodium citrate pH 5.8 containing 0.1 M KCl, and the peak elution volume of each measured.

TABLE 7. Purified Proteins used in the Calibration of the Sephadex G-75 Column.

Protein	Mol. wt.	*Quantity applied	Wavelength used to estimate
Bovine serum albumen	67,000	6 mg.	280 nm
Ovalbumen	45,000	6 mg.	280 nm
Pepsin	35,000	6 mg.	280 nm
Chymotrypsinogen	25,000	6 mg.	280 nm
Trypsin	24,000	6 mg.	280 nm
Myoglobin	17,800	4 mg.	407 nm
Cytochrome C	12,400	4 mg.	412 nm

* Quantities referred to were dissolved in 2 ml. of eluting buffer.

bovine serum albumen (mol. wt. 67,000) and cytochrome C (mol. wt. 12,400) showing that for these proteins (all of which are assumed to be globular in shape) the peak elution volume is directly proportional to the molecular weight.

Fig. 29 shows the elution pattern obtained on applying 2 ml. of a purified low molecular weight adenosine deaminase solution (10 µg/ml.) to the Sephadex G-75 column. Enzyme activity in the eluate fractions was measured spectrophotometrically at 265 nm. A value of 32,000 was obtained for the molecular weight of this form of the enzyme.

THE STOKES RADIUS OF THE LOW MOLECUALR WEIGHT FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE

The peak elution volumes of globular proteins after elution from a Sephadex column are also directly related to the Stokes radii of the molecules (Andrews, 1970). Fig. 30 shows a plot of the Stokes radii of the pure proteins used to standardize the Sephadex G-75 column against their respective peak elution volumes. A value of 24.8 Å was obtained for the Stokes radius of the low molecular weight form of the deaminase.

DETERMINATION OF THE MOLECULAR WEIGHT OF THE PURIFIED HIGH MOLECULAR WEIGHT FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE.

Since the high molecular weight form of the deaminase eluted at the void volume of the Sephadex G-75 column, a gel of higher porosity was used to determine the molecular weight of this form. A Sephadex G-200 Superfine column (45 cm. x 2.5 cm.) was prepared as described in the Methods section and equilibrated with 0.002M sodium citrate pH 5.8 incorporating 0.1 M KCl. Table 8 lists the pure proteins used to standardize the column.

TABLE 8. Purified Proteins used in the Calibration of the Sephadex G-200 Column.

Protein	Mol. wt.	* Quantity applied	Wavelength used to estimate
Thyroglobulin	670,000	10 mg.	280 nm.
γ-Globulin	160,000	10 mg.	280 nm.
Pepsin	35,000	6 mg.	280 nm.
Myoglobin	17,800	6 mg.	407 nm.

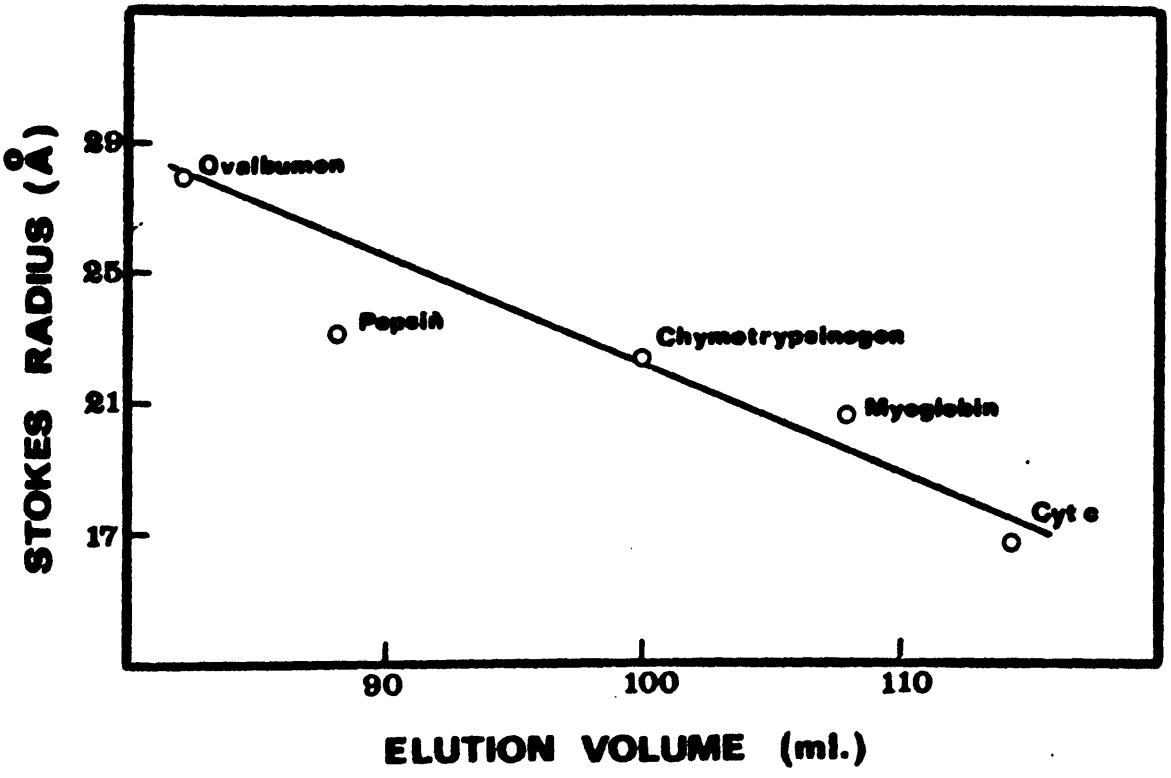


FIG. 30. Standard graph illustrating the relationship between the Stokes radii of a number of pure proteins and their respective peak elution volumes after elution from a column of Sephadex G-75.

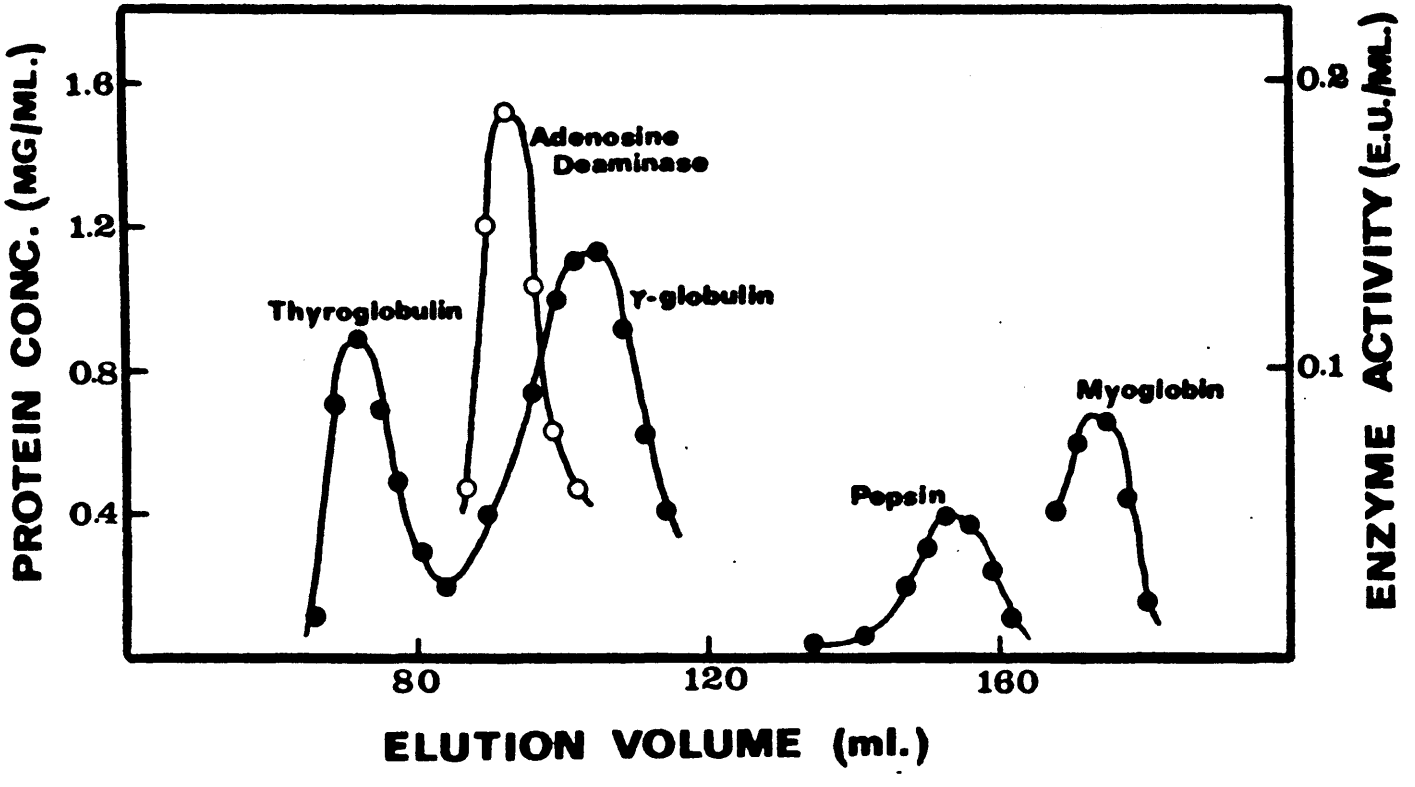


FIG. 31. Elution pattern of a number of pure proteins of known molecular weight from a Sephadex G-200 (Superfine) column equilibrated with 0.002 M sodium citrate pH 5.8 containing 0.1 M KCl 3 ml. of each protein was applied to the column and they were detected by ultraviolet absorption at the appropriate wavelength. 3 ml. of a 0.2 mg./ml. solution of purified high molecular weight rabbit deaminase was also applied and activity estimated spectrophotometrically at 265 nm (pH 7.0).

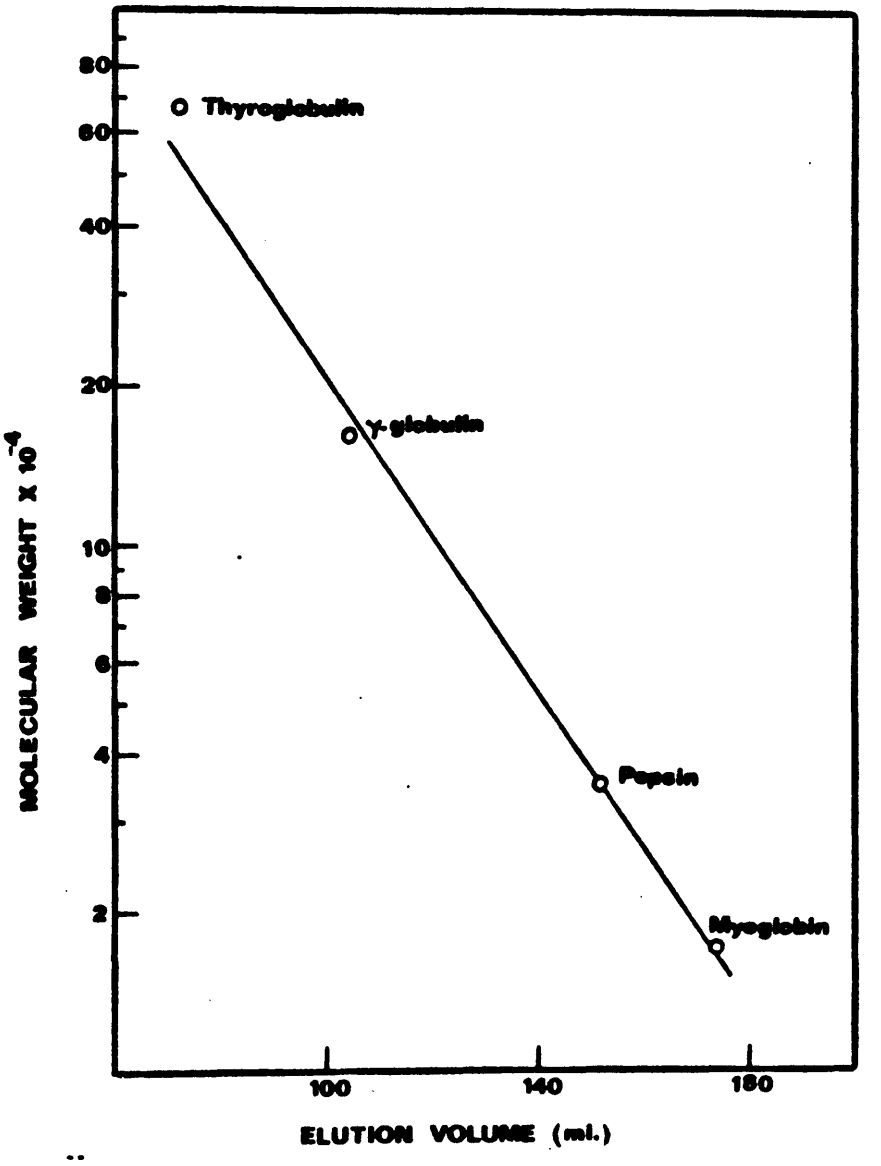


FIG. 32. Standard graph for molecular weight determination on Sephadex G-200 (Superfine). Pure proteins (3 ml. volumes) were applied to a Sephadex G-200 column equilibrated with 0.002 M sodium citrate pH 5.8 containing 0.1 M KCl and the peak elution volume of each measured.

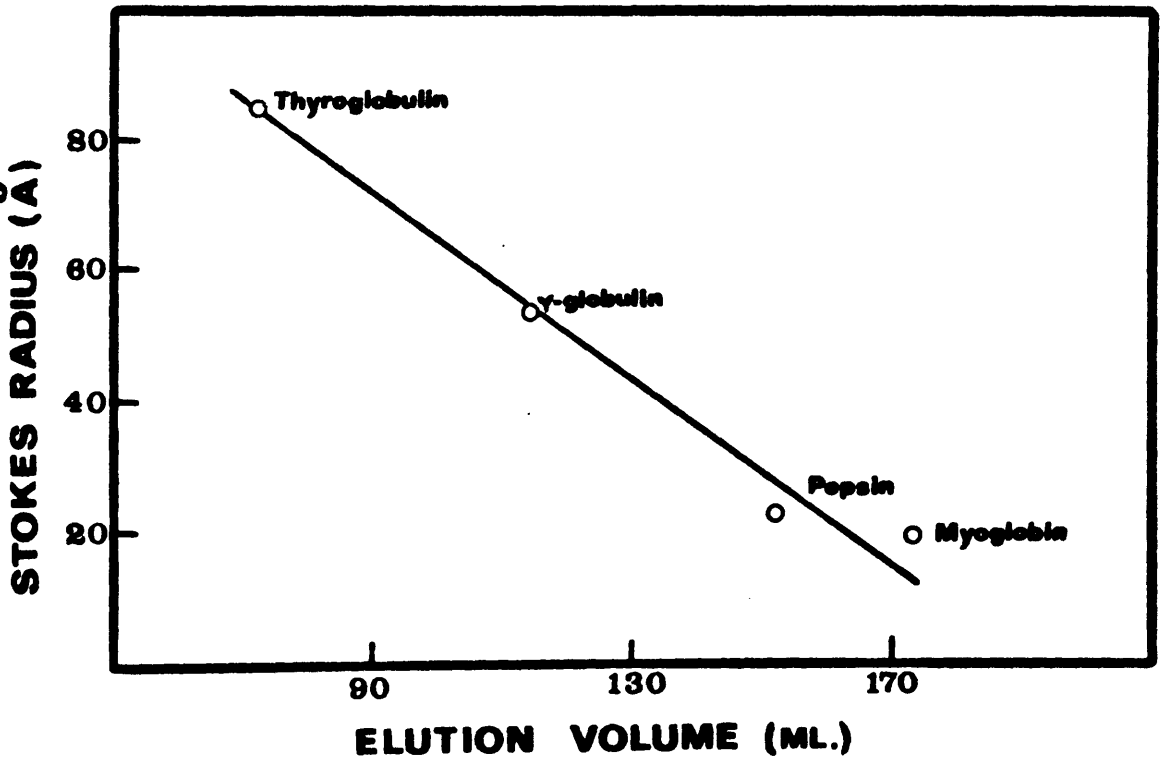


FIG. 33. Standard graph illustrating the relationship between the Stokes radii of a number of pure proteins and their respective peak elution volumes after elution from a column of Sephadex G-200.

* Quantities referred to were dissolved in 3 ml. of eluting buffer.

Fig.31 shows the elution pattern of the pure proteins listed in Table 8 and also shows the elution pattern of the high molecular weight form of the deaminase (3 ml. of a 0.2 mg./ml. solution was applied and assays performed spectrophotometrically at 265 nm).

Fig. 32 shows the relationship which exists between the molecular weights of these proteins and their respective peak elution volumes. A value of 265,000 was obtained for the molecular weight of the high molecular weight form of the enzyme.

The values obtained for the molecular weights of the purified forms of the deaminase show good agreement with the molecular weights obtained using crude intestinal extracts. This indicates that there was no apparent change in molecular weight during the purification of the enzymes and also illustrates the feasibility of molecular weight determinations using crude tissue extracts.

DETERMINATION OF THE STOKES RADIUS OF THE PURIFIED HIGH MOLECULAR WEIGHT FORM OF THE DEAMINASE

Fig. 33 shows a plot of the Stokes radii of the pure proteins applied to the Sephadex G-200 column and their respective peak elution volumes. A value of 69 Å was obtained for the Stokes radius of the high molecular weight form of the enzyme.

ABSORPTION SPECTRA OF THE PURIFIED HIGH AND LOW MOLECULAR WEIGHT FORMS OF ADENOSINE DEAMINASE

The absorption spectra were obtained by scanning 3 ml. solutions of the purified forms of the deaminase between 200 nm and 400 nm in a Sp 800 Unicam spectrophotometer (1 cm. cuvettes were used).

The patterns obtained are shown in Fig. 34. It is evident that both forms exhibit typical protein spectra with maxima at 280 nm and minima at 253 nm. The absorabancy ratios (A_{280}/A_{260}) for the high and low molecular weight forms were 1.3 and 2.0 respectively.



THE EXISTANCE OF MULTIPLE ELECTROPHORETIC FORMS

It has already been shown that the high and low molecular weight forms of rabbit intestinal adenosine deaminase each appear as two active protein bands when subjected to electrophoresis on cellulose acetate paper and polyacrylamide gels. Each molecular weight form would therefore appear to consist of two components differing in charge. Adenosine deaminases from a number of other species such as the mouse and the cat also gave a two-isoenzyme pattern after electrophoresis (Brady and O'Donovan, 1965). In the bovine species however, five and occasionally six isoenzymes can be detected (Murphy et al., 1969a). For the purpose of identification, these have been numbered 1 to 6, the isoenzyme showing greatest anodal migration being No. 1 and the one showing least migration No. 6. In Plate 8 the isoenzyme pattern of the low molecular weight rabbit adenosine deaminase is compared with the isoenzymes of purified bovine lung and spleen deaminases. (These were

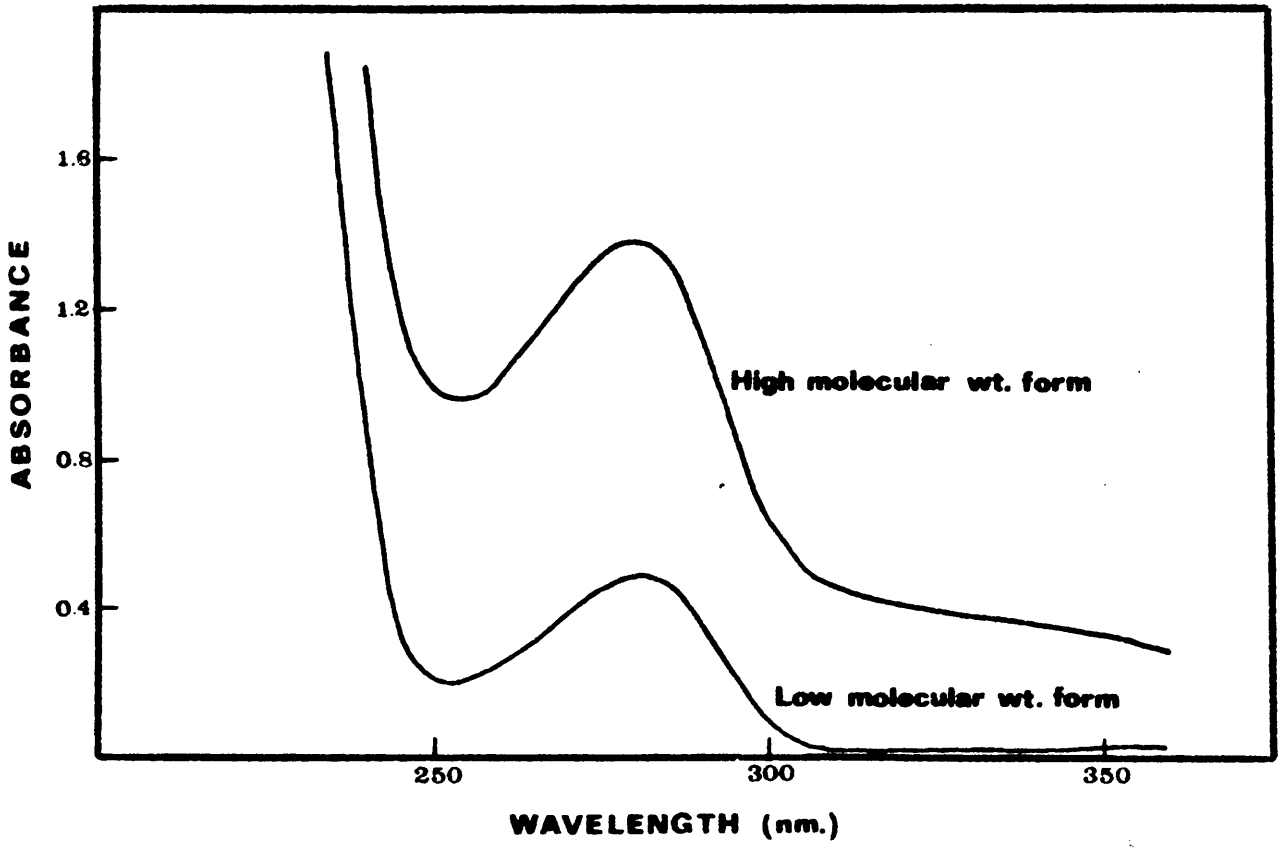


FIG. 34. Ultraviolet absorption spectra of the purified high and low molecular weight forms of rabbit intestinal adenosine deamonase.

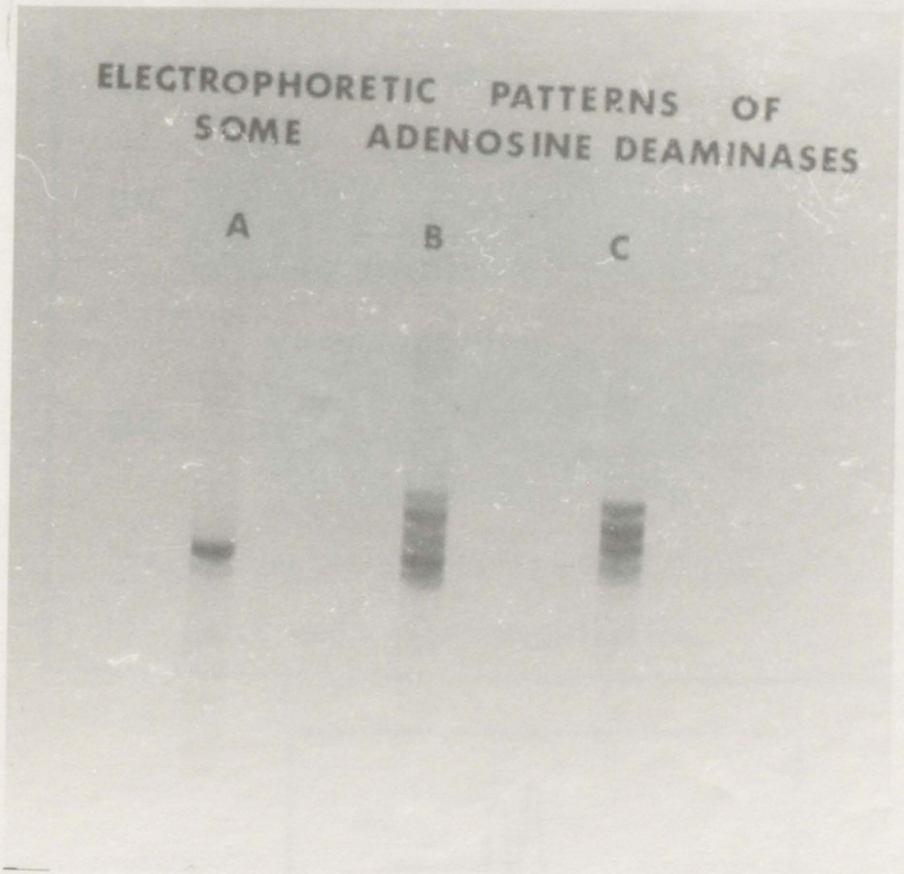
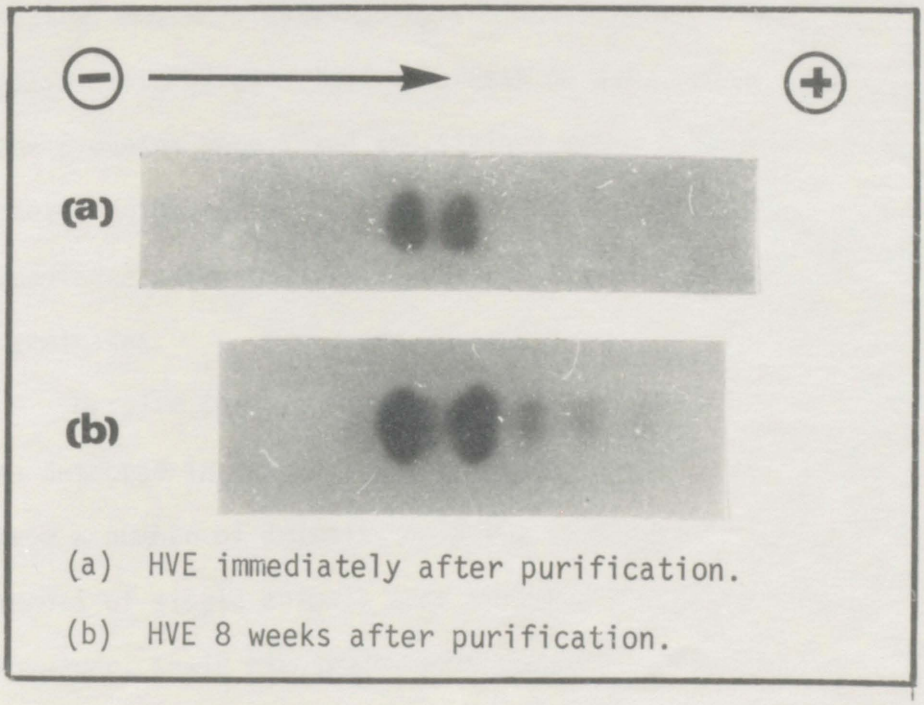


PLATE 8. POLYACRYLAMIDE GEL ELECTROPHORETIC PATTERNS OF SOME PURIFIED ADENOSINE DEAMINASES.

