STUDIES ON
SOME BIOLOGICAL REACTIONS OF
AGRICULTURAL INTEREST

Summary of

THESIS

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by

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The general introduction outlines briefly, with particular reference to ribonucleases, the character of the enzymes which depolymerise nucleic acids. The publication by H. S. Kaplan and L. A. Heppel in J. Biol. Chem. 222 907 (1956) that a heat stable ribonuclease M.W. 2,000 - 5,000 could be isolated from calf spleen led to the research work presented in this thesis. These workers reported the purification of a ribonuclease similar to pancreatic ribonuclease in heat stability and specificity. Publications by other workers revealed that several ribonuclease activities could be extracted from calf spleen. The work reported here describes the procedures taken to purify the low molecular weight ribonuclease.

Since several ribonucleases were reported, the original purification scheme of Kaplan and Heppel was adhered to initially. Section I outlines this procedure and the salient points are highlighted. In addition to a heat treatment there were four fractionations by conventional precipitation techniques, a lengthy dialysis and an Amberlite resin treatment.

Section II, in addition to drafting the criteria for enzyme isolation and purification, reports the investigation of the techniques used originally by these workers. The unfavourable results obtained are presented in detail. Although an equivalent purification was achieved, the yield of heat stable ribonuclease activity was poor. Each of the steps rejected $\simeq$ 50% of the activity. With the exception of the heat treatment which was considered essential, this original system was abandoned in favour of preliminary fractionation and concentration by precipitation with ammonium sulphate before and

after the heat treatment. In this way ≃ 75% of the heat stable activity was concentrated ready for molecular sieve and ion exchange chromatography.

Section III reports the chromatography procedures undertaken to develop a purification scheme for the heat stable spleen ribonuclease. Some early experiments on the gel filtration behaviour of the active sample, particularly with respect to pancreatic ribonuclease, are described. Gel filtration on a Sephadex G-75 column, 5cm x 75cm, was developed to desalt the crude spleen sample. This measure eliminated the lengthy dialysis and achieved a complete recovery of activity with some purification. The desalting technique was followed by ion exchange chromatography.

Chromatography on a Carboxymethyl-cellulose column fractionated the heat stable sample into two ribonuclease active peaks "A" and "B". A satisfactory adsorption of the crude sample on the C.M. cellulose was difficult to effect initially thus the sample was reduced by passing it through Diethylaminoethyl-cellulose as a pretreatment. All the ribonuclease activity passed through the column leaving ≃ 37% of the contaminants adsorbed. The preliminary column work necessary to achieve the fractionation on C.M. cellulose using a gradient elution system is described. This column method was scaled up ten-fold to cope with the large quantity of crude spleen preparation and prepare sufficient amounts of the active peaks "A" and "B" for rechromatography.

Rechromatography on Carboxymethyl-Sephadex revealed an elution

irregularity at the chromatography on C.M. cellulose. Although ribonuclease active peak "B" was eluted as a single peak, the active peak "A" on rechromatography split into two peaks, one of which was eluted at a similar position to active peak "B". This indicated a distribution of activity for peak "A" similar to the chromatography of the crude preparation. On subsequent chromatography the rechromatographed peak "A" did not split again. These results indicated that calf spleen contains two heat stable ribonucleases.

The activity "A" amounted to 16% of the total heat stable ribonuclease as determined by the general assay method. This activity was shown to be as heat stable as the bulk of the preparation. Attempts are made to explain the irregular chromatography effect.

Section IV outlines the merit of disc electrophoresis on polyacrylamide gels as a technique for estimating the purity of protein samples. The spleen ribonuclease fractions "A" and "B" were examined by this technique. It was demonstrated that active fraction "B" had been extensively purified and had an electrophoretic mobility similar to pancreatic ribonuclease. Active fraction "A" though considerably purified contained at least three contaminants.

An estimate of the molecular weights of the two ribonucleases is presented in Section V. A linear relationship exists between elution volume and log. (molecular weight) for globular proteins at gel filtration. To carry out the estimation, a Sephadex G-75 column was calibrated by protein standards of known molecular weight and gel filtration behaviour. After determining the elution volumes for the

heat stable spleen ribonucleases, molecular weights of $\simeq$ 24,000 and 10,000 were attributed to activity "A" and activity "B" respectively. No evidence could be found to support the previous report that a heat stable ribonuclease M.W. 2,000 - 5,000 was present in calf spleen.

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P R E F A C E .

The experimental work described in this thesis was carried out in the Agricultural Chemistry Section of the Chemistry Department, University of Glasgow, under the direction of Dr. W. R. Rees, from April 1961 to October 1966.

During this time I was a member of the teaching staff in the Agricultural Chemistry Section.

I wish to express my grateful thanks to Professor J.M. Robertson, C.B.E., F.R.S., who generously made available the facilities in the Chemistry Department as well as those in the Agricultural Chemistry Section.

I would like to record my sincere thanks to Dr. W. R. Rees, who initiated and supervised this work. I am grateful to Dr. Rees for his constant advice, inspiration and guidance in the general approach to scientific research.

I am indebted to Mr. T. Baird for photographic reproductions included in this thesis and Mr. J. J. Brown for constructing the disc electrophoresis power pack.

C O N T E N T S .

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ABBREVIATIONS USED

R.N.A.	Ribonucleic acid
D.N.A.	Deoxyribonucleic acid
RNase	Ribonuclease
Poly-U	Polyuridídylic acid
Poly-A	Polyadenylic acid
Poly-C	Polycytidylic acid
Poly-G	Polyguanylic acid
Poly-I	Polyinosinic acid
E.DTA,	Disodium salt of ethylenediamine-tetraacetic acid
T.C.A.	Trichloracetic acid
Tris-	tris(hydroxymethyl)aminomethane
C.M.-	Carboxymethyl-
DEAE-	Diethylaminoethyl-
M or M.W.	Molecular weight
R.T.	Room temperature
	All temperatures are expressed in degrees centigrade
I.d.	Internal diameter
O.D.	Optical density
G.M.	General method
A.R.	Analar reagent grade

General Introduction

Enzymes that catalyse the depolymerization of nucleic acids through the cleavage of phosphodiester linkages are widely distributed in nature. It is unlikely that the action they manifest *in vitro* operates *in vivo* without control. There appears to be little degradation of nucleic acids going on in most cells, except in the turnover of that fraction thought to contain messenger RNA [1,2,3]. Some investigators have found that there is a correlation between nuclease activity and the rate of cell growth and development. Consequently, it is not without justification that many workers have sought to show that the abundant variety of nucleases have a significant role in cell function other than the purely degradative action as diagnosed by *in vitro* assay [4,5,6,7].

The multiplicity of ribonucleases in nature suggests a divergence in structure of the enzymes from one type to another, which may be reflected in their catalytic properties. Some, from different tissues, resemble each other closely in properties and action, whilst others seem only to have in common the ability to depolymerise RNA [8,9,10,11,12,13,14]. This picture is complex in that some tissues contain several different kinds of ribonucleases. The purpose of such an intricate arrangement has yet to be discovered.

The nomenclature and classification of the nucleases proposed by the International Union of Biochemistry [15] in 1964 divides them into two basic groups. This division is necessary because nucleic

acids can be cleaved by two essentially dissimilar mechanisms, although the products are often similar after the reactions are complete.

These nucleases are either:

(i) the transferases which act by transferring phosphorus-containing groups to bring about the depolymerization of RNA (page 3)

or (ii) the hydrolases which act by hydrolysing the phosphoric diester bonds to bring about the depolymerization of RNA and/or DNA (page 13).

The confusion arising from the fact that pancreatic ribonuclease could be classed as a phosphodiesterase and as a ribonuclease while other enzymes carried the name phosphodiesterase has therefore been eliminated, removing considerable nomenclature difficulties. The term phosphodiesterase was originally used for most nucleases that cleaved phosphodiester bonds by hydrolytic action. According to the new classification, ribonucleases now belong to the transferase group and the term phosphodiesterase is reserved exclusively to describe certain enzymes which hydrolyse nucleic acids e.g. spleen phosphodiesterase [26].

Nucleases can also be classified depending on whether:

A they react on RNA and/or DNA as substrate

B their mode of action is that of an endonuclease or exonuclease

C the products formed are exclusively the 5', 3' or 2' monophosphate esters, or the appropriate cyclic phosphate esters

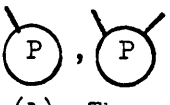
D there is an absolute specificity for the cleavage of particular phosphodiester linkages directed by the type of nucleoside moiety on the phosphodiester chain

The classification becomes evident when each group is considered in greater detail later (pages 3 - 15).

Ribonucleases and deoxyribonucleases are endonucleases in that they attack the molecule of nucleic acid by splitting the linkages for which they are specific, located at points anywhere on the chain. As the attack progresses, fragments of intermediate size are formed and these in turn become the substrate until all sensitive bonds are cleaved. Exonucleases which include the phosphodiesterases of spleen and snake venom, for example, attack the chain of nucleic acid by a consecutive splitting off of mononucleotides from one end of the chain. It is believed that there is a preference for the terminal residues of nucleic acids, though this preference is not absolute [16]. Nucleases attack RNA and DNA polymers, though a preference may be exhibited for a particular substrate [16,145] or substrate state [17, 18,174].

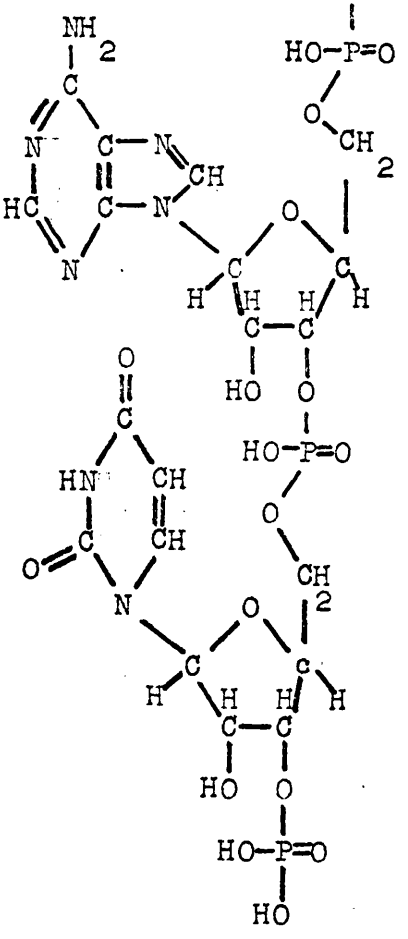
Depolymerization by transferase action

The enzymes that bring about the depolymerization of RNA by transferase action liberate mononucleotides and sometimes oligonucleotides. They act by transferring the 3' phosphate of the nucleotide residue of the polymer, from the 5' position of the adjoining nucleotide to the 2' position of the nucleotide itself forming a cyclic phosphate, resulting in the liberation of the free

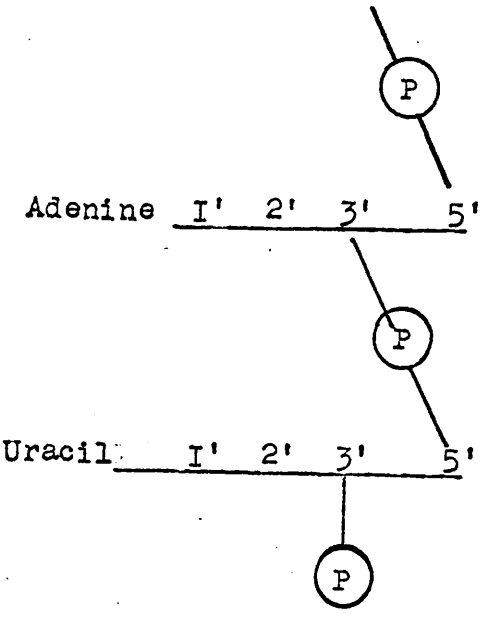
nucleotide or oligonucleotide. Further action can take place in that the transfer of the phosphate group from the 2' position in the cyclic phosphate to water is catalysed. The various enzymes of this group differ by virtue of their specificity requirements for different bases in the substrate chain. To illustrate transferase action on RNA it is convenient to cite examples of enzymes whose modes of action are sufficiently well established to allow the assignment of systematic names. In the diagrams that follow, to illustrate these points a portion of an RNA chain is represented by referring to the base by name, the ribose by straight lines upon which are marked the functional hydroxyls according to the carbon atom involved and the mono, cyclic and diesterified phosphate by  , and  respectively (Fig.1).

(1) The enzyme coded by the numbers E.C. 2. 7. 7. 16 and systematically defined by the International Commission on enzymes [15] as the ribonucleate pyrimidine-nucleotido-2'-transferase (cyclising) from bovine pancreas is involved in the specific transfer action represented diagrammatically in Fig. 2. The enzyme is commonly known by its trivial name as bovine pancreatic ribonuclease [50]. It is now recognised that pancreatic ribonuclease brings about the reaction in two consecutive steps. There is a transesterification to a 2' 3' cyclic diester and the breakdown of this diester to a 3' mono-nucleotide [19]. The attack is specific to phosphodiester linkages which are attached to pyrimide nucleosides at the 3' position. This statement imples that the linkages between pyrimidine nucleoside 3' phosphoryl groups and the 5' hydroxyl groups of adjacent purine or

Fig.I.

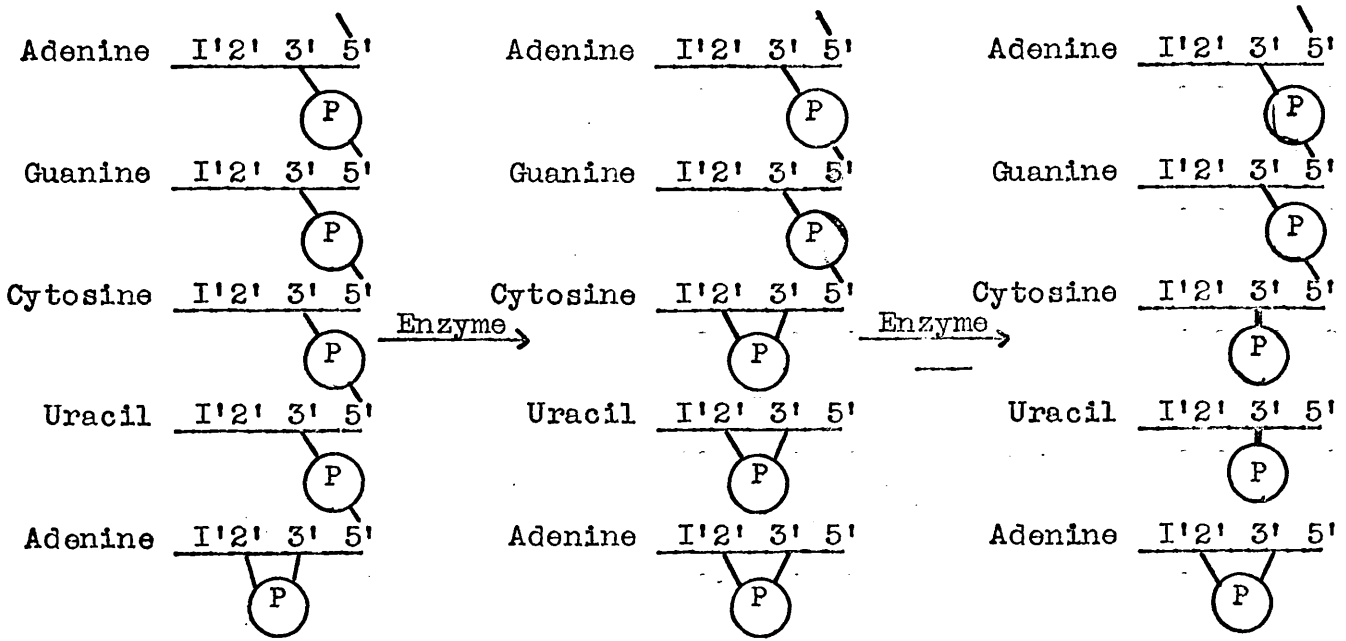


Dinucleotide section of R.N.A. polymer.



Diagramatic representation of R.N.A. polymer.

Fig.2.

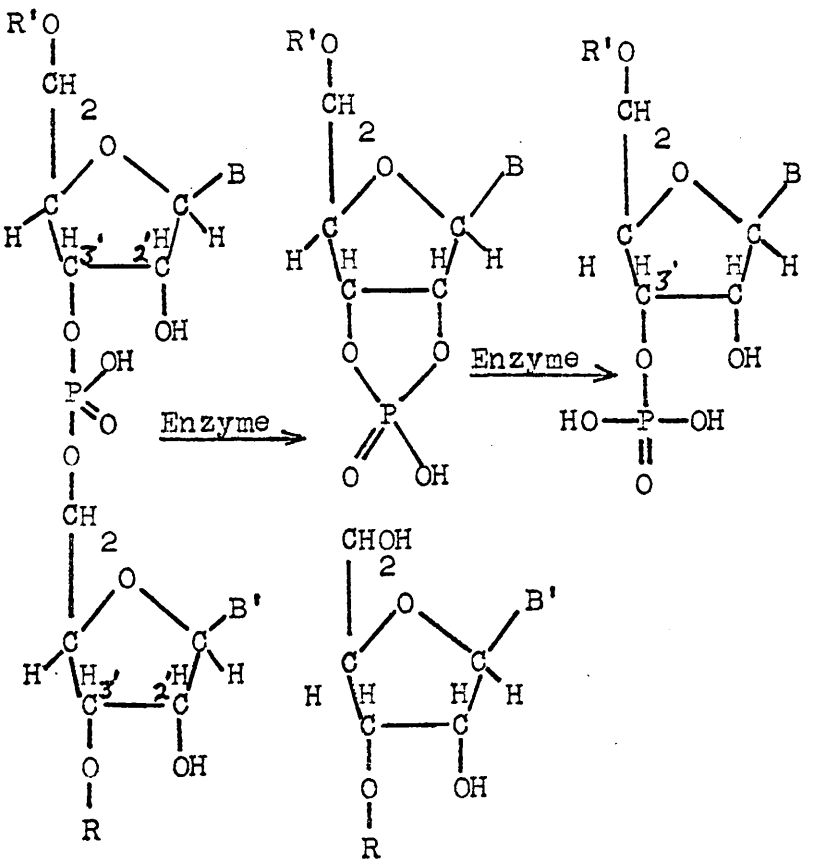


Diagramatic representation of the cleavage of R.N.A. by Pancreatic Ribonuclease.

pyrimidine nucleotides are cleaved. Neither the linkage between adjacent purine nucleotides or those between purine nucleoside 3' phosphoryl groups and 5' hydroxyl groups of adjacent pyrimidine nucleotides are cleaved. At cleavage the 3' phosphate is rapidly transferred from the 5' position of the adjacent nucleotide to the 2' position to produce an intermediate cyclic 2' 3' phosphate of the pyrimidine nucleotide. This transfer is followed more slowly by the transfer of the 3' phosphate from the 2' position to water to yield the pyrimidine nucleoside 3' phosphate only. It can be observed in the more detailed Fig. 3 where B represents the pyrimidine base uracil or cytosine and B_1 is any of the four bases uracil, cytosine, adenine or guanine, that the transfer described above takes place as depicted. Where B is the purine base adenine or guanine, no cleavage takes place irrespective of whether B_1 is a purine or pyrimidine base. Purine nucleoside 2' 3' cyclic phosphates are not substrates for the transfer reaction to water [139,143,144]. The products of the transfer are, therefore, uridine 3' phosphate and cytidine 3' phosphate representing a large proportion of the pyrimidine content of the RNA. Di -, tri - nucleotides and oligonucleotides, forming the "resistant core" to complete enzymic cleavage, are composed of purine nucleosides terminating in either uridine 3' phosphate or cytidine 3' phosphate. Each "limit" oligonucleotide contains one pyrimidine nucleotide at the terminal position because the phosphodiester linkage between the 3' phosphate of the purine nucleoside and 5' hydroxyl of the pyrimidine nucleo-

Fig.3.

Phosphotransferase Action



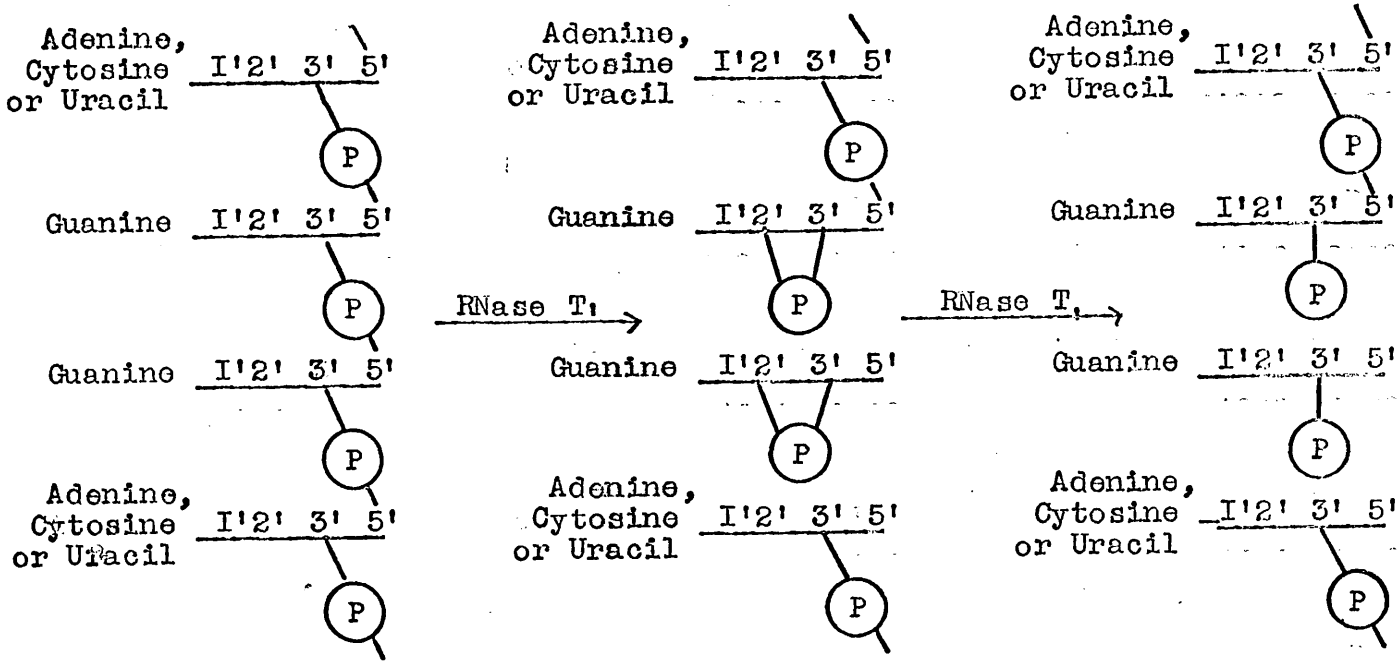
Cleavage of the pyrimidine nucleoside phosphodiester linkage by the two stage action of Pancreatic Ribonuclease.

tide cannot be cleaved. All other phosphodiester linkages are between purine nucleotides.

(2) The enzyme E.C. 2. 7. 7. 26, ribonucleate guaninenucleotide-2'-transferase (cyclising) from Aspergillus oryzae, better known as taka-diastase ribonuclease T_1 [31], acts on RNA similarly to pancreatic ribonuclease but transfers only at the 3' position of the guanylate residues. This guanyloribonuclease transfers the 3' phosphate of the guanosine nucleotide residue of a poly-nucleotide from the 5' position of the adjacent nucleotide to the 2' position of the guanosine residue forming a cyclic nucleotide. The catalysis of the transfer of the phosphate from the 2' position in the cyclic phosphate to water follows. The overall reaction (Fig.4) brings about RNA depolymerization with the production of guanylic acid monomers and oligonucleotides bearing terminal guanylic acid residues on completion of the reaction. Taka-diastase contains other nuclease activities [31,20].

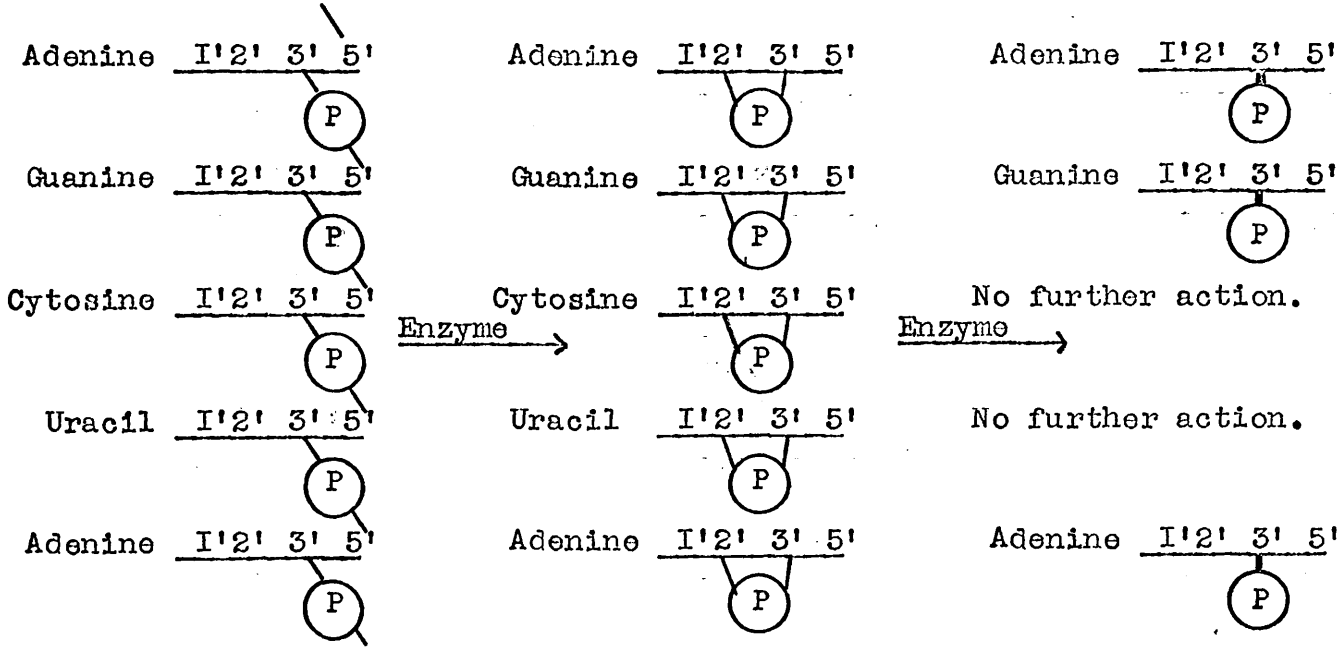
(3) Other ribonucleases which are classed as ribonucleate nucleotido-2'-transferase (cyclising) bring about the complete depolymerization of RNA, no "core" material remaining. Phosphate transfer occurs at all the internucleotide linkages. Differences are exhibited in the rates of phosphate transfer at particular internucleotide linkages, in that certain nucleoside phosphate groups are transferred much more rapidly than others [21,23] and can be terminated at the cyclic phosphate stage with no further transfer to give the free nucleotide (Fig.5). Ribonucleases from

Fig.4.



Diagrammatic representation of the cleavage of R.N.A. by Guanyloribonuclease

Fig.5.