A. LOW MOL. WT. FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE.
B. BOVINE LUNG ADENOSINE DEAMINASE.
C. BOVINE SPLEEN ADENOSINE DEAMINASE.

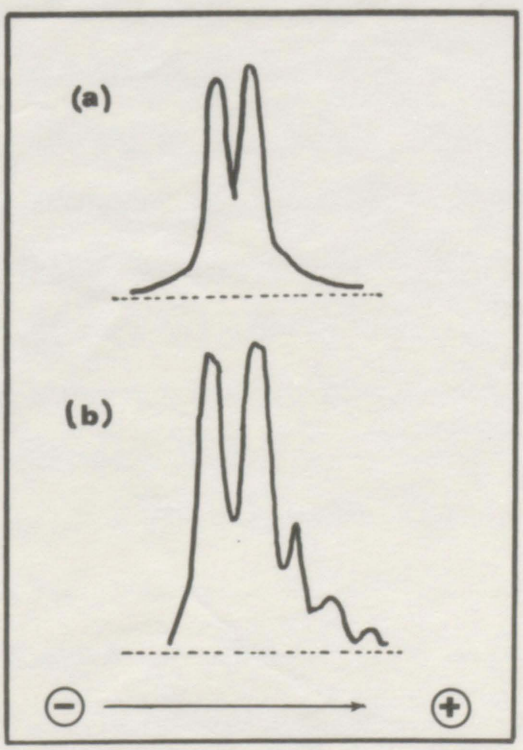
THE GELS WERE STAINED FOR PROTEIN USING 1% AMIDO BLACK IN 7% ACETIC ACID.

PLATE 9. SEPARATION OF THE ISOENZYMES OF THE LOW MOL. WT. FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE USING HIGH VOLTAGE ELECTROPHORESIS (HVE) ON CELLULOSE ACETATE. THE ZYMOGRAM (PHENOL VIOLET) STAIN FOR ADENOSINE DEAMINASE WAS USED.



(a) HVE immediately after purification.

(b) HVE 8 weeks after purification.



DENSITOMETER TRACINGS OF THE ZYMOGRAMS SHOWN ABOVE.

purified by the method of Noonan, 1969). The electrophoretic separations were obtained by subjecting the purified enzymes to polyacrylamide gel electrophoresis pH 8.1. After electrophoresis the proteins were fixed and stained using 0.1% Amido Black in 7% acetic acid. It may be observed that the isoenzymes of the rabbit deaminase correspond with isoenzymes 3 and 2 of the bovine adenosine deaminases.

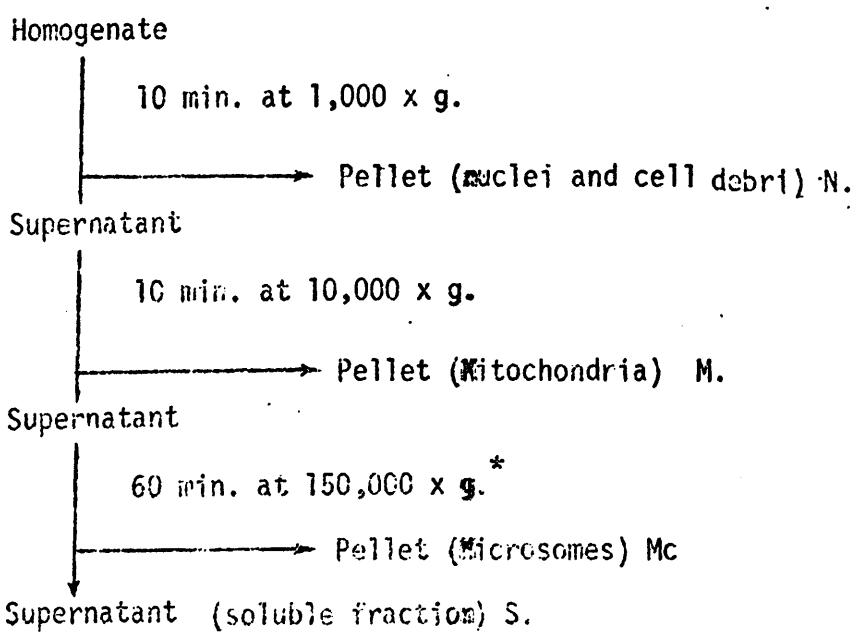
Despite the fact that in the bovine species six isoenzymes can be detected in extracts prepared from mixed intestinal mucosa, derived from a number of animals, only two isoenzymes were present when the mucosa of single animals were examined immediately after death. Moreover, their electrophoretic position varied from animal to animal. It has been observed with these single mucosa that storage at $4^{o}C$ or at $37^{o}C$ leads to an increase in the number of multiple forms from two to four or more. (Murphy et al., 1969a). The possibility that the rabbit intestinal adenosine deaminase might behave in a similar manner was investigated by storing a sample of partially purified low molecular weight form (sp. activity, 80 e.u.) at $4^{o}C$ for several months. Plate 9, shows the electrophoretic patterns obtained using the high voltage cellulose acetate technique before and after storage. On staining for enzyme activity (Methods section p. 33) the number of isoenzymes had increased from two to five. The possibility that these "new" isoenzymes might correspond to the high molecular weight form of the deaminase is ruled out by the fact that the latter enzyme has a slower electrophoretic mobility than the low molecular weight form whereas the isoenzymes produced on storage had a greater electrophoretic mobility.

CELL FRACTIONATION STUDIES

Although the intracellular location of adenosine deaminase has been studied in a number of mammalian tissues such as brain (Jordan et al., 1959) and liver (Stern and Mirsky, 1953), its subcellular distribution in the intestine appears to have received little attention. In the following experiment the location of the enzyme in rabbit intestine was investigated.

Experimental Procedure:

The classical differential centrifugation technique of de Duve (1964) was used. A 0.25M sucrose homogenate of rabbit intestine was obtained by homogenising 20 g. of tissue with 100 ml. of 0.25 M sucrose. (All operations were carried out at 4°C). The homogenate, after being allowed to stand for 30 min., was then centrifugally separated (using a Sorvall Superspeed RC2-B Refrigerated Centrifuge) into various fractions by the following procedure:



* This fraction was obtained using an MSE Superspeed 40 Refrigerated centrifuge.

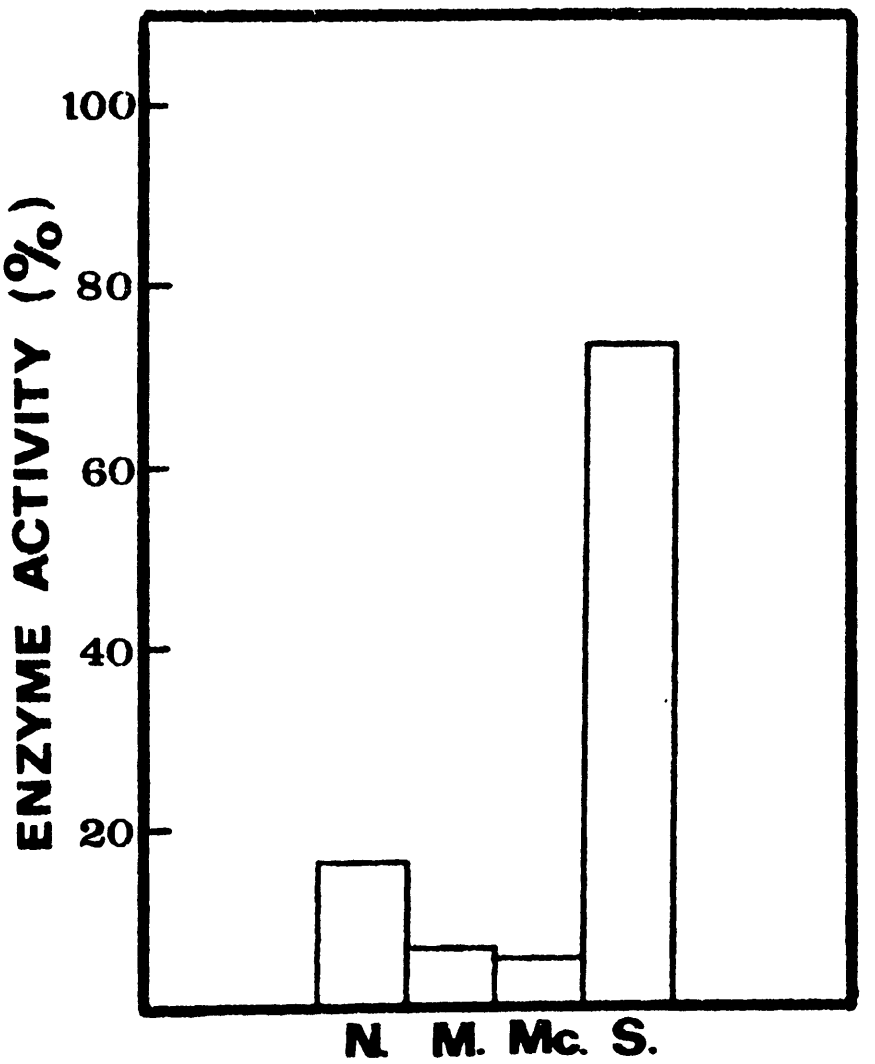


FIG. 35. Differential centrifugation of a 0.25 M sucrose homogenate of rabbit intestine. The following fractions are plotted in abscissa: N.(nuclei and cell debri), M. (mitochondria), Mc. (Microsomes) and S. (soluble fraction).

This method yielded four fractions: (N) cell debri and nuclear fraction, (M) mitochondrial fraction, (Mc) microsomal fraction, and (S) soluble fraction. The pellets were resuspended in appropriate volumes of 0.1M phosphate buffer pH 7.0 incorporating 1% Triton x-100, and allowed to stand at $4^{o}C$ overnight. (The Triton X-100 was added to rupture the various membrane structures). The resuspended pellets were then centrifuged at 16,000 r.p.m. for 30 min. to remove insoluble material, and assayed spectrophotometrically at pH 7.0 for adenosine deaminase activity. The results obtained are illustrated in Fig.35. It can be seen that 73% of the recovered activity was in the soluble fraction and 16% in cell debri and nuclear fraction. The high level of activity in the latter fraction probably represents soluble adenosine deaminase as the pellet obtained at the first centrifugation step did not clearly separate from the supernatant.

Unfortunately, the intestinal tissue used in the experiment did not possess any high molecular weight form of the deaminase. This was observed when samples of the various fractions were applied to a Sephadex G-75 coated thin-layer plate, and adenosine deaminase activity located after chromatography. However earlier results(p have indicated that the high and low molecular weight forms cannot be differentially extracted from the intestine, indicating that both forms are present in the same cellular fraction.

IMMUNOCHEMICAL CHARACTERIZATION OF THE HIGH AND LOW MOLECUALR WEIGHT FORMS OF THE DEAMINASE

Rabbits are currently being used in this laboratory to obtain antiserum against purified bovine intestinal adenosine deaminase and the serum is being used in a comparative study of mammalian adenosine deaminases purified from a variety of sources. In the following experiment the effect of the antiserum on the high and low molecular weight forms of rabbit intestinal adenosine was examined.

Experimental conditions:

0.4 ml. of the antiserum (obtained from a New Zealand White variety of rabbit) was incubated at 37^{o}C with 0.4 ml. of the high and low molecular weight forms and with 0.4 ml. of the bovine intestinal deaminase. The enzymes were suitably diluted (using 0.1M phosphate pH 7.0) to give a reasonable fall in absorbance on assay. Controls were also included substituting 0.1 M phosphate pH 7.0 for the enzyme solutions. After various time intervals 0.1 ml. aliquots were withdrawn and the deaminase activity measured spectrophotometrically at pH 7.0. The results obtained are illustrated in Fig. 36. It is evident that the serum caused inhibition of the bovine intestinal deaminase as expected and also inhibited the high and low molecular weight forms of the rabbit adenosine deaminase. The high and low molecular weight forms used in this experiment were purified from the wild variety of rabbit whereas the antiserum was obtained from the domestic variety (New Zealand White Rabbit) The reaction between the antiserum and the rabbit deaminases would there-

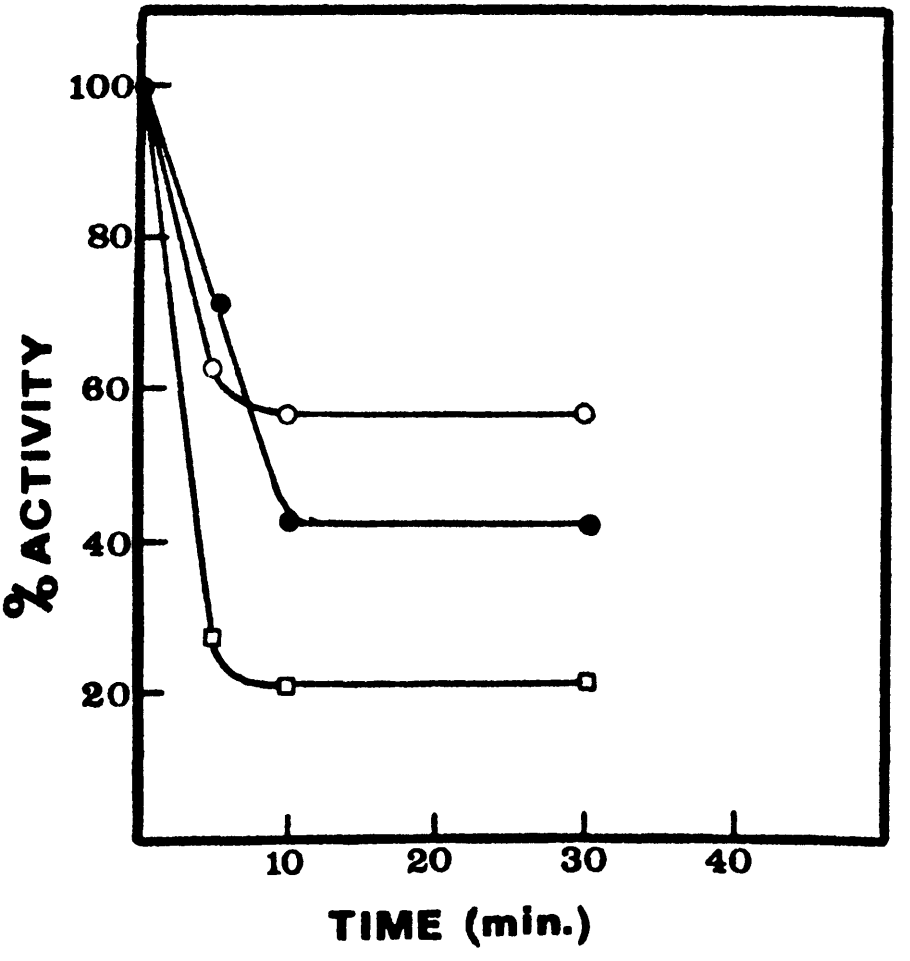
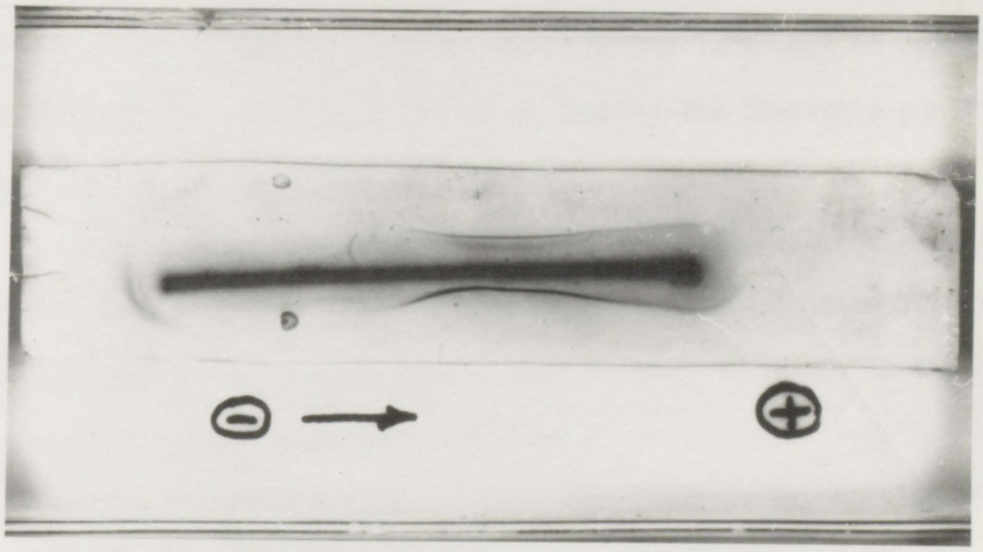


FIG. 36. The effect of anti-serum (rabbit) on the activity of bovine intestinal adenosine deaminase (□——□) and the low molecular weight (○——○) and the high molecular weight (●——●) forms of rabbit intestinal adenosine deaminase.



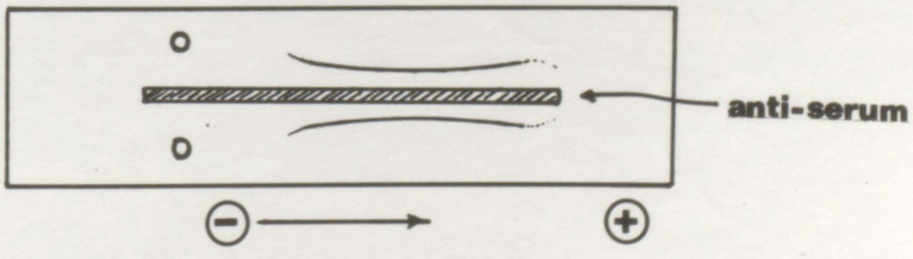


PLATE 10. Immunoelectrophoretic pattern of bovine intestinal adenosine deaminase. Electrophoresis was performed on cellulose acetate paper at 200 V for 35 min. using 20 mM phosphate buffer pH 7.0. After electrophoresis the anti-serum was allowed to diffuse for 24 h. and staining carried out using the Nigrosine stain for protein.

fore appear to be due to a difference in the variety of the rabbits used. This assumption is also based on the fact that in repeat experiments the antiserum failed to inhibit the deaminase purified from the domestic variety of rabbit.

The above experiment demonstrates, however, that the high and low molecular weight forms of the rabbit intestinal adenosine are in some way structurally related both to each other and to the bovine intestinal adenosine deaminase.

Plate 10 shows the precipitin lines obtained by reacting the antiserum against the bovine intestinal adenosine deaminase.

THE EFFECT OF FREEZING AND THAWING ON ENZYME ACTIVITY

It has been observed in previous experiments (p.143) that there is little or no loss in enzyme activity when the partially purified rabbit adenosine deaminase was stored in the frozen state. However, it has been found in this laboratory that the bovine intestinal adenosine deaminase looses its activity when subjected to freezing and thawing. In the following experiment the effect of freezing and thawing on both the purified bovine deaminase and the purified low molecular weight rabbit deaminase is studied.

Experimental conditions:

Diluted solutions of both enzymes (in 0.1M phosphate pH 7.0) were kept at -10°C for several days. At various time intervals the enzymes were removed, allowed to thaw, and 0.1 ml. aliquots removed for enzyme assay at pH 7.0. The results are illustrated in Fig. 37. It is evident that the low molecular weight rabbit deaminase is more stable to this treatment than the bovine adenosine

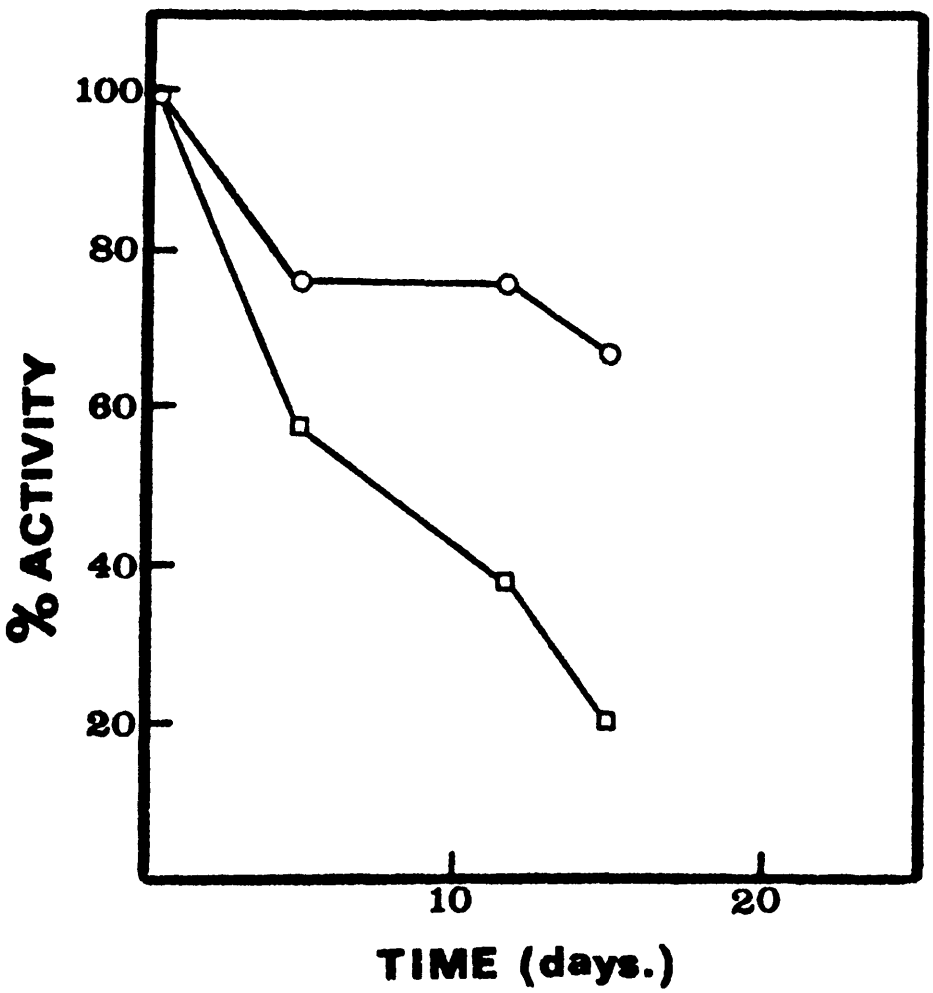


FIG. 37. The effect of freezing and thawing on the activity of the low molecular weight form of rabbit intestinal adenosine deaminase (O——O) and bovine intestinal adenosine deaminase (□——□).

deaminase, the bovine enzyme losing 80% of its activity when frozen and thawed three times, whereas the rabbit enzyme lost only 30% of its activity.

The work described so far involved a comparative study of the general properties of the high and low molecular weight forms of the deaminase. As was shown, differences other than the molecular weight have been detected between them. The question of the nature of the high molecular weight form must now be considered. In general, it seems that proteins consisting of a single polypeptide chain appear to be of low molecular weight, whereas proteins that have been reported to have molecular weights in access of 100,000 have been shown to consist of several subunits. In some instances these subunits may be joined by covalent links, and in others the bonds joining the subunits have been ruptured by means which do not affect covalent links. In Table 9 a selected number of enzymes, which exist in multiple molecular forms, are listed.

TABLE 9. Some Examples of Multichain Enzymes

Enzyme	Mol. wt.	Subunits		Reference
		No. of subunits	Mol wt.	
Lactic dehydrogenase	150,000	4	35,000	Appella, 1961
Threonine deaminase	160,000	4	40,000	Whiteley, 1966
Pyruvate kinase	237,000	4	57,200	Morawiecki, 1960
Catalase	250,000	4	60,000	Schroeder, 1954
Adenosine triphosphatase	284,000	10	26,000	Penefsky, 1965

The fact that an enzyme may possess a very high molecular weight does not necessarily mean that it consists of a number of subunits having the same molecular weight as in the case of the enzymes listed above. Other possibilities must also be considered since many enzymes

have been shown to exist in the form of multienzyme complexes with different activities (e.g. the fatty acid synthetase complex) and some enzymes are known to associate with non-enzymatic cellular material, thus showing an apparent high molecular weight.

The possibility that the high molecular weight form of rabbit intestinal adenosine deaminase might be some type of multienzyme complex was investigated. The purified enzyme was assayed (as described in the Methods section) for the following enzymatic activities:

Adenosine monophosphate deaminase
Alkaline phosphatase
Acid phosphatase
5'-nucleotidase
Xanthine oxidase

The results of the assays showed, however, that none of the above activities were associated with the high molecular weight form.

The possibility also exists that this high molecular weight enzyme might be a type of adenosine deaminase similar to the non-specific Takadiastase deaminase isolated by Minato (1968). The Takadiastase enzyme has been shown to deaminate a variety of natural substrates including adenosine and as already mentioned, it has a molecular weight in the region of that of the high molecular weight adenosine deaminase being studied in the present investigation. The following compounds were therefore tested as substrates of the enzyme:

Adenosine triphosphate
Adenosine diphosphate
Adenosine monophosphate
Cyclic AMP
Nicotinamide adenine dinucleotide

0.1 ml of the purified high molecular weight form was added to 3 ml. of each substrate (0.1 mM in 0.1 M phosphate pH 7.0) and the fall in absorbance at 265 nm measured spectrophotometrically at 37°C. The results showed that deamination of the above substrates did not occur indicating that the high molecular weight enzyme does not resemble the Takadiastase adenosine deaminase in its specificity towards substrates.