Diagramatic representation of the cleavage of R.N.A. by certain unspecific ribonucleases

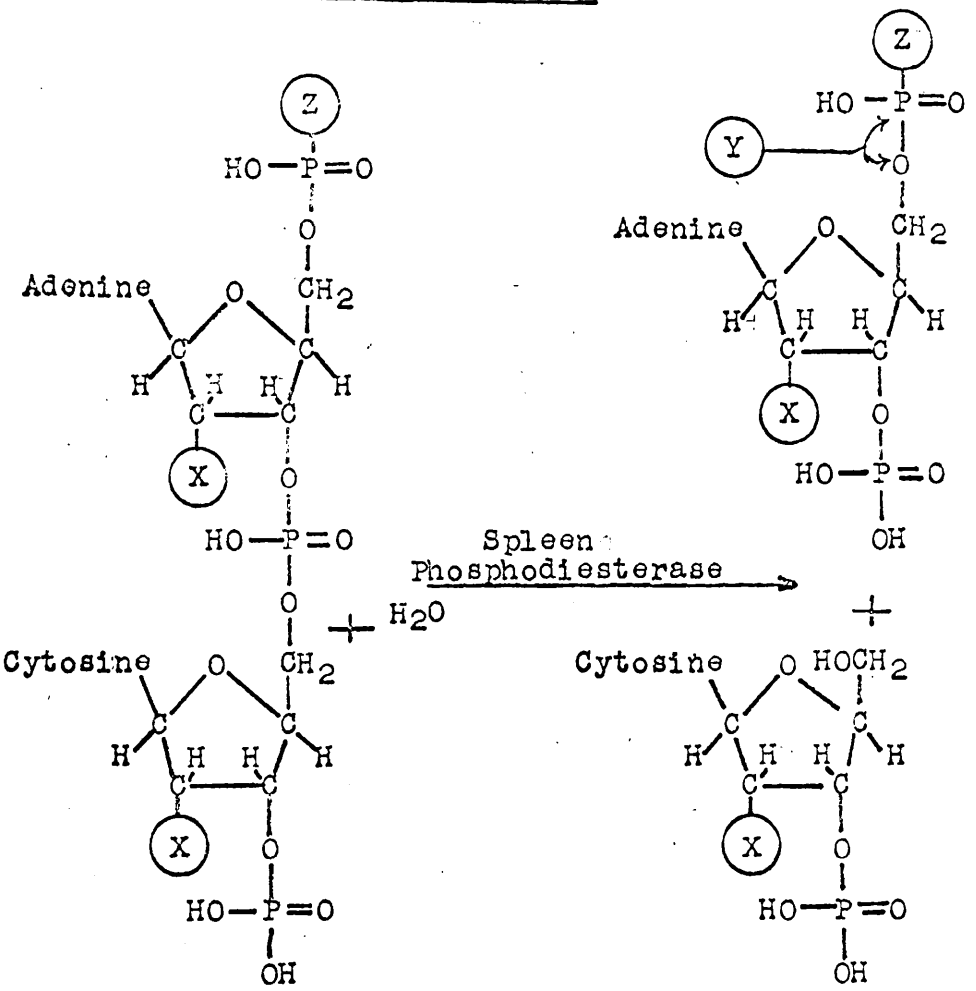
pea leaves [21] and from tobacco leaves [22,23] are involved in transfer at all the internucleotide phosphate linkages. From the 2' 3' cyclic mononucleotides formed, only those with purine bases are involved in further transfer action. The 3' phosphates of the 2' 3' cyclic purine nucleotides are transferred from the 2' position to water. Rate specificity for internucleotide linkages is exhibited. Guanylic acid is released at the fastest rate while cytidylic acid is released at the slowest rate of all. Another ribonuclease from soya bean [24] liberates the adenylate residue before the guanylate residue and differs in specificity from the two plant ribonucleases already mentioned, only in this respect. Spinach ribonuclease [14] brings about the depolymerization of RNA by the complete transfer of 3' phosphate from the 5' position to the 2' position followed by the further transfer of the 3' phosphate of all the cyclic mononucleotides thus formed, from the 2' position to water. The rate of mononucleotide release is in the order guanylate > adenylate > uridylate > cytidylate. The ribonuclease of Bacillus subtilis [25] splits the 3' 5' phosphodiester bond of polynucleotides by transfer as already described. In experiments using synthetic substrate polymer containing a single base, the rates of transfer drop in the order poly G > poly I > poly A > poly U > poly C by a difference in magnitude of x 10 to x 100 between each of them. The products of this transfer are first of all oligonucleotides ending in 2' 3' cyclic phosphate esters then finally 3' guanylic acid, 3' inosinic acid and the 2' 3' cyclic phosphates of the other nucleosides.

Depolymerization by hydrolase action

The hydrolase enzymes which catalyse phosphodiester hydrolysis resulting in nucleic acid depolymerization, are as varied as the ribonucleate nucleotide-2' transferases, (ribonucleases). The spleen [11,26] and snake venom [16] phosphodiesterases are unspecific and both RNA and DNA are attacked. They belong to the orthophosphoric diester phosphohydrolase group of enzymes [15]. They attack the nucleic acid by a consecutive splitting off of mononucleotides from one end of the chain (page 14), and the action of spleen phosphodiesterase is represented in Fig. 5A as an example. Of the four nucleotide bases possible in any nucleic acid chain, adenine, guanine and cytosine are common to both DNA and RNA, uracil is found only in RNA and thiamine is found only in DNA. In Fig.5A, X represents H or OH depending on whether the polynucleotide is DNA or RNA respectively, Y is the next bond which is hydrolysed in the consecutive action while Z represents the rest of the nucleic acid chain. In the general reaction phosphoric diester + water → phosphoric monoester + an alcohol, the spleen enzyme forms the 3' mononucleotides and the venom enzyme the 5' mononucleotide from polynucleotides.

The deoxyribonucleases belong to this class of hydrolase. The enzyme designated E.C. 3. 1. 4. 5. deoxyribonucleate oligonucleotidohydrolase cleaves DNA to yield oligonucleotides [27,28]. Deoxyribonuclease II systematically named deoxyribonucleate 3' nucleotidohydrolase forms 3' nucleotides from DNA [27,28].

Fig.5A. Hydrolase Action



i.e.

Terminal phosphodiester bond of any polynucleotide. + Water. → Polynucleotide chain ending in phosphoric monoester ONE nucleotide less in length. + Primary alcohol on 5' Of the mononucleotide released.

Micrococcal nuclease [27] attacks RNA and DNA forming 3' nucleotides and DNA is attacked with a preference for the adenine thymine nucleotide pair. Azotobacter nuclease [29] attacks RNA and DNA forming 5' nucleotides. These nucleases are grouped as ribonucleate (deoxyribonucleate) 3' nucleotidohydrolase and 5' nucleotidohydrolase respectively.

Some of the aspects of phosphotransferase enzymes are reviewed in Annual Reviews of Biochemistry, Volume 33, by J. P. Hummel and G. Kalnitsky [30], Progress of Nucleic Acid Research and Molecular Biology, Volume 2, by Herbert Witzel [19]. The ribonucleases of Taka-diastase have been recently reviewed in Progress in Nucleic Acid Research and Molecular Biology, Volume 3, by Fugio, E., Kenki, T., and Tsuneko, U.[31]. Much wider reviews on the enzymes responsible for the cleavage of phosphate esters are set out in Volume 5 of The Enzymes [27].

The paragraphs already presented outline, very briefly in some instances, the magnitude of the field of enzymatic study related to nucleic acid degradation. In the following account, which sets out the premises on which the work described in this thesis was founded, ribonuclease enzymes only will be discussed and no further direct reference to the other nucleases will be made.

Several intracellular ribonucleases have been examined since it was established that ribonucleases from other sources can differ from pancreatic ribonuclease in many of their properties. The ribonuclease T_1 from Aspergillus oryzae and the plant ribonucleases

from pea, soya bean and spinach already briefly described are suitable examples from other sources. Two intracellular ribonuclease activities from animal tissue, e.g. in kidney, liver and spleen, are described by Zytko, de Lamerande, Cantero and Allard [32], Roth [33], Stevens and Reid [34] and Maver and Greco [35]. The ribonuclease activities are differentiated on the basis of pH optimum, one exhibiting optimal activity in the acid range (pH 5.2-5.8) the other in the alkaline range of pH (7.8-8.2) using different ribonuclease assay methods [33]. The terms "acid ribonuclease" and "alkaline ribonuclease" are used to denote this property and it appears that these ribonucleases can be further differentiated. The "acid ribonucleases" are heat and acid labile while the "alkaline ribonucleases" are heat and acid stable. A further complication exists, however, in that other ribonuclease activities localised in, or associated with cell particles may be different from those already described as intracellular "acid and alkaline ribonucleases" [33,138,140,142]. It has been shown that "alkaline ribonuclease" cleaves high molecular weight RNA more rapidly than partially degraded commercial yeast RNA, whereas the reverse appears to be true of the acid ribonuclease [34] and conflicting reports have appeared on their modes of action [34,36]. It is of interest that Roth [10,37,38] has shown that the "alkaline ribonuclease" in liver is frequently associated with a natural inhibitor which may be destroyed by acid, heat and mercuribenzoate treatment. This inhibitor appears to be a highly labile protein [37,39].

H. S. Kaplan and L. A. Heppel [8] have prepared from calf spleen an intracellular ribonuclease which closely resembles pancreatic ribonuclease in its catalytic properties and heat stability. According to Kaplan and Heppel's studies the enzyme does not depolymerise RNA completely. By a cleavage similar to pancreatic ribonuclease action, pyrimidine mononucleoside 3' phosphates and pyrimidine nucleoside 3' phosphate terminated oligonucleotides are products of the reaction. The enzyme differs from pancreatic ribonuclease in its more acid pH optimum on RNA as substrate and in its behaviour on the cation exchange resin amberlite I.R.C. 50. (X.E.64) where it behaves as a more basic entity. The paper [8] also presents a report on an ultracentrifuge examination of the purest sample of the enzyme and shows the preparation to exhibit a very low molecular size compared to that of pancreatic ribonuclease which is of M.W. $\simeq$ 13,700. To quote from the paper "Under the conditions of centrifugation a single slowly sedimenting, rapidly spreading boundary, separated from the miniscus only partially after 240 minutes. The sedimentation constant calculated from this run was 0.6×10^{-13} (S.20.W.). Without attempting to measure quantitatively the boundary spreading, there was no evidence of asymmetry and it was rapid enough to be compatible with a small particle of perhaps M = 2,000-5,000. At a higher concentration of protein no heavier components could be detected in a second trial carried out. Samples taken from the top, centre and bottom of the cell showed no gross variation in specific

activity indicating that the boundary seen in the centrifuge represented the active principle or at least was closely associated with it."

Maver et al.[36,40] are also involved in the study of ribonucleases of calf spleen and have prepared an "acid" and "alkaline ribonuclease" from this source by a very different method to that of Kaplan and Heppel [8]. Heat treatment of these ribonuclease activities at 70° for 10 minutes at pH 4.2 show the "alkaline ribonuclease" to be much more stable than the "acid ribonuclease". The purified "acid ribonuclease" lost 85% of its activity compared with a 20% loss of activity of "alkaline ribonuclease" under the same conditions. In contrast, Kaplan and Heppel [8] destroyed 70% of the ribonuclease activity of the homogenate in the initial heat treatment step of their purification procedure by heating at 60° for 10 minutes at pH 3.5. Specificity trials by Maver et al.[40], Maver and Greco [36], on both spleen ribonuclease activities from their purification procedure show the liberation of cyclic and non-cyclic purine and pyrimidine nucleotides in such quantities that a preferential cleavage of the phosphodiester linkages is suggested. They conclude [36] that since their chromatographically purified ribonucleases cleave RNA to purine and pyrimidine nucleotides, they differ from pancreatic ribonuclease and from the ribonuclease of spleen as isolated by Kaplan and Heppel [8].

A much more detailed account of the findings of the two groups who have published reports on the ribonucleases of spleen will be

presented in a later section of this thesis. Sufficient evidence is published [8,36,40,41] on the ribonuclease activities of calf spleen to suggest that the tissue is a source for several different ribonuclease active components. A further examination of the ribonuclease fraction of calf spleen is justifiable from several points of view.

(1) Molecular size

The report from W. R. Carroll in the appendix to Kaplan and Heppel's paper [8], that the spleen ribonuclease could be of molecular weight 2,000 to 5,000 a value $\simeq$ 30% that of pancreatic ribonuclease, the smallest active enzyme extensively studied structurally, warranted further investigation. If this were true then the consequences could be highly interesting. Structural examination of the entity by X-ray crystallography would be less complicated. The molecule comparable in size with a polypeptide of some 30-40 units would present a much more interesting species for sequence analysis and synthesis studies and would presumably be preferable to existing active macromolecular proteins for these purposes.

(2) Specificity

The considerable advance in our knowledge of the structure, function and amino acid sequence of proteins owe a great deal to the selective way in which certain proteases [172], e.g. trypsin and chymotrypsin, can break down proteins into simpler well defined moieties for further study. The peptide linkages at which a protease may act are very limited. For instance, trypsin hydrolyses

peptide bonds involving the carboxylic group of L-arginine and L-lysine. On the other hand, chymotrypsin hydrolyses peptide bonds especially those involving the carboxylic group of aromatic L-amino acids. In the first instance steric restrictions about the molecule and peptide bond regulates the access of the protease. The amount of cleavage will depend on the presence of suitable linkages which is governed by the position, and sequence permutation of the twenty or so different amino acids that could be the constituents of a protein chain. This means that there are very many different types of linkage available for specific cleavage. By the successive use of many protease enzymes on a particular protein species, well defined protein fragments are made available for study.

By direct comparison, the RNA molecule with a varied permutation of only four monomer units throughout the chain, offers less of a variety of specific inter unit linkage for cleavage attack. Because of the extensive chain length with limited unit content, a greater requirement for a singular specificity in cleavage rather than non-specificity exists. Pancreatic ribonuclease and Guanyloribonuclease are the only ribonucleases available with singular specificity (General Introduction). Enormous advances in sequence elucidation will be promoted if an activity with similar singular specificity is uncovered. As already pointed out without giving much detail, the specificities exhibited by the preparations from calf spleen tissue appear to be of a

complex nature. Any ribonuclease is of potential value if it can assist in the determination of nucleotide sequence. The concerted effort to determine the complete nucleotide sequence of purified RNA, especially particular polymers responsible for a specific amino acid transfer have already produced an accumulation of techniques based on enzymatic and chromatographic procedures [146,147]. Any addition to the battery of useful enzymes, where the basic operation is the enzymatic cleavage of RNA into specific oligonucleotides which are then separated and characterised, would be of the greatest value. The chances of an unequivocal sequence determination are greater the more different ways there are to split RNA and this depends on the degradative specificities of the ribonucleases available. Thus an enzyme with a new specifity would be very useful to supplement existing specificities and all the alternative approaches to RNA sequence studies.

Scope of the present work

The study of the properties of an enzyme in impure preparations is fraught with difficulties and though some conclusions may prove to be correct, the inaccuracies and inherent uncertainties of such an approach are obvious. This investigation was concerned with the attempts to purify extensively the calf spleen ribonuclease described by Kaplan, Heppel and Carrol [8]. The approach decided on as first priority after the preliminary investigation, was to use the best protein purification systems currently available to their limit in order to obtain pure samples

before trying to ascertain any of the enzymes properties. This course of action was essential, since the reports available on calf spleen ribonucleases were conflicting. Maver and Greco [36] did not locate the heat stable ribonuclease enzyme purified by Kaplan and Heppel [8] from spleen tissue. However, Maver and Greco's [36] spleen preparation contained a heat stable "alkaline ribonuclease" activity. Accordingly there should be two heat stable ribonuclease activities in calf spleen (page 18).

Two other ribonuclease active components are the heat labile "acid ribonuclease" described by Maver and Greco [36] and the spleen phosphodiesterase described by Hilmoe [42]. Since Maver and Greco did not report a third ribonuclease active component in their spleen preparation, and four components of spleen tissue ought to exhibit ribonuclease activity, it was considered essential to retain the initial extraction and purification steps put forward by Kaplan and Heppel [8]. In this way the low molecular weight ribonuclease component exhibiting pancreatic ribonuclease specificity should be located and retained. Much of the preliminary research undertaken was based on the extraction and several purification steps outlined by Kaplan and Heppel [8]. This work was examined in detail. Several alterations were made as a consequence of the experimental findings and observations, and these are presented towards the end of section II. These same alterations in the extraction and purification steps were adopted to suit the purification techniques introduced later and described after section II.

The report on the investigations carried out is presented in five sections.

Section I is devoted to an abstract of the purification procedure reported in the paper by Kaplan and Heppel [8]. The important features of the methods are outlined to facilitate the discussion and comparison presented in section II.

Section II describes the results of the early extraction and purification methods of Kaplan and Heppel [8]. From such information the methods for obtaining enzyme samples suitable for column chromatography purification procedures was developed.

Section III. An account is given of the trial chromatographic studies undertaken to purify the spleen heat stable ribonuclease sample. A scaled up procedure adopted to secure greater quantities of ribonuclease active sample for a rechromatography step is described. Two methods for the removal of specific inactive components from the enzyme sample with complete retention of the ribonuclease active components are described. This feature was implemented to reduce the load applied at cation exchange chromatography.

Section IV describes the technique adopted to estimate the extent of purification of the ribonuclease active samples procured by the fractionation on cation exchange columns.

Section V. An estimate of the molecular weight of the two heat stable ribonuclease active components is presented. Certain column elution irregularities were observed and are reported.

The significance of the experimental findings and observations made are discussed.

SECTION I

Abstract of the Procedure Presented by Kaplan and Heppel [8] for the Purification of Calf Spleen Ribonuclease

It will be apparent from what has already been written that it was of the greatest importance to carry out the procedure of Kaplan and Heppel to secure the ribonuclease they described. This was considered necessary because of the danger of inadvertently becoming involved in a purification of some other entity with ribonuclease activity also present in the spleen tissue [36,42]. Hence the following account is a fairly detailed synopsis of the extraction and purification steps described by Kaplan and Heppel. Information not directly quoted in their text concerning the purification steps, e.g. protein concentrations, the ribonuclease active content and the percentage of the total ribonuclease active content available or retained at each stage, is presented here. These values were calculated from the tabulated results of a typical fractionation experiment carried out by these workers. A complete interpretation is rendered difficult because various items of information obviously obtained by these workers have not been included in the text. Thus the acetone fractionation at stage VI is the only occasion in the procedure when a working protein concentration is specified.

(a) The Kaplan and Heppel procedure as described in J. Biol. Chem. 222, 907, 1956.

All operations were carried out at 0-3° unless otherwise directed.

Step I. Homogenization. Fresh calf spleen was brought to the laboratory carefully packed in ice. The outer capsular layer was removed; the spleen tissue cut into small pieces and homogenised in a Waring blendor with 3 volumes of 0.05M sodium acetate 10^{-4}M EDTA pH 7.2. This stage was completed within 2 hours.

Step II. Fractionation of the homogenate media at pH 3.5 by the direct addition of ammonium sulphate.

Three litre aliquots of the homogenate mixture were adjusted to pH 3.5 with 1N HCl. Each litre of acidified mixture was brought to 40% saturation by adding 266g of ammonium sulphate and the protein precipitate rejected at filtration overnight. Each litre of filtrate was brought to 80% saturation by the addition of 258g of ammonium sulphate. Filtration was repeated, the filtrate discarded and the precipitate dissolved in 0.05M sodium acetate 10^{-4} MEDTA pH 7.2.

Step III. Heat treatment at pH 3.5

The protein solution containing that fraction precipitated within the 40% to 80% saturation limits in Stage II was reacidified to pH 3.5 with 1N HCl and 500 ml. aliquots heated to 60° by immersion in a water bath at 80°. The mixture was retained at 60° for 10 minutes, cooled and the pH adjusted to 6.5-7.0 with 1N NaOH. Denatured protein was removed by centrifugation and washed by suspending it in 2 volumes

of 0.05M sodium acetate 10^{-4} MEDTA pH 7. The supernatant and wash solution were combined, then fractionated according to step IV.

Step IV. Fractionation of the heat stable protein at pH 7

The residual ammonium sulphate concentration, usually 10%, was determined with a Barnstead conductivity meter. The saturation was brought to 50% by the addition of usually 23.3g ammonium sulphate per 100 ml. and the precipitate discarded by centrifugation. The saturation of the supernatant was brought to 85% by adding 23g of ammonium sulphate per 100 ml. After centrifugation the precipitate was dissolved in 0.05M sodium acetate 10^{-4} MEDTA pH 7.2 and dialysed.

Step V. Dialysis

Rocking dialysis was carried out overnight against running 10^{-4} MEDTA. A granular brown precipitate had to be removed by centrifugation before the solution could be subjected to acetone fractionation at step VI.

Step VI. Acetone fractionation

The supernatant from the dialysed solution was diluted to contain from 6-10 mg protein per ml. 18 ml. of M. sodium acetate was added to every 72 ml. aliquots of the diluted protein solution. Sufficient acetone (72 ml.) at -10° was added to each sample to bring the solution to 44.4% acetone by volume at -15°. The precipitate was removed by centrifugation at 13,000 x g in polyethylene tubes for 3 minutes. By the further addition of 108 ml. acetone the supernatant solution was brought to 66.6% acetone by volume and the second precipitate retained at centrifugation. The solid was allowed to

drain for 30 minutes at -10° then dissolved in 0.05M sodium acetate 10^{-4} MEDTA pH 7.2 and fractionated according to step VII.

Step VII. Fractionation with solid ammonium sulphate at pH 2.

The saturation of the enzyme solution was brought to 30% by adding 16.4g ammonium sulphate per 100 ml. then the pH adjusted to 2 with 1N HCl. Three or four fractions were now collected by the stepwise addition of solid ammonium sulphate and most of the ribonuclease activity was precipitated between 70% and 80% saturation. The precipitates were dissolved in 0.05M sodium acetate 10^{-4} MEDTA pH 7.2.

Step VIII. Treatment with Amberlite IRC 50 (X E.64) Resin.

Fractions obtained by ammonium sulphate precipitation at pH 2 with specific activities of 20 units per mg. protein or more were combined and adsorbed on Amberlite IRC 50 (X E.64) as described by Hirs, Moore and Stein [43]. 20 to 35 ml. of enzyme solution containing 1 to 5 mg. protein/ml. was added to 4-5 ml. packed resin in a 50 ml. centrifuge tube. The active component was adsorbed on to the resin at pH 6.6 in the presence of 0.2M sodium phosphate buffer, which was then used to wash the resin. The concentration of the phosphate buffer was increased to 0.34 M at pH 7.4 and sufficient 2N NH_4OH was added to bring this buffer as supernatant in the presence of the resin to pH 7.4. The ribonuclease active enzyme was collected in the supernatant.

An alternative procedure listed was to apply the resin treatment described as Step VIII after Step IV, the fractionation with ammonium sulphate at neutral pH procedure. In this case the supernatant from

the resin treatment was fractionated with ammonium sulphate at [illegible] in Step VII. This modification permitted the elimination of dialysis and fractionation with acetone. Almost as good a purification, as recorded with the main procedure, was obtained. Kaplan and Heppel revealed that occasionally the specific activity was lower than usual after dialysis and fractionation with acetone but this was invariably compensated for by an improved purification in later stages. A second alternative treatment suggested is to use a 1.1 x 20.5 cm. column of Amberlite IRC 50 (X E.64). 2 ml. enzyme samples from the dialysis Step V were chromatographed with the equilibrating and eluting buffer 0.35M phosphate at pH 7, according to the method of Hirs, Moore and Stein [43]. Eluent fractions containing the ribonuclease active component were pooled and concentrated by precipitation with ammonium sulphate.

(b) Some details relating to the procedure are shown in Tables 1, 2 and 3 on pages 32 and 33.

Table 1 tabulates the important details of the various steps from the typical purification experiment outlined in a table by Kaplan and Heppel [8]. Ribonuclease units per ml. and protein concentration per ml., vertical columns 3 and 4 respectively, were calculated from the information supplied and now presented in columns 1, 2 and 5. It was found that a serious discrepancy existed in the value attributed by Kaplan and Heppel to the specific activity of the heat stable ribonuclease activity of the homogenate, Step 1a. The 700 fold overall purification which they claimed was based on this

value. The total heat stable ribonuclease activity of the homogenate was given as 55,000 units. This value is likely to be correct as it was obtained by direct assay of the homogenate after a heat treatment as described in Step III. The specific activity (degree of purity) of the heat treated spleen homogenate was given as 0.1 units per mg. protein and the total volume of homogenate was 20,200 ml. From these three values it can be calculated that the protein concentration of the homogenate after heat treatment is 27.23 mg. per ml., (vertical column 4, Step 1A, Table 1) which is exactly the same as that for homogenate not heat treated (vertical column 4, Step 1) at 27.23 mg. per ml. This is not possible. The volume of homogenate should not change at heat treatment, though the protein concentration should decrease considerably as heat labile proteins were precipitated. In the present study - 41% of the protein of the homogenate was eliminated by heat denaturation (page 75). This means that the claim of 0.1 units per mg. protein for the specific activity was underestimated and should in reality be nearer 0.2 units per mg. protein if almost 50% of the protein were heat labile. Certainly all of the protein could not be retained as illustrated. The data in Table 1 shows that if the protein content were lowered by 40% which is perfectly reasonable for the heat treatment the specific activity is 0.18. The overall purification claimed by Kaplan and Heppel and based on a specific activity of 0.1 to read 700 fold is not valid, and should be much less. An overall purification of 420 fold is more probable when an allowance of 40%

is made for the removal of protein by heat treatment and the specific activity is 0.18.

Tables 2 and 3 summarize the fate of the heat stable ribonuclease activity at the various stages of the purification procedure. The percentages are calculated directly from the total activity values presented in Table 1 (vertical column 2) and succinctly illustrate the extent of ribonuclease loss at each stage.

The significant features of the procedure are the heat treatment and the many fractionation steps, three by salting out with ammonium sulphate the other by precipitation with acetone. The heat treatment was recognised as being absolutely essential. These steps are discussed in Section II.

Table, I.

The relevant information pertaining to the purification scheme of Kaplan & Heppel.[8]

Column No.	I	2	3	4	5
Step No.	Total volume ml.	Total activity units	Activity units per ml.	Mg. protein per ml.	Specific activity as units/ml
I Homogenate	20,200	I87,000	9.25	27.23 *	0.34
IA Homogenate heat treated as in step 3.	(20,200)	55,000	(2.72)	(27.23) *	0.I(?)
2 $(NH_4)_2SO_4$ precipitation at pH 3.5	900	83,000	92.2	27.I	3.4
3 Heat treatment at pH 3.5	I,0I5	3I,400	30.9	I2.57	2.5
4 $(NH_4)_2SO_4$ precipitation at pH 7.	230	3I,000	I34.8	23.64	5.7
5 Dialysis	255	27,800	I09	I8.I7	6.0
6 Acetone fractionation	255	20,200	79	6.28	I2.6
7 $(NH_4)_2SO_4$ precipitation at pH 2.	90	20,200	224.4	7.57	29.6
8 Amberlite IRC 50 X.E. 64 treatment	90	I2,I00	I34.4	I.87	72

Table, 2.

The yield of spleen ribonuclease activity at the first salting-out, heat treatment and final purification step.

Ribonuclease activity retained after the steps listed below, as a percentage of ---	(a) the total ribonuclease activity in the homogenate.	(b) the heat stable ribonuclease activity in the homogenate.
Step IA. heat treatment of the homogenate.	29.4	I00
Step 2. $(NH_4)_2SO_4$ precipation at pH 3.5	44.4	--
Step 3. The heat treatment at pH 3.5	I6.78 *	57.I
Step 8. The final step - amberlite resin treatment.	6.4	22

* Step 3 retained 37.8% of the ribonuclease activity salted-out at pH 3.5 by step 2.