Another possibility which was considered was that the high molecular weight form consisted of the low molecular weight enzyme bound or adsorbed on to some non-enzymatic cellular material such as nucleic acid, carbohydrate or protein. This possibility was investigated by analysing the purified enzyme for these components.

Test for Nucleic Acid: DNA was estimated by the following methods:

a) Indole Test (Ceroitti, 1952)

b) Diphenylamine Reaction (Burton, 1956.)

a) 0.4 ml. of purified high molecular weight enzyme was incubated with 0.2 ml. of 0.04% indole and 0.2 ml. of concentrated HCl in a boiling water bath for ten minutes. The solution was then cooled and its absorbance at 490 nm measured against a water blank treated in an identical manner.

b) 0.4 ml. of purified enzyme was added to 0.4 ml. of diphenylamine (1.5% (w/v) in acetic acid incorporating 1.5 ml. of conc. H_2SO_4), and the mixture incubated overnight at 30°C. The optical density at 600 nm was then measured against a blank treated in the same manner.

The results of the tests showed that there was no increase in optical density thus indicating that there was no DNA in the preparation.

RNA was estimated using the Orcinol reaction by the method of Hatcher and Goldstein (1969). 1 ml. of purified high molecular weight enzyme (containing 0.3 mg. protein) was treated with $CdCl_2$ so that the final concentration of reagent was 1M. The solution was then incubated at 40^0C for 5 min., centrifuged at 3,000 r.p.m. for 8 min. and the supernatant fluid discarded. To the precipitate was added 1 ml. orcinol reagent (2 g. resorcinol in 35 ml. ethanol made up to 50 ml. with butanol), and 3 ml. $FeCl_3$ solution (0.5 g. $FeCl_3$ in 100 ml. conc. HCl). The mixture was allowed to stand at 90^0C for 60 min., cooled and its absorbance at 660 nm measured against a water blank treated in a similar manner.

The analytical tube had an absorbance of zero which indicated that there was no RNA in the high molecular weight enzyme preparation.

Test for Carbohydrate:

The copper sulphate-phosphomolybdate test (French et al, 1960) was used to determine if any polysaccharides were present in the high molecular weight enzyme preparation. Two working solutions were prepared:

Solution (A) consisted of 40 g. Na_2CO_3 (Anhyd.) + 20 g. $NaHCO_3$ + 25 g. Rochelle salt + 7.5 g. $CuSO_4 \cdot 5H_2O$ + H_2O to 1 litre.

Solution (B) was prepared by dissolving 150 g. molybdic acid + 75 g. Na_2CO_3 (anhyd.) in 500 ml. H_2O; heat and filter; add 300 ml. 85% H_3PO_4 + H_2O to 1 litre.

A sample of purified high molecular weight enzyme was spotted on to a piece of filter paper which was sprayed with solution (A). The paper was heated at 105^{o}C for 5 min. and then sprayed with solution (B). The appearance of a blue spot on a white background indicates the presence of a polysaccharide.

This was not observed in the above test indicating the absence of polysaccharide material from the high molecular weight enzyme preparation.

THE EFFECT OF VARIOUS REAGENTS WHICH MIGHT INDUCE DISSOCIATION OF THE HIGH MOLECULAR WEIGHT FORM OF THE ENZYME

It is evident from the study of the properties of the high and low molecular weight forms of the deaminase that there are a number of similarities between them. This suggests that the two forms may embody a common protein core with an identical active centre(s) or the high molecular weight enzyme could probably be a polymeric form of the low molecular weight adenosine deaminase. In the following experiments various reagents were incubated with the purified high molecular weight enzyme in an attempt to cause conversion to the low molecular weight form.

Initially reagents were used which do not have protein denaturing properties in the hope that the dissociation products, if obtained, would have enzymatic activity. In the first experiment the following compounds (prepared in 0.1M phosphate pH 7.0) were used:

25 mM Mercaptoethanol
25 mM Dithiothreitol
1 M $CaCl_2$
30% Sucrose

(These were the final concentrations after addition to the enzyme).

0.05 ml. of purified high molecular weight deaminase was incubated with 0.05 ml. of the reagents and allowed to stand overnight at room temperature. Samples were then applied to a previously prepared Sephadex G-75 coated thin-layer plate and after chromatography the gel was stained for deaminase activity. The patterns obtained are illustrated on Plate 11. It is evident that mercaptoethanol, dithiothreitol or $CaCl_2$ did not bring about dissociation but a slight conversion was observed in the sample treated with 30% sucrose. Mercaptoethanol and dithiothreitol are both known to effect inter-molecular disulphide bridges so that the high molecular weight enzyme would not appear to be stabilized by this type of bonding. $CaCl_2$ was used following reports that 1M solutions of this reagent dissociates nucleic acid material from basic proteins (Yamazaki and Kaesberg, 1963; Mohberg and Rousch, 1969). The fact that dissociation was not observed is a further indication that nucleic acid is not associated with the high molecular weight enzyme. The effect of the sucrose is somewhat unusual since this reagent has not been known to dissociate protein structures. However, Ma and Fisher (1969) have reported that sucrose also dissociated the high molecular weight adenosine deaminase found in bovine liver, into a low molecular weight form.

In another experiment the effect of the following reagents (prepared in 0.1M phosphate pH 7.0 was investigated):

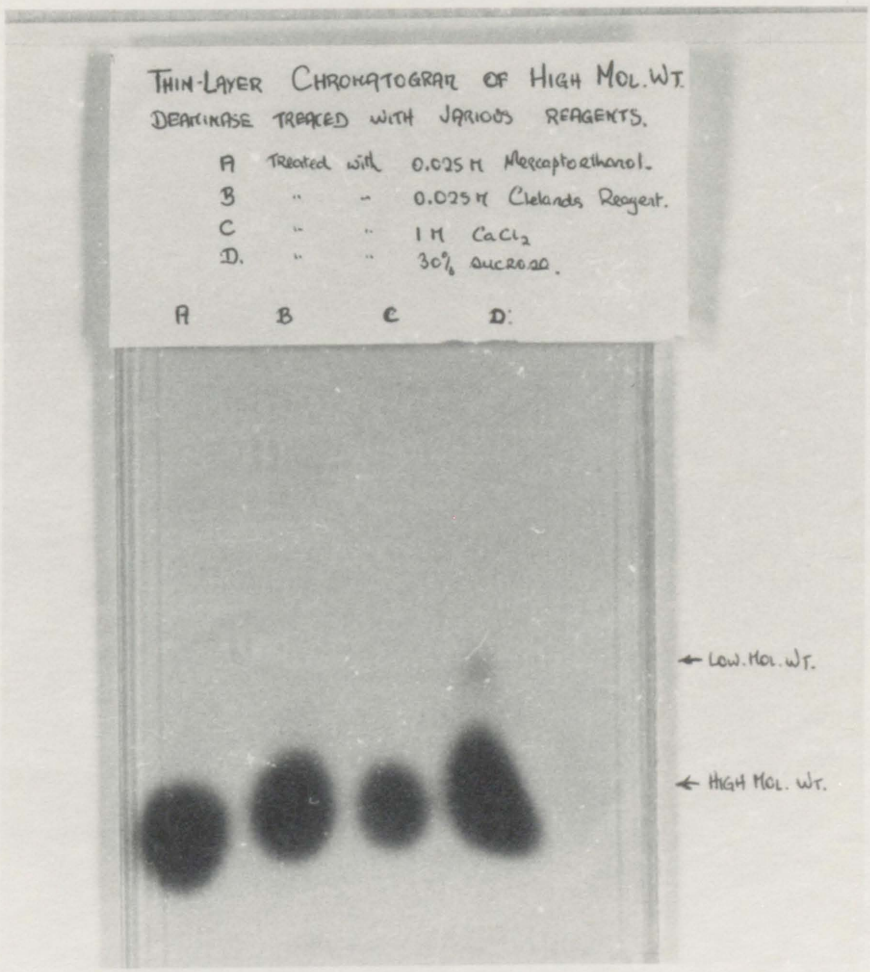


PLATE 11. THIN-LAYER GEL FILTRATION PATTERNS OF THE HIGH MOL. WT. FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE AFTER TREATMENT WITH VARIOUS REAGENTS. THE ZYMOGRAM (PHENOL VIOLET) STAIN FOR ADENOSINE DEAMINASE WAS USED.

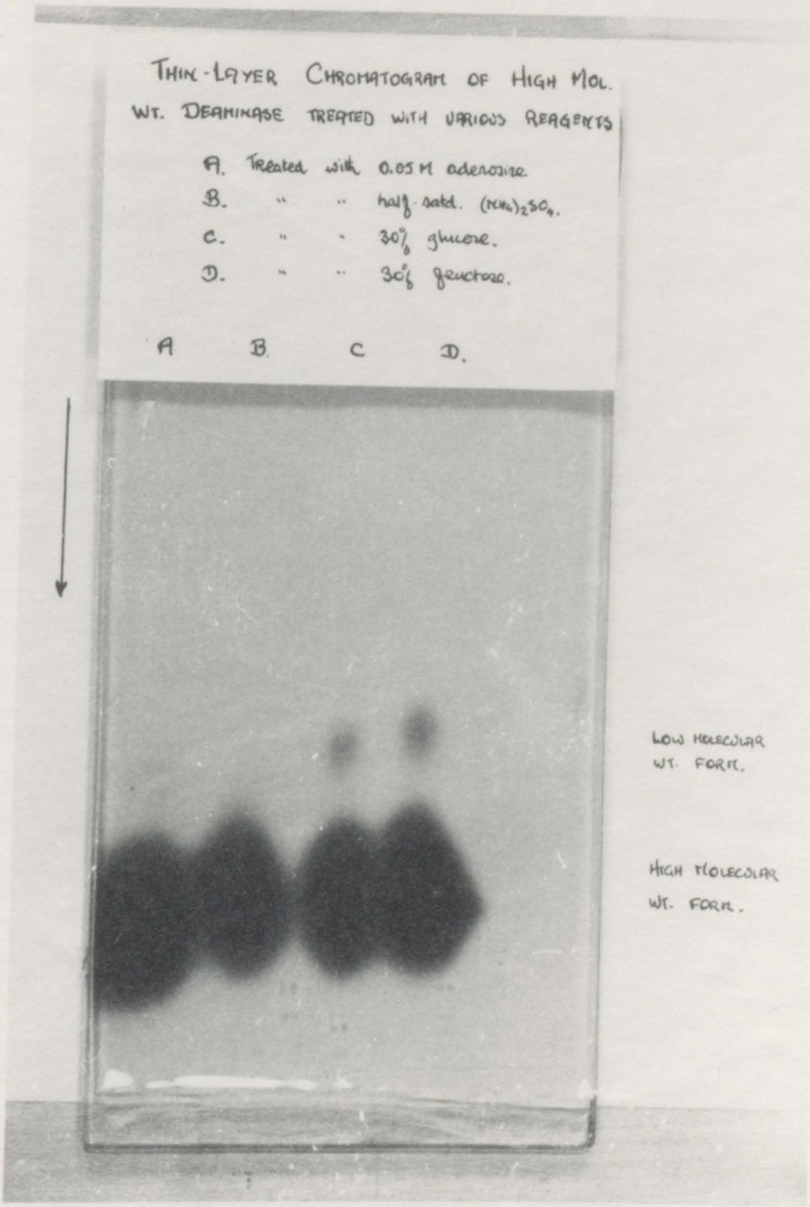


PLATE 12. THIN-LAYER GEL FILTRATION PATTERNS OF THE HIGH MOL. WT. FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE AFTER TREATMENT WITH VARIOUS REAGENTS. THE ZYMOGRAM (PHENOL VIOLET) STAIN FOR ADENOSINE DEAMINASE WAS USED.

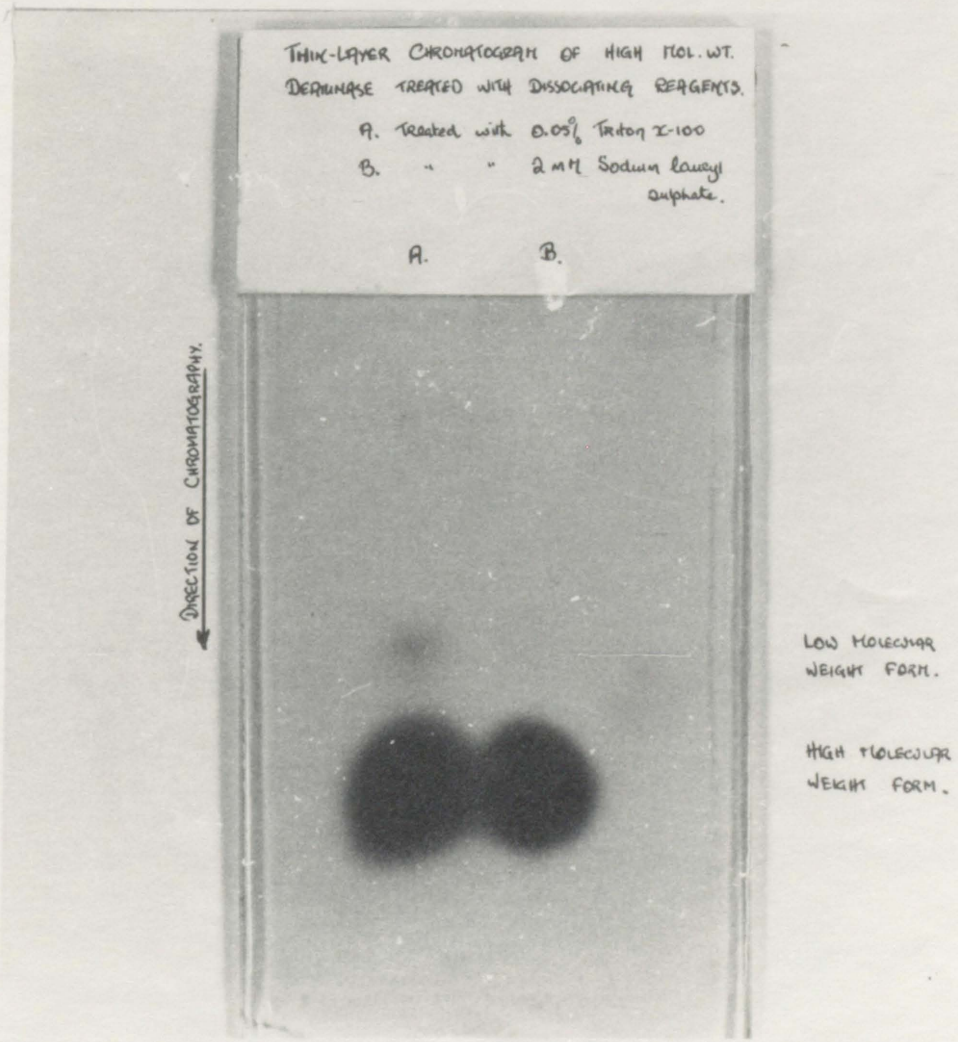


PLATE 13. THIN-LAYER GEL FILTRATION PATTERNS OF THE HIGH MOL. WT. FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE AFTER TREATMENT WITH DISSOCIATING AGENTS. THE ZYMOGRAM (PHENOL VIOLET) STAIN FOR ADENOSINE DEAMINASE WAS USED.

50 mM Adenosine
50% $(NH_4)_2SO_4$
30% Glucose
30% Fructose
0.05% Triton-x-100
2mM Sodium lauryl sulphate (SDS)

(These were the final concentrations after addition to the enzyme).

0.05 ml. of each reagent was incubated with 0.05 ml. of purified high molecular weight enzyme and the solutions allowed to stand overnight at room temperature. The patterns obtained after application to Sephadex G-75 coated thin-layer plates are illustrated in Plates 12 and 13. It can be seen that both glucose and fructose gave some dissociation but the effect was only barely detectable. The adenosine and $(NH_4)_2SO_4$ treated high molecualr weight enzyme samples showed no apparent alteration. Some conversion is also evident in the Triton x-100 treated enzyme. However, on increasing the concentration of the reagent and the incubation time (1% Triton x-100 for 3 days) the pattern obtained was the same. Sodium lauryl sulphate (SDS) at a concentration of 2 mM did not appear to affect the enzyme, but as will be seen later, higher concentrations of this reagent completely inactivated both the high and low molecular weight forms and also caused some dissociation of the high molecular weight enzyme.

ATTEMPTS AT CONVERTING THE LOW MOLECULAR WEIGHT FORM INTO THE HIGH MOLECULAR WEIGHT FORM

It has been observed with some enzymes that their corresponding subunits or low molecular weight components undergo association in

solutions of high protein concentration. For example, beef liver glutamic dehydrogenase is known to undergo a freely reversible, concentration dependant, association-dissociation reaction, the changes in molecular weight having been observed to occur mostly in the range of 0.1 - 2 mg./ml. (Olson and Anfinsen, 1952; Frieden, 1958; Kubo et al., 1959). In the present study the highest concentrations of the low molecular weight form of the adenosine deaminase obtainable were approximately 2 mg/ml. This was due to the fact that only 1-2 mg. of purified enzyme could be obtained from a single purification. However, when these preparations of the low molecular weight form (2 mg./ml.) were subjected to electrophoresis on cellulose acetate paper and in polyacrylamide gels there was no apparent difference in electrophoretic mobility, compared to that obtained with dilute enzyme solutions, indicating that association to the high molecular weight form did not take place at this protein concentration.

An association reaction of a different nature has been reported to take place with the adenosine deaminase (mol. wt. 50,000) purified from human stomach (Akedo et al., 1970). This low molecular weight deaminase was the only type found in the stomach, whereas human lung possesses both high and low molecular weight adenosine deaminases (mol. wt. 200,000 and 50,000 respectively). Akedo reported that the presence of the high molecular weight enzyme in the lung was due to a"converting factor" which was present in the lung and was absent from the stomach. Moreover, incubation of the low molecular weight stomach deaminase with partially purified "converting factor" brought about association to a high molecular weight form.

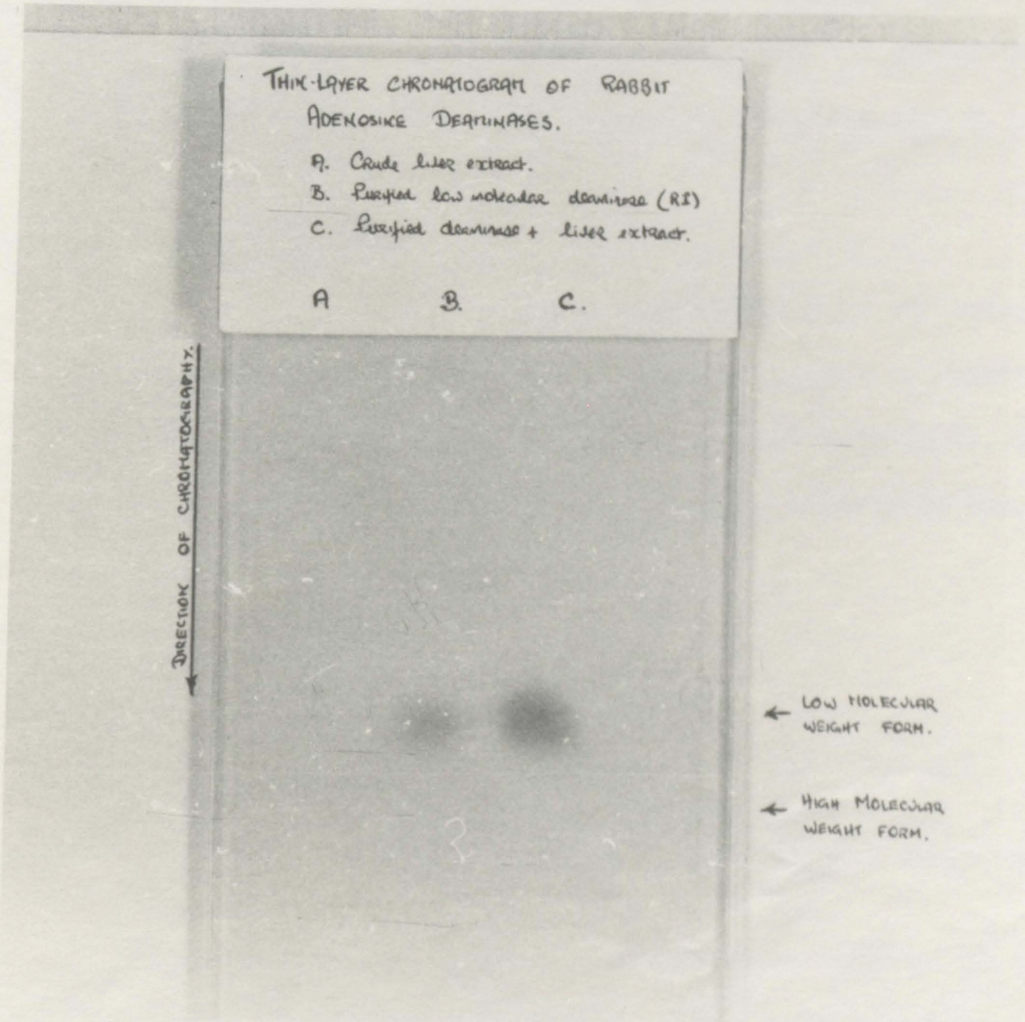


PLATE 14. THIN-LAYER GEL FILTRATION PATTERN OF THE LOW MOL. WT. FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE AFTER INCUBATION WITH A RABBIT LIVER EXTRACT. THE ZYMOGRAM (PHENOL VIOLET) STAIN FOR ADENOSINE DEAMINASE WAS USED.

The possibility that the rabbit adenosine deaminase was undergoing a similar type of association reaction was investigated. A crude extract of rabbit liver was used as a possible source of "converting factor" as this tissue possesses all its adenosine deaminase activity in the high molecular weight form (see p.

Experimental Procedure :

Purified low molecular weight rabbit intestinal adenosine deaminase was used in the study. An aqueous extract of rabbit liver was prepared by homogenising 5 g. of liver with 20 ml. distilled water. Three tubes were set up as follows and allowed to incubate overnight at room temperature :

Tube	Contents
A	0.5 ml. liver extract + 0.5 ml. H_2O
B	0.5 ml. deaminase + 0.5 ml. H_2O
C	0.5 ml. deaminase + 0.5 ml. liver extract

After incubation a sample from each tube was applied to a previously equilibrated Sephadex G-75 coated thin-layer plate. The patterns obtained after staining for enzyme activity are illustrated in Plate 14. As expected no activity could be detected in the sample from Tube A (it has already been mentioned that the level of deaminase activity in rabbit liver is too low to be detected by this method). It is apparent, however, that there was no difference in mobility between the enzymes in Tube B and C , indicating that association to a high molecular weight form had not occurred.

THE EFFECT OF SODIUM SULPHITE ON RABBIT INTESTINAL ADENOSINE DEAMINASE

The effect of sodium sulphite (a disulphide splitting reagent) on the high and low molecular weight forms of the deaminase was investigated in the following experiments. This substance reacts with disulphides according to the following reaction:

$$RSSR + SO_3^{--} \rightleftharpoons RS^- + RSSO_3^-$$

Murphy (1967) found that sodium sulphite inactivated adenosine deaminase from bovine intestine by reacting with the disulphide bonds present in the enzyme. The reaction was reversible at neutral pH values, but at alkaline pHs the equilibrium was far to the right.

In the first experiment the effect of the reagent on the activity of the high and low molecular weight forms was investigated.