Table 3.

The loss of heat stable ribonuclease activity after each step at purification.[8]

Step	Percentage heat stable ribonuclease activity discarded at each step
4. Salting-out at pH 3.5	2
5. Dialysis	I0.4
6. Acetone fractionation	27
7. Salting-out at pH 2	0
8. Amberlite resin treatment	40

SECTION II

A Preliminary Study of the Conventional Techniques

1. The Protein Molecule.

The properties of the protein molecule, especially those used as criteria for separation techniques, are appraised briefly as an introduction to this section.

Proteins exist as specific and definable molecular substances [48,151]. They are naturally occurring high molecular weight polymers of optically active ∝L-amino acids joined by peptide linkages and often have various amounts of carbohydrate or lipid covalently bonded. Most proteins now recognised have been defined on the basis of biological origin or activity and the evidence so far available convincingly supports the concept that each individual protein is a unique molecular entity [44,45,46,47]. The distinguishing characteristics of individual proteins are due to their large size, to the unique composition and sequence of twenty different amino acids that make up their primary structure and to their specific three dimensional configuration. This particular molecular composition endows the protein with its peculiar biological usefulness and determines the properties that are exploitable for identification and separation.

The enzymes, which selectively catalyse most of the chemical reactions essential for normal cellular function, comprise one category of proteins. Other proteins are, the hormones which are key

regulators of metabolic processes, the antibodies elaborated by an organism to counteract agents harmful to it, contractile proteins, respiratory proteins and bacterial toxins etc., all of which contribute to the manifold variety of physiological function exhibited by them. All enzymes are proteins and are differentiated by virtue of the functional property they possess of biological catalytic activity. The sensitivity of enzymes to environmental change is measured by the condition of this catalytic activity. The catalytic function has set them as a group apart from the other proteins. Justification for any alternative arrangement must await the advancement of protein science. Ordinary proteins are considered robust and as a result have been easily manipulated in isolation procedures. They may, in fact, only be the more stable members of a sensitive class analagous to those of the more stable enzyme types. Enzymes are temperamental chemically, yet their endowed sensitivity which imposes restrictions upon experimentation, enables them to be assayed and this property is thus an asset in this respect. The existence of very labile functional entities corresponding to the most sensitive enzymes may be obscured by the absence of a specific property [37,39,134]. The methods will eventually be at hand for the study of all proteins. It is only a matter of time, sophisticated technique and employing the classical methods of physical chemistry before the isolation of all the protein constituents of any given tissue, their characterisation as chemical substances and a knowledge of their interaction as biochemical components will be made possible.

The units of protein structure, the amino acids, exhibit many properties all of which contribute to the ultimate molecular arrangement. Briefly, each exhibits optical activity as a consequence of asymmetry in the molecule, thus proteins are optically active and this property can be further modified as the result of the helical content of the peptide chain comprising the protein. Amino acids or electrolytes exhibiting amphoteric properties and depending on how the charge is distributed can be classed as acidic, basic or neutral. Most of the charged groups of the amino acids are condensed in the peptide bonds of the protein molecule and those amino acids which have other polar groups free to interact with each other are responsible for the protein possessing a net charge. The electron distribution at the peptide bonds and the helical structure permit hydrogen bonding between suitable loci. Proteins are thus multivalent electrolytes with provision for diverse electrostatic interaction [137,153]. The presence of neutral amino acids with hydrocarbon side chains exert structural influences. The distribution and proportion of non-polar and semi-polar to polar regions in the protein molecule has a major effect in influencing the three dimensional configuration of the molecule. Hydrophobic interactions [176,195] occur and this means the interior regions of the protein molecule favours non-polar side chain groups and non-polar regions of polypeptide chain, all of which suggest that lyophilic bonding forces are possible.[196] Other forces not mentioned here may be involved [152]. Recent studies [45,46,60] suggest that these intrinsic

thermodynamic properties of the amino acids in the unique peptide sequence, already predetermined by the genetic mechanism, directs the chain to adopt a particular spatial arrangement at biosynthesis. This spatial arrangement is recognised by the terms secondary tertiary and quatenary structure [47,152,154,156]. The simplest polypeptide form is the primary structure, known as the nascent state [47] which is no more than a linear representation of the sequence of amino acids in the polypeptide chain. It can be imagined as the linear alignment of amino acids which complete the polypeptide immediately at its release from the ribosome on biosynthesis [47]. The polypeptide adopts immediately the conformation of the native protein and prefers to exist mainly in the form of an α helix linear coil. There are 3.7 amino acids residues per coil and the arrangement is stabilised by hydrogen bonding. This is recognised as the secondary structure [155]. Tertiary structure exists when the helix takes up a three dimensional conformation, the extent of which depends on the proportion of helical to non-helical segments on the chain and the configurational restrictions imposed. The tertiary structure is stabilised by specific bondings, e.g. disulphide, lyophilic, electrostatic and hydrogen bonding. Quaternary structure exists when more than one polypeptide unit makes up the native protein molecule [154,135]. These polypeptides have secondary and tertiary structure and the system is stabilised by specific intermolecular bonding believed to be of a similar nature to those which intramolecularly bond the functional tertiary structure of the native protein molecule.

The protein molecule exhibits properties dependent on the consequences of interaction of spatially neighbouring side chain groupings. As a result the protein may exist in several equilibrium forms with transitions taking place caused by changes in the environment that may favour a particular conformation [156]. Conformational fluctuations and the like may temporarily alter an immediate property yet the protein is native in the sense of existing as a singular molecular entity [47,156]. Heterogeneity of molecular species cannot be overlooked when a protein is thought of in this light. Natural microheterogeneity has been revealed with the discovery of multiple forms of certain proteins which appear to be functionally homogeneous [48,141,148]. These proteins are, in fact, limited groups of well defined but chemically differing entities containing sharply specified components, the composition and amino acid sequence of which have been rigidly designated genetically [48]. Microheterogeneity can be produced by variation in <u>in vivo</u> biological production. This manifestation is a product of the imperfect performance of the genetic replicating system whether by induced or accidental evolution, and a similar entity is produced which exhibits a slight molecular variation. Microheterogeneity can occur as a result of structural modification where a slight chemical change or rearrangement has taken place. This appearance of artifacts is most likely to occur during protein isolation as a result of harshly imposed conditions [149]. Excessive manipulation of the protein may lead to incomplete reversible alteration, which in effect signifies

that the protein has been denatured [152,156], albeit slightly. Denaturation whatever its character describes a change of protein structure with concomitant change of property [47]. The existence of multiple protein forms does not conflict with the concept that each individual protein is a unique molecular entity.

It is considered that the diagnostic techniques available for determining protein purity are of limited application [49]. From a strictly logical approach homogeneity is best sought and defined as lack of heterogeneity, and purification should be stringent in this respect. After an exhaustive examination the entity under scrutiny may yet exhibit contamination by secondary components. The removal of fifty percent of all contaminants from a mixture of proteins would give a boost to purity estimates if specific activity was the only measure of purification, yet it provides no measure of homogeneity as all the original proteins could still be present.

When a complex protein mixture is to be studied the special properties of the protein molecule are of first consideration in the choice of isolation and purification procedures. The more obvious properties have been exploited in methods of purification to advantage and include solubility, size, charge, lability, density and biological activity. The more elusive properties, for example, a propensity for interaction with specific reagents, selective site location and deformability are sophisticated features that await a breakthrough in technical applicability for general use to ensue [49]. No simple method will suffice for protein separation. The successive application of

several techniques based on different properties may be sufficient to resolve and account for all secondary components. Each separation criterion that is applied divides the mixture under study into groups that contain components resembling each other with respect to the criterion applied, but likely to differ materially with respect to one another. It is thus entirely possible that fractionation into individual components can be generally achieved by the successive application of separation principles that depend on different molecular properties. The methods that are required for the separation of uncharacterised proteins from unknown protein contaminants cannot be predicted and an empirical approach must be taken, applying one method after the other until the limits of technical applicability are exhausted.

With these primary considerations in mind as a basis for subsequent procedure and with the knowledge that the theory and explanation for each step has yet to be presented, a critical appraisal of the existing methods applied to the particular entity under scrutiny is warranted.

2. Extraction

(a) Survey of the methods used to extract ribonuclease, particularly ribonuclease from calf spleen

The first problem is to modify the in vivo protein environment and extract the proteins into a medium which will allow subsequent purification. The techniques involved in the liberation of proteins from cellular tissue, followed by information on the separation and isolation of proteins have been thoroughly reviewed in a laboratory manual of Analytical Methods of Protein Chemistry, Volume I, 1960, edited by Alexander, P., and Block, R.J., Pergamon Press, London 1960, where a broad spectrum of methods has been covered. These techniques are also adequately presented in section I of Methods in Enzymology, Volume I, edited by Colowick, S.P., and Kaplan, N.O., Academic Press Inc., New York 1955. In the survey of extraction procedures for ribonucleases from animal tissue it is sufficient to confine attention to methods already outlined by workers in this field while bearing in mind the vast amount of literature available on similar work. A study of the methods already adopted served to some extent as an aid to deciding on the significant features of an extraction.

One of the classic examples of extraction, purification and crystallisation of an enzyme was the work carried out by Kunitz [50], later modified by McDonald [51], to produce crystalline pancreatic ribonuclease. This enzyme is particularly robust, withstanding a low pH at extraction and a 100° heat treatment at a later stage, all of which would be harmful to more sensitive entities. Ice cold pancreas

tissue was minced into two volumes of 0.25 N H_2SO_4 then set aside with occasional stirring for 48 hours, extracted and fractionated by salting out with ammonium sulphate at a pH $\angle$ 1.

Ribonucleases present in other tissues were mentioned by Bain and Rusch [52] who assayed and compared the activities of aqueous homogenates. Hirs, Moore and Stein [43] were involved in the chromatographic purification of pancreatic ribonuclease. In this report they considered it of interest to investigate the tissue of other mammalian organs for ribonuclease activity. Dilute H_2SO_4 extracts were prepared from bovine liver, spleen and thymus. These extracts were obtained and chromatographed by the same procedures as applied to the pancreatic extract. Unlike the pancreatic extract ribonucleases were not detected against RNA from yeast in the column effluent fractions. The failure to detect activity was attributed to the fact that either the ribonuclease activity previously detected in spleen and liver was associated with an enzyme not chemically related to pancreatic ribonuclease or the quantities were so small they could not be measured by the procedures adopted. Maver and Greco [55] investigated the ribonuclease activity present in cathespin preparations from calf spleen. The method used for the preparation of the cathespin material was described in an earlier paper by Maver [53] and was essentially similar to that used by Edsall [54] for extracting muscle protein. The material was stored in ice until used. 400g of tissue was minced then ground in a mortar. This homogenate was immediately extracted with 10 volumes of ice cold 1.2M KCl with mechanical stirring for 1 hour.

Edsall [54] revealed that an extractant containing KCl (1.2M) brings more protein into solution than does 0.05M phosphate buffer and this information has been used with the spleen extraction [53]. After adjusting the pH to 7 the spleen suspension was filtered through fine gauze then centrifuged and the supernatant was ready for fractionation at the next stage. In the 1949 [55] paper the extracting solution was reduced to 4 volumes of water but the procedure is similar. In a later paper [56] by these authors the ribonucleases which were separated with the calf spleen cathepsin preparation were now almost free from proteolytic activity. The tissue was disrupted and extracted with water as described for the 1949 [55] paper except that the nucleoproteins were centrifuged off, following which the supernatant from this tissue homogenate was dialysed for 48-66 hours to precipitate inactive protein. After a second centrifugation the supernatant was ready for fractionation. In yet another paper [35] on calf spleen ribonucleases further alterations were made on the extraction procedure. This particular method was used in subsequent papers by Maver, Peterson, Sober and Greco [40] and by Maver and Greco [36]. These workers reintroduced KCl at a lower concentration and used less extractant than originally applied. The KCl concentration in the disrupted tissue was $\simeq$ 0.25M which suggested that the workers were using isotonic conditions similar to that obtained by using 0.25M sucrose. Isotonic KCl conditions may facilitate extraction and retain the standard physiological state in cell particles. It is to be expected that this measure lessens the possibility of interaction

between the components of the extract. They introduced a long emulsifying period of 15 minutes and retained a lengthy dialysis. Frozen tissue was suspended in 2.5 volumes of cold 0.325M KCl and emulsified in a Waring blendor for 15 minutes. The emulsion was filtered through gauze then centrifuged at 600 x g for 45 minutes. The sedimented nucleoproteins and cellular debris were washed twice with cold dilute KCl. The wash and supernatant fluids were combined and dialysed against cold 9° running tap water for 48-66 hours. A coarse protein precipitate was removed by centrifugation and discarded and the supernatant was now ready for salt fractionation. Nuclease preparations were prepared in this way, further purified then lyophilised ready for use in the chromatographic examination by Maver and Greco [36].

Hilmoe and Heppel [41] described three fractions with different ribonuclease activity from calf spleen but gave no details of the extraction procedures used to obtain them. Brown, Heppel and Hilmoe [57] revealed that one of the fractions, described as fraction III was prepared by homogenising the spleen with 3 volumes of cold 0.25M sucrose. The mixture was then adjusted to pH 5.1 and a bulky precipitate which formed, was collected then converted to an acetone powder. This powder was extracted with 20 parts v/w 0.2M acetate buffer pH 6 centrifuged and the resulting supernatant was ready for fractionation. This fraction III was used by Heppel, Markham and Hilmoe [58] and the method of extraction is set out in greater detail in Methods in Enzymology [26]. The spleen was homogenised in a Waring

blendor for 2 minutes with 8.5% sucrose. Before precipitation at the isoelectric point pH 5.1, connective tissue fragments and cellular debris were removed by centrifugation. The precipitate was converted to an acetone powder by homogenisation in a Waring blendor for 15 seconds with 5 volumes of acetone at -10°. The powder was dried, stored then extracted as previously described [57].

The extraction and first ammonium sulphate fractionate step were combined in the method adopted by Kaplan and Heppel [8] (Section I). The tissue was disrupted in 0.05M sodium acetate 10^{-4} MEDTA, pH 7.2 and the pH of the homogenate adjusted to 3.5 with 1N HCl. Ammonium sulphate was added directly to the mixture to bring the saturation to 40% by adding 226g and the filtrate obtained overnight after filtration. The filtrate contained ribonuclease activity for further fractionation by taking the saturation to 80% by the addition of 258g ammonium sulphate per litre. The precipitate was collected by filtration overnight for a second time then dissolved in 0.05M sodium acetate pH 7.2 with a 20 fold reduction in volume. Although there was considerable variation in the many extraction techniques described, no comprehensive method was available. The information confirmed points of similarity and established practice on which conditions for extraction could be based. These aspects are discussed in the next few paragraphs which consider the conditions for extraction in the present work.

(b) Conditions for extraction

A study of the extraction methods already adopted by workers on

ribonucleases from calf spleen assisted with practical points which arose and were not fully described in any one report. The following conditions were considered to be of major consequence and the extraction scheme settled on for this work embraced them where possible.

(i) Speed and ease of manipulation

It was essential that the material should be processed expeditiously to a stage where the conditions were such that autolysis by intrinsic proteolytic action or extraneous contamination was less probable. Initial extraction trials could be on a pilot scale utilising a few grams of material. However, even at an early stage the approach should be such that the method permitted adjustment to a grander scale with ease. These conditions set some limit on the volume applied, time taken and number of manipulations the extraction step required, to produce a solution suitable for exploitation by methods employing specific separation criteria.

(ii) Control of the homogenising medium

The use of a buffer as homogenising medium should promote uniformity of sample in that some control was exerted over the pH of the resulting mixture. Protein solubility varies greatly with pH change and for each protein there is a value at which it is least soluble. Variation in protein solubility was restricted by pH control of the medium and the condition should be reproducible. It would be beneficial if unwanted proteins were removed by precipitation especially if there was no loss of active component by co-precipitation

effects. Temperature control was essential for reproducibility even at extraction. Generally it is better to operate near the freezing point of the extracting system.

(iii) Protein concentration and yield

The concentration of protein should be as high as possible, consistent with as complete an extraction as possible. Excessive dilution could cause denaturation by surface tension effects and ease of manipulation would not be facilitated by large volumes.

(iv) Some considerations of the molecule under study

Any pertinent information available concerning the required active protein was considered for the incorporation or exclusion of certain extraction procedures. The enzyme might be adversely affected by heavy metals [8] immediately cell disruption took place. The inclusion of a chelating agent, e.g. EDTA in the extracting buffer could alleviate this possibility. Clearly the use of deionised water for the preparation of buffers and the exclusion of any source of heavy metal contamination was desirable. The complications presented by the likelihood of there being several ribonucleases in spleen tissue had to be considered. If separation and rejection of material took place at extraction, e.g. by the salting out method used by Kaplan and Heppel [8], the limits adopted were determined by assay. The activity measurements for the enzyme of significant interest could be a small portion of all the ribonuclease activity and optimal isolation of the required enzyme at extraction and subsequent stages could be jeopardised by the masking effect of other ribonucleases on assay.

The known properties of the small molecular weight spleen ribonuclease might be put to advantage or have disadvantages. Kaplan and Heppel [8] found that their spleen ribonuclease was very much smaller than pancreatic ribonuclease and exhibited greater basic properties. With a complex mixture of diversely charged polymers, e.g. lipid, protein, nucleic acid, carbohydrate, about to be released upon the disruption of organised cell tissue the possibilities for indiscriminate interactions to take place were unlimited.[179] This spleen ribonuclease was suitably primed for interaction if indeed it was not already involved in a specific complex in vivo. In the first instance, any extraction procedure which ignored the molecular dimensions of the molecule to be isolated would be ill conceived. It would be unwise to submit the immediately extracted material to extensive dialysis against tap water as did Maver and Greco [56], in view of the information on the suspected molecular weight and hazard from heavy metal contamination by tap water. Considerable time and volumes were involved in an operation that might not be all that essential at this stage.

The treatise by Craig and King [61] on dialysis illustrates some of the limits of exclusion of molecules to porous membranes. They demonstrated that the loss of small proteins to the diffusate with ordinary cellophane membranes was serious with molecules below a molecular weight about 15,000 (page 95). Consequently, a ribonuclease of molecular weight lower than pancreatic ribonuclease in a mixture of several enzymes active against ribonucleic acid, could be easily

lost without detection at the early stages of purification.

(c) Spleen as a source of ribonuclease

Very little comparative information was available on the various ribonuclease sources. Greenstein and Thompson [62] examined various tissue sources of the mouse for ribonuclease activity. Bain and Rush [52] by a different technique compared the ribonuclease sources in rat tissue. Zittle and Reading [63] published comparative studies using the same techniques as Bain and Rush on the various tissues of the rat and rabbit. The findings of these various authors regarding distribution are shown in Table 4. The values in each case were expressed with reference to the most active tissue taken as 100. No comparison could be made where assay conditions were different.

Table 4.

Ref.	Source	Liver	Kidney	Spleen	Pancreas	Small Intestine	Estimation
62	Mouse	15	10	36	100	87	
52	Rat	2.2	9.78	12.4	100		Can be compared
63	Rat			27	100		
	Rabbit			28.2	14.4		

With these small animals it was generally indicative that of the organs tested, the spleen was the next best tissue to pancreas as a source of ribonuclease activity though certain other sources, e.g. bone marrow and small intestine, were reasonably attractive.

From several publications it appeared that nucleic acid catabolising enzymes were present in higher than average concentration in spleen. Acid and alkaline ribonucleases were reported high in the rat and mouse by de Lamerande and Allard [64]. Heppel and Hilmoe [26], Maver and Greco [35,40], and Hilmoe [42] all published reports on several ribonuclease and phosphodiesterase activities in calf spleen. Significantly, it also contained deoxyribonuclease [35] and the catabolically related enzymes nucleoside deaminase and phosphatase [65].

A comparison of spleen and pancreas from bovine origin as a ribonuclease source could be gauged from the report by Kaplan and Heppel [8] who described the amount of spleen ribonuclease per unit weight of spleen as being much less than that of ribonuclease in pancreas but compared favourably with that of other enzymes in spleen, liver and thymus that act on polyribonucleotides. They also stated that the activity of spleen and liver ribonuclease was "weak" compared with pancreatic ribonuclease. As illustrated in Section I the amount of heat stable spleen ribonuclease retained after purification by these workers was only 6.4% of the total ribonuclease content of spleen. If these comparisons made by Kaplan and Heppel [8] were based on the amount of heat stable spleen ribonuclease, then the spleen tissue was a rich source of ribonuclease activity. A comparison of ribonuclease content in the same tissue or different tissues using impure extracts, whether assayed under standard or suitably different conditions, could be extremely unreliable because of the presence of several ribonuclease activities, differences in the degree of activity

and the probability that there might be inhibitors like the labile protein suggested by Roth [37] for alkaline ribonuclease in liver. Each tissue ribonuclease is an individual entity and it would be extremely hazardous to make generalisations based on several isolated examples.

(d) The availability of calf spleen samples

The procedure for the collection of raw material for experimentation is described in G.M.I. (page 282). For the present work sufficient material was available from local abattoirs to fulfil the requirements of any experimental trial on the pilot scale. However, when samples of over 20 spleen were required, journeys to a calf market and abattoir which dealt with 250 calves on certain days, had to be made. The spleen was effectively stored and transported in several stainless steel ice buckets using crystal tips as the ice supply, without difficulty. A restriction was imposed to control uniformity of sample. Only spleen from calves 3-8 days old was acceptable. The co-operation of the abattoir personnel was indispensable in the control of this supply. Spleen from calves over one week old were very much greater in size and were rejected. This measure prevented the material at extraction being contaminated with superfluous fat and ensured that the samples were free from tough membranous tissue.

The desire to standardize conditions at an early stage with a view to obtaining reproducibility of method was supported by the findings of Czok and Bucher [66] who reveal that the success and reproducibility of enzyme preparation is influenced by certain general

factors. The breed and age of the animal play an important role with respect to the composition of the tissue extract. These workers recommend a homogeneous breed within a well defined age limit as tissue source. This condition apparently results in a greater uniformity in the amount of extractable protein and enzyme activity. Of particular interest is the fact that depending on the animals physiological state vast differences can exist in enzyme content. These facts are of importance when consideration is given to them in the light of the findings of Dixon and Webb [67] discussed on page 61 that salting out fractionation limits vary considerably with the concentration of the particular protein investigated.

(e) The spleen organ

Spleen is a very complex organ as it is part of the lymphatic and circulatory systems. It acts as a store for blood and in certain circumstances can hold 1/5 to 1/3 of all blood in the animal. It regulates the volume of blood elsewhere in circulation. The spleen is believed to produce lymphocytes and antibodies and to destroy erythrocytes and platelets [68]. It is thus a heterogeneous organ with respect to cell type. From this it could be assumed that the extent of exsanguination would influence the quality of the extract. Czok and Bucher [66] point out that haemoglobin and plasma proteins are contaminants at the last stage of the purification procedure for muscle myogen. With spleen tissue which serves as an active centre for very many physiological functions a complex protein extract could be expected.

(f) The system of extraction retained

The apparatus, various solutions, reagents, the preparation of raw material and the measures taken to ensure uniformity of homogenate sample are described in experiment I (page 353). This detailed account of the extraction stage was the outcome of many trials involving various quantities of spleen. The homogenization of tissue was carried out by a procedure similar to that described by Kaplan and Heppel [8] Section I (page 26). Since the extraction included the first stage of an ammonium sulphate fractionation only the disruption of the spleen tissue is considered in this paragraph. The fresh calf spleen, carefully packed in ice crystal tips were processed in pairs in the cold room. The outer capsular membrane was removed, the tissue sliced into small pieces and homogenized in a Waring blendor capacity 600 ml. at 12,000 R.P.M. for 1 minute with 3 volumes of 0.05M sodium acetate 10^{-3} MEDTA pH 7.2. After the homogenate was sieved through clean muslin to remove membranous tissue, 3 litre portions were adjusted to pH 3.5 by the dropwise addition of $\simeq$ 200 ml. IN HCl. The required amount of ammonium sulphate to bring the mixture to the saturation limit of the first precipitation was then added. Disruption of the tissue took place into the same extractant solution as that used by Kaplan and Heppel [8] at a neutral pH. Extraction followed at exactly pH 3.5 after the pH was carefully adjusted. The addition of $\simeq$ 200 ml. of cold N HCl at a dropwise rate down the inside wall of the beaker, with continuous mechanical stirring to pH 3.5 ensured thorough mixing, promoted extraction and reduced the

danger of denaturation by local concentration effects.

Many enzymes are inactivated in solution when the pH is taken below 5 [150]. It could only be assumed that lowering the pH in this way protected the homogenate from enzymes that may have a deleterious effect on sensitive components. It could also be recognised that indigenous R.N.A. is precipitated normally at this pH [150] and removed with the first fraction at centrifugation. A deep smooth stirring action was maintained to prevent turbulence. The inclusion of the chelating agent EDTA ensured the removal of harmful divalent cations that might be released into the medium by preferentially forming a more stable complex with this agent. Precautions were exercised in the choice of suitable utensils, dionised water and analytical grade reagents.

Kaplan and Heppel [8] made no mention of a homogenisation time. The homogenization time varied from one paper [42] to another [35]. A 15 minute duration to "emulsify" with a Waring blendor as advocated by Maver and Greco [35] seemed too long and suggested excessive froth production and the possibility of protein denaturation by surface tension effects. In the present work the tissue which was not membranous or tough in any way, appeared adequately dispersed after homogenizing for 1 - 1½ minutes [42]. The sieving of the homogenate at this stage removed blood vessels, particularly a bulky splenic artery which often remained as a stringy mass and interfered with centrifugation operations. The removal of these blood vessel fragments produced a more uniform sample for the first salting out with

ammonium sulphate, especially when reproducibility had to be taken into account. Removal by centrifugation of cellular particles and debris, components precipitated at pH 3.5 and by the first salting out with ammonium sulphate from the homogenate completed the extraction (page 65).

3. Salting out and heat treatment

(1) The salting out technique

(a) Theory

The theoretical aspects of the salting out process and of the variables which determine the solubility of proteins in salt solution have been presented recently by Czok and Bucher [66] and Dixon and Webb [67] and earlier by Green and Hughes [69] in comprehensive reviews of the subject.

Electrolyte progressively added to a protein solution causes two effects, as the ionic strength increases. First of all there is an increase in solubility as the activity coefficient of the protein decreases, i.e. a "salting in" effect. As the ionic strength becomes higher, the second effect causes a diminishing of solubility and this predominates after the solubility passes through a maximum then decreases until precipitation is complete. In "salting out" the salt anions are important while the cations play a secondary role. Salts with di- and trivalent anions are especially effective for precipitating proteins, e.g. $(NH_4)_2SO_4$, Na_2SO_4, Na_3PO_4 and K_3PO_4. Exactly why proteins are salted out is not completely understood. It has been suggested [69] that the salt ions on dissolving become hydrated, thus

"removing" part of the water which is then "unavailable" as solvent and "dehydration" of the protein molecule is brought about. The solvent becomes organised preferentially about the salt ions to an extent that its normal organisation around the protein molecule is decreased. This decrease in solvation enables the protein molecules to associate in the solid phase as a precipitate.