Experimental Conditions:

The diluted enzymes in 0.05 M sodium borate buffer pH 9.3 were incubated at 37°C with sodium sulphite (0.05 M, 0.1 M and 0.5 M) for various time intervals. Assays were performed spectrophotometrically at pH 7.0 and the results obtained are graphically illustrated in Fig. 38. It can be seen that inhibition of the deaminases occurred, the rate of loss in activity becoming more rapid as the sodium sulphite concentration was increased. It was also evident that the reagent had a differential effect on the two forms, the high molecular weight form retaining 30% of its activity after 220 min. in 0.5 M sodium sulphite, whereas the low molecular

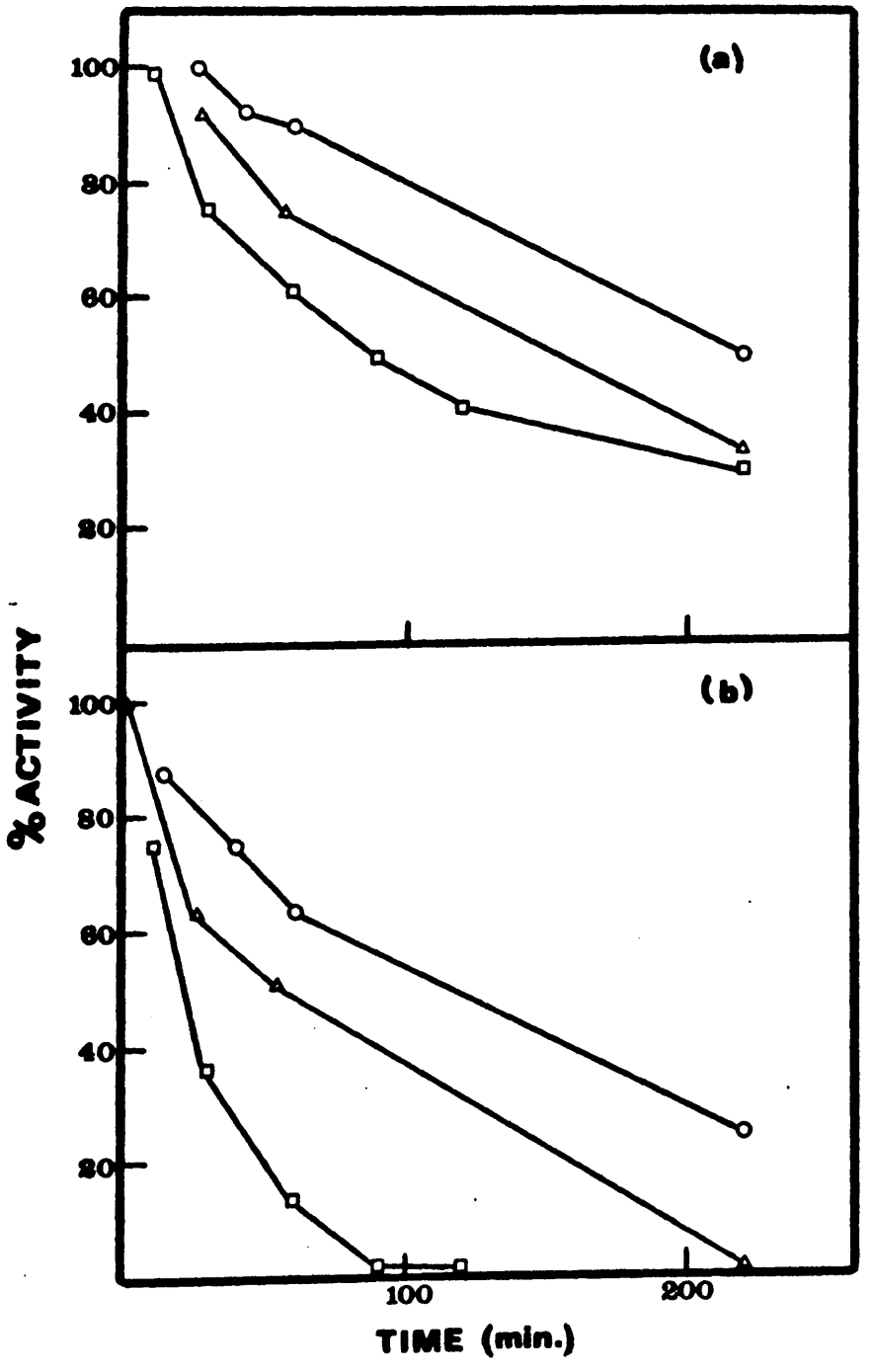
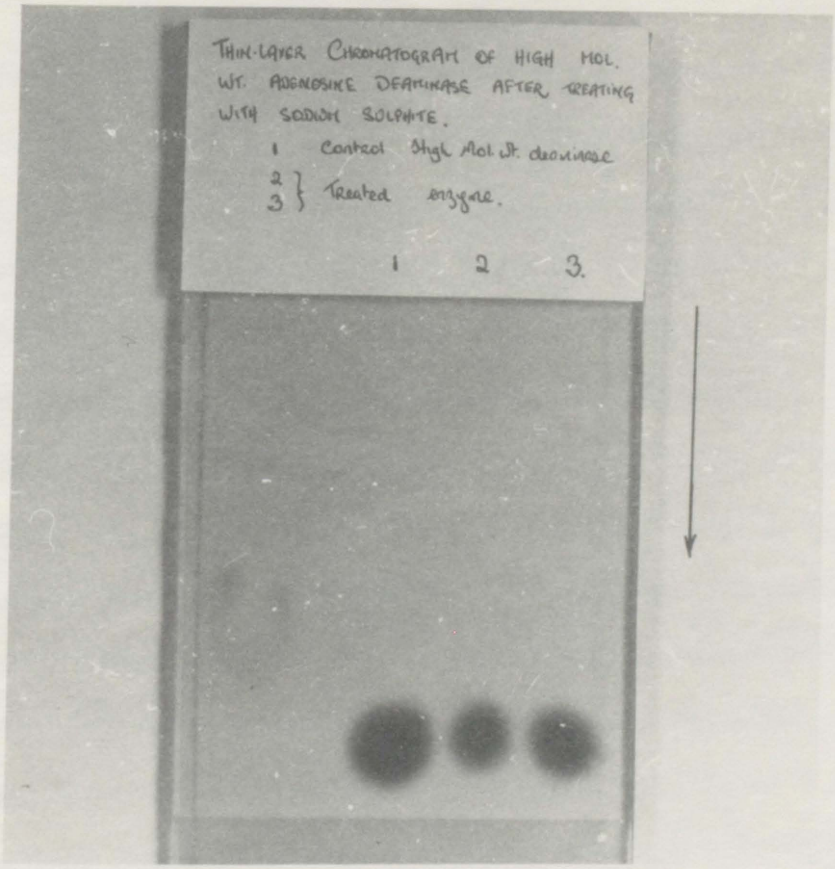
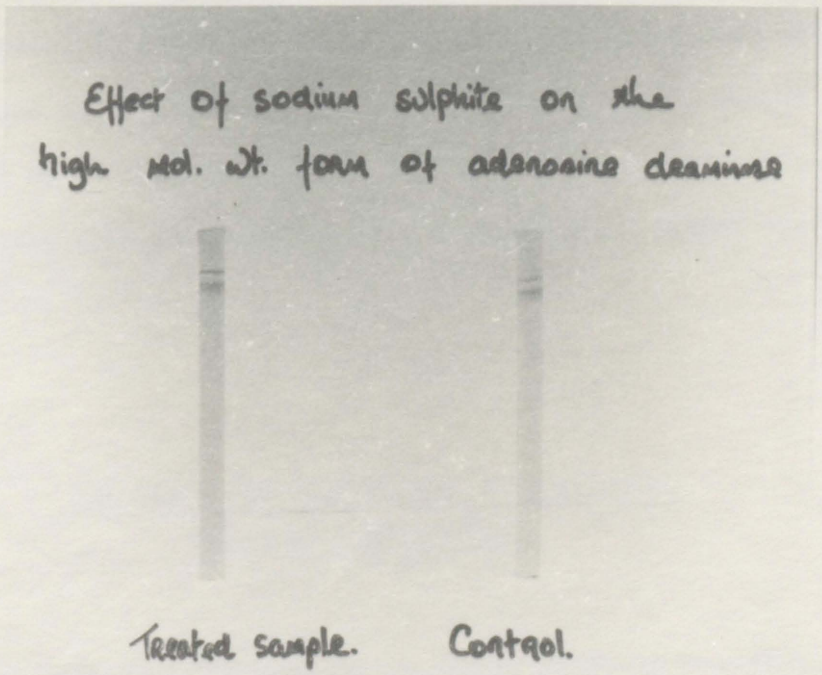


FIG. 38. The effect of sodium sulphite on (a) the high and (b) the low molecular weight forms of rabbit intestinal adenosine deaminase. The concentrations of reagent (in 0.05M borate buffer pH 9.3) used were:
○—○ , 0.05 M; △—△ , 0.1 M and □—□ , 0.5 M.
The enzymes were incubated at 37°C with the reagent and assays performed spectrophotometrically at 265 nm (pH 7.0).

PLATE 15



THE ZYMOGRAM (PHENOL VIOLET) STAIN FOR ADENOSINE DEAMINASE WAS USED.



POLYACRYLAMIDE GEL ELECTROPHEROGRAM. THE GELS WERE STAINED USING 1% AMIDO BLACK IN 7% ACETIC ACID.

weight form was completely inactivated using the same concentration of reagent after only 90 min. The high molecular weight form would therefore appear to be more stable this being possibly due to the fact that the disulphide bridges in the molecule are in some way masked or hindered and may not be exposed to the same extent as those present in the low molecular weight form.

So as to investigate if the reagent was having a dissociating effect on the high molecular weight form, the enzyme (2 mg./ml.) was incubated at 37°C with 0.05 M sodium sulphite (in borate buffer pH 9.3). After 1 h. incubation a sample was withdrawn and applied to a Sephadex G-75 coated thin-layer plate, and the plate stained for enzyme activity after first being developed. After 5 h. incubation another sample was withdrawn and subjected to electrophoresis in polyacrylamide gels at pH 8.1. The gels were stained for protein using 1% Amido Black in 7% acetic acid. The patterns obtained are illustrated in Plate 15. It can be seen that using both techniques there was no apparent change in the mobility of the enzyme compared to that of a control which had not been treated with sodium sulphite indicating that dissociation to a low molecular weight species did not occur.

THE EFFECT OF TEMPERATURE AS A POSSIBLE MEANS OF DISSOCIATION

The effect of temperature on enzyme activity has already been investigated and it was shown that both forms of the deaminase are stable when heated to 60°C for 15 min., but lose all activity when

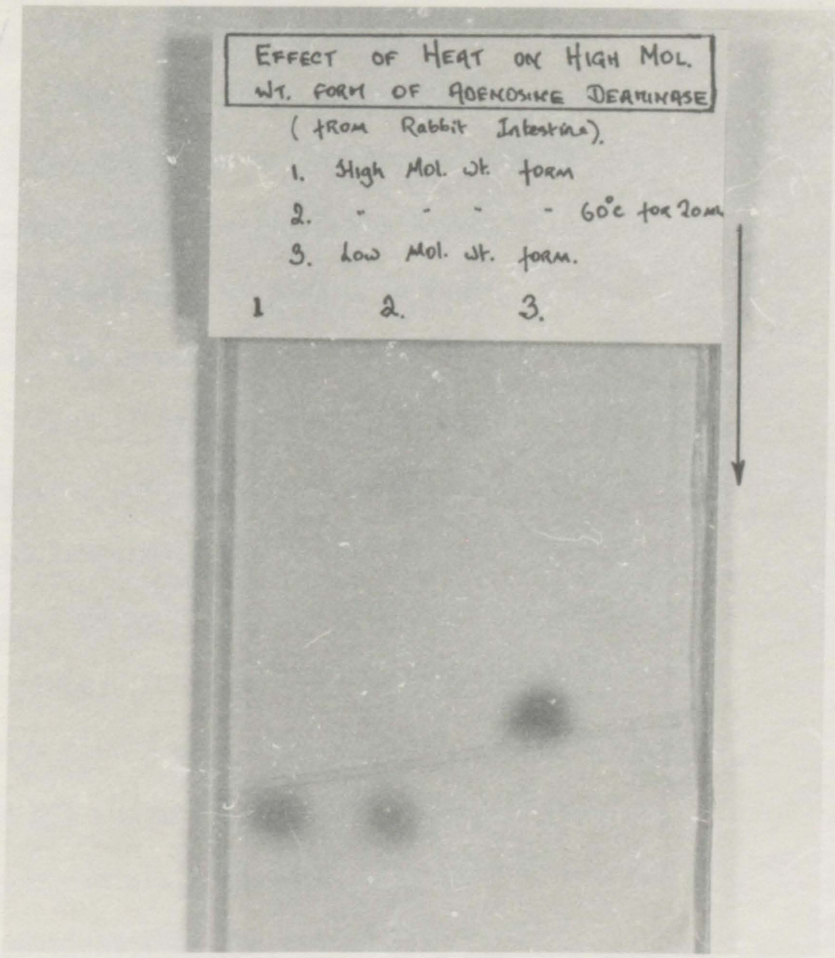


PLATE 16. THIN-LAYER GEL FILTRATION PATTERN OF THE HIGH MOL. WT. FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE AFTER HEATING TO 60°C FOR 20MIN. THE ZYMOGRAM (PHENOL VIOLET) STAIN FOR ADENOSINE DEAMINASE WAS USED.

heated to 70°C. Dissociation of many polymeric enzymes and proteins is facilitated by elevating the temperature. For example, Jolley et al. (1969) have shown that mushroom tyrosinase can be dissociated into a low molecular weight species when a solution of the enzyme is heated to 50°C, and porcine thyroglobulin dissociates on heating to 70°C for 5 min. at pH 9.8 (Reithel, 1963). The following experiment was designed to study the effect of heat on the high molecular weight form of rabbit intestinal adenosine deaminase.

0.1 ml. of an aqueous solution of the high molecular weight form (2.2 mg./ml.) was heated to 60°C for 20 min., and an aliquot then applied to a previously prepared Sephadex G-75 coated thin-layer plate. Purified high and low molecular weight forms which were not heated were also applied. The plate was developed with 0.05 M KCl and was stained for adenosine deaminase activity using the zymogram stain (p.29). The results are illustrated in Plate 16 It is evident that the heated high molecular weight enzyme showed the same pattern as the unheated sample indicating that dissociation did not occur on heating to 60°C for 20 min.

THE EFFECT OF ACID AND ALKALINE pH ON THE HIGH MOLECULAR WEIGHT FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE

Many high molecular weight enzymes have been shown to undergo dissociation when maintained at extremes of pH for a period of time. (Mann and Vestling, 1969; Saha et al., 1969). In the following experiment the high molecular weight form of adenosine deaminase

was subjected to high and low pH values in an effort to bring about dissociation of the enzyme. The buffers used in the experiment were:

0.2 M Na_2HPO_4 - citric acid pH 2.4

0.1 M Na_2CO_3 - $NaHCO_3$ pH 10.6

These pH values are well outside the pH stability range of both the high and low molecular weight forms of the deaminase. 0.1 ml. of an aqueous solution of the purified high molecular weight enzyme was incubated with 0.1 ml. of each buffer for 5 h. at 20°C. The solutions were then dialysed overnight against Tris-glycine electrophoresis buffer pH 8.1.

Following dialysis the samples were subjected to electrophoresis at pH 8.1 in polyacrylamide gels. Untreated high and low molecular weight forms of the deaminase were also analysed and used as controls in the experiment. After electrophoresis the proteins were fixed and stained with 1% Amido Black in 7% acetic acid. The patterns obtained showed that there was no apparent difference in electrophoretic mobility between the treated and untreated high molecular weight enzyme which indicated that dissociation had not taken place. Acid and alkaline pH therefore do not appear to have any effect on the structure of the high molecular weight deaminase.

THE EFFECT OF PROTEOLYTIC ENZYMES ON THE HIGH MOLECULAR WEIGHT FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE

The following investigation was carried out to determine if proteolytic enzymes have a dissociating effect on the high molecular

weight form of adenosine deaminase. In the first experiment the proteolytic enzyme trypsin was used.

0.1 ml. of purified high molecular weight deaminase (2 mg./ml.) was incubated at 37°C with 0.025 ml. trypsin solution (0.2 mg./ml.) in 0.05 M ammonium acetate buffer pH 8.5. The final ratio of trypsin to deaminase was 1:50. After 20h. and 40h. aliquots were withdrawn and subjected to electrophoresis in polyacrylamide gels at pH 8.1. The proteins were fixed and stained with 1% Amido Black in 7% acetic acid. Plate 17 illustrates the patterns obtained where it can be seen that the 20 h. and 40 h. samples had the same electrophoretic mobility as an untreated high molecular weight preparation indicating that dissociation had not taken place.

In a second experiment a number of proteolytic enzymes were simultaneously incubated with the high molecular weight form of the deaminase. A solution of the following enzymes was prepared in 0.25 M ammonium acetate pH 8.5 (concentration of each enzyme: 0.2 mg./ml.).

Carboxypeptidase A
Fibrinolysin
Ficin
Papain
Chymotrypsin

The solution also contained 10% LiCl (for solublization of the carboxypeptidase A), 5 mM cysteine (required for activation of ficin and papain) and 5 mM $CaCl_2$ (required for the activities of carboxypeptidase A and chymotrypsin). 0.025 ml. of the solution was incubated at 37°C with 0.1 ml. of purified high molecular weight adenosine deaminase (2 mg./ml.). The final concentration of each proteolytic

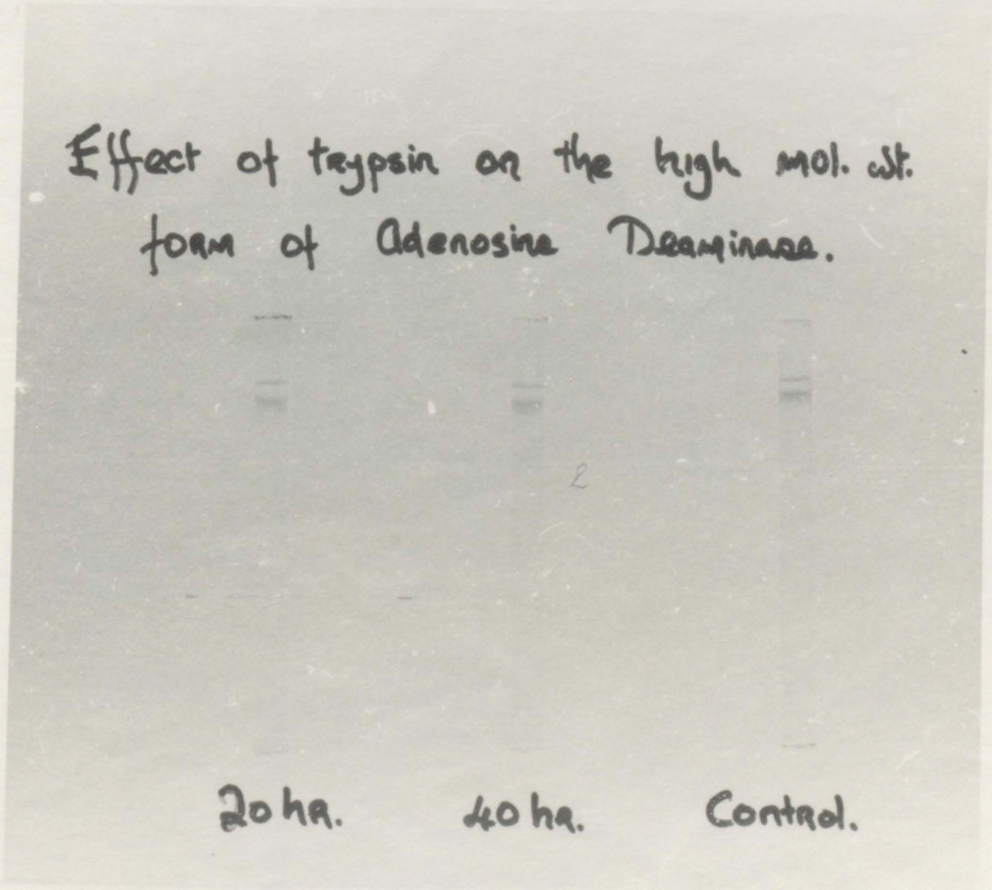
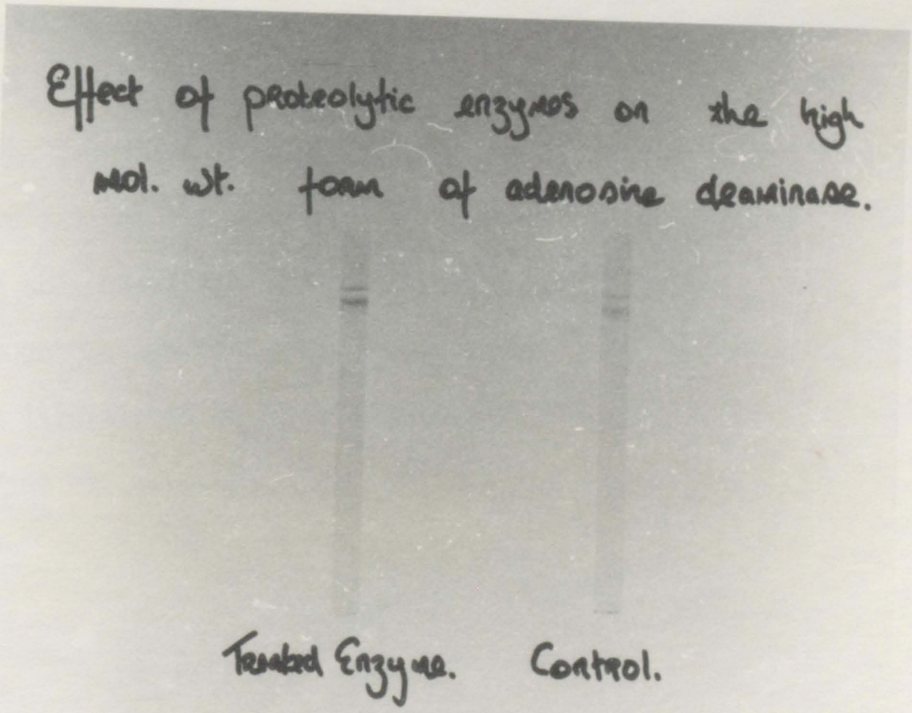
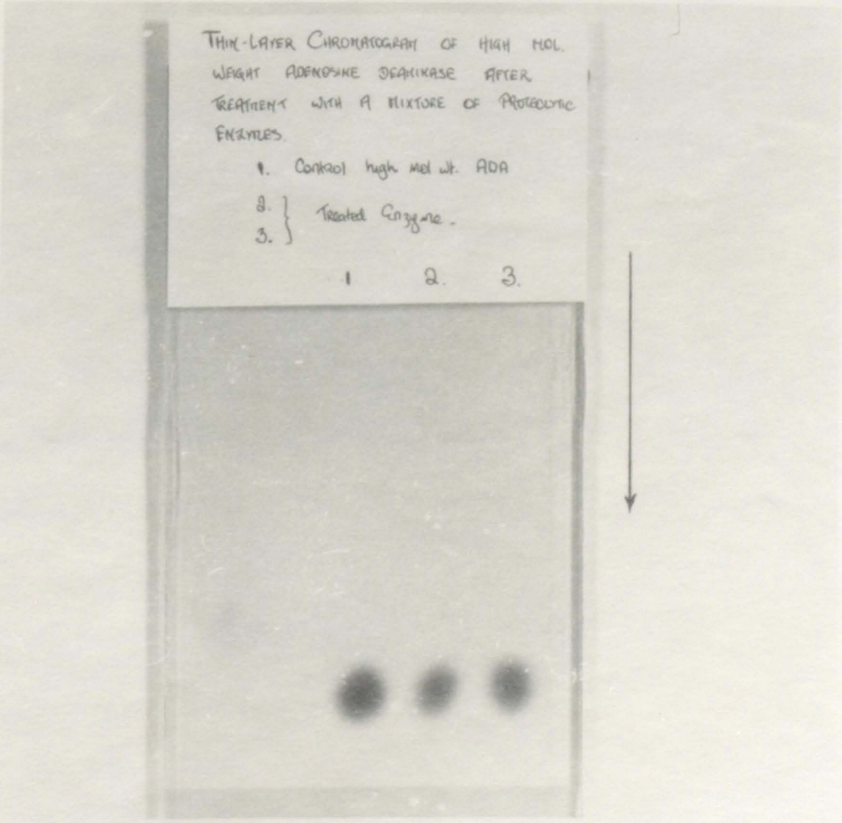


PLATE 17. POLYACRYLAMIDE GEL ELECTROPHORETIC PATTERN OF THE HIGH MOL. WT. FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE AFTER TREATMENT WITH TRYPSIN. THE GELS WERE STAINED USING 1% AMIDO BLACK IN 7% ACETIC ACID.



POLYACRYLAMIDE GEL ELECTROPHEROGRAM. THE GELS WERE STAINED USING 1% AMIDO BLACK IN 7% ACETIC ACID.



THE ZYMOGRAM (PHENOL VIOLET) STAIN FOR ADENOSINE DEAMINASE WAS USED.

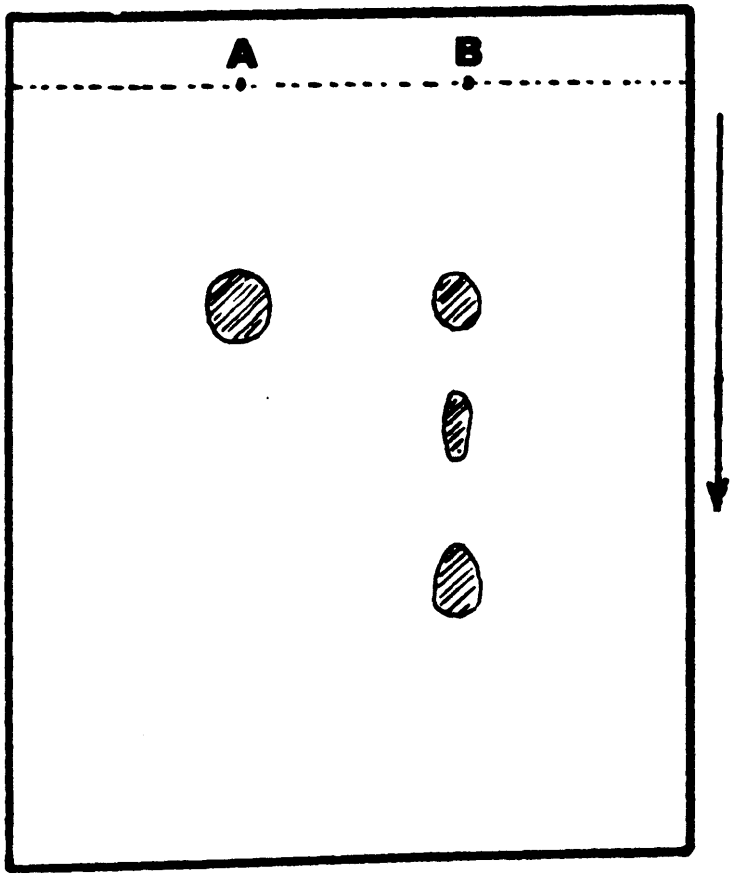
enzyme was 0.04 mg./ml. After 20 h. samples were withdrawn and analysed by polyacrylamide gel electrophoresis pH 8.1 and by thin-layer gel filtration on Sephadex G-75. (The polyacrylamide gels were stained for protein using 1% Amido Black in 7% acetic acid, and the thin-layer plate stained for enzyme activity using the zymogram stain for adenosine deaminase). The patterns obtained are illustrated in Plate 18 where it is again evident that there was no dissociation.

THE EFFECT OF GUANIDINE HYDROCHLORIDE AND UREA AS DISSOCIATING AGENTS

The procedures commonly employed for the dissociation of protein structures involve the use of denaturing agents such as guanidine hydrochloride or urea (Hass and Hill, 1969: Banks et al., 1968; Acton et al., 1969). These conditions are known to produce extensive changes in tertiary structure and the subunits obtained are usually insoluble at neutral pH in the absence of the denaturing agents. In the following experiments the effect of these reagents on the high molecular weight form of rabbit intestinal adenosine deaminase was studied.