A direct relationship has been formulated by Cohn [70] between protein solubility and the ionic strength of the solution. In a concentrated solution of electrolyte the decrease in the logarithm of the solubility of a protein is a linear function of increasing ionic strength. In mathematical terms this has been represented by the formula :

$$\log S = \beta - K\Gamma_2$$

where S = Solubility of the protein in g/Kg water

Γ_2 = Ionic strength in moles/litre of solution

β and K are constants

K represents the slope of the linear function while β is the hypothetical solubility, i.e. log S at zero electrolyte concentration. Temperature, pH, the amount and type of salt, the amount and type of protein are the important variables.

(b) Effect of pH, temperature and salt

Amount and type of salt, pH and temperature can all be adequately controlled. Much less is known about how proteins behave when a mixture is presented for fractionation by the "salting out" technique. The solubility of a particular protein is directly affected by pH,

temperature and the amount of a particular salt on the intrinsic properties of that protein. Exactly where the protein will precipitate at salting out depends not only on these factors but on the nature of the salt and the amount of that protein in solution[67].

The effect of these variables can be briefly delineated.

(i) pH

At constant ionic strength protein solubility increases at pH values acid or alkaline to the isoelectric points of the proteins. Dixon and Webb[67] illustrate the importance of this variable. They show how the relative solubilities of ovalbumin and carboxyhaemoglobin may vary very rapidly with pH. At constant ionic strength a change from pH 5 to 6 is sufficient to alter the ratio of the solubilities of these two proteins by several thousand fold. To retain well defined, reproducible precipitation limits pH must be strictly controlled.

(ii) Temperature

In dilute aqueous solutions of electrolyte most proteins have a positive temperature coefficient and are more soluble as the temperature rises from 0^{o}. In more concentrated electrolyte solutions this condition in some instances is reversed. Some proteins are less soluble and can be precipitated when the solution temperature is raised from 0^{o} without an increase in salt content. This sensitive effect to temperature change is illustrated in the report by Edsall[54] who states that myosin which is soluble at 800 mg of protein nitrogen/litre at 0^{o} under a standard condition of pH and ionic strength has

no measurable solubility at room temperature. The effect is used to crystallise human haemoglobin by Green [71] who shows that carboxy-haemoglobin is ten times more soluble in an ammonium sulphate solution at 0^{o} than it is at 25^{o}. From this it can be realised that protein solubility in the salt fractionating concentration range is affected by changes in temperature. The effects are different for the different proteins of a mixture and the order of precipitation of a series of proteins as the salt content is increased may be entirely different at one temperature from that of another temperature. By implementing a standard temperature condition and strict buffer control of the pH from one fractionation to the next, reproducibility with respect to these two variables is adequately controlled.

(iii) Type of salt

The formula $\frac{\Gamma}{2} = \frac{Cn\ Z^2 n}{2}$ is used to calculate ionic strength. $\frac{\Gamma}{2}$ is the molar ionic strength, C is the molar concentration of each ion n and Z the valency. For each ion of salt in solution CZ^2 can be calculated and the sum of these values for each ion present divided by 2 to give $\frac{\Gamma}{2}$. Divalent and trivalent salt ions produce much higher ionic strengths and are generally more effective as precipitants. Dixon and Webb [67] show that some are better than others in the efficiency of precipitation. These authors illustrate that the salts are more effective in the order potassium phosphate > sodium sulphate > ammonium sulphate > sodium citrate > magnesium sulphate when precipitating carboxyhaemoglobin. All the chlorides are much less effective. They maintain that the salt anion and its valency state

are more important than cations in influencing salting out and that cations, particularly di- and trivalent cations, are more effective in the salting in process, except at very low ionic strengths. Chlorides are listed as extremely inefficient. Though sodium sulphate is more effective than ammonium sulphate it is less soluble and has a limited application. Magnesium sulphate is not very effective as it causes the protein to precipitate over wide ranges of salt addition. Phosphates are particularly good protein precipitants and act as buffers at the same time. Pennell [72] who notes that sodium chloride, magnesium sulphate and various acetates and chlorides are considered as specific precipitants for individual proteins, points out that it has not been demonstrated that their use is more specific than that of phosphate or ammonium sulphate under controlled conditions of pH, temperature and ionic strength.

A salt suitable for protein fractionation must be highly soluble, reasonably cheap, pure, and without any direct effect on proteins [178]. The most popular choice of salt is ammonium sulphate which can fulfil these requirements and has been most commonly used in enzyme fractionations despite certain disadvantages. The pure salt is slightly acid in solution and certain protein estimations can be difficult because of the ammonium ion [67].

(iv) Amount of salt

With the addition of large amounts of solid ammonium sulphate at successive fractionation steps, allowance must be made as considerable volume changes accompany the addition of the salt to the protein

solution to increase the salt concentration from a lower to a higher value. A knowledge of these changes is necessary for the calculation of salt concentration. Kunitz [73] recognised this fact and published the following formula which standardises the salting out procedure in relation to the salt addition and concentration:

$$X = \frac{533\ (S_2 - S_1)}{1 - 0.3\ S_2}$$

X = g solid ammonium sulphate added to 1 litre of solution of saturation S_1 to bring it to saturation S_2

S_1 = initial fraction of saturation

S_2 = final or desired fraction of saturation at 23°

This formula enables the calculation of the amount of salt necessary to produce any increase in concentration. Green and Hughes [69] present a table from which the amount of ammonium sulphate necessary to increase the concentration of 1 litre of solution already at a particular salt concentration to a higher salt concentration can be read direct. Dixon [74] published the equivalent information in the form of a nomogram. These charts are convenient to use, and avoid lengthy calculations. The concentration values are calculated as a percentage saturation of 4.1M ammonium sulphate at 25°. A saturated solution of ammonium sulphate at 25° is 4.1M and requires 767 g of salt for each litre of water. The values listed [69,74] are exact concentrations in terms of moles/litre.

(c) Nature of the protein

Dixon and Webb [67] discuss the theory of protein interaction and

salting out and describe some experimental work carried out by other workers on simple protein mixtures. The results and their interpretation indicate that in the absence of interaction between proteins it is expected that the different proteins of a mixture will precipitate sharply and independently upon salting out. They note, that the assumption is frequently made that a precipitating protein will tend to carry down a certain amount of the other proteins, so that the range of salt concentration over which any one protein will be precipitated, will depend on the other proteins present and a sharp separation is prevented. Though it is well known that under certain conditions interaction between different proteins can be observed [179], Dixon and Webb [67] believe that this has never been demonstrated in the presence of a high concentration of salt. The basis of this argument is that the main factor in determining interaction viz. the surface potential, is reduced to very low values at high salt concentration and the argument therefore predicts that a specific protein will precipitate independently of other proteins. Furthermore, proteins do not always precipitate within fixed limits of salt concentration characteristic for the proteins at a given pH, temperature and salt type. The precise limits of salt concentration for a specific protein vary with the concentration of that protein. An enzyme can be precipitated within fixed saturation limits of salt concentration from one fractionation trial to the next by diluting the enzyme solution to the same concentration of that enzyme on each occasion. Dixon and Webb [67] illustrate the effect of protein

concentration at salting out by demonstrating, using the known solubility constants of serum albumin, that with a protein concentration of 30 mg. per ml., the serum albumin will mostly precipitate in the range 30-40% saturation with ammonium sulphate. If the serum albumin solution is diluted to 10 mg/ml. then the saturation limits for precipitation are at higher values and the serum albumin will be mostly precipitated in the range 35-45% saturation with ammonium sulphate. As part of the evidence that proteins precipitate independently of each other Dixon and Webb [67] cite the work of Roche and Darrien [75] who experimented on an artificial mixture of pure crystalline horse serum albumin and dog haemoglobin. They precipitated them with ammonium sulphate from separate solutions and from a mixture at standard concentration and conditions. The proteins in the mixture are brought out of solution at exactly the same salt concentrations which precipitates them when in solution alone. It would be desirable if more information along these lines were available.

With enzymes the point of first precipitation can be determined by assay of activity. However, by altering the concentration of enzyme by dilution, completely different precipitation limits in no way related to that of the previous limits for the enzyme can result. Dixon and Webb [67] advise that in the absence of any knowledge of the behaviour and precipitation limits of contaminating proteins it is probably best to secure a range of salt saturation which precipitates 75% of the enzyme activity required and this ought to be accomplished

by a 6-10% increase in saturation from the point of first precipitation. The concentration of a specific enzyme is a variable that must be taken into account in finding the best conditions for the precipitation of that component. Since the amount and proportion of the components making up a particular protein solution may vary considerably from one occasion to the next (page 52) a reproducible fraction at salting out may not be obtained even if the concentration of the specific enzyme component is controlled. Certain components may be included in the fraction that are excluded on other occasions because they are present in lower or higher concentrations which means they will precipitate within different salting out fraction limits each time. It can be concluded from Dixon and Webb[67] that as the concentration of a particular protein decreases from a specific amount the quantity of ammonium sulphate required to precipitate it will be greater and the precipitation limits increase to higher values. At higher concentrations of the component much less salt will be required to precipitate it from solution thus the precipitation limits decrease. This effect is different with different proteins thus a reproducible fraction component content within well defined salt saturation limits is not possible unless the source solution is uniform in its content from one experiment to the next. No account has been taken of the contribution, if any, that the varying concentration of the other protein components make to the precipitation conditions of the solution, especially if they are present in large quantities compared to the amount of protein or enzyme component under

study, e.g. contribution to ionic strength.

Although the processes are not fully understood much the same type of action is responsible for the precipitation of proteins by organic solvents. Askonas [76], who used acetone to fractionate enzymes from a rabbit muscle source, found that these proteins under controlled conditions of temperature, pH and ionic strength precipitate out of solution in a definite order. Each protein tends to come out of solution after a constant percentage of the total protein of the sample has precipitated regardless of the concentration of the precipitant at which this level may be reached. It can be concluded from this observation that a particular component of the mixture will be precipitated in a particular fraction if the protein concentration of the samples to be fractionated are very similar in constitution and content from one preparation to the next. This observation can also be interpreted to mean that the amount or variation in proportions and properties of the other components will have an effect on the precipitation point of a particular component. It suggests that if a major protein contaminant which precipitates early in the series is trebled in content, where the total content of the whole sample is unchanged, the protein or enzyme also present at the usual concentration will precipitate later and not when expected as a larger percentage of the whole sample must be precipitated first in this case. Should an analogous situation exist during salt fractionation with solutions which can be variable in component content from a non-reproducible source of supply, unpredictable difficulties are expected especially if attempts are made to precipitate the fraction in narrow

precipitation limits on each occasion. The relative proportions of other proteins in a mixture, whether or not all the proteins precipitate independently of each other, may well influence the saturation of salt required to precipitate a particular component.

Czok and Bucher[66] write that the protein precipitate may be considered as a mixture of "n" proteins in "m" different states of order in the range of micellar dimensions and point out that this is influenced by the numerous possibilities in altering the variables applied to the solution before the addition of the salt. Quantity of ions, hydrogen ion concentration, temperature and time all affect the composition of the precipitate and proteins in solution before the removal of the precipitate. No mention is made of the influence of the varying amounts of the different proteins unless quantity of ions embraces this. Many of the constituents of a protein extract, e.g. heparin, nucleic acid and the proteins themselves can increase or decrease greatly with the degree of exsanguination or physiological state of the animal[66] before the tissue is obtained. The effect of an unpredictable and possibly reactive component content from one extract to the next on how the solutions generally behave at salting out may produce fractions of undefined and even unreliable component content.

(d) The salting out of calf spleen ribonucleases at pH 3.5

As mentioned on page 55 the extraction procedure adopted here was complete after the homogenate was brought to 40% saturation with ammonium sulphate and insoluble material removed by centrifugation. Several salting out operations were carried out using the same ammonium

sulphate saturations advised by Kaplan and Heppel [8] (page 26). The method settled upon is outlined in Experiment 2 (page 357). The $\simeq$ 3.2 litre portions of homogenate at pH 3.5 were brought to 40% saturation by the addition of 226 g per litre of solid ammonium sulphate, and the very large bulk of solid material removed by centrifugation at 15,000 x g for 10 minutes at 0°. The clear supernatent was measured then brought to 80% saturation by the addition of 258 g ammonium sulphate per litre and the precipitate collected by centrifugation at 30,000 x g for 10 minutes at 0°. The supernatant was discarded and the solid dissolved in 0.05M sodium acetate 10^{-4} MEDTA pH 7.2 to a twenty fold reduction in the volume from that of the homogenate. Standardisation of technique with a view to reproducibility and uniformity of sample was undertaken at every opportunity. The addition of ammonium sulphate was carried out slowly and at an even rate to the homogenate or solution with continuous mechanical stirring to eliminate local salt concentration effects. In the case of the homogenate after the salt addition is complete, the mixture was stirred for 1/2 hour to ensure thorough and even mixing, then left standing for 1 hour before centrifugation. After the salt addition was complete with the addition of 258 g ammonium sulphate to raise the saturation of the supernatant from 40% to 80%, the mixture was stirred for 1/2 hour to ensure all the ammonium sulphate was dissolved. The mixture was left untouched overnight before centrifugation. It became standard practice to repeat experiments, conscious that the various factors of pH, temperature, salt content, effect of time and

details of procedure, if controlled contributed to reproducibility.

In the present work before an appraisal could be made of the efficacy of the extraction and salting out with ammonium sulphate at pH 3.5 the sample retained by the fractionation had to be heat treated then assayed to reveal the amount of heat stable component secured. To facilitate the discussion on the salting out of the heat stable spleen ribonuclease at pH 3.5 the information on heat treatment is presented first.

II. The heat treatment at pH 3.5

Since the heat stable spleen ribonuclease activity described by Kaplan and Heppel [8] is a small portion of all the spleen ribonuclease activity (page 50) a heat treatment was included as an essential operation in the purification procedure undertaken at this investigation. To be absolutely certain of experimenting with this particular ribonuclease activity the fractions which were obtained by precipitation within the saturation limits 40-80% with ammonium sulphate at pH 3.5 were heat treated at every trial. The heat treatment recommended by Kaplan and Heppel [8] remained unaltered and the general method applied, is presented in Experiment 3 (page 358).

(a) Some generalizations on heat treatment

Fractional denaturation by heating where a fairly heat-stable component is known to exist is a valuable preliminary step to any purification procedure. Many enzymes are purified by controlled heating at a well defined protein content in the range 50-70° for a limited number of minutes (Taylor [77]). Heat treatment involves

selective denaturation with precipitation and removal of formerly active and inert protein from the preparation. Heat denaturation of proteins has a large temperature coefficient; consequently the destruction temperature is usually quite sharply defined and is different for each protein. The conditions laid down for the heat treatment are vital in obtaining the desired effect. The variables, pH, temperature, time and details of procedure are of major consequence in standardizing conditions from one trial to the next. The state of purity of the protein mixture at the time of heat treatment is also a critical factor [192]. Apparently less pure extracts or mixtures are more resistant to heat denaturation and generally greater purification is obtained by the first treatment than by successive ones. Less stable proteins are those which are removed first thus the temperature, duration of the treatment and the amount of each of these proteins will have considerable influence on the effectiveness of this step.

(b) Conditions for the heat treatment

Kaplan and Heppel [8] (page 26) did not reveal the protein content of the sample prior to heat treatment in their details of the procedure. It could be calculated from data available (page 32) to be 27 mg. protein/ml. This is a very high concentration for the operation as solutions containing this amount of protein are quite viscous. For the present work the heat treatment procedure outlined in Experiment 3 (page 358) was used. The protein fraction precipitated between 40% and 80% saturation with ammonium sulphate was dissolved in 1 litre of 0.05M sodium acetate 10^{-4} MEDTA pH 7.2

per 25 extracted spleen and the protein concentration was between 10-17 mg/ml. The pH, time and temperature conditions were implemented with precision and an exact technique was followed through closely with careful manual stirring to promote even mixing and constant temperature distribution over the specified periods for each portion of the extract. The whole protein solution was taken to pH 3.5 by the careful addition of 1N HCl. Because of the large volumes generally involved 500 ml. portions of the acidified solution were taken to 60° by immersing the container in a water bath at 80°. On reaching 60° the container was transferred to a second water bath[136] at 60° for exactly 10 minutes. The mixture was cooled rapidly to 0-3° by transferring it to a 4 litre beaker immersed in an ice bath. Before removing insoluble protein by centrifugation at 30,000 x g for 7 minutes at 0° the mixture was adjusted to pH 7 after all the portions were heat treated and cooled. The precipitate was extracted by resuspending it in 2 volumes of 0.05M sodium acetate 10^{-4} MEDTA pH 7 and the mixture centrifuged as before. The initial supernatant and wash were combined.

(c) A survey of the information available on the heat treatment of calf spleen preparations

Some important inferences could be drawn from the literature on which enzyme activities were most likely to survive or be eliminated by the heating conditions. Kaplan and Heppel[8] claimed that most of the ribonuclease activity extracted and fractionated was due to other ribonuclease activity which was destroyed by the

acid heat treatment and that the ribonuclease activity which was heat stable at the early stage remained heat stable after further purification (see Table 5). They showed that the pH of the solution was important. A 10 minute heat treatment of the spleen homogenate over the temperature range 60° to 95° and an 80° temperature treatment over durations within 10-40 minutes all at pH 3.5 had little effect on the amount of activity that was retained. In comparison, heat treatment at 80° for 10 minutes at pH 5 left only 5% of the original activity. Hilmoe [42] showed that spleen phosphodiesterase was very much less stable at pH 3.5 than at pH 5.

Maver and Greco [35] found that purified ribonuclease activity from calf spleen, heat treated, by the method of McDonald [51], was very sensitive to a temperature of 100° for 15 minutes in 0.1 N acetic acid. All the deoxyribonuclease activity was destroyed. Of the ribonuclease activity 10% remained while 34% resisted denaturation after heating at 70°. Maver, Peterson, Sober, and Greco [40] comparing their findings with those of Kaplan and Heppel [8] wrote that most of their preparation would be destroyed at 60° for 10 minutes at pH 3.5. They stated that most of their preparations had activity with pH optima in the acid range pH $\simeq$ 5.7 and very little in the alkaline range pH $\simeq$ 7.2. Apparently much less than 20% of the "acid ribonuclease" activity remained after heating while the "alkaline ribonuclease" activity was unaltered either by heat treatment for 15 minutes at 70° pH 3.5 or at 80° for 10 minutes at pH 3.5. These investigators claimed that the greater sensitivity

Table 5.

Summary of details for the heat treatments applied to the calf spleen ribonuclease preparations.

Source Ref.	Details					%age of RNase in the sample heat stable at assay pH		
	Stage of purification of sample	Temp °C	Time in min.	pH	Conditions in the protein solution.	6.5	5.7 5.8	7.8 8.2
Kaplan & Heppel ref.8	Homogenate	60	10	3.5	0.05M. Na acetate 10^{-4}M.EDTA pH7 & 27 mg. protein/ml.	45		
		70	10	3.5		30		
		95	10	3.5		30		
		80	40	3.5		25		
		80	10	5		5		
	Fraction salted out at pH 3.5 (step 2)	50	10	3.5	same buffer 27 mg/ml protein 10% $(NH_4)_2SO_4$	50		
		60	10	3.5		45		
	Pure sample from step 7.	60	10	2	same buffer 7.6 mg/ml. protein	100		
		80	10	3.3		80		
Maver & Greco ref. 35.	Extensively purified sample exhibiting two activities	100	15		0.1N acetic acid		10	
		70	15				34	
	ditto.	70	15	3.5	0.005M sodium phosphate		20	100
		80	10	3.5			20	100
Maver et al ref.40	Spleen RNase activity not retained on DEAE cellulose at pH 7.	70	15	7	ditto		40	85
	RNase held and purified on DEAE	70	15	7	ditto		15	
Maver & Greco ref.36	"Acid RNase" purified	70	10	4.2	1 mg protein/ml.		15	
	"Alkaline RNase" pure	70	10	4.2	1 mg protein/ml.			80
Hilmoe ref.42	Pure spleen phosphodiesterase.	60	5	3.3	0.23 mg protein/ml.	at pH 6.6/7.1		
			10			12		
			20			5		
						4		

of the "acid ribonuclease" was one of the characteristics that differentiate it from the spleen ribonuclease described by Kaplan and Heppel [8] and from pancreatic ribonuclease.

It was of interest to note from the report by Maver et al [40], that of the ribonuclease activity which passed through a D.E.A.E. cellulose column equilibrated with 0.005M phosphate buffer (pH 7.0), 85% of the original activity assayed at pH 8.2 resisted that heat denaturation at 70° for 15 minutes at pH 6.5-7 while only 40% of that same activity was evident on assay at pH 5.8 with the same heat treatment. In the most recent paper by Maver and Greco [36] describing the separation of "acid" and "alkaline ribonucleases" from spleen they stated that the purified "acid spleen ribonuclease" lost 80% of its activity when heated at 70° for 10 minutes at pH 4.2 at a concentration of 1 mg. protein/ml. while under the same conditions the purified "alkaline ribonuclease" activity lost only 20% of its original activity. This information presented a confusing variation in times, temperatures, degree of purity of samples tested and pH of the medium, thus diverse conclusions must be expected. Maver and Greco [36] demonstrated that the "alkaline ribonuclease" activity could also be assayed in the range of pH 5.7-7.0, optimally at 7.0 especially when mg^{++} ions were supplied. Mg^{++} caused an increase in activity which was concomitant with a lowering of the pH optima from pH 7.8-8.2 down to pH 7.0-7.2 under the conditions of assay. In contrast Mg^{++} caused a reduction in the activity of the "acid ribonuclease". Kaplan and Heppel [8] found that Mg^{++} ions caused a

25-35% stimulation of the activity of the heat stable spleen ribonuclease which had a pH optimum between pH 6-7 with activity expressed in the range of pH 5-8 with Mg^{++} ions present. The percentage of acid ribonuclease activity surviving heat treatment in certain reports [36,40] must be of provisional value when it is known that both the heat stable "alkaline ribonuclease" activity and the heat stable spleen ribonuclease Kaplan and Heppel [8] described, exhibit activity in this acid range. It could be judged that a heat treatment of the crude spleen preparation would destroy most, if not all, of the activity described as acid ribonuclease while most of the activity described as "alkaline ribonuclease" would survive.

Spleen phosphodiesterase, first examined by Heppel and Hilmoe [41] as one of the three ribonuclease fractions from spleen and lately purified by Hilmoe [42], should not be present to any extent after heat treatment at 60° for 10 minutes at pH 3.3. Hilmoe [42] has published a table which illustrated that heat treatment at 60° for 10 minutes or 20 minutes leaves as little as 5% and 4% respectively of the original spleen phosphodiesterase activity; Table 5 gives a composite record of these findings.

III. Distribution of total ribonuclease activity, heat stable ribonuclease activity and protein during extraction, salting out and heat treatment at pH 3.5.

In the present study it was possible to estimate tentatively the amount of heat stable ribonuclease and protein in the spleen homogenate. On certain trials a portion of the spleen homogenate was centrifuged

to remove cellular debris and the clarified solution divided into two portions. One portion was heat treated at pH 3.5 as described in Experiment 3 (page 358). Untreated and heat treated homogenate solution were assayed for ribonuclease activity (page 341) and the protein concentrations estimated by the Biuret method (page 346). The bulk of the homogenate was fractionated by salting out at pH 3.5 with solid ammonium sulphate as described on page 357, then the active fraction was heat treated according to the procedure described in Experiment 3 (page 358). Samples of the spleen solution before and after the heat treatment were assayed and the protein content estimated. Columns 1-4 of the table 15 (page 114) give the full results in detail for a typical extraction and heat treatment. It was possible to relate the amount of ribonuclease activity, heat stable ribonuclease activity and protein in the original homogenate prior to extraction to that amount in the ammonium sulphate fraction retained before and after the heat treatment. In this way some indication of the efficiency of these essential procedures could be gauged.

Table 6 illustrates the distribution of the protein content and Table 6a gives some indication of protein obtained per 100 ml. of spleen homogenate in the various trials. Table 7 illustrates the allotment of total ribonuclease activity at the salt fractionation and the recovery of heat stable ribonuclease in various fractions, An accompanying column provides similar data where possible from the original publication of Kaplan and Heppel [8].

TABLE 6

PROTEIN DISTRIBUTION

DESCRIPTION	PERCENTAGE*
Protein discarded on heat treatment of the homogenate (typical value)	41
Protein discarded on heat treatment of the 40-80% $(NH_4)_2SO_4$ precipitated fraction (typical value)	44
Protein rejected in the 0-40% $(NH_4)_2SO_4$ fraction at extraction	50
Protein retained in the 40-80% $(NH_4)_2SO_4$ fraction precipitated	34 (34 15)
Protein discarded in the supernatant of the 40-80% saturation precipitate	16
The amount of protein retained as heat stable protein after heat treatment as a percentage of the heat stable protein of the homogenate	14 (14.8 7.8)

* The percentages are from one typical experiment. The percentages in brackets give an indication of the variation found for these values in four experiments. The values for protein discarded at heat treatment did not vary greatly from trial to trial.

Table 6A

The amount of protein obtained per 100ml of spleen homogenate before and after the heat treatment of (a) the homogenate and (b) the fraction salted out at pH 3.5

Trial	1	2	3	4	5	6
Description						
Gm. protein released per 100ml of untreated homogenate. step I	0.38	-	0.64	-	-	0.53
Gm. protein/100ml of untreated homogenate heat stable in the homogenate after a heat treatment step IA	-	-	0.38	0.85	0.54	
Gm. protein/100ml of untreated homogenate extracted and retained by salting-out at pH 3.5 within the 40-80% $(NH_4)_2SO_4$ fractionation limits step II	0.12	0.06	0.1	-	-	-
Gm. protein/100ml of untreated homogenate heat stable in the fraction salted-out at pH 3.5 after the heat treatment of experiment 3. step III	-	-	0.056	0.07	0.08	0.11

In all the trials the amount of protein was measured by the Biuret test. G.M.15a.

Table 7.

Distribution of the spleen ribonuclease activity.

Description of stage.		Present work.	Cf data from table 2, page 33.
%age of the total RNase activity which was found to be heat stable in the spleen homogenate.		26.6	29.4
%age of the total RNase activity in the homogenate precipitated by increasing the $(NH_4)_2SO_4$ concentration of the homogenate from 40-80%.		44 (39.5)	44.4
%age of the total RNase activity in the homogenate discarded by increasing the $(NH_4)_2SO_4$ concentration from zero to 40%.		(50)	not available
%age of the total RNase activity in the homogenate discarded in the supernatant of the 40-80% ppted. fraction.		(10.5)	not available
%age of the RNase activity in the fraction ppted. by 40-80% $(NH_4)_2SO_4$ to survive the heat treatment of Exp 3.		35 (32)	37.8
%age of the RNase activity which was present at the end of the heat treatment stage as a %age of -	(a) the total RNase activity of the original spleen homogenate.	15.5 (10)	16.78
	(b) the heat stable RNase activity present in the heat treated homogenate.	58 (41.5)	57

The %ages were obtained from a typical spleen extraction experiment investigated at all stages.

The values in brackets were from a similar experiment which exhibited less favourable results.