Effect of Guanidine Hydrochloride

The thin-layer gel filtration technique was used to study the effect of this denaturing agent. 0.1 ml. of both purified high and low molecular weight forms were separately dialysed overnight against 6 M guanidine hydrochloride prepared in 0.5 M Tris pH 7.0

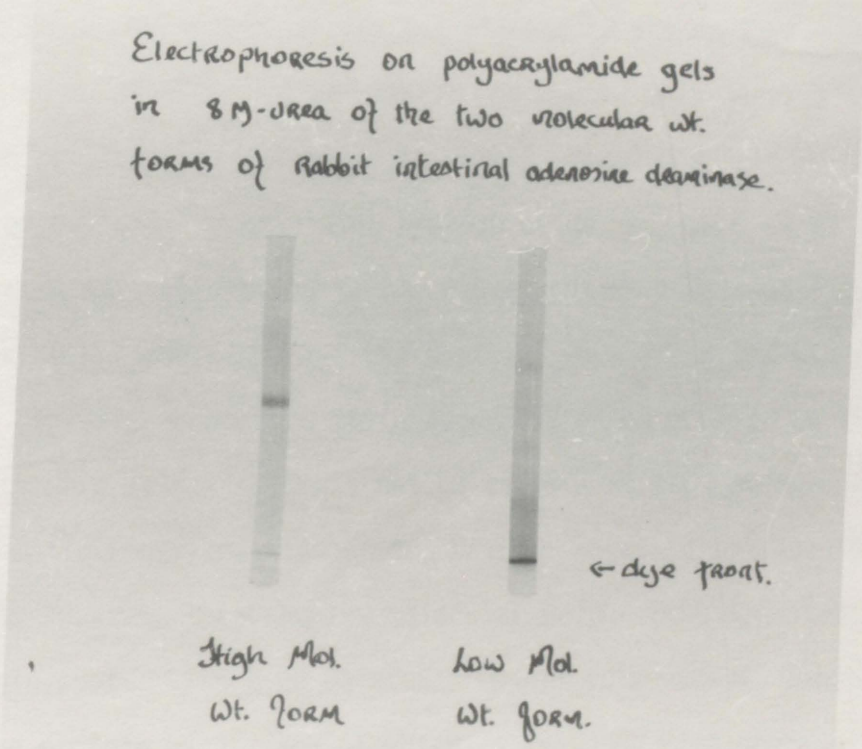


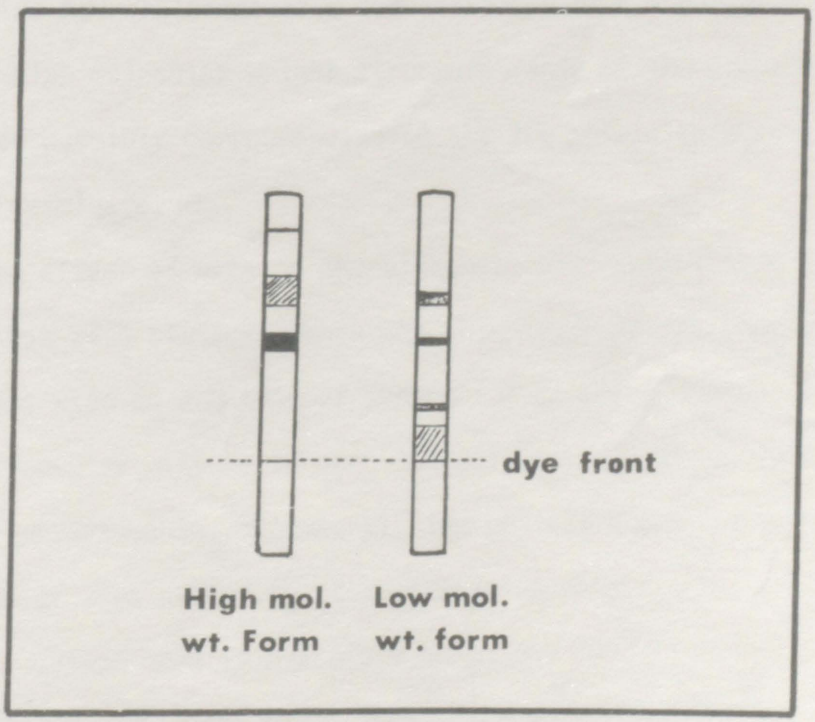
A Low mol. wt. form

B High mol. wt. form

FIG. A. Thin-layer gel filtration pattern of the low and high molecular weight forms of rabbit intestinal adenosine deaminase after treatment with 6M guanidine hydrochloride. The enzymes were incubated with the reagent (in 0.5 M Tris pH 7.0) for 24 h. at 37°C and applied to a Sephadex G-75 coated thin-layer plate equilibrated with 0.5 M Tris pH 7.0 containing 6 M guanidine hydrochloride. After chromatography the plate was stained for protein using the Ponceau S-Nigrosine stain.

PLATE 19





THE GELS WERE STAINED USING 1% AMIDO BLACK IN 7% ACETIC ACID.

The guanidine hydrochloride was allowed to act on the enzymes for 24 h. at 37°C. A Sephadex G-200 coated thin-layer plate was then prepared, equilibrated overnight with 0.5 M Tris pH 7.0 incorporating 6 M guanidine hydrochloride, and samples of the treated enzymes applied to the plate. After development (which took 48 h. approxiamtely) the proteins were fixed and stained using the nigrosine-ponceau S stain as described on P. 34. As the protein spots were faint and could not be satisfactorily photographed a schematic representation of the pattern obtained is shown in Fig. A. It is apparent that the low molecular weight form migrated as a single spot. The high molecular weight form however exhibited three protein bands, one of which corresponds with the position of the low molecular weight form. It is evident therefore that guanidine hydrochloride at a concentration of 6 M caused dissociation of the high molecular weight structure, some of the subunits so obtained possibly corresponding to the low molecular weight form of the deaminase.

The effect of urea on the high molecular weight form of the enzyme was also studied. When the thin-layer gel filtration technique was used as a method of separation as above, the flow rate of the buffer-urea mixture through the gel was very slow, making the method impracticable. Polyacrylamide gel electrophoresis in the presence of urea proved to be successful however.

The method used is described on p. 40. 0.2 ml. of the purified high and low molecular weight forms were dialysed overnight against 8 M urea in 0.1 M Tris pH 8.1 and the urea was allowed to act on the enzymes for 18 h. at 40°C. Samples were then applied to polyacrylamide gels and after electrophoresis the proteins were fixed and stained using 1% Amido Black in 7% acetic acid. The patterns

obtained are illustrated in Plate 19 (Both photographic and schematic representations are shown). As in the case of the guanidine hydrochloride treated enzyme, three major protein bands were obtained when urea was allowed to act on the high molecular weight deaminase, two of which had the same electrophoretic mobility as the two slowest moving bands of the denatured low molecular weight enzyme. In comparison with the guanidine hydrochloride (where only a single band was obtained) several protein bands were apparent in the urea denatured low molecular weight form.

The results indicate however that both the high and low molecular weight forms may in some way be structurally related to each other.

DISSOCIATION OF THE HIGH MOLECULAR WEIGHT FORM OF ADENOSINE DEAMINASE USING SODIUM DODECYL SULPHATE.

Sodium dodecyl sulphate (SDS) is an anionic detergent which is widely used in the dissociation of high molecular weight protein structures. Detergents occupy a unique position among protein denaturants, in that they are able to produce a drastic cooperative conformational change in proteins at remarkably low reagent concentrations. A concentration as low as 1% (w/v) sodium dodecyl sulphate has been shown to dissociate oligomeric proteins into their constituent subunits (Shapiro et al., 1967; Sheys and Doughty, 1971). Before investigating the effect of SDS on the high molecular weight form of adenosine deaminase an experiment was performed to study the

effect of various types of detergent on deaminase activity.

The following detergents were used:

Neutral:	Triton x-100
	Tween 80
Cationic:	Cetyltrimethylammonium bromide (CTAB)
Anionic:	Sodium dodecyl sulphate (SDS)

1% solutions of all detergents in 0.1M phosphate pH 7.0 were used. Assays were performed spectrophotometrically (pH 7.0) after incubating both the high and low molecular weight forms of the deaminase with each detergent for 1h. at 37°C. The results showed that the neutral detergents had no effect on enzyme activity whereas both the cationic and anionic detergents gave 100% inhibition almost immediately. The effect of these detergents was the same for the high and low molecular weight forms of the enzyme.

EFFECT OF SODIUM DODECYL SULPHATE ON THE HIGH MOLECULAR WEIGHT FORM

0.2 ml. of purified high molecular weight deaminase was dialysed overnight against a solution of 0.01M sodium phosphate pH 7.0 containing 1% SDS and 1% β-mercaptoethanol. After dialysis the enzyme was incubated at 37°C for 2h. The solution was then dialysed overnight against 0.01M sodium phosphate pH 7.0 containing 0.1% SDS and 0.1% β-mercaptoethanol and subjected to electrophoresis in SDS-polyacrylamide gels as described on p. 38. The pattern obtained in one of these gels is illustrated in Plate 20 (upper photograph). Two protein bands are apparent, Band 11 probably corresponding to a low

molecular weight dissociation product. The lower half of Plate 20 shows a densitometer tracing of the protein stained gel. Integration of the quantity of Amido Black bound to the two bands gave an estimate of 63% and 37% respectively for the proportion of total protein contained in Bands I and II.

SDS polyacrylamide gel electrophoresis also offers a simple and rapid method for the determination of the molecular weights of protein molecules (Weber and Osborn, 1969). Polypeptide chains migrate through the SDS gels at a rate which is proportional to their molecular weight. A plot of relative migration against log. molecular weight gives a straight line relationship from which unknown molecular weights of protein subunits and polypeptides can be estimated.

In an effort to determine whether Band II (in the above experiment) corresponded to the low molecular weight form of the enzyme a second experiment was performed in which the purified low molecular weight deaminase was also treated with SDS. A number of purified proteins were simultaneously treated and subjected to electrophoresis so as to estimate the molecular weight of the protein contained in Band II.

Procedure:

The pure proteins used in the study are listed in Table 10. 0.5 mg. of each protein was dissolved in 1 ml. of 0.1 M phosphate pH 7.0 containing 1% SDS and 1% β mercaptoethanol and treated as already described (p.38.). 0.1 ml. of aqueous solutions of the purified high and low molecular weight forms of the deaminase were also treated in a similar manner (The enzyme solutions were dialysed against the SDS). After allowing the detergent to act on the proteins they were subjected to electrophoresis in SDS polyacrylamide gels.

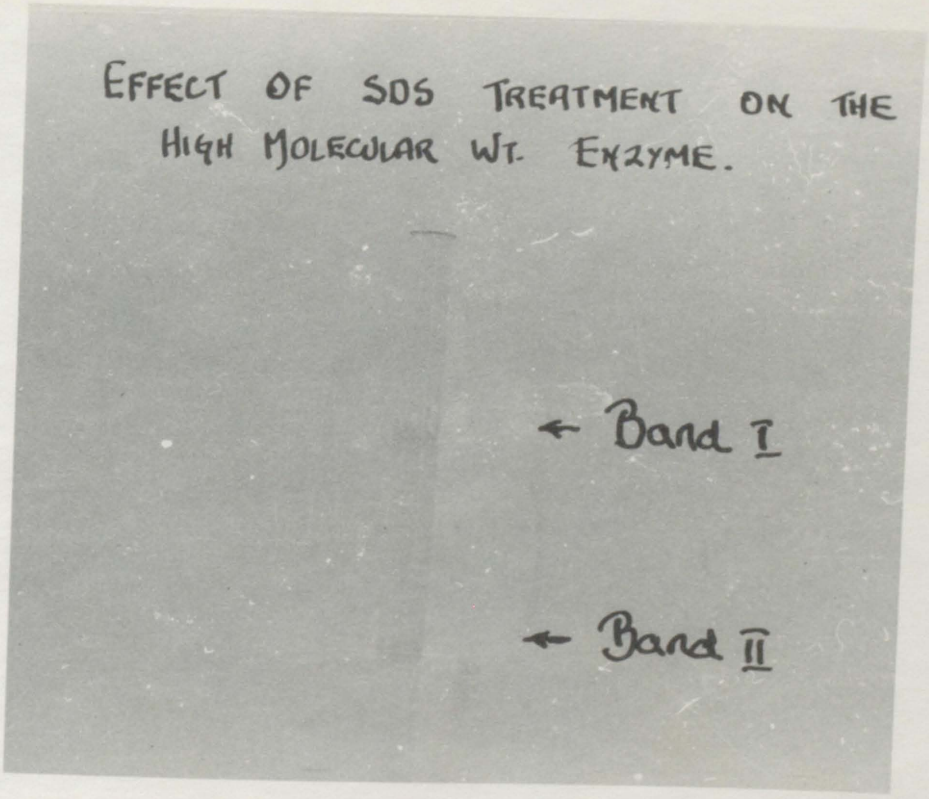
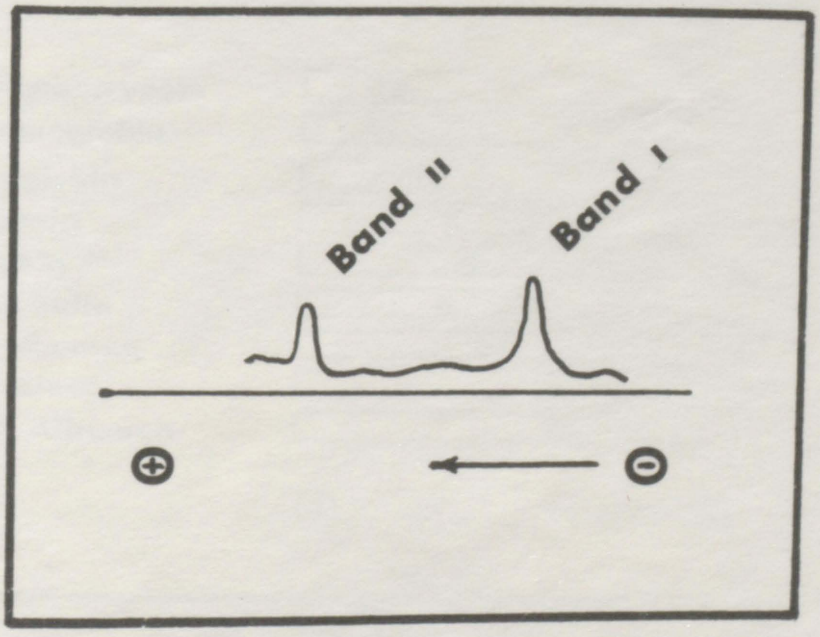


PLATE 20. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE HIGH MOLECULAR WEIGHT FORM OF RABBIT INTESTINAL ADENOSINE DEAMINASE. THE GELS WERE STAINED USING 1% AMIDO BLACK IN METHANOL-ACETIC ACID.



DENSITOMETER TRACING OF ELECTROPHEROGRAM SHOWN ABOVE.

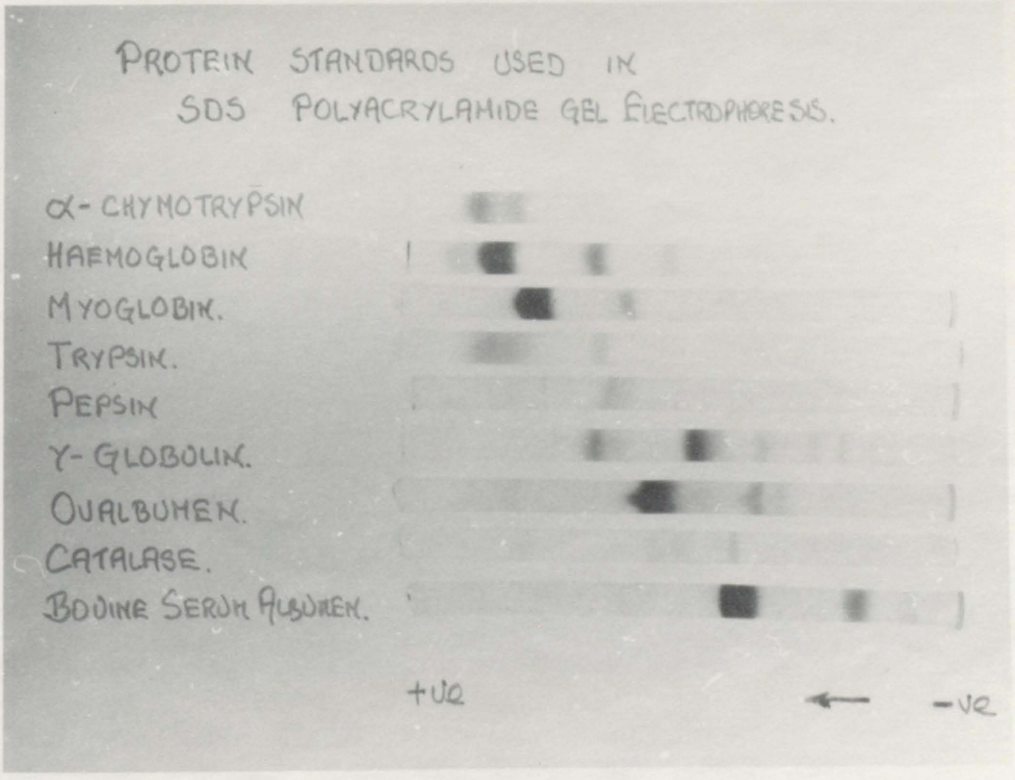
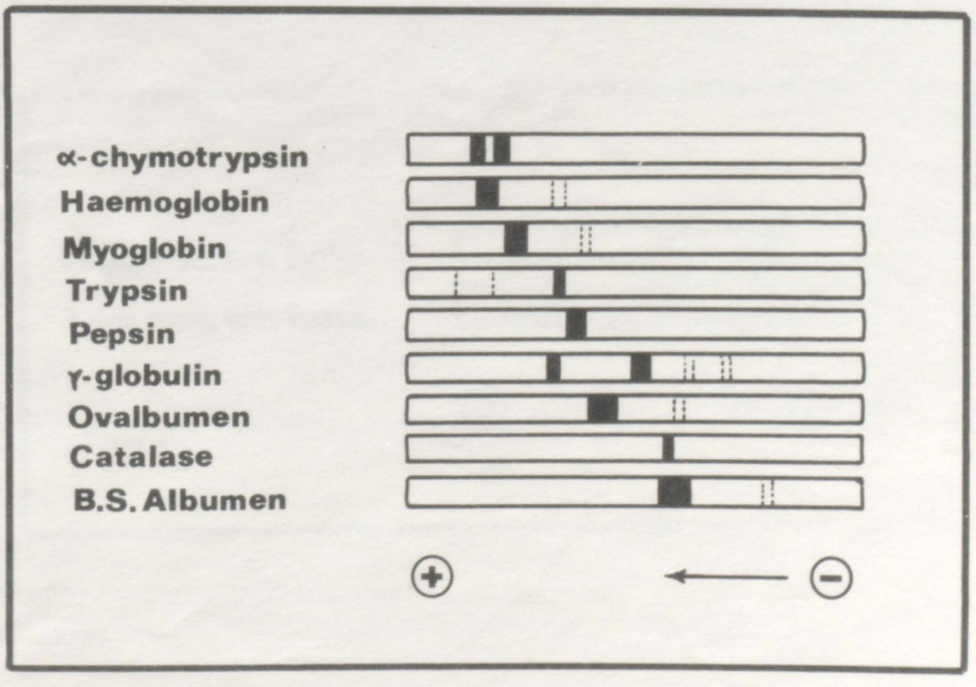


PLATE 21. SDS-POLYACRYLAMIDE GEL ELECTROPHORETIC PATTERNS OF SOME PURIFIED PROTEINS. THE GELS WERE STAINED USING 1% AMIDO BLACK IN METHANOL-ACETIC ACID.



SCHEMATIC REPRESENTATION OF ELECTROPHEROGRAMS SHOWN ABOVE.

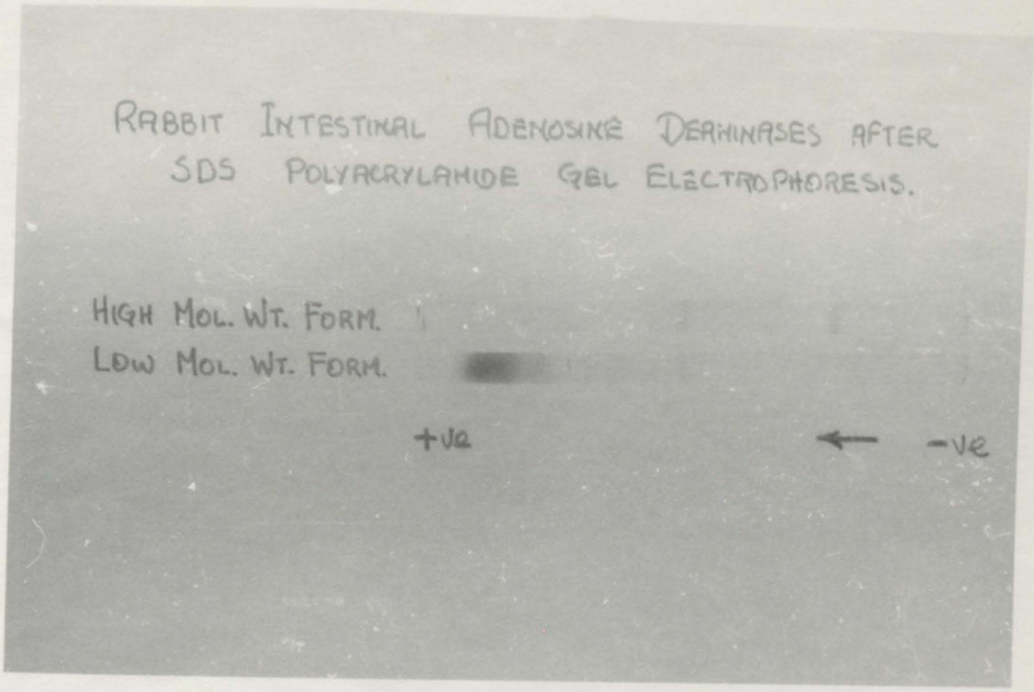
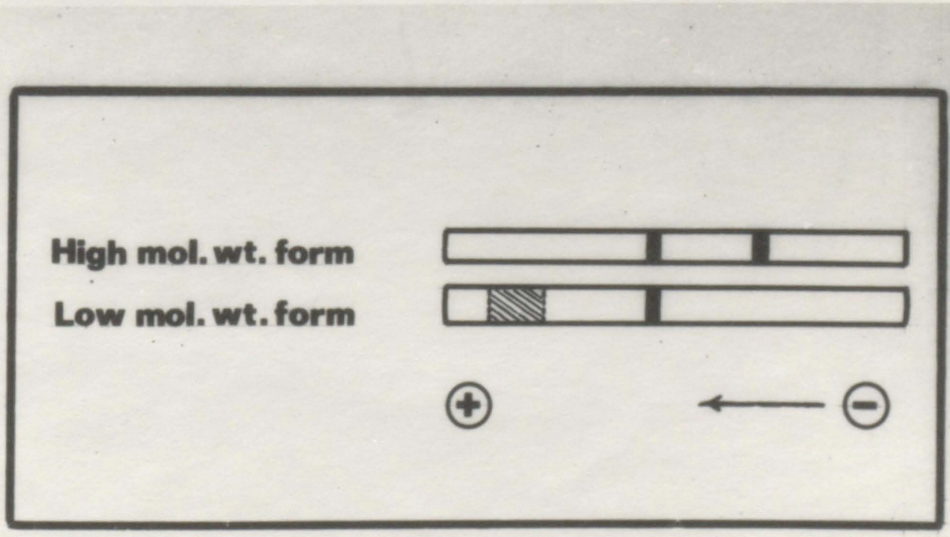


PLATE 22. SDS-POLYACRYLAMIDE GEL ELECTROPHORETIC PATTERNS OF THE HIGH AND LOW MOL. WT. FORMS OF RABBIT INTESTINAL ADENOSINE DEAMINASE. THE GELS WERE STAINED USING 1% AMIDO BLACK METHANOL-ACETIC ACID.



SCHEMATIC REPRESENTATION OF ELECTROGRAMS SHOWN ABOVE.

TABLE 10. Pure Proteins Studied by SDS Polyacrylamide Gel Electrophoresis.

* Protein	Subunit mol. wt.	Reference
Serum albumen	68,000	Tanford et al., 1967
γ-Globulin (Heavy	50,000	Rutishauser et al.,1968
and light chains)	23,500	"
Catalase	60,000	Sund et al., 1967
Pepsin	35,000	Bovey et al., 1960
Chymotrypsinogen	25,700	Dayhoff , 1967
Trypsin	23,300	"
Myoglobin	17,800	"
Hemoglobin	15,500	"



* In repeat experiments both ovalbumen and ∝-chymotrypsin (mol. wts. 45,000 and 21,500 respectively) were also used.

Plate 21 shows the patterns obtained after staining where it is evident that in some cases dissociation into subunits was not complete. A schematic representaiton of the protein bands is shown in the lower half of Plate 21.

Fig. 39 illustrates the relationship which exists between the molecular weights of the sodium dodecyl sulphate-treated proteins and their relative migration in the polyacrylamide gels. It can be seen that the relationship is a linear one.

Plate 22 depicts the patterns obtained after treatment of the high and low molecular weight forms of the rabbit adenosine deaminase with sodium dodecyl sulphate. As in the previous experiment two protein bands were obtained with the high molecular weight enzyme.

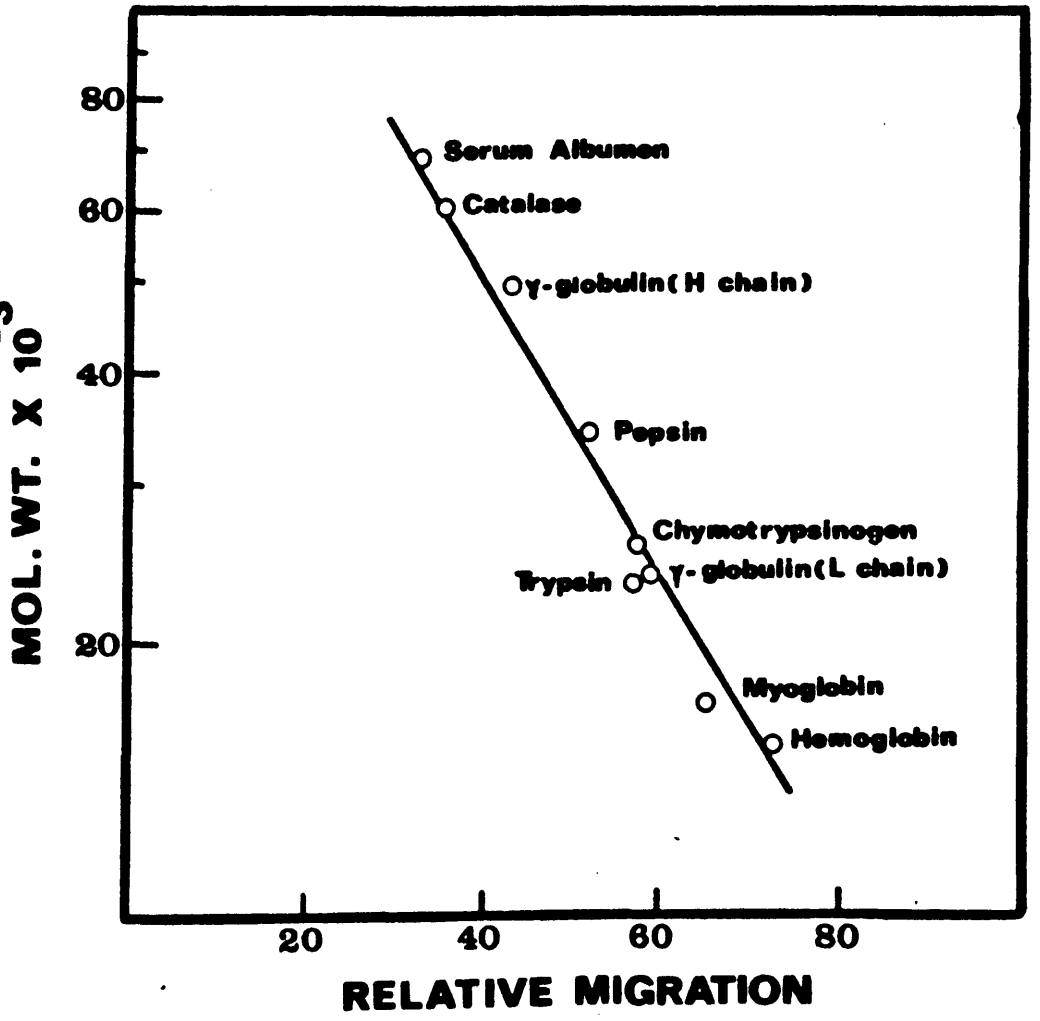


FIG. 39. The relationship between electrophoretic mobility and molecular weight for a number of standard proteins after SDS-polyacrylamide gel electrophoresis. The molecular weights of the proteins are shown in Table 10.

the fastest moving band having the same electrophoretic mobility as the slow moving band of the SDS-treated low molecular weight form. The low molecular weight enzyme unexpectedly gave a sharp band possessing a slow electrophoretic mobility and also a diffuse fast moving protein band. The molecular weights of the various bands which were estimated using the standard graph (Fig. 39) shown in Table 11.

TABLE 11. Molecular Weights of Various Bands obtained after Electrophoresis of the high and low Molecular Weight Forms of the Deaminase in SDS Polyacrylamide Gels.

Enzyme Form	Protein Band	Molecular Weight
High mol. wt.	Slow	>100,000
High mol.wt.	Fast	39,000
Low mol. wt.	Slow	39,000
Low mol. wt.	Fast (diffuse)	≈17,000

It is apparent that the high molecular weight form of the deaminase has been partially dissociated using SDS treatment into a protein of molecular weight 39,000 which is in the same region as the molecular weight of the purified low molecular weight deaminase as determined by gel filtration (32,000). The molecular weight of the slow moving band of the high molecular weight form (> 100,000) could not be accurately estimated since it was outside the range for the particular gel used in the experiment (5% polyacrylamide gel). The pattern obtained with the low molecular weight enzyme (a slow moving band, mol. wt. 39,000 and a fast moving diffuse

band, mol. wt. ≈ 17,000) was unusual. The fast moving band may correspond to a dissociation product of the enzyme or perhaps could represent contaminating low molecular weight components. It is interesting to compare these results however, with those obtained when another hydrolytic enzyme found in the intestine (trypsin) was treated with sodium dodecyl sulphate and subjected to electrophoresis (see Plate 21). It can be seen that the patterns obtained with the trypsin and the low molecular weight rabbit adenosine deaminase were very similar. This diffuse protein band of apparently very low molecular weight has also been observed by other workers when trypsin was treated with sodium dodecyl sulphate (Shapiro and Maizel, 1969).

ATTEMPTS AT DISSOCIATING THE HIGH MOLECULAR WEIGHT FORM OF ADENOSINE DEAMINASE USING MALEIC ANHYDRIDE

The introduction of negative charges into proteins by succinylation (Klotz and Keresztes-Nagy, 1963; Hass, 1964) is a mild procedure, in which the peptide chains remain soluble at neutral pH (Klotz, 1967). During succinylation (using succinic anhydride) the free amino groups of proteins (NH_3^+) are replaced with a $NHCOCH_2CH_2COO^-$ function. Butler et al. (1967) have suggested maleic anhydride instead of succinic anhydride as a blocking agent for amino groups in proteins for peptides. Following this, Sia and Horecker (1968) have shown that proteins can be smoothly and completely dissociated into soluble subunits with maleic anhydride.

It was considered of interest therefore to investigate the effect of this reagent on the high molecular weight form of rabbit intestinal adenosine deaminase. Most studies carried out using maleic anhydride have employed the use of protein solutions of fairly high concentration. Due to the limited amount of enzyme available in the present study, the method of Bruton and Hartly (1968) was used. These workers determined the subunit structure and molecular weight of methionyl-tRNA synthetase from E.coli with only 0.2 mg. of protein using C^{14} maleic anhydride.

The C^{14} maleic anhydride used in the study was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England.

Procedure:

The enzyme-reagent incubation mixture was prepared as follows:

0.175 ml. enzyme (containing 0.2 mg. protein)
0.05 ml. C^{14} maleic anhydride (Benzene solution containing 5μCi)
0.1 ml. of 60 mM borate buffer pH 9.5

The mixture was allowed to stand at room temperature for 1h. with intermittent shaking. After the reaction was completed the volume was brought up to 1 ml. using 60 mM borate pH 9.5 and the solution applied to a standardized Sephadex G-75 Superfine column (40 cm. x 2.5 cm.) equilibrated with 10 mM phosphate pH 7.0 containing 10 mM mercaptoethanol. 3 ml. fractions were collected and the level of radioactivity in each determined as described in the Methods section (p.

In the first experiment purified bovine intestinal deaminase (mol. wt. 35,000) was used. The elution pattern obtained is illustrated in Fig. 40. It is apparent that the maleic anhydride reacted

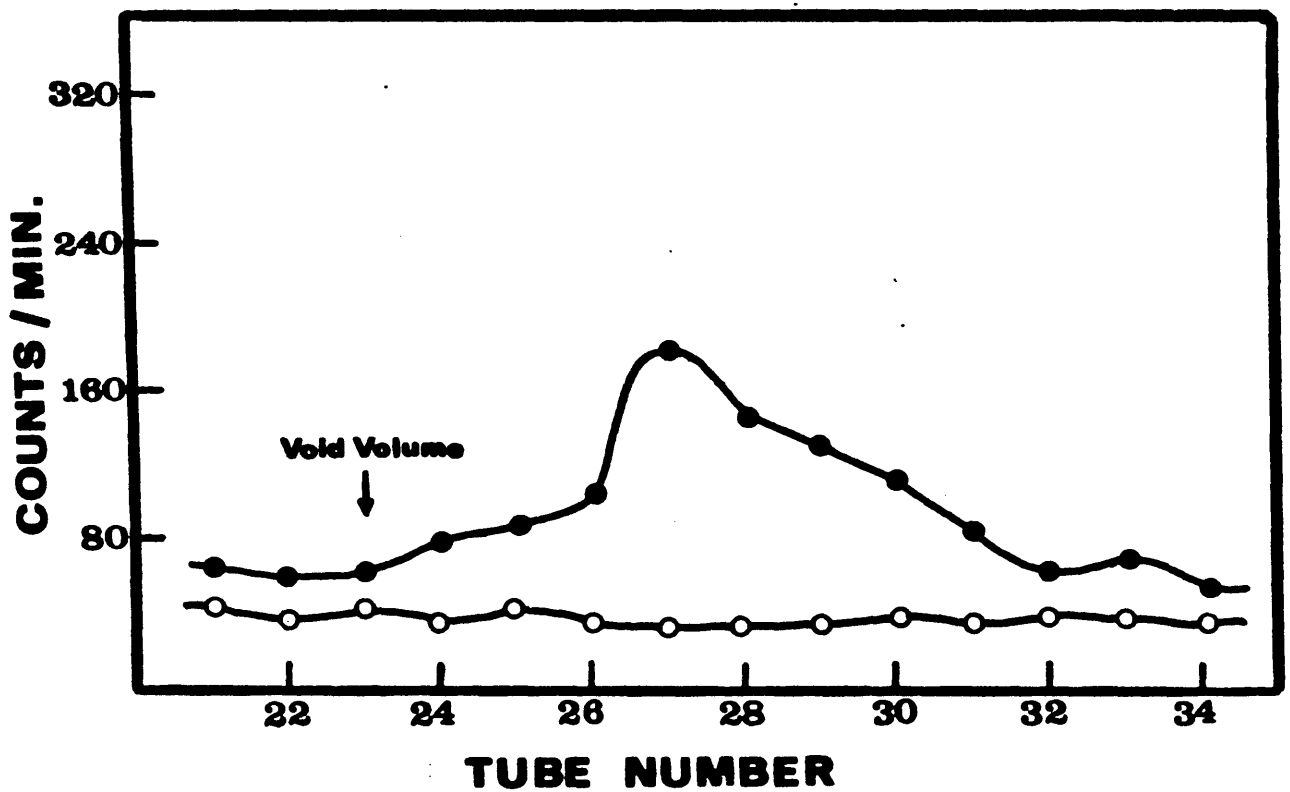


FIG. 40. Gel filtration of adenosine deaminase (treated with C^{14}-maleic anhydride) on Sephadex G-75 (Superfine). Bovine intestinal deaminase (●——●) and the high molecular weight form of rabbit intestinal adenosine deaminase (○——○) were treated with C^{14}-maleic anhydride and applied to the Sephadex column equilibrated with 10 mM phosphate pH 7.0 containing 10 mm β-mercaptoethanol. 3 ml. fractions were collected and radioactivity measured using a nuclear Chigago Gas Flow Detector.

with the enzyme. It was hoped that if dissociation of the high molecular weight rabbit deaminase took place when reacted with the reagent, a similar pattern might be obtained. However, as can be seen from Fig. 40, the maleic anhydride failed to react with this deaminase. The experiment was repeated three times and in no case was radioactivity detected after elution from the Sephadex column.

THIOL CONTENT OF RABBIT INTESTINAL ADENOSINE DEAMINASE

It has already been found that sulphydryl groups are necessary for the activity of both the high and low molecular weight forms of rabbit intestinal adenosine deaminase. In the following study the number of -SH groups in the denatured deaminases was estimated.

Estimation of sulphydryl groups by DTNB

5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was first used by Ellman (1959) to determine the sulphydryl content of tissue extracts and purified proteins. The total protein sulphydryl groups of the two forms of rabbit intestinal adenosine deaminase were estimated in the following manner:

Two 1 cm. cuvettes were set up containing the following:

Analytical

1.7 ml. of guanidine hydrochloride (9M) in 0.1M phosphate pH 8.0

0.1 ml. of 0.1M phosphate buffer pH 8.0 containing 10 mM EDTA

* Enzyme solution
25 µl of 10 mM DTNB in 0.1 M phosphate pH 8.0
Make up to a volume of 3.025 ml. with 0.1M phosphate pH 8.0

* In the case of the high molecular weight form 0.4 ml. enzyme containing 0.358 mg. protein was used, and in the case of the low molecular weight form 0.3 ml. enzyme containing 0.516 mg. protein was used.

Control

Same as the analytical except that 0.1 M phosphate buffer pH 8.0 was substituted for the enzyme solution.

The absorbance of the analytical was measured at 412 nm using the control as a blank. The number of sulphydryl groups was calculated from this with the use of a molar extinction coefficeint of 13,600 (Ellman, 1959). The following values were obtained for the thiol content of the two forms of the enzyme.

THIOL CONTENT OF RABBIT INTESTINAL ADENOSINE DEAMINASE

(moles of SH/mole of enzyme)

High Mol. Wt. Form	Low Mol. Wt. Form
18.7 (19)	2.4 (2)

The figures in parenthesis represent values obtained to the nearest whole number.

The above values were determined using molecular weights of 260,000 and 34,000 respectively for the high and low molecular weight forms. It is interesting to note that when the thiol content of the high molecular weight was calculated using a molecular weight of 34,000 (which could possibly represent a subunit molecular weight) a value of 2.55 was obtained. This is almost identical with the value determined for the low molecular weight form of the deaminase.

Estimation of SH groups by 2,2'-Dithiodipyridine

2,2'-dithiodipyridine (Trade name, Aldrithiol-2 obtained from Aldrich Chemical Co. U.S.A.) has also been used for the determination of sulphydryl groups in simple compounds and in biological materials (Grassetti and Murray, 1967). The reagent reacts readily and completely with thiols with the formation of 2-thiopyridone. As in the case of DTNB the course of the reaction can be followed spectrophotometrically. The 2,2'-dithiodipyridine absorbs maximally at 343 nm, while the reaction product 2-thiopyridone has virtually no absorption at that wavelength.

The SH content of the denatured high and low molecular weight forms of the deaminase were estimated in the following manner.

Two 1 cm. cuvettes were set up containing the following:

Analytical

1.7 ml. guanidine hydrochloride (9M) in 0.1 M phosphate pH 7.0

0.5 ml. of 5 mM aldrithiol -2 in 0.1M phosphate pH 7.0 containing 20% methanol.

* Enzyme solution

Make up to a volume of 3 ml. with 0.1 M phosphate pH 7.0

* In the case of the high molecular weight form 0.4 ml. enzyme containing 0.358 mg. protein was used, and in the case of the low molecular weight form 0.3 ml. enzyme containing 0.516 mg. protein was used.

Control

Same as the analytical except that 0.1M phosphate buffer pH 7.0 was substituted for the enzyme solution.

The absorbance of the analytical was measured at 412 nm using the control as a blank. The SH content of the enzymes was calculated using a molar extinction coefficeint of 7,600 (Grassetti and Murray, 1967). The following values were obtained:

THIOL CONTENT OF RABBIT INTESTINAL ADENOSINE DEAMINASE
(moles of SH/mole of enzyme)

High Mol. Wt. Form	Low. Mol. Wt. Form
5.75 (6)	1.77 (2)

The figures in parenthesis represent values obtained to the nearest whole number.

As in the case of the reaction with DTNB two SH groups were detected in the low molecular weight enzyme. The value obtained with the high molecular weight form however (6 SH groups) is much lower than the figure determined using DTNB. This could possibly be due to incomplete reaction of the aldrithiol-2 with the SH groups in the enzyme molecule.

Again, when the thiol content of the high molecular weight form

was calculated using a molecular weight of 34,000 a value of 1.28 was obtained which is lower than the value of 1.77 obtained for the low molecular weight enzyme.

THE AMINO ACID COMPOSITION OF THE HIGH AND LOW MOLECULAR WEIGHT FORMS OF RABBIT INTESTINAL ADENOSINE DEAMINASE.

Investigations carried out on the effect of dissociating agents such as sodium dodecyl sulphate and guanidine hydrochloride have indicated that the high and low molecular weight forms of the deaminase may in some way be structurally related. A study which might provide valuable information about the two forms is the determination of their amino acid composition. Having acquired an amino acid analyser in the laboratory it was decided therefore to carry out this investigation.

Because of the limited quantities of purified enzyme which were available only very small amounts (0.1 mg. approx.) were used for each analysis. The weighed lyophilized enzyme was hydrolysed as described (p.42) for 48h. and 72h. and 0.4 ml. of the hydrolysate (which had been evaporated to dryness and re-dissolved in 0.2 N sodium citrate buffer pH 2.2) was applied to the analyser. A standard amino acid solution (containing 25 nmoles of each amino acid) was also applied and the analyser constants for each amino acid were determined. These constants were then used in estimating the quantity of each amino acid in the analytical solutions. The

values of serine and threonine were determined by extrapolation to zero time and those of valine and isoleucine were taken as the values obtained after the longer hydrolysis time (72h.)

Estimation of cystine and cysteine as cysteic acid.

The combined cystine and cysteine content was determined according to Hirs (1967). The method depends on the oxidation of these amino acids to cysteic acid using performic acid. The oxidizing agent was prepared by mixing 1.0 ml. 30% hydrogen peroxide and 9.0 ml. 88% formic acid, allowing to stand at room temperature for 1h. and the solution then cooled to -4°C using a "Hetofrig" cooling-bath (Heto Birkerød Denmark) and used immediately. The weighed lyophilized protein (0.1 mg. approx. of each molecular form was used) was placed in a Pyrex ignition tube and 1 ml. performic acid reagent added. The capped tube was then left in the cooling-bath for 20h. Excess reagent was destroyed almost at once by the addition of 0.15 ml. 48% HBr in the cold. The tube was then attached to a rotary evaporator and concentrated to dryness. Removal of bromine was facilitated by the presence of a few pellets of sodium hydroxide in an attached condenser. 1 ml. of constant boiling HCl was added to the dry residue and hydrolysis allowed to proceed at 110°C for 20h. The hydrolysate was then concentrated to dryness with a rotary evaporator, dissolved in 0.2N sodium citrate buffer pH 2.2, and applied to the amino acid analyser. Since the yield of cysteic acid is usually 94 $\pm$ 2% using this method, the amount of cysteic acid obtained was divided by 0.94 to give a corrected value. The number of cystine plus cysteine was conveniently calculated with

reference to the amount of a stable amino acid (phenylalanine was used) determined from the same chromatogram.

The results of the amino acid analysis and cysteic acid determination are listed in Table 12. The amino acid composition of bovine intestinal adenosine deaminase as determined by Phelan (1970) is also shown for comparison.

TABLE 12 Relative Amounts of Amino Acids in Bovine and Rabbit Intestinal Adenosine Deaminases.

	Bovine Deaminase (Phelan, 1970)	Rabbit Intestinal Adenosine Deaminase	
		Low Mol.Wt. Form	High Mol.Wt. Form
Cys (O_3H)	6	4	5
Asp	31	23	29
Thr	18	15	16
Ser	18	11	19
Glu	38	35	32
Pro	20	15	13
Gly	19	15	15
Ala	22	19	18
Val	25	25	18
Met	6	(6)	(6)
Ile	13	10	13
Leu	26	26	26
Tyr	12	8	12
Phe	11	11	11
His	9	7	8
Lys	22	14	10
Arg	12	9	12
Trp	3	(3)	(3)

Since both Met and Trp were destroyed during acid hydrolysis of the enzymes the amounts of these could not be determined in the above analysis. The figures in parenthesis therefore represent values obtained for the bovine intestinal adenosine deaminase.

It must be stressed that although the values shown in Table 12 for the bovine deaminase represent the number of amino acid residues per enzyme molecule, the figures shown for the rabbit enzymes may not. The reason for this is because the amount of protein which was used for each analysis was so small that an accurate weight estimation could not be obtained. The figures shown therefore, represent the relative amounts of each amino acid present based on the assumption that the enzymes contain 11 Phe per weight of 34,000. It was necessary to do this so as to obtain a direct comparison of the relative amounts of each amino acid in the high and low molecular weight rabbit deaminases. It is interesting to note that having made this assumption molecular weight values of 33,466 and 35,023 are obtained for the low and high molecular weight forms respectively from the summation of the amino acids.

Examination of Table 12 reveals that although the relative amounts of each amino acid in the low and high molecular weight forms of the deaminase are not identical(which would indicate that the high molecular weight enzyme was an oligomeric form of the low molecular weight enzyme) they appear to be in some cases very similar. For example, the values for Cys (O_3H), Thr, Glu, Pro, Gly, Ala, Leu, His and Arg are practically the same. Furthermore, the general pattern of values appear to be somewhat similar and appear to correspond with the pattern shown by the bovine intestinal adenosine deaminase: Asp and Glu are high in all cases , the value of Glu being greater than

that of Asp; Val and Leu are also high, whereas Cys(O_3H), His and Arg are quite low.

Although no definite conclusions can be drawn from the above results, it was considered of interest to estimate the ratio of non-polar amino acids to polar amino acids for the bovine deaminase and the low molecular weight rabbit deaminase. Several workers (Fisher, 1964; Bieglow, 1967; Van Holde, 1966 and Klotz, 1970) have shown that this ratio can be useful in explaining why some single-chain proteins tend to aggregate while others do not. In general, it appears that a small peptide chain cannot tolerate more than about 30% of hydrophobic residues; a greater number will lead to association. Fig. 41 shows a list (compiled by Van Holde, 1966) of a number of globular proteins for which good data on amino acid composition are available. Proteins known to exist primarily as single-chain entities are listed on the left; the multichain proteins are listed on the right. The only multichain proteins listed are those found as definite aggregates. The vertical scale gives the percentage of a group of hydrophobic residues (proline, valine, leucine, isoleucine and phenylalanine) contained in each. While some overlap occurs, the distinction between the two groups of proteins is clear.

The correlation observed in Fig. 41 has been simply explained by Van Holde as follows:

" Kendrew (1962) has pointed out that in myoglobin, almost all of the hydrophobic residues are in the interior of the molecule, whereas the surface is composed largely of polar groups. If an enzyme of low molecular weight were to contain too large a fraction of non-polar groups, not all of these could be covered by the polar cortex. "Patches" of hydrophobic residues would be in contact with

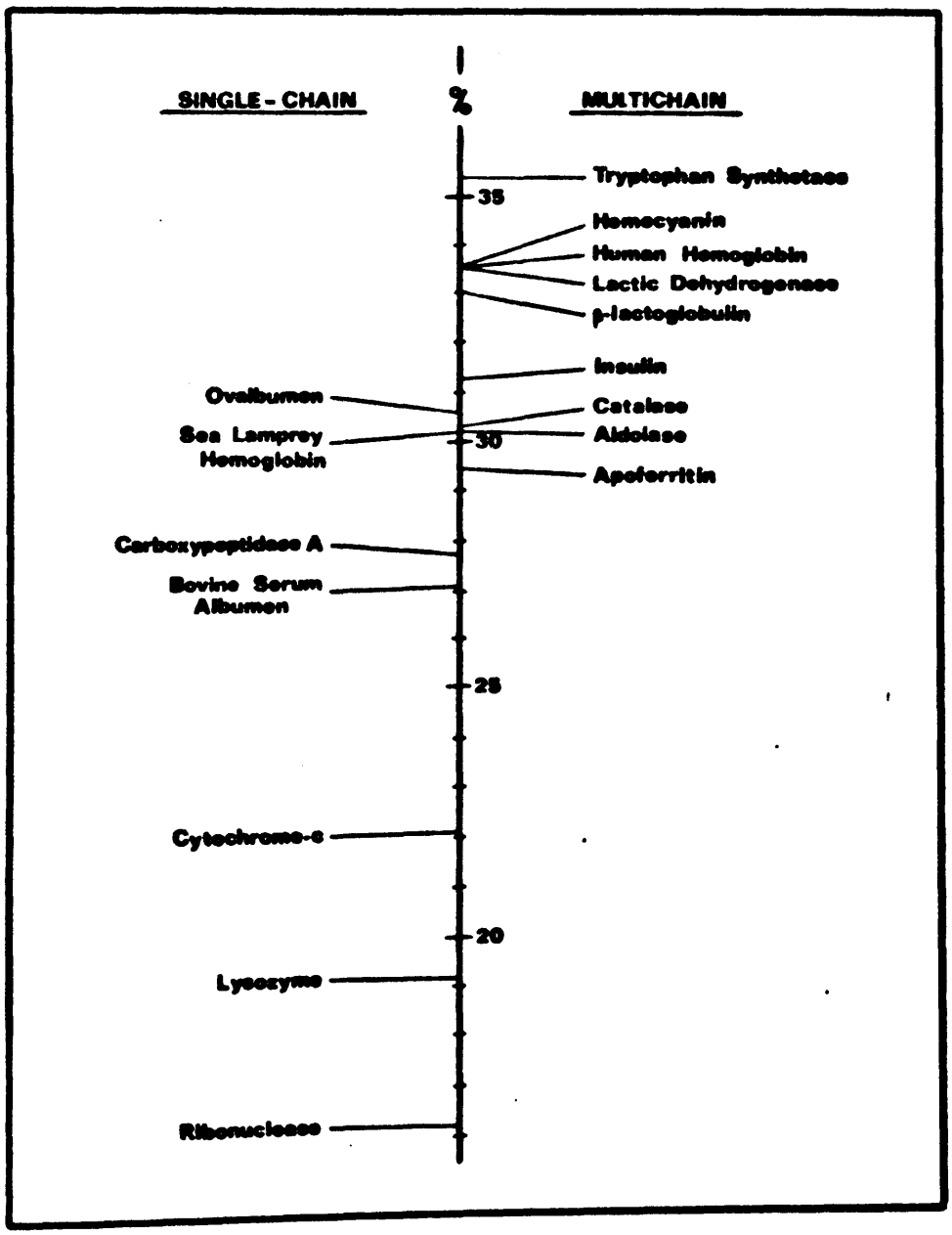


FIG. 41. Globular proteins ranked versus percentage of certain hydrophobic residues (proline, valine, leucine, isoleucine and phenylalanine).

the solvent, and these would provide natural sites for association. In a sense, the association process would put these groups back inside".

For the bovine intestinal adenosine deaminase the content of the five hydrophobic residues is 30.5% which lies at the crossover point on the scale. This enzyme has not been known to associate to higher molecular weight forms. With the low molecular weight form of the rabbit intestinal adenosine deaminase however, a value of 34% is obtained for the hydrophobic amino acid content. This figure is high up the scale shown in Fig. 41 which indicates that this enzyme should have a greater tendancy towards aggregation than the bovine intestinal adenosine deaminase.