(a) The fractionation at pH 3.5

Kaplan and Heppel[8] and Maver and Greco[35], two independent groups of workers showed that 29.4% and 22.7% respectively of the ribonuclease activity in the spleen tissue homogenate could be regarded as heat stable. In the present work these estimates were confirmed and a reasonably equivalent figure of 26.6% was found, Table 7 (page 77). From the information detailed by Kaplan and Heppel (page 32) it could be calculated that the fraction obtained by the first salting out of ribonuclease activity by raising the ammonium sulphate saturation from 40-80% was not efficient. 57% of the ribonuclease activity which was heat stable in the homogenate was precipitated in this fraction. In the present work several extractions were carried out using the same ammonium sulphate saturation limits detailed by Kaplan and Heppel, Experiment 2 (page 357) and it was shown that 58% of the heat stable ribonuclease activity of the homogenate at the most was precipitated. This was in agreement with the previous finding[8]. However, the retention of heat stable ribonuclease activity varied from as low as 30%, range (30-58%) in trials. In fact only about half the heat stable ribonuclease activity was effectively retained after the heat treatment process, and this amount was only 17% of the total ribonuclease activity of the spleen homogenate. The significant feature here was that extraction of the heat stable ribonuclease from the homogenate by salting out with ammonium sulphate at pH 3.5 was far from optimum. Another important aspect was the low yield of protein in the fraction

containing the enzyme after the heat treatment. Only 14% of the heat stable protein of the homogenate, (Table 6)(page 75) was recovered in this fraction. The moderate recovery of enzymic activity at 58% was offset by a satisfactory purification effect as a result of this low protein content. The overall purification to the heat treatment stage was 13 fold in Kaplan and Heppel's report[8] while Table 15 (page 144) shows a 4.4 fold purification for an equivalent experiment in this investigation.

As described in later sections, techniques were introduced where the ribonuclease active components were completely conserved within well defined fractions while certain contaminants were completely rejected. The partition of components, including the enzymic entity over several fractions similar to that obtained at, e.g. salting out was eliminated. To be consistent with this situation, as full a yield of the heat stable ribonuclease as possible was desirable at the preliminary stages after homogenization to start with. The problem was to increase the percentage of heat stable ribonuclease activity retained in the sample prior to the heat treatment with the understanding that an impressive purification at this stage was not required. This sample was obtained by salting out at pH 3.5 direct from the homogenate to concentrate the volume 20 fold before heat treatment and was considered a satisfactory manipulation which should be retained. Ribonuclease activity was rejected at this stage by salting out with ammonium sulphate, Table 7 (page 77), in both the supernatant after 80% saturation and the first precipitate discarded

after the salt content was raised to 40% saturation. The sample retained, precipitated within relatively wide saturation limits from 40% to 80% contained only 58% of the ribonuclease activity required. Now ammonium sulphate, according to the information on page 61, permits precipitation of an enzyme when a specific type of activity reveals well defined limits for its application [67]. Since the above findings were not consistent with the increase in saturation Dixon and Webb[67] recommend for enzyme precipitation (page 63) the wide saturation limits required to precipitate the heat stable ribonuclease activity, suggested the components of the mixture were not precipitating according to the theoretical explanation.

The investigation carried out and described in Section III of this thesis partly explained the poor fractionation of the ribonuclease activity by a narrow salt saturation increment. Two heat stable ribonuclease activities were present and were probably salting out at different levels at each fractionation consistent with the details on salting out theory (page 61). Thus by rejecting protein at extraction, Table 6 (page 75), by ammonium sulphate precipitation within the saturations 40 to 80%, two heat stable ribonuclease activities amounting to 58% of the heat stable ribonuclease activity was carried forward to the next step. As already mentioned, of the total ribonuclease activity present in the homogenate only 26.6% was heat stable thus the restrictions of specific salting out limits could jeopardise precipitation of this minor quantity of ribonuclease activity at those stages prior to the elimination of the greater

part (73.4%) of the total ribonuclease activity which was heat labile.

Clearly some method had to be put into effect which would concentrate greater quantities of the ribonuclease activity which would be heat stable at the heat treatment stage. As proposed earlier (page 25) the extraction and early purification technique should conform to that described by Kaplan and Heppel. It was decided to redistribute and concentrate the spleen ribonuclease activity by adjusting the salting out effect to precipitate more enzyme in the fraction retained.

(b) Refractionation by salting-out with ammonium sulphate at pH 7.0

(i) The effects of a refractionation on a protein mixture at salting-out.

At protein precipitation by salting-out, a particular protein will always precipitate within the same saturation limits of ammonium sulphate concentrations provided the variables described on page 56 are controlled[67]. Until recently, it was believed that the point at which a given enzyme precipitates at a later stage of purification by salting out, could be different from that at which it comes down at the initial precipitation by salting out, simply because it is no longer accompanied by the same mixture of substances. This notion is partly refuted[67] and the situation shown to be much more complicated even when protein interactions are ignored. Dixon and Webb[67] have shown that protein reprecipitation under exactly the same conditions in a simple protein mixture has little additional purification value,

i.e. when the desired fraction is redissolved in the same volume as it originally occupied and the refractionation takes place within the same salt limits, few contaminants may be eliminated from the fraction. If refractionation is carried out in a much reduced volume as is usually done the system becomes much more complicated. According to the theoretical considerations of Dixon and Webb[67] the enzyme can be precipitated by a much lower quantity of salt because of an increase in enzyme concentration per unit volume. What happens to the contaminants depends on how their respective concentration has varied and how effectively they salt out with respect to each other at increased concentrations.

Since the sample volume is reduced after a salt fractionation for reasons of economy and convenience the versatility and effectiveness of a second similar fractionation may be increased by varying the pH and/or temperature. Under these cirumstances a greater degree of purification is expected. (Page 61). It is convenient to take a preparation from one purification step to the next at a constant cold room temperature and in this way the temperature is adequately controlled.

A change of pH has startling effects on the solubilities of individual proteins and this probably offers greater scope for the reproducible alteration of a variable. It is much easier to control the pH of the protein environment thus a refractionation of the protein mixture at a different pH is preferable to any technique based on temperature variation.

(ii) The refractionation of the heat stable ribonuclease preparation

Kaplan and Heppel[8] (page 27) determined the residual ammonium sulphate content of the spleen enzyme sample from the first salting out and heat treatment at pH 3.5 on a Barnstead conductivity meter. After the heat treatment (page 27) they recommended that the pH be taken to 6.5-7.0. The protein content of the sample was 12 mg per ml and the fraction precipitated within the saturation limits 50%-85% ammonium sulphate was collected and dissolved in 0.05M sodium acetate 10^{-4} MEDTA pH7 at a concentration of 24 mg/ml ready for dialysis. An almost complete recovery of heat stable ribonuclease activity at 98.7% was recorded by them, with a two fold purification and five fold reduction in volume. 57% of the protein is eliminated by the fractionation.

In the present work the spleen protein solution was refractionated after the heat treatment of Experiment 3 (page 358). The residual ammonium sulphate concentration was estimated by the modified Nessler's method[175] U.M. (page 338) and the conditions for the refractionation with ammonium sulphate at pH 7 are set out in Experiment 4 (page 359) The spleen protein sample, containing heat stable ribonuclease activity at exactly pH 7, was taken to 50% saturation with ammonium sulphate. After the removal of the precipitated protein the supernatant solution was taken to 85% saturation with ammonium sulphate at the rate of 23 g per 100 ml of enzyme solution. The precipitated protein was brought into solution by the slow addition of small

quantities of 0.05M sodium acetate 10^{-4} MEDTA pH 7 to a protein content of 20 mg/ml. Table 8 illustrates the type of results obtained by this salting out procedure.

According to the ribonuclease assay and protein determinations, considerable ribonuclease activity and protein were discarded in the supernatant by this precipitation step. In the trial B the supernatant was taken to saturation with ammonium sulphate and the precipitate assayed. Only 5.3% of the total activity unaccounted for was recovered by this precipitation. 50% of the total ribonuclease activity presented in the protein sample after heat treatment was recovered in the appropriate fraction on refractionation. The salting out gave a 1.7 fold purification and a fivefold reduction in volume. This very poor yield of ribonuclease activity did not compare favourably with the 98.7% recovery claimed by the original authors[8] (page 84). The 50% ribonuclease activity recovered at this stage compared favourably with the 58% yield of heat stable ribonuclease activity fractionated out at the first salting out at pH 3.5, Experiment 2. At the refractionation, ribonuclease activity was discarded in the first precipitation, e.g. in trial A and in the supernatant which appeared to contain considerable ribonuclease activity not precipitated by ammonium sulphate even at saturation[79], (see page 91). Subsection 6 (page 111) deals further with the outcome of the investigation of the salting out of pH 3.5 already discussed on pages 78 - 82, and refractionation at pH 7 described above.

Table. 8.

The distribution of protein and ribonuclease activity on salting-out with ammonium sulphate at pH 7.

Precipitation limits as a %age of ammonium sulphate concentration.	Distribution of protein as a %age of the total protein applied.		Distribution of RNase activity as a %age of -		
			the total RNase applied.		the total RNase retained.
Trial	A	B	A	B	B
Fraction I Ppted within 10% to 50% saturation.	34.5	25.0	18.4	6.7	10.7
Fraction II Ppted within 50% to 85% saturation.	24.5	33.3	37.0	50.0	84.0
supernatant from 50% to 85% pptation. containing the remainder.	41.0	41.7	44.6	43.3	-
Fraction III Ppted within 85% to 100% saturation.	-	-	-		5.3

IV. The concentration of ribonuclease active protein by salting out

Concentration with limited preliminary fractionation of the spleen ribonuclease activity from the crude spleen preparations by salting out with ammonium sulphate before and after the heat treatment was attempted as an alternative to the salting out system which was presented by Kaplan and Heppel[8] and found to be unsatisfactory when repeated in the present work (pages 78 and 84). The use of ammonium sulphate to concentrate the spleen preparation was successful in conserving ribonuclease activity in the crude precipitates formed. Subsection 6, page 111, summarises how these precipitations were adopted to cope with the preparation of spleen ribonuclease samples at the preliminary stages of purification in addition to presenting briefly the reasons for abandoning most of the purification steps published by Kaplan and Heppel[8].

(a) The concentration of spleen ribonuclease at pH 3.5 before the heat treatment

The method adopted is outlined in Experiment 1 (page 353) which details also the precautions taken to effect reproducibility of spleen extract. This method was a modification of Experiment 2 (page 357).

To 3 litre aliquots of homogenate at pH 3.5, 160 g ammonium sulphate was added per litre (30% saturation) and the bulky precipitate and solids removed by centrifugation at 10,000 x g for 10 minutes at 0°. The bulky precipitate and solid material was re-extracted with an equal volume of 0.05M sodium acetate 10^{-4} MEDTA pH 3.5 made 30% saturated with ammonium sulphate, by stirring as a slurry. The solid

material was rejected by centrifugation as above. The supernatant from the extraction and re-extraction were combined and 356 g ammonium sulphate was added per litre to bring the saturation from 30% to 80.5%. The supernatant was discarded after the precipitate was retained by centrifugation at 30,000 x g for 7 minutes at 0^{o}. The precipitate was dissolved in 0.05M sodium acetate 10^{-4} MEDTA pH 7.2. The difference between trials carried out using this method to that described in Experiment 2, was the introduction of a re-extraction of the rejected homogenate tissue and precipitate and the addition of a greater amount of salt to precipitate the active fraction. It will be recalled that in Experiment 2 considerable quantities of ribonuclease activity, (Table 7, page 77), was rejected (50%) in the homogenate tissue and protein precipitate by raising the salt concentration from zero to 40% saturation. In Experiment 1 only 2% was rejected in comparison. This was done by taking the ammonium sulphate saturation to 30% (c.f.40%) and re-extracting the solids and protein precipitate with an equal volume of extracting buffer made 30% saturated with ammonium sulphate. This constituted a direct re-extraction without dissolving the precipitate, and was intended to collect ribonuclease activity mechanically trapped in the large volume of precipitate and tissue solid.

The re-extraction was not as important a feature as the changes in the amount of solid ammonium sulphate added. Previously in this work, and as used by Kaplan and Heppel[8] a further 258 g ammonium sulphate per litre was added to precipitate 58% of the ribonuclease

activity which was heat stable and was 17% of the total ribonuclease activity of the sample (page 66). In comparison 356 g ammonium sulphate per litre was added in the modified method used here to concentrate the spleen sample from the supernatants after cellular debris and protein precipitated to 30% saturation were removed. This supernatant contained 98% of the ribonuclease activity which had to be concentrated by salting out. As previously discussed (page 60) the amounts of ammonium sulphate required to precipitate proteins from solution ought to be applied at 25°. However, the amounts tabulated[69,74] appear to be used, irrespective of this standard temperature condition, at the temperature specified for the experiment. Reproducibility of protein precipitate depends on repeating procedure exactly, by applying the conditions stated for temperature, salt content, pH and technique.

A check on the quantities of ammonium sulphate used by Kaplan and Heppel to precipitate the protein by raising the salt saturation from 40% to 80% saturation revealed a possible discrepancy based on this observation. Irrespective of the inaccuracy of adding solid salt to a volume of homogenate which contained solid tissue material, the amount of ammonium sulphate added to raise the saturation of the homogenate from zero to 40% was adequate. Kaplan and Heppel[8] then added 258 gms of ammonium sulphate per litre to raise the saturation from 40% to 80%. From the charts referred to on page 60 it could be estimated that the ammonium sulphate saturation was 77% and to bring the saturation to 80% required 285 g not 258 g per litre of ammonium

sulphate, a possible discrepancy of 27 g/litre.

In the modified method of Experiment 1 which concentrated rather than fractionated, 356 g ammonium sulphate was added per litre of supernatant to raise the saturation from 30% to 80.5%. This amount eliminated the above discrepancy and based the salt concentration on the percentage saturation of 4.1M by reference to the charts but applied at the temperature of this experiment at 3°. The last of the solid ammonium sulphate added, dissolved with difficulty and the mixture was stirred for 1 hour. Experiment 1 describes how the mixture was left overnight to allow the fine precipitate to settle out after it aggregated, with the result that much of the clear supernatant could be siphoned off to produce a much reduced centrifugation programme. The mixture centrifuged well despite some doubts that the high salt concentration would cause the precipitate to resist centrifugation. The yield of ribonuclease activity in the fraction collected by centrifugation was 78% with a loss of 20% to the supernatant which was rejected. This was much superior to the 44% recorded as percentage of the total activity of the homogenate extracted in the 40 to 80% precipitated fraction, Table 7, page 77, Experiment 2, page 357. The amount of ribonuclease activity rejected in the supernatant saturated at 80.5% with ammonium sulphate was surprisingly high at 20%.

The significant feature of the modification to the fractionation at pH 3.5 first used in Experiment 2 was that considerably more ribonuclease activity was retained and concentrated before the heat treatment. The scheme to widen the saturation limits to give a

concentration effect was based on the theoretical considerations already discussed on page 61 , particularly the fact that enzymes do not precipitate within fixed limits of salt saturation characteristic of each enzyme. i.e. The limits vary depending on the concentration of the enzyme and the less enzyme present the greater the quantity of ammonium sulphate required to precipitate that enzyme. Thus the less concentrated the enzyme in solution the greater are the chances that an increasing proportion of the enzyme resists precipitation and remains in solution even if the solution is saturated with salt. A very recent paper by Chesbro et al[79] described how 30-40% of a Staphylococcal nuclease was soluble in a solution saturated with ammonium sulphate and this feature was attributed to its small molecular size. The remainder of the Staphylococcal nuclease precipitated between 70-80% saturation with ammonium sulphate. In the present work it was considered that by lowering the saturation to 30% in the modified Experiment 1 the increase in ribonuclease activity redistributed to the supernatant would cause a greater retention of ribonuclease activity at the second salt precipitation which was mainly a concentrating step. However, as already mentioned considerable ribonuclease activity was rejected when it was not precipitated at 80.5% saturation.

The alternative to this concentration step was a conventional investigation of the ammonium sulphate fractionation of the homogenate by well defined increments of salt addition. In this way it might have been possible to detect and secure one heat stable ribonuclease

activity without the other even in the presence of similar heat labile ribonuclease activities. This would have been very difficult to achieve and would have required a heat treatment and fractionation of each sample on CM cellulose as described on page 316 before salt fraction limits for each could have been settled on. However, the precise fractionation conditions for each ribonuclease activity might have been achieved for one batch of raw material and this might not have been standard. Such findings would have been of no significance if the source of the homogenate could not be guaranteed reproducible in the amount of protein present and extracted per spleen and possibly even in the relative proportions of each protein or enzyme in the sample. Although precautions were taken to disrupt the spleen tissue in exactly 3 volumes of 0.05M sodium acetate 10^{-4} MEDTA pH 7 (page 354) the content of protein was not controlled. The concentration of protein and relative proportions of the various proteins would depend on the degree of exsanguination. Table 6a is not complete in detail but sufficient information was available from the trials under Experiment 2 to demonstrate that the extract from spleen was not reproducible from one batch to the next. Also if there was a variation in blood content from one batch to the next, the content of blood protein to spleen protein would vary from one extract to the next, thus the relative proportion of different proteins at purification must vary. The consequences of variation in protein content is described earlier in Section II (page 63).

(b) Concentration of the ribonuclease active sample after heat treatment

A protein concentrating step based on the salting out at pH 7 with ammonium sulphate was introduced at a later stage of the purification after the carboxy-methyl cellulose column fractionation was a functional part of the procedure (Section III). The main purpose of this was to provide a concentrated enough protein solution to make desalting on Sephadex possible, as small concentrated samples were necessary for application to the columns. The heat treatment destroyed 50% of the protein in solution which was then too dilute in content for an efficient and optimum application to the Sephadex columns. After heat treatment the solution had to be concentrated to 40-50 mg protein per ml from 12 mg protein/ml. To comply with some of the proposals described earlier in this section (pages 79, 90) when considering a modification to the salting out procedure at pH 3.5 to retain a greater quantity of ribonuclease activity, a similar adjustment was made at this concentrating step to secure a larger proportion of the ribonuclease activity in the protein fraction retained. This fraction was secured within the saturation limits 40-85% as against 50 to 85% and the amounts of ammonium sulphate were adjusted according to the values given on the published charts (page 60)[69,74]. The procedure is detailed in Experiment 5 (page 361) and was essentially similar in the details of technique to Experiment 4. The ammonium sulphate saturation was taken to 40% and any precipitate formed was removed by centrifugation. The saturation of the supernatant was then

raised to 85% and the centrifuged precipitate retained. It was then dissolved in the particular buffer used to equilibrate the Sephadex column.

No values for purification were available, though assays showed that 2% of the total ribonuclease activity was discarded when a precipitate formed on addition of ammonium sulphate to 40% saturation. Of the original activity 88% was retained in the protein fraction precipitated between 40 and 85% saturation. The remaining 10% of the ribonuclease activity was discarded in the supernatant.

4. The Dialysis Process

Dialysis is reviewed by L. C. Craig and T. P. King in volume 10 of Methods of Biochemical Analysis, edited by D. Glick and published by Interscience Publishers Inc., New York 1962. Dialysis can be described as a manipulation to remove low molecular weight diffusible solutes of varied description from the non-diffusible components of a solution. e.g. Noxious ions like copper, or other ions introduced at salting out can be removed from the protein solution by dialysis against deionised water containing a chelating agent or deionised water respectively. The essential feature in dialysis is a membrane which is permeable to small molecules and solvent and impermeable to large molecules particularly macromolecules. Cellophane which has a suitable pore size for this general purpose is the most commonly used membrane material.

The mechanism currently held to operate is that the membrane has

the functions of a sieve with more or less rigid pores of fixed size. The model existence of the membrane is aptly described by Craig and King[61] who state that when cellophane is wet with water it can be considered to be like a very thin sponge with the expected tortuous anastomosing pores or microcavities of various shapes and sizes. Of considerable significance is the possibility that small proteins or enzymes will not be excluded by the pores. Craig and King[61] have demonstrated that the loss of small proteins to the diffusate with ordinary cellophane membranes becomes serious with molecules below molecular weight 15,000. They illustrate that 50% of pancreatic ribonuclease is lost in 6.6 hours from the retentate in ordinary 20/32" visking tubing.

At dialysis the sample under study is confined to the membrane sac and transfer of solute molecules and solvent is brought about by diffusion pressure induced by the pressure gradient across the membrane. The factors which affect the rate of equilibration are the membrane thickness, temperature and particularly the amount of surface area of the cellophane containing sample presented to the equilibrating medium[80]. The state produced by extensive dialysis against deionised water is extremely unnatural and can cause protein denaturation and precipitation in some instances[72].

Kaplan and Heppel in their paper[8] recommended rocking dialysis overnight against running 10^{-4} MEDTA where the EDTA solution was adjusted to pH 7.2 with NaOH. After dialysis a granular brown precipitate was removed by centrifugation from the retentate. Although

a slight increase in purification was obtained, probably the effect of the removal of euglobulin type proteins precipitated in the absence of salt[72] a loss of ribonuclease activity of 11.5% accompanied by a reduction in the protein content by 15% could be calculated to have taken place. In the appendix to the paper[8] an ultracentrifugal study of the ribonuclease activity suggested a molecular weight in the range 2,000-5,000. In view of the findings of Craig and King[61] with the much larger pancreatic ribonuclease at dialysis the loss of 11.5% spleen ribonuclease activity after an overnight dialysis was surprisingly low. It could only be assumed that ordinary untreated dialysis membrane was used.

In the present work, overnight dialysis was used. A full description of the method is set out in the general procedure for dialysis G.M.3. Ordinary visking tubing was used and up to 40% of the ribonuclease activity was lost from the retentate. The amounts of protein lost by diffusion or precipitation varied from 39% to almost 60% in one instance. An attempt to redissolve the precipitate, which formed within the tubing during dialysis because of the removal of salt, in 0.05M sodium acetate 10^{-4} MEDTA was not completely successful. The proportion of protein which redissolved contained only 2% of the original ribonuclease activity. This amount was not significant and was probably accounted for by contamination at precipitation. Attempts to recover ribonuclease activity from the diffusate by freeze drying or adsorption on C.M. cellulose were not successful.

The possibility of desalting by gel filtration on Sephadex without risk of ribonuclease activity loss, directed operations to this new molecular sieve material (page 119) where mild conditions could be extremely well controlled. Some ideas on adjusting the pore size of cellophane[80,157] to pass low molecular weight proteins which could be recovered from a controlled volume of diffusate, was abandoned in favour of Sephadex before sufficient information on the fate of the ribonuclease activity not accounted for in the retentate could be ascertained. Hardly any ribonuclease activity was recovered, when a 5 litre quantity of primary diffusate, after 6 hours of dialysis, was concentrated by freeze drying and the lyophilised material assayed. Similarly a further 5 litres of diffusate on application to a cation exchange cellulose column at a pH conducive to retaining the spleen ribonuclease (page 165) did not contain sufficient ribonuclease activity to compensate for the high loss experienced. The proteinaceous material retained by the column was removed by eluting with 1M NaCl then assayed for ribonuclease activity. It was estimated that 10% of the 40% loss was accounted for by the presence of ribonuclease activity in these dialysis diffusate samples. It may be that high dilution and a long delay under these conditions was detrimental to the state of the ribonuclease active component. The many aspects relating to loss of ribonuclease activity at dialysis have not been sufficiently probed to comment further without speculation.

5. Protein precipitation with organic solvents

(a) Theory

Enzyme fractionation by precipitation with organic solvent has not been as generally applied as the more widely used salting out precipitation techniques with neutral salts which are described on page 55. The addition of organic solvents to aqueous solutions of proteins produces marked effects on the solubilities of these proteins. Much of the effect is due to the change in dielectric constant of the medium. A uniform medium has a property described as its dielectric constant which determines the magnitude of the forces which act between any two given electrical charges placed at definite distances apart in the uniform medium[83]. The higher the dielectric constant the smaller is the force of attraction or repulsion between these charges. Dipolar ions such as glycine increases the dielectric constant of water while miscible organic solvents, e.g. methanol and acetone, decrease it and these effects can be used to advantage[49]. By decreasing the dielectric constant the coulombic attractive forces between the unlike charges of the protein molecules are increased and solubility is lowered. With prudent control over certain variables precise, selective fractionation, comparable with neutral salt precipitations of the protein components of a mixture is conceivable. The addition of miscible organic solvents to the aqueous medium lowers the dielectric constant, certain protein-protein interactions are favoured and precipitation ensues. It is uncertain whether this effect is of greater importance than more specific interactions in

determining solubility. Completely water miscible organic solvents by virtue of their interaction with the water itself may cause dehydration of the protein molecule. Direct solvation of the protein must also occur where part of this solvation represents exchange with protein bound water. This solvation may increase protein solubility while dehydration decreases it and the net effect of these two factors can never be reasonably predicted for protein mixtures[69].

(b) The variables that influence acetone fractionation

Any reproducible scheme requires careful attention to the variable:

(1) temperature

(2) dielectric constant

(3) protein content

(4) pH

(5) ionic strength

They are all related in some way. Since they affect protein solubility these variables must be controlled during the experimental procedure.

Control of temperature

The lability of proteins particularly enzymes in the presence of organic solvents generally requires that the temperature be kept close to the freezing point of the solution throughout the fractionation. Manipulation involving transfer, e.g. at centrifugation, may be difficult to perform without the uncertainty of a 5^{o} variation in temperature when the temperature is -15^{o}. Variation in temperature

affects protein solubility and any increase in solubility requires a greater quantity of organic solvent to effect the same precipitation. An immediate variation in temperature of a solution can come about by the heat produced on mixing solvent with water.

Dielectric constant

Although ethanol has been used most frequently, e.g. in the serum protein fractionations devised by Cohn[81] where intricate manipulation of the variables earlier listed brings about outstanding separations, acetone, according to Askonas[76], is much superior to other solvents for enzyme separations. This author[76] finds that the precipitation is sharp and a 2% increase in acetone concentration causes a sufficiently large precipitation step. Table 9 shows that the dielectric constant varies slightly with temperature change and of the solvents listed acetone has the lowest dielectric constant. Upon addition of acetone to water a greater reduction in the dielectric constant of the solution can be expected for smaller volume additions than with ethanol or methanol. Table 10 illustrates how the dielectric constant of water is lowered by solvent addition. According to Askonas[76] methanol produces hardly any separation or purification and is the least useful of the miscible solvents tested. It can be seen that very much more methanol has to be added to the aqueous solution to lower the dielectric constant to the values obtained by acetone addition.

TABLE 9. EXAMPLES OF DIELECTRIC CONSTANT

Solvent	Dielectric constant	
	At 20° *	At 25° +
Water	80	78.5
Methanol	32.4	-
Ethanol	25	-
Acetone	19.6	21.2

* Reference 82

+ " 83

TABLE 10. CONTROL OF DIELECTRIC CONSTANT OF AQUEOUS SOLUTION BY ORGANIC SOLVENTS*

Dielectric constant of the aqueous solution	Weight % of solvent in water to produce the dielectric constants listed at 20°		
	Methanol	Ethanol	Acetone
80	0	0	0
70	22	18	18
60	42	34	34
50	62	51	49
40	82	68	64
30	-	88	81
32	100	-	-
25	-	100	-
20	-	-	100

* Reference 69a

Protein content

The point at which a particular protein will be brought out of solution at fractionation by salting out depends partly on the concentration of that protein in solution[67]. Similar effects can be expected to occur at the precipitation of protein by organic solvents[69]. If a particular component is present in lower content than usual, then it can be expected that much more solvent will be required to precipitate it and the acetone concentration limits within which precipitation takes place will not be the same. Askonas[76] finds that proteins precipitate out of solution in a definite order. Each protein comes out of solution when a constant percentage of the total protein has precipitated regardless of the solvent concentration at which this level of precipitation may be reached (page 64). With an extract from the multifunctional spleen organ[68], where the content of blood protein at exsanguination cannot be adequately controlled the relative amounts of the many protein components may vary greatly from trial to trial. Although the precipitation order may remain constant it may not be possible to state with certainty, from one trial to the next, the point at which the major portion of the required component will precipitate. This could happen irrespective of whether the total protein concentration remains unchanged. If on the other hand a reproducible protein composition is possible then the position of precipitation of all the components can be fixed. From this it can be suggested that protein concentration expressed as mg. of protein per ml. is inadequate[8].

pH

At pH values where the protein is less soluble, (near the isoelectric point) protein precipitation by organic solvents is favoured. At pH values away from this pH the protein is more soluble and requires much more organic solvent for precipitation, thus increasing the danger of denaturation.

Ionic strength

A very low ionic strength of ca 0.03[76] facilitates protein fractionation by this technique, although some ions are required for aggregation. At an ionic strength of 0.1 proteins tend to be more soluble, hence greater quantities of organic solvent are required to effect a precipitation. At these higher ionic strengths a decrease in the sharpness of separation with lower recovery of the components is recorded by Askonas[76]. She found that the nature and concentration of the salts influences fractionation profoundly. Certain salts may be required to assist in the formation of complexes which favour precipitation, or where recovery is promoted, some specific protection against denaturation may be involved. Sufficient information on these aspects is not available.

(c) The acetone fractionation of spleen ribonuclease preparations

The purification procedure outlined by Kaplan and Heppel[8] included an acetone fractionation where the second fraction precipitated was retained and was shown to contain between 50-66% of the original activity with a purification between 1.9 and 2.5 fold. The details given for the conditions of the acetone fractionation

were not as complete as could be desired for comparison. The enzyme solution was extensively dialysed against 10^{-4} MEDTA overnight, then diluted to contain 6-10 mg. protein per ml. A quantity of 1M sodium acetate was added to bring the sodium acetate content to 0.2M ($\frac{\Gamma}{2}$ = 0.4) before the addition of cold acetone. By calculation the salt content fell to 0.111M sodium acetate ($\frac{\Gamma}{2}$ = 0.22) at the end of the first precipitation when 44.4% v/v acetone was added. No mention of the pH of the medium was made at any point during the fractionation, though it was presumed to be about neutral and a wide variation in the content of protein from 6-10 mg. per ml., without reference to the active component, was specified. A high molal ionic strength $\frac{\Gamma}{2}$ = 0.22 cf. 0.03[76] was used, although after a precipitation has taken place it is doubtful if ionic strength can be calculated directly on dilution with acetone as the ions are involved in the protein precipitate and deplete the medium of salt[76]. Excess salt of one type, e.g. sodium acetate, may have some effect in overcoming the additional variation caused by salt depletion of the medium at later precipitations. Exact data for temperature control was outlined for the addition of acetone and centrifugation of the first precipitated fraction which was rejected. No information on temperature for further acetone addition and collection of the important second precipitate is given. The second addition of acetone brings the acetone content v/v to 66.6% and the sodium acetate content to 0.066M or $\frac{\Gamma}{2}$ = 0.132 by calculation. This fraction was centrifuged off and drained free of excess solution at -10° for

30 minutes then dissolved in 0.05M sodium acetate 10^{-4} MEDTA to complete the step.

A 22% increase in acetone concentration was a large fractionation step particularly as the return of ribonuclease activity was low at between 50-66% of the original activity with only a 1.9-2.5 fold purification. This would not seem all that satifactory as acetone with its sharp precipitation effect was reported [76] to exhibit almost complete recovery of enzyme activity consistent with fairly sharp separations. (5% increase in acetone concentration and greater purification of the order 5-12 fold).

(d) An assessment of the acetone fractionation

In the present work a purification procedure possibly similar to that used by Kaplan and Heppel[8] was attempted to find out how much of the ribonuclease activity (40-50%) not returned in the second fraction was either distributed active in other fractions or lost by denaturation and rejection in the final supernatant.

The acetone fractionation procedure detailed on page 287 was used at the three experimental pilot trials outlined on pages 361 - 363 The protein samples fractionated in experiment 6 and 7 were from the same extract and directly comparable in protein content at the beginning of the fractionation while the protein sample fractionated in Experiment 8 was the product of a different extraction but obtained by the same procedures. These procedures included the homogenization of the spleen tissue and salting out at pH 3.5 with ammonium sulphate, (Experiment 2), heat treatment (Experiment 3),

salting out with ammonium sulphate at pH 7 (Experiment 4) and finally dialysis against 10^{-4} MEDTA pH 7 overnight.

Table 12 demonstrates the fate of the protein content at fractionation, 40 to 50% of the protein was unaccounted for and was presumed rejected in the supernatant after the three fractions were taken. The protein recovered in fraction B was fairly constant at between 26-29% of the total. Fraction A varied in content quite significantly.

TABLE 12

Percentage of original protein content in each fraction

Fraction precipitated within the % added acetone by volume	% Protein (Biuret)		
	Experiment 6	Experiment 7	Experiment 8
Fraction A 0 → 44.4% Acetone v/v	11.6	26.3	14.5
Fraction B 44.4 → 66.6% Acetone v/v	26.5	29	29
Fraction C 66.6 → 75% Acetone v/v	5.4	8.7	not taken
% protein of original sample returned in the fractions collected	43.5 A + B + C	64 A + B + C	43.5 A + B only

Table 13 presents the distribution of ribonuclease activity in the fractions produced by acetone fractionation. 50% of the ribonuclease activity could be secured in fraction B. In two experiments fraction A apparently contained little activity while Experiment 7 which produced a complete recovery of ribonuclease activity in the three fractions taken and where the protein sample

TABLE 13

Percentage of ribonuclease activity recovered

% Acetone v/v to Produce	Experiment 6	Experiment 7	Experiment 8
Fraction A 0 - 44.4%	8.6	41.5	9.5
Fraction B 44.4 - 66.6%	49	48.7	50
Fraction C 66.6 - 75%	6.1	9.8	not taken
% Ribonuclease activity retained of original sample	63.7	100	59.5

was exactly the same as that for Experiment 6, showed a good yield of ribonuclease activity in fraction A. The recovery of activity almost equalled that of fraction B. There could be no way of accounting for this as all three experiments were carried out in exactly the same manner as far as could be ascertained by the procedure adopted. Experiments 6 and 8 suggested that either the ribonuclease activity not recovered was rejected in the supernatant of fraction C or lost by inactivation, probably at the first fractionation. Experiment 7 certainly suggested that if appropriate measures were in force fraction A could yield the balance of the ribonuclease activity. The fate of the balance of the ribonuclease activity processed was not satisfactorily resolved by the number of experiments performed. Excluding a general inactivation at all

stages one alternative would be the rejection of activity in the supernatant of high acetone content as at least 50% of the protein was lost to this fraction. It was more likely, from the experience with Experiment 7 that in the early stages of fractionation for the production of fraction A when the temperature was at 0^{o} sufficient temperature control consistent with minimal acetone additions had not resulted. Inactivation had taken place in Experiments 6 and 8 but not in 7 where conditions were conducive to optimal fractionation.

The large amounts of acetone used for the fractionation zero to 44%, 44 to 66% v/v were not consistent with the sharp precipitation limits generally associated with acetone fractionation. According to Askonas[76] it is not understood why some proteins show a greater tendency to spread over wide fraction spans. Protein interaction and the adsorption of protein, one with the other may be responsible. Alternatively, one or more of the precipitation variables, e.g. ionic strength, may not be optimal for fractionation (page 99) and these require a thorough investigation. No difficulties were experienced in the natural flocculation, centrifugation or redissolving of the precipitates obtained suggesting sufficient ions were present to facilitate these operations. However, there could be too high an ionic strength to facilitate sharp precipitations (page 103).

Table 14 lists the purifications obtained by the relevant fractions.

TABLE 14

Experiment	Fraction	Purification
6	B	2.1
7	A	1.54
7	B	1.6
8	B	1.7

In Experiment 7 the purification of fraction A was as good as that obtained with fraction B, the fraction normally retained as the ribonuclease active sample. A direct comparison with the yield and purification figures published by Kaplan and Heppel[8] showed that much the same yield and purification had been achieved with an acetone fractionation (page 103). The outcome of the procedure as it stands was not outstanding and unless conditions could be improved to implement a sharper fractionation with much higher yields, e.g. a 10% increase in acetone content to produce a precipitate containing 80-90% of the original ribonuclease activity, the procedure had little advantage over safer salt fractionations performed at temperatures easily controlled. A control of the pH of the environment had not been meaningfully taken into consideration. With respect to protein solubility phenomena, pH is one of the most potent variables and ought to be strictly controlled if a reproducible procedure is to be adopted.

The figures 9 and 10 (pages 131, 133) can be directly compared. Figure 10 exhibits the elution pattern of a gel filtration on Sephadex of the active fraction B from Experiment 8, while Figure 9 depicts the elution pattern of the protein sample before the acetone fractionation. The elution patterns are similar in outline and the effect of the acetone fractionation has been demonstrated by a reduction in the bulk of the inactive protein peaks either side of the ribonuclease active region giving greater prominence to this section of the pattern, Figure 10. It would be of interest to know whether the complete removal of certain protein components had been achieved or whether various proportions of most components were rejected as a consequence of the wide precipitation limits employed.

It was decided not to continue with the acetone fractionation or set about an investigation to attempt an improvement of the fractionation technique by varying ionic strength or controlling pH more effectively. It was thought preferable at this point to embark upon an examination of the mild fractionation techniques of gel filtration on Sephadex, and ion exchange on substituted cellulose materials which were then becoming available commercially. Other reasons for abandoning the acetone fractionation in addition to the unsatisfactory results so far obtained on the pilot scale basis were that the processing of large volumes of spleen preparations would have to be carried out in several similar small scale trials until the total quantity was dealt with, or the uncertainties of a scaled up procedure would have to be dealt with.

6. Summary of the results of the preliminary investigation made on the techniques already employed to purify the spleen ribonuclease

The subsections 2, 3, 4 and 5 of Section II are devoted to an exhaustive study of the first six steps of the original Kaplan and Heppel[8] treatments outlined earlier in Section I. As previously emphasised the inclusion of these steps was considered imperative to ensure that the appropriate ribonuclease activity was purified. Two of the purification steps outlined, VII and VIII (pages 28, 29) were not examined. Step VII was a third salting out with ammonium sulphate at pH 2. Like their purification by refractionation at pH 7 with ammonium sulphate a full yield of ribonuclease enzyme was obtained. In Step VIII highly active samples from Step VII were purified by a bulk treatment with Amberlite I.R.C. 50 (X.E.64) resin. Although these two steps produced a six fold purification, a 40% loss of ribonuclease activity was attributed mainly to the instability of the enzyme to prolonged contact with the concentrated phosphate buffer at pH 7 used to elute the resin.

In the present work a third salting out was not attempted mainly because the disappointing results with the two salting out techniques already investigated did not warrant further trials of this nature. The heat stable ribonuclease was not effectively salted out of solution, Table 16, page 115. At pH 3.5 and pH 7 salting out with ammonium sulphate precipitated only 58% and 50% respectively of the heat stable ribonuclease activity. Table 16

shows a serious loss of about 50% of the heat stable ribonuclease activity for each preliminary step attempted.

For this reason alone Step VIII, the Amberlite treatment, which inactivated a further 40% of the ribonuclease activity was not attempted. Much superior ion exchange media to the synthetic resins used by Kaplan and Heppel were now available and these alternative approaches were investigated, (Section III).

From the results of the early work undertaken and described in Section III, it was apparent that there was the possibility of two heat stable ribonucleases as already postulated on page 22 This possibility was strengthened although not conclusively by the elution patterns from the C.M. cellulose columns, page 185. This finding was in part a pointer for the abandonment of some of the purification techniques carried out as described by Kaplan and Heppel and led to the modification of others as already described on pages 87, 93. Both the ammonium sulphate fractionation procedures precipitated ribonuclease activity over wide saturation limits with a 40% increase in salt concentration and only a recovery of half the ribonuclease activity in each case. A considerable amount of other protein components must fractionate out to contaminate the sample (page 63) and it was apparent the ribonuclease activity was a very small fraction of the protein in the extract. e.g. After the 420 fold purification obtained by the original authors 0.03% of the protein present in the homogenate was retained (page 32). In the present study the excessive losses (Table 16) encountered at

all fractionation steps was attributed to the fact that an attempt was made unknowingly to precipitate the activities of two heat stable ribonucleases within well defined salting out limits.

An analysis of the results of the extraction and initial purification techniques attempted permitted a valid assessment of the efficiency of the methods employed. Table 15 illustrates the results from one of the more favourable trials carried through to completion at the acetone fractionation. Specific activity measurements from the original paper (page 32) are presented in Table 15 for direct comparison. Table 16 lists the percentage of heat stable spleen ribonuclease activity lost at each step and compares them directly with the equivalent values calculated from the original paper[8] (page 33). Kaplan and Heppel pointed out that the specific activity was occasionally lower than usual after dialysis or acetone fractionation, but maintained that this was compensated for by an improved purification at the final stage with Amberlite resin.

In the present study no outstanding purification step consistent with a good yield was accomplished after the heat treatment and little was achieved by the three steps carried out. No attempt was made to improve the conventional fractionations by salting out, at pH 3.5 and pH 7.0, and acetone fractionation by adjusting the variables associated with these procedures to more suitable conditions if this was possible. The specific activities for the purification steps from the heat treatment stage No. 3 to acetone

Table 15.

A typical result from the fractionation steps attempted.*

Step No.	Procedure.	Total volume (ml.)	Total activity units.	Activity units per ml.	Mg. protein per ml. (Biuret)	Specific activity as units per ml.	Cf. col 5 table 1, page 32.
1.	Untreated homogenate.	3,300	37,158	11.26	6.4	1.75	0.34
1A.	Homogenate heat treated as in step 3.	3,300	9,900	3.0	3.8	0.78	0.1° (0.18)
2.	$(NH_4)_2SO_4$ precipitation at pH 3.5	200	16,440	82.2	16.5	5.0	3.4
3.	Heat treatment at pH 3.5	220	5,764	26.2	7.8	3.36	2.5
4.	$(NH_4)_2SO_4$ precipitation at pH 7.	42	2,856	68	11.76	5.78	5.7
5.	Extensive dialysis over a 24 hour period.	122	1,659	13.6	2.7	5.0	6.0
6.	Acetone fractionation.	78	813	10.4	1.0	10.4	12.6

* Presented as suggested in "Enzymes." Ref. 84.

° See page 27.

Table 16.

Details of the significant spleen ribonuclease activity losses.

Stage in the procedure.	%age of the heat stable RNase activity rejected at each step	
	Present work. (col. 1.)	Cf. data from tables 2 & 3. page 33. (col. 11.)
The extraction from the homogenate and salting out at pH 3.5 by increasing the $(NH_4)_2SO_4$ concentration from 40% to 80% Exp. 2.	41.8	42.9
The salting out at pH 7 by increasing the $(NH_4)_2SO_4$ concentration from 50% to 85%. Exp. 4.	50.5	2
The extensive dialysis over a 24 hour period. G.M. 3.	42	10.4
The acetone fractionation where fraction B was secured by an increase in the acetone concentration from 44.4% to 66.6% v/v . Exp. 6.	51	27

After the acetone fractionation 8.2% of the heat stable ribonuclease activity originally present in the homogenate was retained in fraction B.

fractionation No. 6, Table 15, page 114, were in near agreement with the equivalent values from the original paper and this suggested the purification procedure had gone according to plan. However, Table 16 shows a serious loss of ribonuclease activity at each of these steps and similar losses were not evident for the equivalent steps presented by Kaplan and Heppel, of column II, Table 16. This finding could not be explained. Inconsistent values for the specific activity of the heat treated homogenate from trial to trial was obtained. In the example illustrated in No.2 column of Table 15 the specific activity is 0.78 which is 4 fold greater than that of the original authors'[8] findings. In the present study this value varied from much lower values at 0.2 to 0.78 with the different samples which suggested that some samples were purer than others. This effect could be attributed to the variation in amount of protein extracted and was a reflection of the irregular content of the source material, (Table 6a, page 76). This meant that it was very unreliable to base the degree of purification, as calculated from specific activity values for later stages, upon the specific activity value of the heat treated homogenate which should be constant, e.g. the salting out at pH 3.5 followed by heat treatment results in a 4.4 fold purification if the specific activity of the heat treated homogenate was high at 0.78. However, the purification was impressive at 34 fold if the specific activity of the heat treated homogenate was low at 0.1 (Table 15, column 2). Consequently the efficiency of extraction and salting out at pH 3.5

had little to do with the degree of purification developed at this early stage. The degree of purification was more a reflection of the state of the source material. It was concluded that irrespective of the specific activity of the heat treated homogenate, a reasonably constant specific activity value of about 3, (Table 15, column 4), could be achieved after the heat treatment stage and it was at this stage that a reproducible starting point for further steps was effectively based.

As a consequence of the experience gained in these preliminary investigations as discussed in Section II, crude heat stable ribonuclease samples were prepared for the ion exchange column development work of Section III as follows.

Spleen obtained by G.M.1. was treated according to the extraction and salting out procedure of Experiment 2 (page 357). The preparation precipitated from the acidified homogenate within the saturation limits 40 to 80% was heat treated according to the method described in Experiment 3 (page 358) and the heat stable protein sample dialysed as described on page 284. The dialysed solution was freeze dried, then stored in 4 gm. sealed bottles in the deep freeze as an off-white fluffy solid. Stocks of this heat stable protein sample were built up to give a good supply of material for the column development work which is reported in Section III. This alternative approach which was indicated by the success of the column experiments in Section III was slightly modified to fit in with the column procedures adopted. The basic idea prior to column chromatography

was to carry out extraction and concentration of the sample by preliminary fractionations using conventional precipitation methods.

The purification scheme presented in Table 15 and used by Kaplan and Heppel[8] was abandoned in favour of three key operations which have already been described in the appropriate paragraphs.

1. The concentration and optimum retention of ribonuclease activity from the homogenate. This was effected by Experiment 1 (page 353) and was a modification of the first extraction and salting out at pH 3.5. A protein fraction was concentrated direct from the homogenate at pH 3.5 by precipitation where the salting out saturation limits rose from 30% to 80.5%. The arrangement is discussed on pages 87 - 92.

II.. The heat treatment remained unaltered. This was an essential feature of the preparation and had to be retained precisely as described by Kaplan and Heppel. Three quarters of the ribonuclease activity of the spleen homogenate was destroyed. The procedure for the heat treatment is set out in Experiment 3 (page 358) and discussed on page 67.

III. The heat stable ribonuclease preparation was concentrated by salting out at pH 7. Small volumes of concentrated spleen protein solutions were made available for desalting by gel filtration on Sephadex which leads directly to other chromatographic techniques on columns. (Section III). Freeze drying and extensive dialysis were eliminated and the sample was in a concentrated state. The procedure is outlined in Experiment 5 (page 361) and reported on page 93.

SECTION III

Purification of the Calf Spleen Ribonuclease Preparation by Column Chromatography

A. Molecular sieve chromatography:

1. Preliminary studies

(a) Cross linked dextran gels

The favourable scientific publicity [99,100,101] accorded to cross linked dextran gels as a medium for exclusion chromatography, where protein molecules can be distinctly separated by virtue of differences in molecular dimension, made apparent a technique of immense potential for the purification of spleen ribonuclease preparations. The commercial Sephadex types are distinquished in their sieving properties by the lower limits of complete exclusion of neutral dextran polymers, which are selected within narrow fraction limits with respect to molecular weight. These Sephadex types are modified dextrans where cross linking is controlled within well defined limits to give a three dimensional network of pores. The different degree of cross linkage determines the porosity of the network, in that a high degree of cross linkage gives a compact structure with low porosity, while a low degree of cross linkage gives a highly porous structure. The large excess of free hydroxyl groups present on the honeycombed polysaccharide structure enables Sephadex to swell in aqueous solutions to form stable gels. The greater the hydrophilic content which is consistent with high porosity, then the greater the degree of swelling of the

particles.

The solution imbibed by the gel particles after swelling is available as solvent to the different sized solutes which can penetrate the gel. The degree of availability depends on the porosity of the gel grain, thus in the first instant the distribution of solutes inside and outside is determined by the solvent volume inside the gel grain and the total volume. Depending on the degree of cross linkage the pores in the gel grain are able completely to discriminate against the entrance of solutes over certain sizes, thus separation by exclusion depends on the fact that molecules sufficiently different in size will penetrate the gel particles to different extents. These are three classes of penetration:

(i) Some molecular species can penetrate the gel freely.

(ii) Others cannot penetrate the gel and are excluded.

(iii) Certain molecules can penetrate part of the gel but not the entire volume and any effect depends on the extent of penetration.

(b) <u>An attempt to bulk treat the spleen ribonuclease preparation.</u>

<u>Method</u>

The combined "all or nothing" effect exhibited by the penetration classes (i) and (ii) was considered to have great potential. If the spleen ribonuclease[8] was in the range molecular weight 2,000 - 5,000 then the molecular sieve medium offered a unique opportunity of discarding completely all contaminants above a

predetermined molecular dimension by a single manipulation. Conversely a similar manipulation where all contaminants of suitably lower molecular size could be removed by a judicious choice of conditions would be almost equally effective. The earlier a step with this specific action was incorporated into a purification scheme then the greater would be the overall effect from subsequent purification. Without first carrying out a comprehensive fractionation of the spleen extract, it was decided to test the potential of the molecular exclusion technique at an early stage of purification, immediately before and after the heat treatment described in Experiment 3 (page 358). With this in mind Sephadex G-25 and G-50 were purchased. Table 17 details the important particulars of these molecular sieves[110].

TABLE 17

Sephadex	Lot No.	Water Regain g.H_2O/g. Dry Gel.	Approx. Exclusion Limit MW. Dextran	Range of Separation with Dextran Molecules.	Fractionation in the molecular weight range.
G-25	To 6741	2.4	5,000	MW >4000 From MW < 1000	0-2000
G-50 Medium Grade	To 7863	5.1	10,000	MW>10,000 From MW < 3,000	1,000-10,000

It was considered of immense practical value that the dry Sephadex could be added to portions of the spleen protein preparation

and exert exclusion effects while absorbing the low molecular weight solutes into the gel pores on swelling[59,129], until an equilibrium was reached between those solutes distributed between the inner and outer solvent volumes. If large quantities of spleen preparation could be treated effectively in this way then the limitations that exist with molecular sieve column chromatography could be avoided (page 128). The possibility that most of the ribonuclease activity would penetrate the gel pores of the sephadex grains and exclude the major portion of the protein contaminants which could be removed by centrifugation was investigated. Several pilot scale trials were carried out. Experimental details are described on page 366.

Experiment 9 describes the procedure where a ribonuclease sample precipitated by raising the ammonium sulphate concentration from 40% to 80% (Experiment 2, page 357) was treated by the addition of dry Sephadex G-50 powder. The solution had not been heat treated as described in Experiment 3 (page 358). Table 18 illustrates the proportion of protein, determined by the biuret method and the total ribonuclease activity in each fraction. Experiment 10 describes the method whereby the absorbed protein from Experiment 9 was further treated by the addition of dry Sephadex G-25 powder. Experiment 11 describes the method by which a spleen extract, similar to that used in Experiment 9 but which had been exposed to heat treatment as described in Experiment 3, was treated by adding dry Sephadex G-50 powder. It could be concluded that most of the excluded material was removed by the basket centrifugation to spin

off excess liquid. The absorbed protein was obtained by eluting the centrifuged gel in a short column. The centrifugate and eluate from Experiments 9, 10 and 11 were examined for protein content and ribonuclease activity. The results are expressed in Tables 18, 19 and 20 respectively.

TABLE 18.

Results from Experiment 9

Experiment	G-50 Sephadex batch treatment of the fraction precipitated by salting out at pH 3.5 within the $(NH_4)_2SO_4$ fraction limits 40-80% and no heat treatment as Experiment 3		
Fractions	% of protein in each fraction	% of total activity in each fraction	Specific gravity as units/ mg protein
Centrifugate:- mainly excluded proteins and the balance of low mol. wt. proteins	43.8	39	2.81
Eluate:- mainly absorbed proteins and some excluded proteins as a result of contamination	56.2	61	4.8

Conclusions

The batch treatment of large volumes of protein extract appeared practical where the excluded components of a mixture were considerably concentrated[59,129] in a very much reduced volume without expecting the complete removal of the smaller components. Although only the

Table. 19.

The results from Experiment 10.

Experiment	Sephadex G-25 batch treatment of the eluate sample from Exp. 9 where most of the molecules above M.W. "10,000 were excluded.		
Fraction.	%age of total protein in each fraction.	%age of total activity in each fraction	Specific activity as units per mg protein.
Centrifugate. Mainly excluded protein and the balance of low M.W. components.	26	65	6
Eluate. Mainly absorbed protein and some excluded protein as a result of contamination.	74	35	1.4

Table. 20.

The results from Experiment 11.

Experiment	Sephadex G-50 batch treatment of the fraction ppted. by salting out at pH 3.5 with $(NH_4)_2SO_4$ Exp. 2 then heat treated as described in Exp. 3.		
Fraction.	%age of total protein in each fraction	%age of total activity in each fraction	Specific activity as units per mg protein.
Centrifugate. Mainly excluded protein and the balance of low M.W. components	69	41	2
Eluate. Mainly absorbed protein and some exoluded protein as a result of contamination.	31	59	4

smaller molecular sized entities could penetrate the gel on swelling, an equilibrium existed between the solute of the inner pore volume later eluted in a short column and the same solute types in the volume surrounding the gel particles. This outer volume contained most of the excluded proteins and a proportion of the low molecular weight components, due to this equilibrium state after it was removed at centrifugation. Similarly the column eluate from the gel pores after centrifugation was expected to contain some excluded material and partly excluded material as a result of uncentrifuged contamination on, or between gel particles.

Under the conditions of these limited trials no extraordinary displacement of ribonuclease activity or protein content with striking purification was observed, Tables 18 and 20. The cost of several kilos of Sephadex necessary to treat large volumes in bulk is probably not warranted unless a more precise manipulation is possible. With Sephadex G-50, the random displacement of ribonuclease activity proved disappointing as the results suggested that whether the preparation was heat treated or not, the ribonuclease activity was dispersed between the pores at absorption and the outer volume and equal quantities were present in both fractions. The data in Table 19 suggests that most of the ribonuclease activity was excluded by Sephadex G-25. However, the return of ribonuclease activity which could be obtained from the spleen preparation, using Sephadex G-25 solely for a concentrating effect, at 65% was not enough to consider the technique efficient.

The investigation of the batch treatment technique with dry Sephadex was abandoned mainly because of the poor resolution of ribonuclease activity and lack of clear cut fractions especially with Sephadex G-50. Instead it was decided to experiment further with Sephadex and examine the behaviour of the active preparation on molecular sieve column chromatography on Sephadex G-25 and G-50.

(c) Molecular sieve column chromatography

It was appreciated that much greater resolution of the components in the spleen preparation would be achieved by the discriminating sieve action explained by the three classes of penetration, already described (page 120), over a length of Sephadex gel column if the components had different molecular dimensions.

Theory

Molecular sieve action on a suitable gel column length produces a chromatographic separation by restricted molecular diffusion through the bed of cross linked grains. With columns an aqueous solution of the protein sample is placed discretely on the surface of the gel bed, previously equilibrated with buffer or salt solution and allowed to percolate through the column length. As the sample is eluted through the gel length with buffer, the small molecules, which diffuse into the gel, are retarded while the large molecules which are excluded from the gel pores are separated as they move faster in the outer volume surrounding the gel particles. The degree of exclusion from the gel determines the elution volume. At the

completion of the run the smallest particles are eluted last and the column is automatically recyclised ready for the next sample application.