SECTION IV

STUDIES ON THE RABBIT LIVER ADENOSINE DEAMINASE

It has already been mentioned that the intestines of the wild variety of rabbit appear to possess less of the high molecular weight adenosine deaminase than those of the domestic variety. During the early studies on the high and low molecular weight forms of the intestinal deaminase, the former type of rabbit was used, and for this reason very little of the high molecular weight enzyme was available for purification. It has been observed by Ma and Fisher (1969) that the rabbit liver possesses all its adenosine deaminase activity in a high molecular weight form. Before attempts were made to purify the high molecular weight deaminase from the intestine therefore, preliminary steps in the purification of the rabbit liver enzyme were investigated.

An extraction procedure developed by Cohn et al. (1951) for the fractionation of bovine liver proteins which involved the use of ethanol-water mixtures at various pH values (at $4^{o}C$) was applied to the rabbit liver. Although separation of the adenosine deaminase from various non-enzymatic proteins was achieved, the ethanol caused inactivation of the enzyme thus rendering the method unsuitable.

Attempts were also made to fractionate the rabbit liver adenosine deaminase using heat treatment, acetone treatment and polyethylene glycol fractionation. However, none of these proved to be useful as purification steps. (The polyethylene glycol fractionation was carried out using the method of Polson et al. (1964). The ammonium sulphate re-extraction technique which was found to be very successful in the purification of the intestinal deaminases could not be applied in the case of the rabbit liver due to the very low yield of enzyme which was obtained.

The following steps were found to be suitable however, for partial purification of the rabbit liver adenosine deaminase.

STEP 1. Extraction using Glass Distilled Water.

The rabbit liver was homogenised with one volume of glass distilled water. A further four volumes of water was then added and the extract allowed to stand at $4^{o}C$ (with intermittent shaking) for 2h. It was then centrifuged at 10,000 r.p.m. for 20 min. and the supernatant solution retained.

STEP 2. 30%-70% Ammonium Sulphate.

Solid $(NH_4)_2SO_4$ was added to the above supernatant to give a saturation of 30%. After allowing to flocculate for 30 min. the solution was centrifuged and the precipitate discarded. The supernatant fluid was then brought to 70% saturation with ammonium sulphate. The precipitate obtained in this case, after centrifugation, was retained and dissolved in a small volume of glass distilled water.

STEP 3. Dialysis against Running Tap Water

The re-suspended enzyme after ammonium sulphate fractionation was next dialysed against running tap water for a period of two days. (Dialysis against distilled water yielded very cloudy enzyme solutions, the solutions obtained after dialysis against running tap water being quite clear). Insoluble protein was removed by centrifugation at 10,000 r.p.m. for 20 min. and the supernatant fluid retained.

STEP 4. Batch Absorption using DEAE-Cellulose pH 4.0 and Stepwise Elution.

To the dialysed enzyme solution from Step 3 was added DEAE cellulose which had been equilibrated with 0.002M sodium acetate pH 4.0. After stirring for 5 min. the mixture was centrifuged and the supernatant fluid discarded. Finally the enzyme was eluted from the DEAE using a 0.3M NaCl solution.

This procedure was capable of effecting a 30-fold purification of the adenosine deaminase, the specific activity having increased from 0.004 e.u./mg. to 0.12 e.u./mg.

To investigate if the preparation obtained by the above procedure contained more than one molecular form of the deaminase, 3 ml. (0.7 e.u/ml.) was applied to a standardized Sephadex G-75 Superfine column (40 cm. x 2.5 cm.) and the proteins eluted with 0.002M sodium citrate pH 5.8 containing 0.1 M KCl. The pattern obtained is illustrated in Fig. 42 where it can be seen that all of the adenosine deaminase activity eluted at the void volume of the column, there being no evidence for the presence of a low molecular weight form. This is in agreement with the findings of Ma and Fisher (1969). Deoxyadenosine as well as adenosine was used in estimating the activity of the eluate fractions, and it is evident (Fig. 42) that the enzyme has the same deoxyadenosine/adenosine ratio (0.75) as the high and low molecular weight forms found in the intestine.

Ma and Fisher (1969) also reported that the high molecular weight deaminase in bovine liver could be partially dissociated to a low molecular weight form by treating an aqueous liver extract with concentrated $(NH_4)_2SO_4$. To investigate if the rabbit liver deaminase would undergo a similar dissociation, 3 ml. of the preparation from Step 4 was brought to 90% saturation with $(NH_4)_2SO_4$

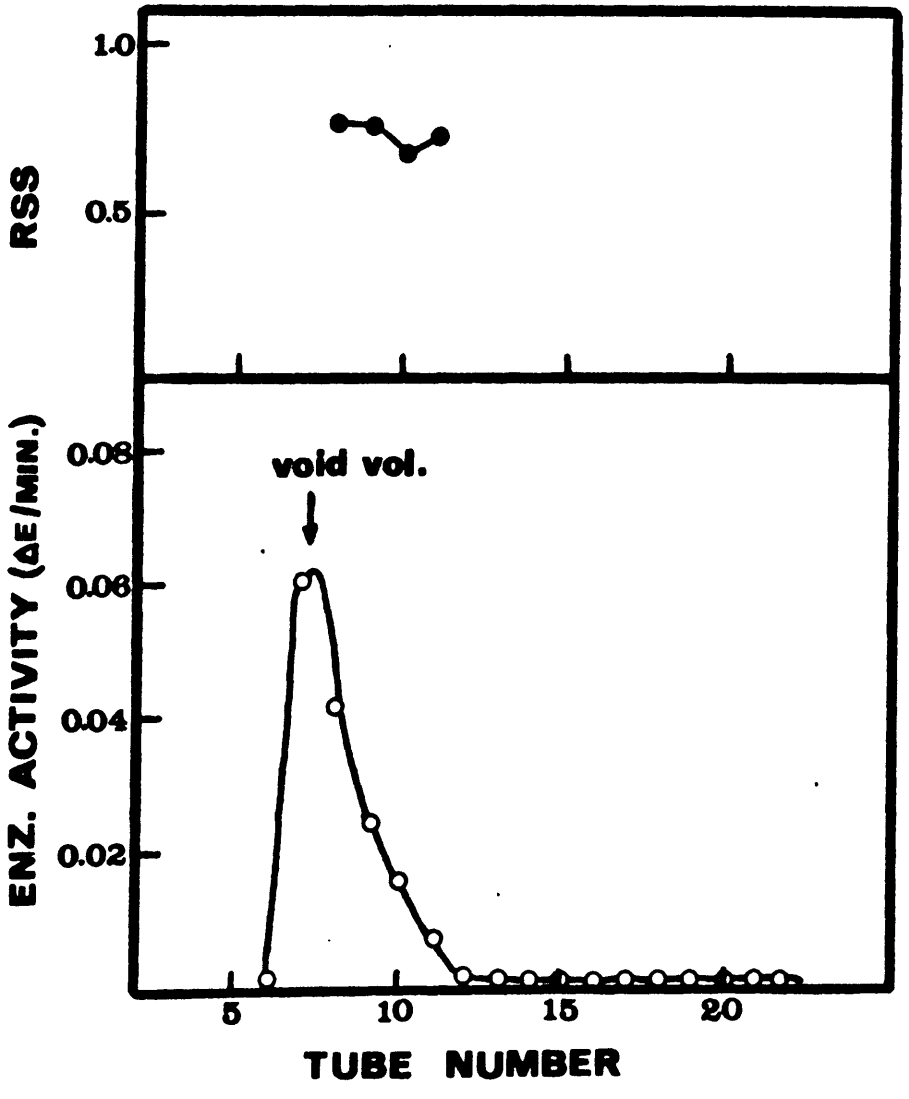


FIG. 42. Gel filtration of rabbit liver adenosine deaminase on Sephadex G-75 (Superfine).

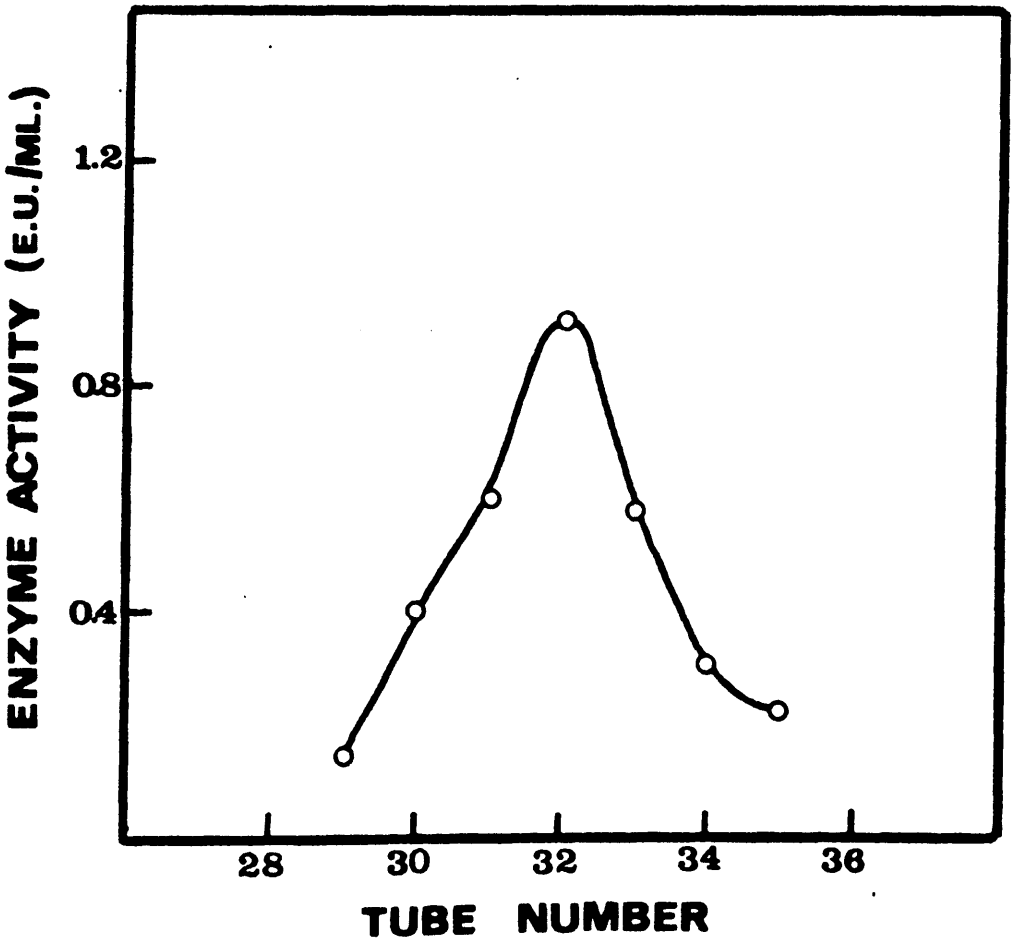


FIG. 43. Gel filtration of rabbit liver adenosine deaminase on Sephadex G-200 (Superfine.)

The precipitated enzyme was then dissolved in 0.002M sodium citrate pH 5.8 and applied to the Sephadex G-75 column. The pattern obtained was identical with that shown in Fig. 42 indicating that dissociation had not occurred. The rabbit liver deaminase would therefore appear to be different from that found in bovine liver.

MOLUCULAR WEIGHT OF THE PARTIALLY PURIFIED RABBIT LIVER ADENOSINE DEAMINASE

Partially purified enzyme from Step 4 was concentrated by precipitation with ammonium sulphate (90% saturation) and re-dissolved in 0.002 M sodium citrate pH 5.8 (+ 0.1M KCl) to give a solution possessing an activity of 2.8 e.u./ml. 3 ml. of the solution was then applied to a previously standardized Sephadex G-200 Superfine column (45 cm. x 2.5 cm.) and the enzyme eluted with 0.002 M sodium citrate pH 5.8 containing 0.1 M KCl.

Fig. 43 shows the elution pattern obtained. From the standard graph (p. 202) a molecular weight of 246,000 was estimated for the rabbit liver adenosine deaminase.

DISCUSSION

A procedure has been developed for the separation and purification of the two molecular weight forms of rabbit intestinal adenosine deaminase, the results of which constitute a major portion of the work reported in this thesis. Rabbit intestine was first shown to possess high and low molecular weight forms of adenosine deaminase by Buggy (1966). Preliminary experiments have indicated that although the two forms of the enzyme appear to be present in the same subcellular fraction (cytoplasm), the relative amounts of each differs in various regions of the small intestine. The deaminase activity of the duodenum and upper intestine is largely due to the low molecular weight form whereas the lower intestine consists mainly of the high molecular weight form. The level of enzyme activity also decreases gradually proceeding from the duodenum to the lower intestine. This fall in activity could possibly be explained by the fact that the high molecular weight enzyme, shown to contribute to most of the deaminase activity in the lower intestine, has a very low specific activity. The level of adenosine deaminase therefore in terms of protein concentration is probably the same throughout the length of the small intestine. It has been shown that in species which do not possess a high molecular weight adenosine deaminase (cat, rat and guinea-pig) the level of enzyme activity is the same in the lower intestine as in the duodenum (Brady and O'Donovan, 1965). Rabbit spleen possesses a small amount of a high molecular weight adenosine deaminase which probably resembles the high molecular weight form found in the intestine. The

spleen of the bovine species has also been reported to contain some high molecular weight adenosine deaminase (Ma and Fisher, 1969).

Preliminary studies on the purification of the rabbit adenosine deaminase have shown that acetone powders of the intestine are unsuitable as starting material. Although acetone powders have been successfully used in the purification of the enzyme from bovine intestine (Brady and O'Connell, 1962) and from chicken duodenum (Hoagland and Fisher, 1967), a 70% loss in activity was recorded when an acetone powder of rabbit intestine was prepared. Extracts of whole intestine (using 0.2M acetate buffer pH 5.5 as extractant) were therefore used as starting material for purification. Fractional precipitation of the deaminase with acetone also caused considerable loss in enzyme activity, the recovery varying from 35% to 64%. Acetone fractionations have been used however, in the purification of bovine heart adenosine deaminase (Rockwell and Maguire, 1966) and in the purification of the Takadiastase adenosine deaminase (Minato, 1968). It would appear therefore, that the rabbit intestinal enzyme is more sensitive to acetone treatment than adenosine deaminases from other sources. Despite the inactivating effect of this organic solvent, it was possible to fractionally separate the two molecular weight forms of the enzyme, the high molecular weight form being precipitated by 50-90% (v/v) acetone and the low molecular weight form being precipitated by 90-170% (v/v) acetone.

Rabbit intestinal adenosine deaminase is also rapidly inactivated at high temperatures, all activity being lost between 60^{0} and 70^{0}C. On heating acetate extracts of the intestine, the inactivation

of the deaminase paralleled the precipitation of non-enzymatic protein which rendered the use of a heat step unsuitable for purification. Bovine lung and spleen adenosine deaminases however, can tolerate a temperature of 70°C for 10 min. and a heat step has been used in the purification procedures developed for these enzymes (Noonan and Brady, 1969).

Although ammonium sulphate fractionation which has been used in the purification of adenosine deaminases from a variety of sources (Hoagland and Fisher, 1967; Pfrogner, 1967) gave considerable purification of the rabbit intestinal deaminase, an alternative procedure (termed the $(NH_4)_2SO_4$ "Re-extraction Method") was found to be more suitable. The high specific activity obtained using this method can be attributed to the large amount of inactive protein which precipitates out of solution following dialysis of the ammonium sulphate treated enzyme. These precipitated proteins appear to be water-insoluble (at 4°C) and presumably require an environment of high ionic strength to remain in solution.

Thin-layer gel filtration experiments followed by densitometry have shown that the ratio of the high and low molecular weight forms does not vary during the first three steps of the purification procedure. This allows both forms to be purified simultaneously without repitition of individual purification steps. The thin-layer gel filtration method (followed by densitometry) has proved to be useful in determining the relative amounts of the two forms of the enzyme in various solutions. The technique has several advantages over the use of Sephadex columns which include speed and adaptibility to very small samples. Results obtained using the Sephadex columns showed excellent agreement with those obtained with the thin-layer

gel filtration method which confirmed the validity of using the latter technique for comparative analysis.

The anion exchanger DE-52 cellulose proved successful in the purification of the enzymes, chromatography having been carried out at pH 5.8. Although considerable purification was achieved, the procedure did not separate the two forms of the enzyme. Besides using the main enzyme peak which was eluted from the DEAE column for the purification of the high and low molecular weight forms, side peak fractions, which were shown to possess a considerable quantity of the high molecular weight enzyme, were also used for purification. This necessitated the duplication of all subsequent steps in the purification of the high molecular weight form.

Sephadex G-75 and Sephadex G-200 gels were found to be very useful in the final stages of purification. Complete separation of the high and low molecular weight forms was achieved on columns of Sephadex G-75 with a simultaneous increase in the specific activity of both forms. This Sephadex gel has also been used in the purification of bovine intestinal adenosine deaminase (Murphy et al., 1969a). In the present purification, the Superfine grade of Sephadex yielded enzyme solutions of much greater specific activity than the corresponding coarser grade.

The specific activity of the purified low molecular weight rabbit deaminase (250-300 e.u./mg.) is in the same order as the specific activities of other mammalian adenosine deaminases of similar molecular weight. Table 13 compares the value obtained with those of other purified deaminases.

TABLE 13. Specific Activities of a Number of Purified Adenosine Deaminases.

Enzyme Source	Sp. Act. (e.u./mg.)	Reference
Bovine intestine	400 - 470	Murphy et al., 1969a
Bovine spleen	400	Noonan and Brady, 1969
Bovine lung	420	"
Bovine placenta	252	Sim and Maguire, 1971
Cat lung	600	Murphy et al., 1969b
Rabbit intestine (low mol. wt. form)	*250 - 300	Present investigation

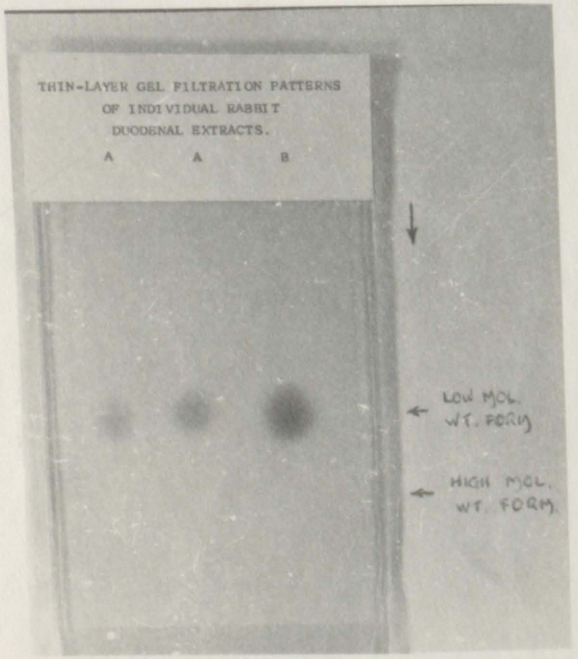
* These estimates of the specific activity of the rabbit deaminase were made using the spectrophotometric method of assay. Because the activity obtained with this method represents only 65% of the true activity determined by the microdiffusion technique, the specific activity of the low molecular weight form was in the region of 400 e.u./mg, and that of the high molecular weight form, 40 e.u/mg.

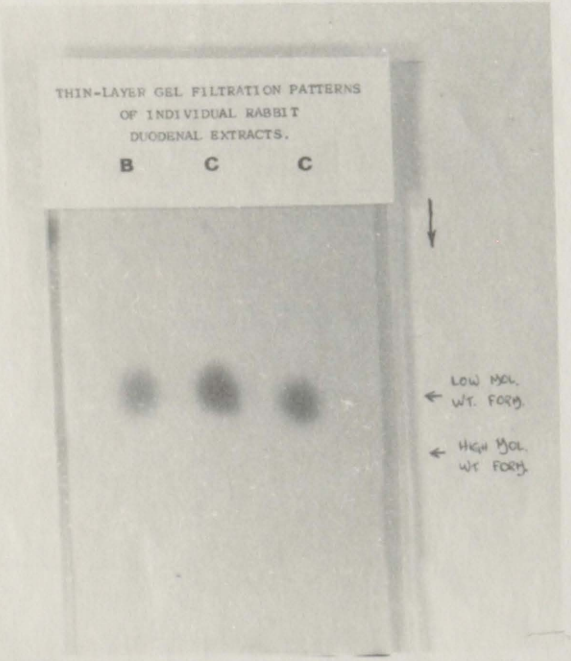
The specific activity of the purified high molecular weight form was approximately ten times lower than that of the low molecular weight enzyme, a value of 25-30 e.u./mg. (using the spectrophotometric method of assay) being obtained. Although other mammalian adenosine deaminases of molecular sizes in the region of that of the high molecular weight rabbit enzyme have not been extensively investigated, a large enzyme (mol. wt. 200,000) which appears to correspond to the type-A enzymes of Fisher's classification has recently been purified from human lung by Akedo et al. (1972) and was shown to have a specific activity of 2.7 e.u./mg. The same workers have purified a type-C adenosine deaminase (mol. wt. 50,000) from human stomach which had a specific activity of 47.4 e.u./mg. This value is considerably lower

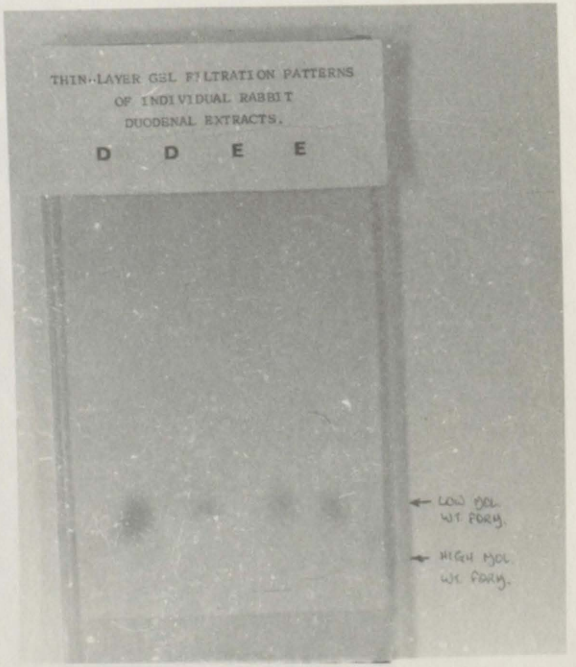
than the specific activities of the low molecular weight enzymes listed in Table 13. An adenosine deaminase with an unusually low specific activity has been purified from bovine serum (Cory et al., 1967). Although the enzyme is of similar molecular weight to the bovine intestinal deaminase (an S value of 3.9 has been reported compared with a value of 3.46 for the bovine intestinal enzyme) the specific activity of the purified enzyme was only 1.1 e.u./mg. Both the human stomach and the bovine serum enzymes appear to be the only low molecular weight mammalian adenosine deaminases reported to possess a low specific activity. A high molecular weight deaminase (mol. wt. 120,000) which closely resembles the type-B enzymes of Fisher's classification has been purified from the bay scallop by Harbison and Fisher (. in the press) which possesses a specific activity of 180 e.u./mg. In general, therefore, it would appear that the high molecular weight type-A enzymes have low specific activities, whereas the type-B (mol.wt. 110,000) and the type-C (mol.wt. 35,000) adenosine deaminases possess high specific activities.

The relative amounts (as estimated by the thin-layer gel filtration method) of the high and low molecular weight forms of the deaminase in individual rabbit intestines varies considerably. Throughout the entire study, it appeared that, in general, the domestic variety of rabbit possessed a greater quantity of the high molecular weight enzyme in the intestine than did the wild variety. However, towards the end of the work reported in the thesis, a survey of a number of domestic types of rabbit was conducted with a view to determining whether or not they had the same level of high molecular weight enzyme. The following varieties of rabbit

PLATE 23. THIN-LAYER GEL FILTRATION PATTERNS OF EXTRACTS OF INDIVIDUAL RABBIT INTESTINES (DOMESTIC VARIETY). THE ZYMOGRAM (PHENOL VIOLET) STAIN FOR ADENOSINE DEAMINASE WAS USED.







were investigated:

Variety of Rabbit	Reference Letter	Number Studied
Californian	A	2
English	B	2
New Zealand Red	C	2
* New Zealand White	D.	2
Chincilla	E	2

* This variety of rabbit was used for the purification of the high molecular weight enzyme reported in the thesis.

Plate 23 illustrates the patterns obtained using the thin-layer gel filtration technique. As can be seen, no high molecular weight enzyme could be detected in any of the rabbit intestines studied despite the fact that the animals were all of the domestic variety. These results were very surprising and appeared to contradict earlier observations. Similar findings have been obtained in this laboratory with porcine intestines, some of which appear to possess about 50% of their adenosine deaminase activity in a high molecular weight form while in others no high molecular weight enzyme can be detected (Noonan, unpublished data). The reason for these differences is not understood.

A comparative study of the properties of the high and low molecular weight forms of the rabbit deaminase revealed that there were a number of differences between them. The pH optima of both forms were the same (pH 6.5) except in the case of the high molecular weight enzyme using deoxyadenosine as substrate where the optimum was at pH 6.0. The rabbit enzyme appears to differ in this respect

from other mammalian adenosine deaminases such as those of the cow, rat and cat which have pH optima at 7.0. The dog however, gives optima of 6.85 and 6.75 using adenosine and deoxyadenosine respectively as substrates (Brady and O'Donovan, 1965). The low molecular weight form of the rabbit deaminase also appears to have a narrower pH stability range than the high molecular weight form.

Both forms of the enzyme have similar isoelectric points which possibly explains why they elute from the DEAE cellulose column during purification at the same position. The values obtained (5.16 for the low molecular weight form and 4.94 for the high molecular weight form) are in the same order as those of a variety of mammalian adenosine deaminases studied by Brady and O'Donovan (1965). The relative substrate specificities (ratio of activity with deoxyadenosine and adenosine as substrates) of the high and low molecular weight forms were also similar. This is in agreement with the results of Ma and Fisher (1969) who found that the relative substrate specificities of the type-A enzymes in a variety of mammalian livers were the same as the corresponding type-C enzymes. The rabbit liver adenosine deaminase also gave a similar value (0.75) to the intestinal deaminases.