The molecular sieve process can be regarded as a chromatography in which the stationary phase is the gel. In direct contrast with true liquid-liquid chromatography the solutes are distributed between a mobile and a stationary phase in which the solvent composition is the same. The stabilising substance is not passive as it is supposed to be in partition chromatography but has a decisive influence on the process. The function of the swollen dextran gel is not only to stabilise the stationary phase but also to provide a three dimensional network having the property to sort molecules according to size. The range of separation increases with decreasing degree of cross linkage of the gel medium.

The gel column

In a packed gel column of total volume Vt, two kinds of aqueous phases are distinguishable. Vo, the void volume is regarded as the phase surrounding the gel particles, i.e. "moving phase", and Vi the pore volume is the inner volume or "stationary phase". Vo and Vi in addition to Vg the volume occupied by the gel matrix make up the column volume Vt. The void volume Vo can be determined by measuring the volume of solvent required to elute a substance that is completely excluded by the grains. It is possible to calculate Vi from known data, i.e. the dry weight of the gel substance and the water regain specified. Since the degree of exclusion of substances by the gel

determines the elution volume, the effluent volume Ve for a particular solute substance depends on the volume Vo external to the gel particles and to the distribution coefficient Kd which is the partition coefficient for a particular solute between Vo and Vi under standard column conditions. The relationship Ve = Vo + KdVi applies and similar to Rf in paper chromatography, a substance subjected to molecular sieving can be characterised by its Kd value. The kd value indicates the fraction of the inner volume accessible to the solute. For example when Kd = 0 the molecules are excluded to Vo only, when Kd = 1 the molecules are freely distributed between Vo and Vi and when Kd $>$ 1 $<$ 0 the solute is partially excluded. Any deviations from these limits indicates that secondary interactions are involved[102,103,130,131].

The greatest restriction envisaged before developing a column technique was a limitation in the volume of sample suitable for application. With normal preparative columns, samples of no more than 10 ml. could be contemplated while trial pilot runs with a 1 ml. sample had to be considered suitable. The simplest of the techniques involving molecular sieving is desalting where large molecules are confined to the void volume and are eluted first, while lesser sized molecules and inorganic salts are completely occluded and eluted last[99].

Procedure.

The techniques used to prepare, treat and manipulate the gel, columns and chromatographic runs are described in pages 292 - 302.

The very early column trials with Sephadex G-25 and G-50 were carried out on spleen ribonuclease samples at various stages of purification. Succeeding experiments improved as difficulties were successively recognised and overcome. Compared to batch volume treatment, small volumes only could be processed, thus molecular sieve column chromatography might not have been the most economical and satisfactory step to introduce at a very early stage when the preparation was bulky. However, it was of prime importance to obtain some idea of how the ribonuclease activity distributed, with respect to the resolving power of the Sephadex types G-25 and G-50 in columns, for future reference.

(d) Molecular sieving on G-25 Sephadex

Experiments 12 and 13 (pages 367, 368) describe the molecular sieve chromatography of two spleen preparations on Sephadex G-25. Experiment 12 describes the sieving of a 3 ml. sample of that fraction precipitated by raising the saturation of ammonium sulphate from 40% to 80% in the acidified spleen homogenate (Experiment 2). The sample was not subjected to heat treatment. All the protein and ribonuclease activity was eluted as a single peak at the void volume of the column. Eluate sample collection difficulties were encountered and the elution pattern is not presented. Exactly the same elution pattern was obtained with a similar G-25 column, Experiment 13, page 368, where a similar spleen preparation to that used in Experiment 12 was molecular sieved after the heat treatment procedure described in Experiment 3. Figure 8 (page 131) illustrates the

elution pattern and shows the protein and ribonuclease activity had eluted as a single peak at the void volume. The considerable length of the column, 1 x 55 cm., when a 1 ml. protein sample was applied, should have been sufficient to separate any lower molecular weight ribonuclease active components. The G-25 gel column excluded the protein and ribonuclease activity of the sample as was partly shown by the batch treatment earlier (page 122) with the same medium.

(e) Molecular sieving on G-50 Sephadex

Five experimental trials with Sephadex G-50 medium in a column of constant dimension 2 x 66 cm. are reported in Experiments 14 - 18, (pages 368 - 369). The preparation of gel, column preparation and column chromatography technique are described on pages 292 - 302. In Experiment 14, 1 ml. of solution from a sample prepared at a stage of greater purification than those used with Sephadex G-25 earlier, was applied, Figure 9, page 131 . The spleen enzyme sample in this case was prepared by salting out pH 7 (Experiment 4) after salting out and heat treatment at pH 3.5. The fraction was dialysed then freeze dried before a portion was taken, dissolved in buffer then molecular sieved. The elution pattern shows that two distinct peaks of protein were obtained and the bulk of the ribonuclease was eluted in the trough region between the peaks. The first peak of ribonuclease activity eluted, appeared along with the largest molecular sized components at the void volume front in a very restricted elution volume compared to the major quantity of ribonuclease activity eluted in the trough region.

FIG. 8.

Gel filtration of the spleen ribonuclease sample on sephadex G-25 (Exp. 13).

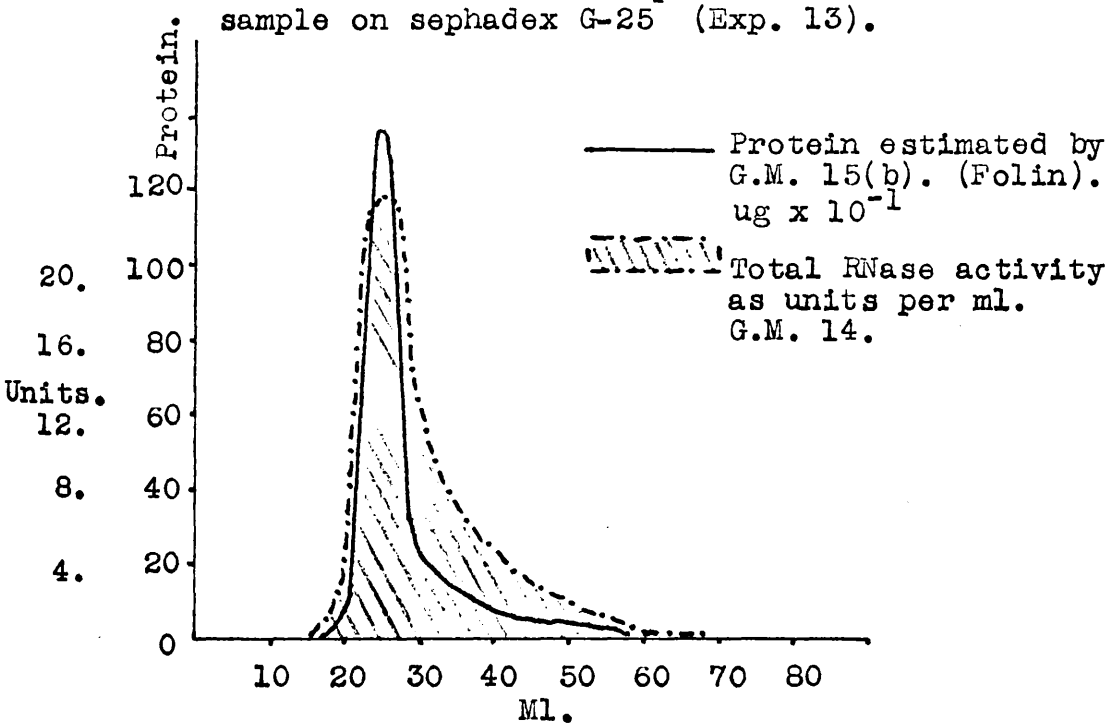
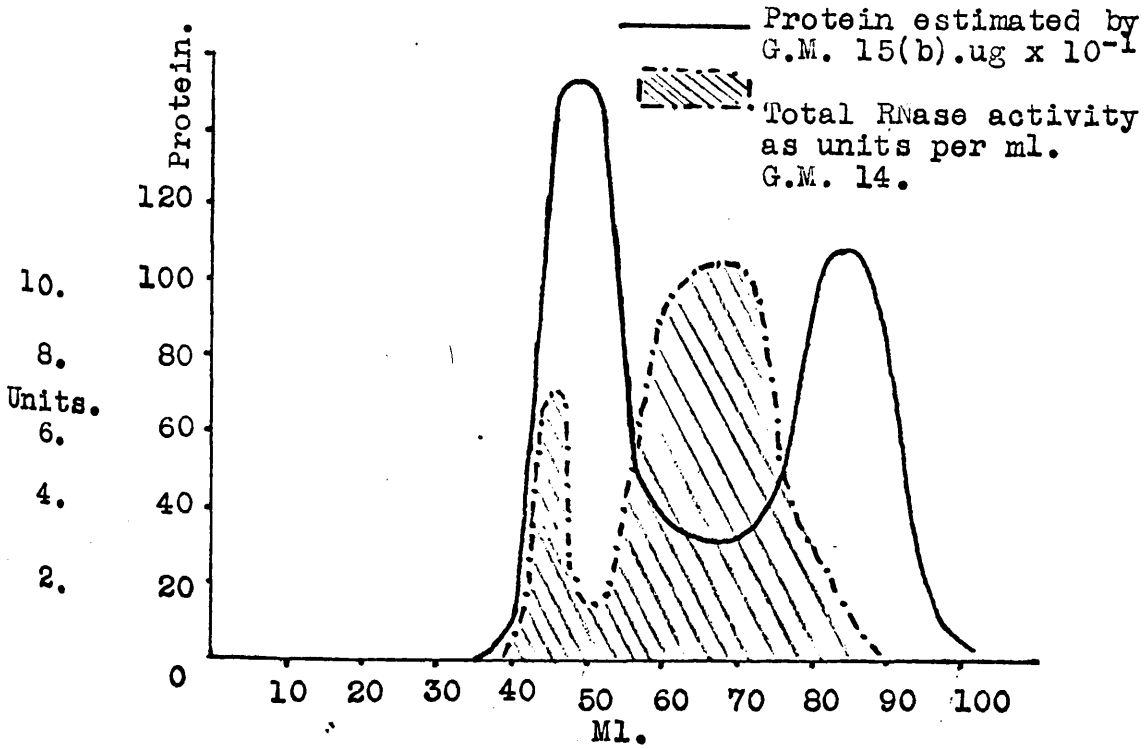


FIG. 9.

Gel filtration of the spleen ribonuclease sample on sephadex G-50 (Exp. 14).



Experiment 15 carried out on the same column, under exactly the same conditions again revealed the two inert protein peaks P and Q. In this experiment a 1.5 ml. protein sample, taken direct from the ribonuclease active fraction without freeze drying, was obtained from the acetone precipitated fraction B of Experiment 8, page 363. On this occasion one ribonuclease active peak R only, was present and was situated between the two inert protein peaks, Figure 10. The enzyme sample molecular sieved in Experiments 14 and 15 were from the same purification trial, the only difference being that the sample applied to Experiment 15 was much purer after an acetone treatment. The two inert protein peaks were much reduced in bulk and the trough region between the peaks, within which the ribonuclease activity was eluted, had greater dimensions. Figures 9 and 10 can be directly compared.

The apex region of the two inert protein peaks P and Q depicted in Figure 10 were bulked, freeze dried to concentrate, the solid taken up in buffer and the total volume was 1 ml. after 1 mg. of crystalline pancreatic ribonuclease was dissolved. This 1 ml. sample was molecular sieved in Experiment 16, page 368 on the same column as used in Experiments 14 and 15. Figure II illustrates the protein and ribonuclease activity pattern. The position of elution of the pancreatic ribonuclease activity in relation to the two inert protein peaks can be observed and compared directly with the spleen ribonuclease activity pattern presented in the previous Figure 10.

Figure 12 depicts the elution pattern of protein and

FIG. 10.

Gel filtration of the spleen ribonuclease sample on sephadex G-50 (Exp. 15) after purification by the acetone fractionation.

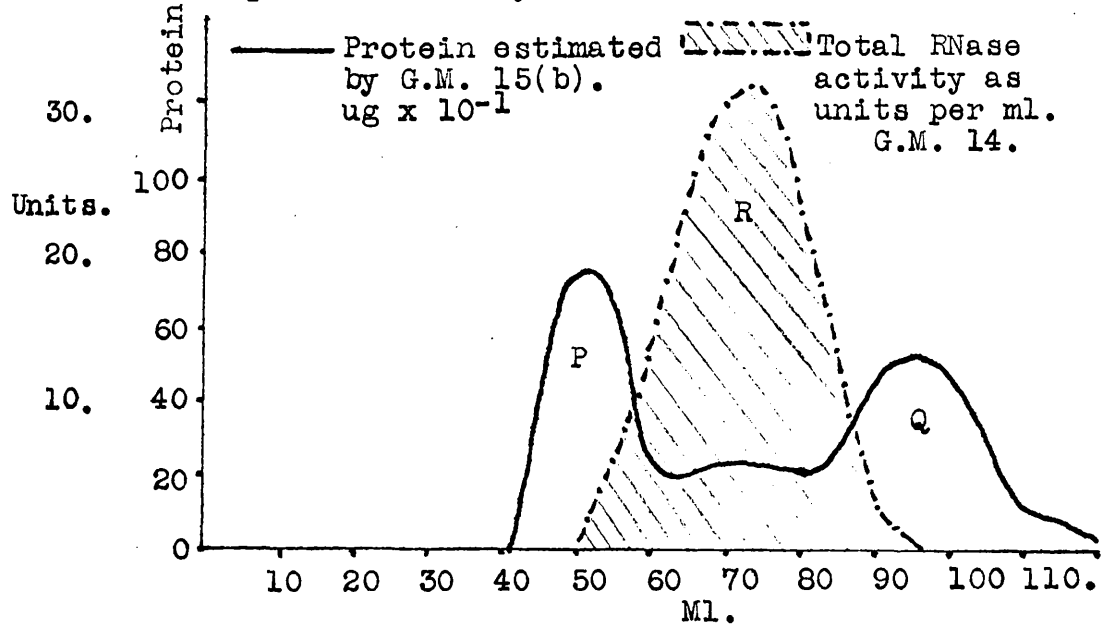
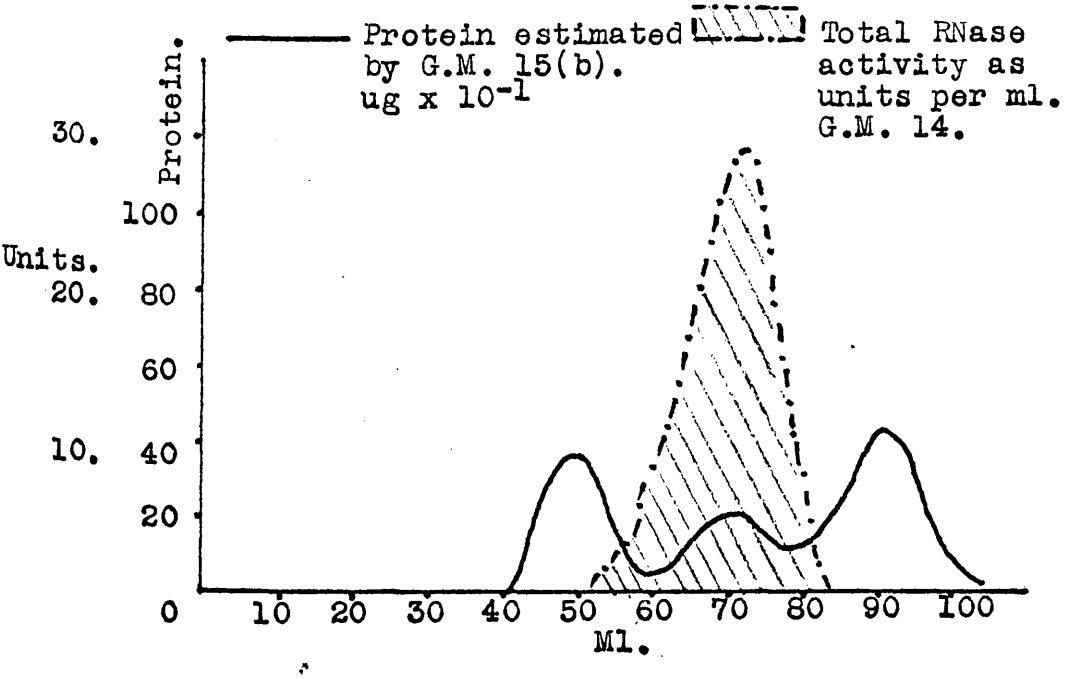


FIG. 11.

Gel filtration of the two spleen protein peaks P and Q as markers and 1mg (crystalline) pancreatic ribonuclease on sephadex G-50. (Exp. 16).



ribonuclease activity after the molecular sieving of a crystalline ribonuclease sample on the same Sephadex G-50 column and is described in Experiment 17. The 0.5 ml. sample applied contained 5 mg. pancreatic ribonuclease only.

Figure 13 presents the elution pattern obtained in Experiment 18. In this experiment the spleen ribonuclease active region from Experiment 14, Figure 10, was bulked, concentrated by freeze drying, then re-sieved on the same column after dissolving in a small volume of buffer. Remnants of the two inert protein peaks appeared on the elution profile irrespective of the fact that the ribonuclease active fractions selected from the effluent of Experiment 14 were from the trough region and little of the two inert peaks could be included in the sample. However, there must have been sufficient present to redistribute in this way. Similar to the elution pattern of Figure 9, (Experiment 13), a small ribonuclease active peak appeared at the void volume front. The major proportion of the spleen ribonuclease activity present, persisted in the trough region between the two inert protein peaks.

The purpose of Experiments 14 - 18 was to establish in a simple manner whether in fact the spleen ribonuclease activity was of low molecular dimension and to find out if the gel filtration technique could be used as an effective purification step. Figures 9 - 13 depicting the molecular sieve elution patterns of various enzyme samples on the same column, are presented together for direct comparison.

FIG. 12.

Gel filtration of a 5mg sample of (crystalline) pancreatic ribonuclease on sephadex G-50 (Exp. 17).

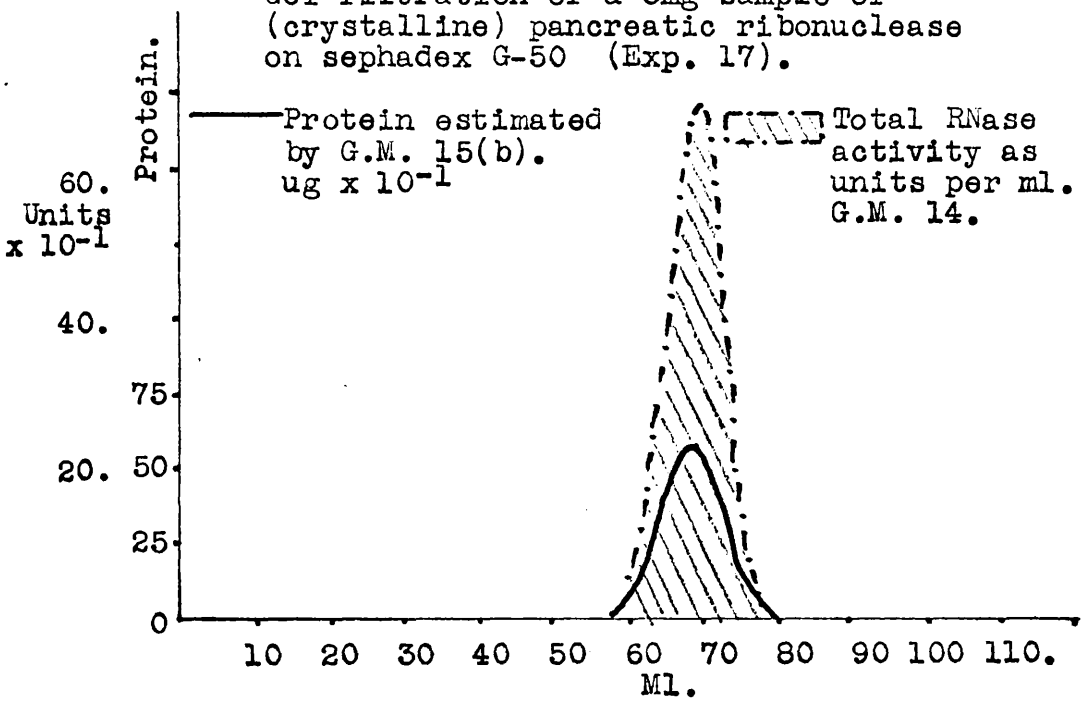
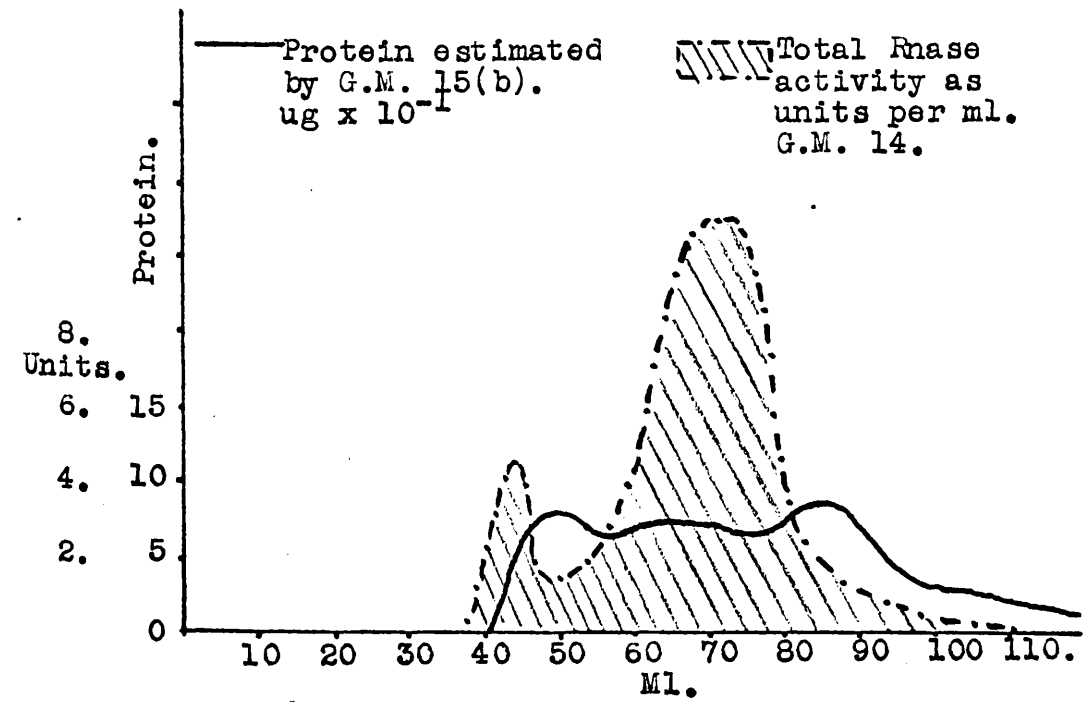


FIG 13

Gel filtration of the spleen ribonuclease active peak R from Fig. 10 on sephadex G-50 a second time (Exp. 18).



A major criticism was the inaccuracy involved in estimating the fraction volume. The Locarte "drop counting" fraction collector at a level of 1 ml. fraction size, did not seem accurate enough, e.g. 0.1 ml. error per tube increased the elution span by 10 ml. after 100 1 ml. samples were collected. This was sufficient to put the latter part of the elution pattern out of focus and made exact comparisons less favourable. Another factor which contributed to a non-reproducible elution pattern was the rate of flow. The column flow rate determines the drop size and this determines the sample volume which has an effect on the elution pattern from one run to the next. Rate of flow, size of the sample, viscosity of sample all influence the elution pattern.

(f) Some remarks on the molecular size of the ribonuclease activity as indicated by these experiments.

The ribonuclease activity was excluded by Sephadex G-25, Figure 8, Experiment 13, and according to the manufacturers data presented on page 121, most probably exceeded a molecular weight of 4,500. Experiment 14, Figure 9, suggested the ribonuclease activity was fractionating on G-50 Sephadex. If the molecular weight range for fractionation with this media at 1,000-10,000 as determined by sieving limit dextrans of established molecular weight could be applied to globular protein for comparative purposes, then the ribonuclease activity was certainly of low molecular weight. Two columns were ran with pancreatic ribonuclease, Experiment 16,

Figure 11, and Experiment 17, Figure 12, for direct comparison purposes. Pancreatic ribonuclease was eluted on the fractionating range of the elution pattern and the pancreatic ribonuclease activity peak compared favourably with that for the spleen ribonuclease activity. Pancreatic ribonuclease has a molecular weight $\simeq$ 13,700 which is above the estimate for complete exclusion of 10,000 and fractionating range 1,000-10,000 as determined with dextran polymer fractions. Since molecular size and molecular weight are relatively closely related properties it appeared there may be sufficient difference between the polymer species to suggest the discriminating sieve action responded differently. The behaviour of particular polymer species to sieving may be such that the range of protein molecular weights which are excluded or fractionate on a particular gel type are greater than that expressed by dextrans. The spleen ribonuclease activity, Figure 15, compared very well in its pattern of elution to the patterns obtained with pancreatic ribonuclease activity sieved in combination with other protein, Figure 16, then sieved alone, Figure 17. The findings that the two activities fractionated in close proximity indicated a similar molecular dimension, though it could not be gauged whether one was smaller or larger than the other. This difficulty was brought about by the ribonuclease activity peak of spleen ribonuclease, Figure 10, being very wide, much wider than expected and wider than the pancreatic ribonuclease activity, e.g. the first protein peak eluted spanned only 20-25 ml. while the spleen ribonuclease activity

spanned 35-40 ml. This did not compare favourably with Figure 12 where the pancreatic ribonuclease activity peak was only slightly broader than the first protein peak at 25 ml. This difference in the pattern of elution made a direct comparison in an attempt to estimate whether spleen ribonuclease was larger or smaller than pancreatic ribonuclease less favourable. The variable elution patterns, particularly with the spleen ribonuclease activity, Figures 9 and 10, could be attributed to the effect of viscosity differences in the samples applied. This was not the case as the inert protein peaks in the same patterns did not have correspondingly great elution spans.

An unusual ribonuclease activity pattern was produced in Figure 9 and to a lesser extent in Figure 13. A minor amount of ribonuclease activity appeared with the first protein peak eluted. In both these experiments and unlike the sample applied to produce the elution pattern of Figure 10 the spleen ribonuclease samples were prepared by redissolving, after freeze-drying to concentrate, before they were sieved. The sample applied to produce Figure 13 was obtained from the ribonuclease activity of the trough region of Figure 10 only. It could be that the redissolved sample was over-concentrated at 30-50 mg. per ml. for Figure 9 and viscosity effects prevented a complete resolution of the compounds. This explanation could be rejected particularly in considering Figure 9 where the two inert protein peaks were distinctly resolved without tailing and Figure 13 where the sample applied was not over concentrated at

re-sieving. An alternative suggestion at this time was that the freeze-drying process might encourage strong protein interactions which did not disperse completely at redissolving, especially as the solution was concentrate and applied immediately to the column. It could be expected that the associated molecules were eluted earlier in the void volume as they were of larger molecular dimensions. The preparation applied to produce the elution pattern presented in Figure 10 was not freeze-dried prior to application yet this same ribonuclease activity after freeze-drying to concentrate it for Experiment 18, gave the elution pattern presented in Figure 13. The preparation did not molecular sieve to reproduce the same pattern as before, Figure 10, but eluted similar to the preparation applied to give Figure 9. All the preparations were heat treated and according to Kaplan and Heppel's[8] report, only the heat stable spleen ribonuclease survived this treatment. The possibility that two ribonuclease active enzymes were present was dismissed. Although the preparations which were molecular sieved, Figures 9 and 10, were from different stages of purification and the removal of one of the enzymes was possible, i.e. by the acetone treatment, the appearance of a small amount of larger molecular weight component, Figure 13, from the re-sieving of the ribonuclease active region selected from Figure 10 which did not exhibit a second ribonuclease activity suggested only one ribonuclease peak. It was not until the carboxymethyl cellulose columns were introduced to fractionate the spleen preparation that two ribonuclease active components were detected in

the ribonuclease sample which was eluted over a span of 30-40 ml. in the trough region of the gel column. At this time this ribonuclease peak was considered atypical in span because of some interactions at gel filtration. A critical appraisal of these columns revealed advantages and disadvantages to molecular sieving as a suitable technique to incorporate into a purification scheme.