The high and low molecular weight forms of the rabbit deaminase appear to be inactivated to the same extent by urea and dimethylformamide solutions. 8M urea causes complete loss in activity at pH 7.0 which is similar to the findings of Brady and O'Sullivan for the bovine intestinal adenosine deaminase (Brady and O'Sullivan, 1967). Guanidine hydrochloride causes inactivation at a much lower concentration (2M), the high molecular weight form being less stable towards the reagent than the low molecular weight form. Both forms

of the enzyme show very different activation energies and Km values. The activation energy of the low molecular weight form (7.8 k cal.) lies within the range of 4-10 k cal. which has been reported by Ma and Fisher (1966) to be characteristic of vertebrates in general. The figure obtained for the high molecular weight form, however, (1.15 k cal.) is considerably lower than this range of values. Ma and Fisher (1968a) also found that the high and low molecular weight adenosine deaminases in chicken liver (mol. wt. 110,000 and 30,000 respectively) differed in their activation energies. The value for the high molecular weight enzyme (4 k cal.) was considerably lower than that of the low molecular weight form (17 k cal.). The Km values of the chicken enzymes also differed by a factor of seven (Ma and Fisher, 1968a) compared with a factor of ten for the high and low molecular weight forms of the rabbit adenosine deaminase (using adenosine as substrate values of 33.3×10^{-5} and 3.3×10^{-5} were obtained for the high and low molecular weight forms respectively). The figures obtained for the low molecular weight form is in the same order as values reported for other adenosine deaminases of similar molecular weight. Some metal ions also inhibited the high and low molecular weight forms of the rabbit intestinal deaminase, but to different extents. While Cu^{+} and Cu^{++} gave 100% inhibition in each case, Cd^{+} and Co^{+} inhibited the high molecular weight form, but did not effect the activity of the low molecular weight enzyme. Fe^{3+} and Cr^{3+} also inhibited the high molecular weight form to a greater extent. These results indicate that the high molecular weight enzyme is more sensitive to metal ion inhibition than the low molecular weight form.

Studies with thiol reagents have indicated that sulphydryl groups

are necessary for the activity of both forms of the enzyme. This is in keeping with observations made on most adenosine deaminases investigated to date. The pattern of reactivity with the thiol reagents is very similar to that reported for the adenosine deaminase isolated from the bovine species (Murphy, 1967; Ronca et al., 1967; Maguire and Sim, 1971). 5,5'-dithiobis-(2-nitrobenzoic acid DTNB), iodoacetic acid and its amide iodoacetamide failed to inhibit the enzymes. DTNB does however, react with the denatured enzymes and has been used to determine the sulphydryl content of the high and low molecular weight forms of the rabbit deaminase. Evidence that sulphydryl groups are necessary for enzyme activity was obtained by reacting the deaminases with the organomercurials, p-chloromercuriphenylsulphonic acid, p-chloromercuribenzoate and phenylmercuric acetate, and subsequently, reactivating the enzymes with β-mercaptoethanol, dithiothreitol and dithioerythritol. The fact that the inactivation and reactivation patterns were very similar for both forms of the rabbit deaminase suggests that the active sites of both may be very similar. It has been suggested for the bovine intestinal deaminase that the lack of reactivity with alkylating reagents (iodoacetic acid and iodoacetamide) was due to the fact that the essential sulphydryl group may be contained in a hydrophobic region of the enzyme molecule. By using competitive inhibitors and from pH-velocity studies, Maguire and Sim (1971) have proposed a mechanism of action for the bovine placental adenosine deaminase (mol. wt. 35,480) involving a cysteine, a histidine and a lysine residue at the active site of the enzyme. It was suggested that binding of substrates (and competitive inhibitors) is via hydrogen bonding from the enzyme

sulphydryl to N_I of the substrate and that an attacking water molecule is bound to the tertiary nitrogen of the histidine imidazole. The histidine residue is so orientated in the active site as to permit the stereospecific attack of the water oxygen on C_6 of the substrate. The role of the ammonium group of the lysine residue is envisaged as one of facilitating removal of the leaving group ($-NH_2$). Photooxidation studies have also indicated that a sulphydryl group, and possibly a tryptophan residue, may be necessary for the activity of the rabbit intestinal deaminase. Both forms of the enzyme were strongly inhibited by n-bromosuccinimide, a reagent which attacks tryptophan residues. The non-specific takadiastase adenosine deaminase was also found to be inactivated by n-bromosuccinimide and by photooxidation in the presence of methylene blue (Minato, 1968), and it was suggested that a tryptophan residue was necessary for the activity of this enzyme.

As regards the nature of the high molecular weight form of the rabbit deaminase, a number of tentative suggestions were made which were subsequently tested. The possibility that the macromolecule might be some type of multienzyme complex possessing a number of enzymatic activities was investigated, but only adenosine, 2'-deoxyadenosine and 3'-deoxyadenosine were deaminated. These substrates are also hydrolysed by the low molecular weight form of the enzyme. The possibility that the high molecular weight form might be a similar type of adenosine deaminase to the Takadiastase deaminase which deaminates a variety of naturally occurring substrates was also investigated. However, although both enzymes have similar molecular weights, they differ widely in their substrate specificities. The possibility existed that the high

molecular weight enzyme might consist of the low molecular weight form bound to some non-enzymatic cellular material, thus giving an apparent high molecular weight. However, a test for polysaccharide showed that this substrate was absent from the preparation. Despite the fact that an absorbancy ratio (A_{280} : A_{260}) of 1.3 was obtained with the high molecular weight enzyme (compared with a value of 2.0 for the low molecular weight form) neither DNA nor RNA could be detected in the purified enzyme. The high molecular weight form would therefore appear to be entirely protein in nature.

Attempts at dissociating the high molecular weight deaminase using reagents which might induce conversion into active subunits were largely unsuccessful. Triton x-100 and sucrose (also glucose and fructose) did however, give a slight conversion to a low molecular weight species possessing activity. As the effect in each case was only barely detectable, these reagents cannot be said to be of any great significance as dissociating agents for the enzyme. Ma and Fisher (1969) found however, that during sucrose density gradient experiments, the type-A adenosine deaminase (mol. wt. 200,000) of bovine liver dissociated into an active low molecular weight species. A similar dissociation effect was observed with the same enzyme during ammonium sulphate concentration experiments. In contrast, in the case of the rabbit intestinal deaminase, treatment with ammonium sulphate did not affect the high molecular weight form of the enzyme. Similar results were obtained with the rabbit liver adenosine deaminase (mol. wt. 246,000) when partially purified preparations of the enzyme were concentrated using ammonium sulphate and subsequently chromatographed on a Sephadex G-75 column.

Elevated temperatures, acid and alkaline pH, high salt concentrations, incubation with proteolytic enzymes and treatment with maleic anhydride failed to bring about dissociation of the high molecular weight rabbit intestinal deaminase. Since these procedures are commonly used for the dissociation of multichain proteins and enzymes it would appear that the high molecular weight rabbit adenosine deaminase possesses an unusually stable structure.

Guanidine hydrochloride, a well known denaturant and dissociating agent, did however convert the enzyme into low molecular weight components, one of which had the same mobility on Sephadex G-75 coated thin-layer plates as the guanidine hydrochloride treated low molecular weight adenosine deaminase. The fact that Sephadex gels were used indicated that the dissociation product had a similar molecular weight to that of the low molecular weight form of the enzyme. Guanidine hydrochloride has also been found to dissociate the large adenosine deaminase (mol. wt. 214,000) from Takadiastase powder (Minato, 1968) the dissociation products having molecular weights of 111,000 and 29,000 respectively. Recently, Akedo et al. (1972) have reported that the human lung adenosine deaminase (mol. wt. 200,000) is dissociable using guanidine hydrochloride into an active low molecular weight species (mol. wt. 50,000).

Urea and sodium dodecyl sulphate were also found to dissociate the high molecular weight form of the rabbit intestinal adenosine deaminase. On treating the enzyme with sodium dodecyl sulphate, a protein of molecular weight 39,000 was obtained, which had the same electrophoretic mobility as the SDS treated low molecular weight deaminase. Although the products of these treatments with dissociating agents have to be confirmed as identical with the native

low molecular weight adenosine deaminase, the findings suggest two possible interpretations of the structural relationship between the two forms of the enzyme: (i) The high molecular weight form is a polymeric form of the low molecular weight enzyme. (ii) The high molecular weight form is a complex of the low molecular weight enzyme and some other protein(s). Similar interpretations have been put forward for the large (E_l) and small (E_s) adenosine deaminases found in human tissues by Akedo et al. (1970). By the finding of the apparent conversion of E_s to E_l in the presence of normal lung sap, they supposed the existence of some unknown factor(s) capable of stimulating conversion of E_s to E_l and termed it tentatively the "conversion factor". Furthermore, Akedo et al. (1972) have succeeded in obtaining electrophoretically nearly homogenous conversion factor which had a molecular weight of 140,000 as estimated by gel filtration with Sephadex G-200. The authors have suggested that the large enzyme (E_l) is composed of the small adenosine deaminase (E_s) bound to the conversion factor. This view of the structure of a high molecular weight adenosine deaminase is different from that proposed by Ma and Fisher (1969) who have suggested that the type-A adenosine deaminase (mol.wt. 200,000) found in mammalian livers is a polymeric form of the low molecular weight enzyme (mol. wt. 35,000) present in the same tissue. The presence of a "conversion factor" in rabbit liver has not been detected in the present investigation. It cannot be stated definitely therefore at the present time which of the two interpretations fits the high molecular weight form of the rabbit intestinal adenosine deaminase. Studies on the sulphydryl content using 5,5'-dithiobis-(2-nitrobenzoic acid) have indicated a number of sulphydryl groups in the enzyme

molecule which would be consistent with the view that the enzyme is a polymeric form of the low molecular weight enzyme. However, a much smaller number of sulphydryl groups were detected using aldrithiol-2. Two sulphydryl groups were detected in the low molecular weight form which is consistent with the findings for other mammalian adenosine deaminases of similar molecular weight.

The results obtained for the ratio of polar to non-polar amino acids present in the low molecular weight rabbit adenosine deaminase compared to the value obtained for the bovine intestinal adenosine deaminase offers a plausible explanation as to why the rabbit enzyme should have a tendancy towards aggregation. The percentage hydrophobic residues present (34%) is higher than the value obtained for the bovine intestinal deaminase (30.5%) and is also higher than the hydrophobic amino acid content of a number of well known enzymes which exist as multiple molecular forms (glutamic dehydrogenase, aldolase and catalase). The large percentage of hydrophobic amino acid residues in the rabbit adenosine deaminase could also account for the remarkable stability of the high molecular weight form of the enzyme.

BIBLIOGRAPHY

Acton, R.T., Bennet, J.C., Evans, E.E. and Schrohenloher, R.E. (1969) J. Biol. Chem. 244, 4128.

Akedo, H., Nishihara, H., Shinkai, K. and Komatsu, K. (1970) Biochim. Biophys. Acta 212, 189.

Akedo, H., Nishihara, H., Shinkai, K., Komatsu, K. and Ishikawa, S. (1972) Biochim. Biophys. Acta 276, 257.

Andrews, P. (1964) Biochem. J. 91, 222.

Andrews, P. (1970) Methods of Biochemical Analysis 18, 1.

Appella, E. and Market, C.L. (1961) Biochem. Biophys. Res. Commun. 6, 171.

Baer, H.P., Drummond, G.I. and Duncan, E.L. (1966) Mol. Pharmacol. 2, 67.

Baer, H.P. and Drummond, G.I. (1966) Biochem. Biophys. Res. Commun. 24, 584.

Baer, H.P., Drummond, G.I. and Gillis, J. (1968) Arch. Biochem. Biophys. 123, 172.

Banks, B.E.C., Doonan, S., Gauldie, J., Lawrence, A.J. and Vernon, C.A. (1968) Eur. J. Biochem. 6, 507.

Barker, S.A., Gray, C.J. and Jolley, M.E. (1971) Biochem. Biophys. Res. Commun. 45, 654.

Bellin, J.S. and Yankus, C.A. (1968) Arch Biochem. Biophys. 123, 18.

Bieglow, C.C. (1967) J. Theoret. Biol. 16, 187.

Brady, T. (1942) Biochem. J. 36, 478.

Brady, T.G. and O'Connell, W. (1962) Biochim. Biophys. Acta. 62, 216.

Brady, T.G. and O'Donovan, C.I. (1965) Comp. Biochem. Physiol. 14, 101.

Brady, T.G. and Hegarty, V.J. (1966) Nature 209, 1027.

Brady, T.G. and O'Sullivan, M. (1967) Biochim. Biophys. Acta 132, 127.

Bruton, C.J. and Hartley, B.S. (1968) Biochem. J. 108, 281.

Buggy, M. (1966) M.Sc. Thesis, National University of Ireland.

Butler, P.J.G., Harris, J.I., Hartley, B.S. and Leberman, R. (1967) Biochem. J. 103, 78p.

Burton, K. (1956) Biochem. J. 62, 315.

Ceriotti, G. (1952) J. Biol. Chem. 198, 297.

Clarke, D.A., Davoll, J., Phillips, F.S. and Brown, G.B. (1952) J. Pharmacol. Expt. Therap. 106, 291.

Coodington, A. (1965) Biochim. Biophys. Acta 99, 442.

Cohen, L.A. (1968) Annual Review of Biochemistry 37, 716.

Cohn, E.J., Surgenor, D.M. and Hunter, M.J. (1951) Enzymes and enzyme systems: their state in nature (Edsall, J.T. ed.) p. 105. Harvard University Press, Cambridge, Massachusetts.

Conway, E.J. and Cooke, R. (1939) Biochem. J. 33, 479.

Cooper, D.M.F. (1971) M.Sc. Thesis, National University of Ireland.

Corri, G.T., Slein, M.W. and Cori, C.S. (1948) J. Biol. Chem. 173, 605.

Cory, J.G. and Suhadolnik, R.J. (1965). Biochemistry 4, 1733.

Cory, J.G., Weibaum, G. and Suhadolnik, R.J. (1967) Arch. Biochem. Biophys. 118, 428.

Cory, J.G., Carreno, A.LJ. and Rich, M.A. (1970) Enzymologia 41, 232.

Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404.

de Duve, C. (1964) J. Theor. Biol. 6, 33.

Dierick, W., Olislaegers, P. and Stockx, J. (1967) Arch. Internat. Physiol. et Biochim. 75, 623.

Ellman, G.L. (1959) Arch Biochem. Biophys. 82, 70.

Fiers, W. and Vandenoriessche, L. (1960) Arch. Internat. Physiol. et Biochim. 68, 203.

Fisher, H.F. (1964) Proc. natn. Acad. Sci. Wash. 51, 1285.

Fisher, J.R., Ma, P.F. and Chilson, O.P. (1965) Comp. Biochem. Physiol. 16, 199.

Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375.

Flodin, P., Gelotte, B. and Porath, J. (1960) Nature 188, 493.

French, D., Knapp, D.W. and Pazur, J.H. (1950) J. Amer. Chem. Soc. 72, 5150.

Frieden, C. (1962) J. Biol. Chem. 237, 2396.

Galanti, B. and Giusti, G. (1968) Minerva Med. 59, 5867.

Galanti, B. et al. (1968) Giorn. Mal. Parass 20, 982.

Garen, A. and Levinthal, C. (1960) Biochim. Biophys. Acta 38, 470.

Ghosh, N.K. and Fishman, W.H. (1968) Biochem. J. 108, 779.

Giusti, G. (1970) Pol. Arch. Med. Wewn. 44, 525.

Glazer, A.N. (1970) Annual Review of Biochemistry 39, 110.

Goldberg, D.M. (1965) Brit. Med. J. 1, 353.

Grassetti, D.R. and Murray, J.F. (1967) Arch Biochem. Biophys. 119, 41.

Gustafson, T. and Hasselberg, I. (1951) Exptl. Cell Research 11, 642.

Harris, H., Spencer, N. and Hopkinson, D.A. (1968) Ann. Hum. Genet. Lond. 32, 9.

Hass, L.F. (1964) Biochemistry 3, 535.

Hass, G.M. and Hill, R.L. (1969) J. Biol. Chem. 244, 6080.

Hatcher, D.W. and Goldstein, G. (1969) Analyt. Biochem. 31, 42.

Hirs, C.H.W. (1967) Methods in Enzymology 11, 59.

Hoagland, V.D. and Fisher, J.R. (1967) J. Biol. Chem. 242, 4341.

Ipata, P.L. and Rossi, C.A. (1962) Boll.Soc. It. Biol. Sper. 38, 1117.

Itoh, R., Mitsui, A. and Tsushima, K. (1967) Biochim. Biophys. Acta 146, 151.

Jolley, R.L. Jr., Robb, D.A. and Mason, H.S. (1969) J. Biol. Chem. 244, 1953.

Jordan, W.K., March, R., Hovchin, O.B. and Popp, E. (1959) J. Neurochem. 4, 170.

Jori, G., Galiazzo, G. and Scoffone, E. (1969) Int. J. Prot. Res. 1, 289.

Kalckar, H.M. (1947) J. Biol. Chem. 167, 429.

Kaplan, N.O., Colowick, S.P. and Ciotti, M.M. (1956) 194, 579.

Kendrew, J.C. (1962) Brookhaven Symp. Biol. 15, 216.

Klein, W. (1961) Z. Physiol. Chem. 324, 163.

Klotz, I.M. and Keresztes-Nagy, S. (1963) Biochemistry 2, 445.

Klotz, I.M. (1967) in "Methods of Enzymology" Vol. 11 (C.H.W. Hirs, ed.) p. 576 Academic Press, New York.

Klotz, I.M. (1970) Arch Biochem. Biophys. 138, 704.

Koehler, H. and Benz, E.J. (1962) Clin. Chem. 8, 133.

Koch, A.L. and Vallee, G. (1959) J. Biol. Chem. 234, 1213.

Krawczynsky, J. et al. (1965) Clin Chim. Acta 11, 227.

Kubo, H., Iwatsubo, M., Watasi, H. and Soyama, T. (1959) J. Biochem. Tokyo 46, 1171.

Layne in Colowick, S.P. and Kaplan, N.O. (1957) Methods in Enzymology 3, 451. Academic Press, New York.

Lennox, F.G. (1941) Austral. Council Sci. Ind. Res., Pamphlet No. 109, 37.

Lineweaver, H. and Burke, D. (1934) J. Amer. Chem. Soc. 56, 658.

Ma, P.F. and Fisher, J.R. (1966) Comp. Biochem. Physiol. 19, 799.

Ma, P.F. and Fisher, J.R. (1968a) Biochim. Biophys. Acta 159, 153.

Ma, P.F. and Fisher, J.R. (1968b) Comp. Biochem. Physiol. 27, 105.

Ma, P.F. and Fisher, J.R. (1968c) Comp. Biochem. Physiol. 27, 687.

Ma, P.F. and Fisher, J.R. (1969) Comp. Biochem. Physiol. 31, 771.

Ma, P.F. and Fisher, J.R. (1972) Comp. Biochem. Physiol. 41b, 469.

Maguire, H.M. and Sim, M.K. (1971) Eur. J. Biochem. 23, 22.

Maizel, J.V. Jr. (1966) Science 151, 988.

Malysheva, M.K., Polyakova, N.M. and Emchuk, T.I. (1964) Urk. Biokhim. Zh. 36, 323.

Mann, K.G. and Vestling, C.S. (1969) Biochemistry 8, 1105.

Martinez-Carrion, M., Turano, C., Riva, F. and Fasella, P. (1967) J. Biol. Chem. 242, 1426.

McLaren, A.D. and Shugar, D. in Alexander, P. and Bacq, Z.M. (1964) Photochemistry of Proteins and Nucleic Acids p. 156. Pergamon Press, London.

Meegan, E.P. (1968) M.Sc. Thesis, National University of Ireland.

Meighen, E.A. and Schachman, H.R. (1970) Biochemistry 9, 1167.

Miller, A.L. and Levy, H.R. (1969) J. Biol. Chem. 244, 2334.

Minato, S. (1968) J. Biochem. Tokyo 64, 815.

Mohberg, J. and Rusch, H.P. (1969) Arch. Biochem. Biophys. 134, 577.

Moore, S. and Stein, W.H. (1963) Methods in Enzymology 6, 819.

Morawiecki, A. (1960) Biochim. Biophys. Acta 44, 604.

Morris, C.J.O.R. (1964) J. Chromatog. 16, 167.

Muller, M. and Moller, K.M. (1969) Com. Rendus Des Travaux du Laboratoire Carlsberg 36, 463.

Murphy, P.M. (1967) Ph.D. Thesis, National University of Ireland.

Murphy, P.M., Noonan, M., Collins, P., Tully, E. and Brady, T.G. (1969a) Biochim. Biophys. Acta 171, 157.

Murphy, P.M., Brady, T.G. and Boggust, W.A. (1969b) Biochim. Biophys. Acta 188, 341.

Mustafa, S.J. and Tewari, C.P. (1970) Biochim. Biophys. Acta 198, 93.

Nishihara, H., Akedo, H., Okada, H. and Hattori, S. (1970) Clin. Chim. Acta 30, 251.

Noonan, M. and Brady, T.G. (1969) Biochem. J. 115, 15p.

Northrop, J., Kunitz, M. and Herriot, R.M. (1948) Crystalline Enzymes. Columbia University Press.

O'Brien, E. (1968) M.Sc. Thesis National University of Ireland.

Olson, J.A. and Anfinsen, C.B. (1952) J. Biol. Chem. 197, 67.

Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, 321.

Penefsky, H.S. and Warner, R.C. (1965) J. Biol. Chem. 240, 4694.

Pfrogner, N. (1967) Archives Biochem. Biophys. 119, 141.

Phelan, J., McEvoy, F., Rooney, S. and Brady, T.G. (1970) Biochim. Biophys. Acta 200, 370.

Polson, A., Potgieter, G.M., Largier, J.F., Mears, G.E.F. and Joubert, F.J. (1964) Biochim. Biophys. Acta 82, 463.

Porath, J. and Flodin, P. (1959) Nature 183, 1657.

Powell, J.F. and Hunter, J.R. (1956) Biochem. J. 62, 381.

Purzycka, J. (1962) Acta Biochim. poln. 9, 83.

Rao, G.J.S. and Ramachandran, L.K. (1962) Biochim. Biophys. Acta 59, 507.

Raymond, S. and Weintraub, L. (1959) Science, 130, 711.

Reithel, F.J. (1963) Advances in Protein Chemistry 18, 123.

Rich, M.A., Geldner, J. and Meyers, P. (1965) J. Nat. Cancer Inst. 35, 523.

Rockwell, M. and Maguire, M.H. (1966) Mol. Pharmacol. 2, 574.

Roush, A.H. and Betz, R.F. (1956) Biochim. Biophys. Acta 19, 579.

Rutishauser, U., Cunningham, B.A., Bennet, C., Konigsberg, W.H. and Edelman, G.M. (1968) Proc. Nat. Acad. Sci. U.S.A. 61, 1414.

Saha, A., Sawh, V.L. and Chen, A.B. (1969) Arch. Biochem. Biophys. 133, 178.

Sanger, K.C.S. and Mustafa, S.J. (1971) Biochem. Biophys. Res. Comm. 43, 1056.

Schmidt, G. (1928) Hoppe-Seyl. Z. 179, 243.

Schneider, W.C. and Hogeboom, G.H. (1952) J. Biol. Chem. 195, 161.

Schroeder, W.A., Shelton, J.R., Shelton, J.B. and Olson, B.M.(1964) Biochim. Biophys Acta 89, 47.

Shapiro, A.L., Scharaff, M.D., Maizel, J.V. Jr., and Ohr, J.W. (1966) Proc. Nat. Acad. Sci. 56, 216.

Shapiro, A.L., Vinuela, E. and Maizel, J.V. (1967) Biochem. Biophys. Res. Comm. 28, 815.

Shapiro, A.L. and Maizel, J.V. Jr. (1969) Analyt. Biochem. 29, 505.

Sheys, G.H. and Doughty, C.C. (1971) Biochim. Biophys. Acta 235, 414.

Sia, C.L. and Horecker, B.L. (1968) Biochem. Biophys. Res. Comm. 37, 731.

Siegler, R., Geldner, J. and Rich, M.A. (1964) Cancer Res. 24, 444.

Sim, M.K. and Maguire, M.H. (1971) Eur. J. Biochem. 23, 17.

Smithies, O. (1955) Biochem. J. 61, 529.

Spande, T.F. and Witkop, B. (1967) Methods in Enzymology 11, 506.

Spikes, J.D. and Livingston, R. (1969) Adv. Radiation Biol. 3, 29.

Stern, H. and Mirsky, A.E. (1953) J. gen. Physiol. 37, 177.

Straub, F.B., Stephaneck, O. and Acs, G. (1957) Biokkimiya 22 118.

Tanford, C. (1962) Brookhaven Symp. Biol. 17, 154.

Tanford, C., Kawahara, K. and Lapanje, S. (1967) J. Amer. Chem. Soc. 89, 729.

van Holde, K.E. (1966) in Hayashi, T. and Szent-Gyorgyi, A.G.(editors), Symposium on molecular architecture in Cell Physiology, Prentice-Hall, Inc. New Jersey. Englewood, Cliffs, p. 81.

Vinuela, E., Algranati, I.D. and Ochoa, S. (1967) Eur. J. Biochem. 1, 3.

Wagner, R.P. and Mitchell, H.K. (1948) Arch. Biochem. 17, 87.

Warburg, O. and Christian, W. (1941) Biochem. Z. 310, 384.

Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406.

Westerfield, W.W., Richert, D.A. and Bloom, R.J. (1959) J. Biol. Chem. 234, 1889.

Westhead, E.W. (1965) Biochemistry 4, 2139.

Whiteley, H.R. (1966) J. Biol. Chem. 241, 4890.

Wills, E.D. (1952) Biochem. J. 50, 421.

Yamazaki, H. and Kaesberg, P. (1963) J. Mol. Biol. 7, 760.

Zittle, C.A. (1946) J. Biol. Chem. 166, 499.