Conventional dialysis with cellophane sacs, where considerable loss of ribonuclease activity by excessive diffusion was already experienced (page 96) and reported by Craig and King[61], could be replaced by molecular sieving as a desalting process[99]. A 100% return of ribonuclease activity from Sephadex columns should be expected. The process could fit in admirably after precipitation stages where the enzyme preparation was concentrated. When desalting by column techniques was introduced an attempt to adjust the conditions to enable a purification by the molecular sieve fractionation process was also considered. The complete removal of vastly different molecular sized components would add substantially to the overall effectiveness of later purification techniques adopted. A dual effect of desalting with protein fractionation by molecular sieve action, involved the combination of two stages into one, Sephadex G-25 was suitable only for the desalting process. Sephadex G-50 did not seem to be quite adequate for the present conditions, i.e. although the ribonuclease activity in the trough region, Figure 10, page 133 was collected and the material re-sieved after concentrating, a considerable amount of the two inert protein peaks either side was taken and appeared at the re-sieving, Figure 13, page 135.

The columns ran in Experiments 14 and 15 were important when the purification efficiency was considered. In Experiment 14 a heat stable ribonuclease sample from Experiment 4 (page 359) was molecular sieved on Sephadex G-50. When the fractions of the trough section between the two protein peaks, Figure 9, page 131, were bulked rejecting most of these protein peaks and any ribonuclease activity associated with them, only 40% of the ribonuclease activity eluted was retained. This sample exhibited a 3 fold purification, Table 22, page 142.

In Experiment 15 the sample applied to the column was a portion of fraction B from Experiment 8 (page 363). In Experiment 8 the heat stable ribonuclease active sample from Experiment 4 similar to that molecular sieved in Experiment 14 above, was dialysed then fractionated with acetone. The sample applied to the column was purer than the previous sample and again the fractions eluted in the trough section, Figure 10, between the two protein peaks were bulked, rejecting most of the protein peaks and any ribonuclease activity associated with them. On this occasion 75% of the ribonuclease activity eluted was retained. (see Table 22). The acetone fractionation was abandoned (page 110) thus the outcome of Experiment 14 was important in deciding whether Sephadex G-50 could be used in a purification step. After salting out with ammonium sulphate at pH 7, Experiment 4, page 359, desalting was essential before the sample could be processed further either by ion exchange column chromatography or acetone fractionation[8]. The stage directly after Experiment 4 was

Table. 22.

Purification and recovery of spleen ribonuclease after gel filtration on the sephadex G-50 column.

	Experiment 14.	Experiment 15.
Total ribonuclease activity units applied to the column.	330	221
Ribonuclease activity units bulked from the column effluent in the section between the two protein peaks.	132	165
%age of the total ribo-nuclease activity recovered.	40	74.7
Specific activity of the sample applied to the column.	5.7	10.4
Specific activity of the bulked ribonuclease active effluent.	17.9	29.6
Extent of purification.	3 fold	2.8 fold.

considered the most favourable for the introduction of a Sephadex column treatment which would desalt the protein sample as a minimum purification effect. In Experiment 14 the 1 x 66 cm. Sephadex G-50 column gave insufficient resolution and was of too low a capacity (Figure 9). To obtain the resolution achieved there, the 1 ml. sample applied containing 57 mg. protein was a very small portion of the entire sample which required desalting. In addition the amount of ribonuclease activity satisfactorily bulked after the elution was only 40% of the total ribonuclease activity which was eluted over a wide elution span between the two protein peaks. The results from this series of experiments with G-25 and G-50 Sephadex made it clear that the resolution obtained was not sufficiently great, hence further experiments with Sephadex G-75 were advisable.

2. Preparative scale molecular sieve chromatography

(a) Sephadex G-75 and column chromatography

Sephadex G-75 separates dextran molecules of 10,000 molecular weight from those of 50,000[110]. The dextran polymers are fractionated in the range 1,000 to 40,000 and those above molecular weight 50,000 are completely excluded[110]. This Sephadex grade was expected to have greater resolving properties thus contaminants in high and low molecular weight ranges might be excluded from the ribonuclease active fraction when a more extensive elution pattern than that found with Sephadex G-50 was obtained.

A major disadvantage appeared to be the very limited capacity of the column used, with a large amount of protein preparation in

process the molecular sieve desalting stage could be severely rate determining. It was obvious that the volume applied should be low in relation to the column volume if optimum resolution was to be obtained. Viscosity is related directly to the protein concentration and has an effect on the resolution, thus there is a limit to the concentration which can be applied. To overcome the fact that the desalting stage could become a rate determining step in a purification procedure, very large columns were considered where several large columns could be in use at the same time and enable the successive application of samples. The alternative to molecular sieving at an early and bulky stage in a purification scheme was to postpone the treatment until the samples had reduced in size and concentration as the result of other purification techniques. To do this it would be necessary to desalt earlier and the advantages from a dual effect might be lost. Some of these observations were not put into effect immediately. After the ion exchange celluloses were investigated for purification purposes, molecules sieve chromatography was reintroduced at a preparative scale on G-75 Sephadex.

(b) Gel filtration on a Sephadex G-75 column of large dimensions

Sephadex G-75 was examined to find out if a more extensive elution pattern could be developed. It was also considered that if gel filtration was to be effective in the dual role of desalting and removing completely certain components for purification purposes, the column should be able to cope with a substantial amount of material. A large column of Sephadex G-75, 5cm. ld x 75 cm. long was prepared

in accordance with the gel filtration procedures described in G.M.5 and G.M.6 and its potential gauged after three trials as follows. Experiment 30 was conducted to determine the void volume and assess the potential range of the elution pattern. Blue dextran "2000" is a dyed dextran of high molecular weight (2 million) and is totally excluded from all Sephadex gels[173]. The 0.2% w/v aqueous solution, applied to the large column to establish the void volume or origin of any elution pattern at the point 490-500 ml. was eluted within a span of 70 ml. (460-530 ml.). Sucrose at a concentration of 5 mg/ml was included in the sample and indicated the end of the elution pattern. At a molecular weight of 342 the sucrose molecules would be freely admitted to the gel pores. The elution pattern, Figure 32, page 149 , shows that sucrose was eluted over a range of 300 ml. between 1,300 to 1,600 ml. in the elution region. This data indicated the volume and position any salt contaminants would occupy at desalting if the concentrations were reasonable. Fractionation could extend over 1,000 ml. in which the maximum span of elution exhibited by a single component should be no more than that exhibited by sucrose. Salts and very low molecular weight substances would be expected to have the greatest width of elution distribution as they would be totally occluded and free to diffuse during the sieve action. The isosceles outline of the eluted peaks in Figure 32 for blue dextran and sucrose indicated satisfactory gel stability and packing throughout the column length.

Experiment 31 (page 381) was designed to test the system with

a standard protein sample. 70 mg. of crystalline bovine plasma albumin (mw. 67,000) (Armour Laboratories) in 7 ml. was eluted as described in Experiment 30. The elution pattern Figure 33 (page 149) exhibited a double peak effect. A minor quantity of protein having a greater molecular size was eluted first. The major albumin peak was eluted over a range of 150 ml. at the 550 ml. effluent point. Both peaks were eluted close together over 200 ml. at a point a little after the void volume (see Figure 32). The extent of elution proved satisfactory for a protein concentration and sample volume of this order. The elution pattern revealed that bovine plasma albumin contained a contaminant of higher molecular weight. The lesser peak eluted first could be attributed to higher albumin polymers[96,97,98] which have been shown to be present in most samples of bovine plasma albumin not highly fractionated.

Experiments 30 and 31 were essential preliminary investigations of the gel filtration behaviour of standard compounds on the large Sephadex G-75 column. The results, in addition to calibrating the column, provided the information necessary to submit a sample of the spleen ribonuclease preparation to the same procedure.

In Experiment 32 (page 382), a heat stable spleen ribonuclease sample prepared by the preliminary purification procedure, was molecular sieved. The sample was prepared by extracting and salting out at pH 3.5 followed by a heat treatment, then concentrating by salting out at pH 7 according to the three key procedures referred to on page 118 . The spleen ribonuclease preparation which was

centrifuged to remove any undissolved protein after the fraction from the concentrating step of Experiment 5 (page 361) was dissolved in 0.05M tris HCl 10^{-4} MEDTA pH 7.5 7 ml. at about 50 mg. protein per ml., was layered on to the column by the method already outlined, then the column eluted with buffer to develop the molecular sieve chromatogram. 10 ml. samples were collected and the protein concentration recorded by measuring the O.D. of each tube at 280 mμ on the spectrophotometer against the buffer as reference. The ribonuclease activity pattern was determined by assaying each fraction (page 341). Figure 34 (page 149) illustrates the elution pattern obtained.

A volume of solution 7-10 ml. has to be chromotographed per column as many columns would have to be run to cope with the volume of solution at this stage, especially with bulky preparations, e.g. with the example tabulated on page 114 , 42 ml. containing 11 mg. per ml. from only eleven calf spleen would require 4 columns to process this volume. This quantity could be fractionated at one run after concentration to 50 mg. per ml. Nevertheless, with preparations on a larger scale (100 spleen) several column runs were required to cope with the sample before the next stage would be operational and this was a restriction. The elution pattern, Figure 34, exhibits little resolution of the protein components. It was realised that many protein contaminants were present as a result of the wide salting out limits applied at concentration and this pattern suggested the presence of very many components in equal quantities (see page 62).

The ribonuclease activity pattern has an extensive elution span from 700 ml. - 1,200 ml., i.e. 500 ml. in all which was considerably outwith the 300 ml. exhibited by sucrose, Figure 32, and that which should be typical for a single entity. The major portion of the ribonuclease activity peak was eluted at lower molecular weight limits and this region could be bulked to give a preponderance of lower molecular weight ribonuclease activity in the sample. There was no requirement for this as later fractionations on cation exchange columns achieved a complete separation of two ribonuclease components. All fractions containing ribonuclease activity were bulked when the Sephadex G-75 column was put into general use for desalting with limited fractionation.

A considerable amount of large molecular weight material and contaminants of lower molecular weight than the ribonuclease activity were eliminated. That section of the elution pattern equivalent to where sucrose was eluted, Figure 32, was the region inorganic salts, e.g. $(NH_4)_2SO_4$ appeared in the effluent and meant that the ribonuclease active fractions were desalted. Two important features were apparent from the results of the large scale gel filtration. The heavy load applied, total volume 7 ml. at 47-50 mg. protein/ml. of crude preparation, was responsible for the very poor resolution of components, Figure 34, page 149 . The wide elution span in which ribonuclease activity was eluted, cf. Figure 9 for Sephadex G-50, meant that the complete bulking of these fractions to give a 100% yield of ribonuclease activity resulted in a low purification at 1.6 fold only, Table 23.

FIG. 32.

Gel filtration of "blue dextran 2000" and sucrose on sephadex G-75 (Exp. 30).

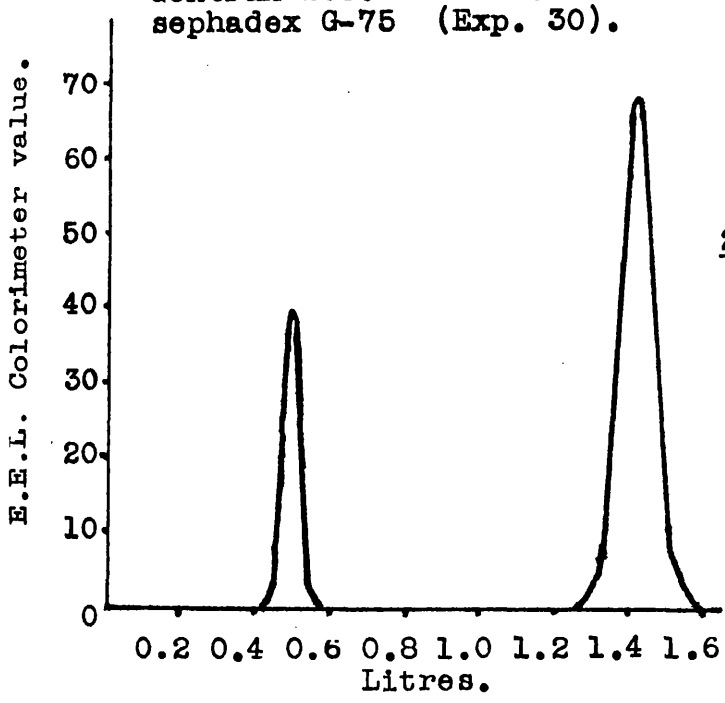


FIG. 33.

Gel filtration of (crystalline) bovine plasma albumin. (Exp. 31).

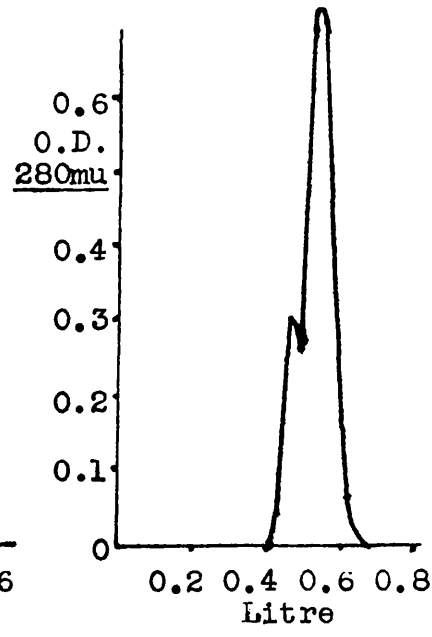


FIG. 34.

Gel filtration of the concentrated crude spleen ribonuclease active sample on the preparative scale sephadex G-75 column. (Exp. 32).

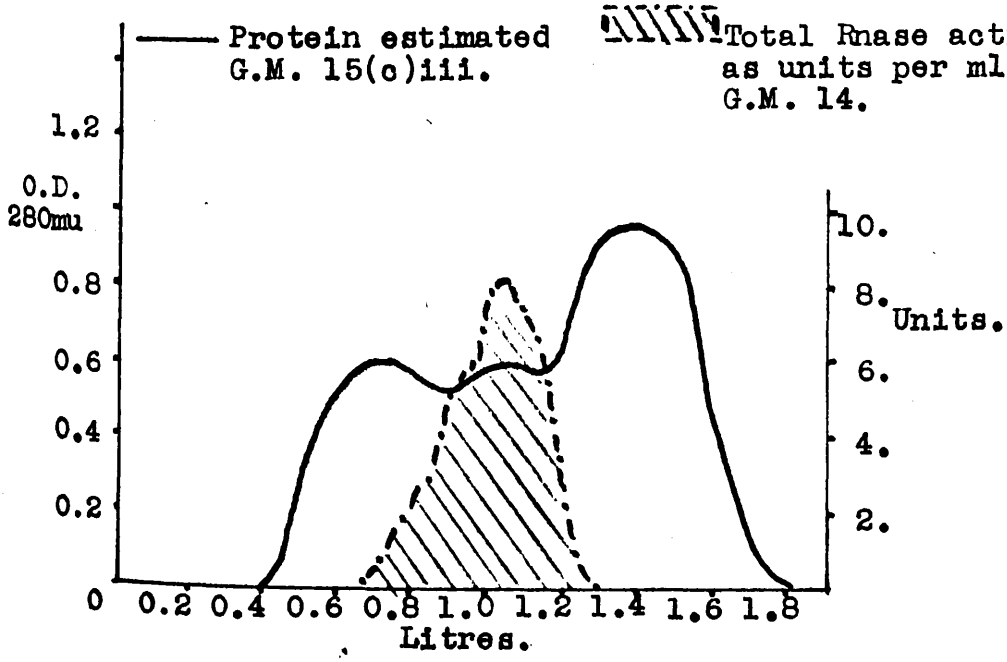


TABLE 23

Gel filtration on a column of Sephadex G-75

	Total Volume Ml	Total Activity Units	Specific Activity
Spleen preparation applied	7	1,000	3.0
Bulked column fractions containing ribonuclease activity	420	960	4.8

In the column techniques adopted for the purification sequence described on page 209 , crude ribonuclease samples were molecular sieved on the preparative scale Sephadex G-75 column as described in Experiment 32 to desalt the preparation, with as complete a return of ribonuclease activity as practicable, irrespective of the low purification effect.

B. Ion exchange chromatography

1. General Introduction

The many ion exchange processes are discussed fully by several authors in Calmon and Kressman's book "Ion Exchangers in Organic and Biochemistry" published by Interscience Publishers, New York 1957. The ion exchange resins available at this time were of limited use for adsorbing proteins because of their small pore size properties. It was not until the introduction of the cellulose ion exchangers by Sober and Peterson[85] that a revolution in the chromatography of large molecules particularly proteins was brought about. This fact and the

scope of their potential is demonstrated by the comprehensive reviews of their use by Peterson and Sober[86], Boardman[87] and Boman[88]. Boman discusses the purification of enzymes by ion exchange chromatography.

The chromatography of proteins on ion exchangers involves the establishment of multiple electrostatic bonds between charged sites on the adsorbent and sites bearing the opposite charge on the surface of the protein[86]. The procedure in its most general form involves the use of a columnar bed of uniformly packed particles of adsorbent with the interstitial spaces completely filled with liquid that is in equilibrium with the adsorbent with respect to pH, salt concentration and temperature.

A protein sample, in similar equilibration to the column with respect to pH, salt content and temperature, can be applied to the column. The number of bonds established depends on the coloumbic forces acting between the proteins in the moving phase solution and stationary phase adsorbent. Adsorption chromatography involves the differential concentration of substances on the surface of the selected adsorbent, then their differential desorption at elution[88]. The cellulose ionic exchangers are shown to cope adequately with protein mixtures and have justified the claims of the inventors[158,159]. They contain a limited number of ionisable groups and retain much of the original micro-structure of the cellulose from which they are prepared. The open structure permits ready penetration by large molecules resulting in a high capacity for protein adsorption[86]. It

is this property which makes them superior to synthetic resins which are amorphous and relatively hydrophobic. An additional advantage may be the hydrophilic nature of the cellulose skeleton since it may be less harmful to enzymes than the hydrophobic synthetic resins which may cause protein denaturation[88].

No simple relationship exists between different types of proteins and chromatographic behaviour, thus no significant predictions have been made[88]. The affinity of a protein for an ion exchange adsorbent is a function of the number of bonds that can be established between the protein and the adsorbent under the conditions employed[161]. Proteins differ significantly in charge density, or number of charges due to their size, or extent of charge distribution, The total effect determines the affinity of these molecules for the adsorbent and appears unpredictable. Elution can be accomplished by raising or lowering the pH to alter the number or sign of the charges on the protein or adsorbent. The salt concentration of the eluting medium can be increased and this causes a decrease in the effectiveness of the existing electrostatic bonds between protein and adsorbent. To achieve a multistage desorption process analogous to that responsible for the high resolution observed in the ion exchange chromatography of small molecules, e.g. anion exchange resins and nucleotides[89,105], is considered much more difficult with proteins[160,169]. In comparison to nucleotides, protein molecules are very large containing many charges capable of forming bonds with the ionised adsorbent. Differential elution is

accomplished by reducing the number of forces on the protein molecule through appropriate changes in pH and/or by decreasing the effectiveness of existing bonds by increasing the salt concentration to produce a competing ion effect. However, not all the many bonds holding a protein dissociate at the same time, thus as the chromatography develops there will be a less well defined resolution with the successive distribution of the components of the mixture along the column length.

2. Ion exchange and the present work

Kaplan and Heppel's[8] report that spleen ribonuclease could be retained much longer than pancreatic ribonuclease on Amberlite IRC 50 (X.E.64), a resin preparation used to chromatograph basic proteins[43], indicated that a suitable adsorbent might be the cation exchanger carboxymethyl cellulose. C.M. cellulose is a weak cation exchanger with a large capacity to retain proteins, especially neutral and basic proteins[86]. The material has a pK 3.5-4.2 depending on the salt present and an exchange capacity of 0.70, m.eq. per gram.

Taborsky[90] has published the details for the ion exchange chromatography of commercial crystalline pancreatic ribonuclease. This protein was resolved into five components on a C.M. cellulose column at constant pH 8 using a tris buffer[106] and increasing sodium ion gradient as competing ion. Four of the components were active and the other component was an inert contaminant. This example of chromatography at a most sensitive resolution level was inspirational in suggesting that a very similar procedure might be devised to

isolate the spleen enzyme. The pancreatic ribonuclease sample was crystalline, free from major contaminants, and the refined chromatography revealed heterogeniety of ribonuclease active component mainly[90]. The difficulties major contaminants could present at chromatography might be appreciable in comparison.

The chromatographic method detailed by Taborsky[90] was repeated as a prototype to introduce and instruct on the technique before a procedure was developed in the present work for the spleen preparation. The ideal situation envisaged was that the spleen enzyme might be one of the most tightly bound components of the preparation. The enzyme was a minor part of the mixture and of small molecular dimension, thus the effective capacity of the C.M. cellulose for the entity might be great. Also by applying the mixture in large quantities most of the unwanted protein components, even though all were adsorbed initially, might be displaced from the column first. The intention was to have the protein sample bound on the first 2% to 5% of the adsorbent as a band leaving the remainder of the column length free for redistribution as the elution developed.

3. Conditions for cation exchange chromatography

If the protein components of the spleen preparation are immobilised at the start of the fractionation, the resolving power of the developing system devised must selectively remove the most loosely bound molecules first. The increased competition from the eluting agent then proceeds to induce the next group of molecules to leave and so on. With a single run several components can be

resolved at maximum capacity. The requirement then must be to have maximum resolution in that region of the elution containing the ribonuclease component by readjusting the conditions to effect this. Considerable trial and error experimentation must be undertaken to establish these conditions. To secure a reasonable resolution without ambiguity of elution pattern, the gradient elution technique, where a continuously changing agent is applied, suggested a superior arrangement to the alternatives of developing at constant buffer strength elution or stepwise elution[91].

The limits to the range of pH and salt within which a given protein can enter into adsorption equilibrium with the adsorbent is quite small[86]. However, the affinity of the different proteins for the adsorbent can be quite wide, thus the selection of a suitable constant buffer strength elution when many proteins are present constitutes a difficult trial and error procedure, especially to obtain a complete and effective resolution in a single run. Discontinuity in the flow of the eluent by changing the eluent strength can result in the artifactitious distribution of proteins and this is an added complication at the interpretation of elution patterns[88]. A gradient can encompass all the requirements automatically and can be adjusted to suit the properties of the protein mixture. First of all, it is necessary to find the conditions required to permit the formation of many bonds between the adsorbent and the protein molecules where the bonds remain in force so long as the eluting conditions remain the same. Any tendency for protein

bands to spread out at constant strength elution or stepwise elution can be curbed by the use of gradients of suitable increasing eluting power. The slightly higher content of competing ion effect when the protein is eluting resists any tendency for the last of those molecules to tail at chromatography.

Cation exchange can be represented by the formula which is possibly an over simplification of the reaction[88].

FORMULA 1.

Preparation of ion exchanger in the Na^+ form.

$$R\text{-}\ COOH + Na^+ + H_2O \underset{\longleftarrow}{\xrightarrow{\text{Regenerate}}} R\text{-}\ COO^-\ Na^+ + H_3O^+$$

FORMULA 2.

The exchange reaction.

$$R\text{-}\left[COO^-\right]_n n\ Na^+ + Pr^{\alpha+} \underset{\text{Desorb}}{\overset{\text{Adsorb}}{\rightleftharpoons}} R\text{-}\left[COO^-\right]_n Pr^{\alpha+} + nNa^+$$

R- represents the insoluble cellulose structure.

$Pr^{\alpha+}$ represents an amphoteric protein with α positive charges.

Any protein which exhibits α positive charges will participate in the reversible exchange with the sodium salt of the ion exchanger. Adsorption is facilitated by low salt cations, i.e. competing ion and a large positive charge on the protein, while displacement of protein is facilitated by the reverse effect of a decrease in the positive charge of the protein molecule or increase in content of the competing ion. The simplest gradient types are based on this and are recognised as the most useful for protein chromatography. It

is possible to increase the ionic strength as a competing ion effect and change the pH, or possibly for greater effect combine both. The interdependent variables of ionic strength, protein charge, ionic exchanger type and temperature[102] can all be controlled.

Many technical details other than the fact that the protein adsorption properties are sensitive to pH and ionic strength must be considered with a gradient development to obtain good resolution. The first $\simeq 2\%$ of the column only must be used for adsorption leaving the remainder of the length available for resolution. The effective length of the gradient compared to the dead volume of the column must be very much greater to produce a good resolution. Protein adsorption and displacement can be complicated by complex protein interaction[88]. Resolutely held protein components are displaced by sodium ions which compete for the exchange sites. However weakly held proteins can be displaced by proteins having a greater tendency to be adsorbed. Thus a protein-protein desorption effect also contributes to the separation[88]. Another factor which may be important is the protein concentration. With a particular elution scheme the eluting strength at which particular proteins can be eluted may depend on the amount of protein applied to the column[88].

4. <u>The application of cation exchange chromatography to the spleen enzyme preparation</u>

In view of the necessarily repetitive nature of much of this

account and because of the complexity of the detailed comments, the reader's attention is drawn to the fact that in the figures that follow, the elution patterns illustrated in Figures 28, 29, 30 and 31 represent the culmination of this part of the work. The other figures relate to subsidiary but nevertheless essential preliminary work. Figures 28 - 31 are of central interest as they demonstrate that calf spleen possesses two heat stable ribonuclease activities. The results are discussed in sub-section 6(b), page 193. The two heat stable ribonuclease activities are examined further in Sections IV and V.

(a) Development of the conditions for chromatography

The prototype column mentioned earlier (page 154) and reported in Experiment 19 (page 370) was partially successful, but attempts to fractionate the calf spleen ribonuclease preparation in the same way were not satisfactory. The spleen ribonuclease preparations used were from samples stored in the deep freeze after the heat treatment stage, Experiment 3, page 358 , dialysed then freeze dried, (page 351). The lack of success was traced to a technical failing in the gradient assembly, Figure 35, page 312 . The gradient elution system of Alm et al[91] used by Taborsky[90] was replaced by a linear gradient system, Figure 35, page 312 , which is used regularly in this laboratory for the ion exchange column chromatography of nucleotides[105]. On this occasion the tube used to connect the mixing chamber and stock reservoir chamber had a bore of 0.5 cm. diameter. Consistent with the slow flow rate used, three days were required to

run the column with the gradient applied and during this time interchange by diffusion had taken place between the two chambers. The gradient was ineffective. The mixing caused a dilution and though there was an initial rise in ionic strength, the gradient could never reach the final salt concentration intended at the completion of the run. The 0.5 cm. bore glass tube was replaced by a short length of 1 M.M. bore glass tubing which adequately controlled the flow, from the reservoir to the mixing chamber only, as both solvent levels fell evenly at an appropriate flow rate. From this experience it was taken as a point of importance that adequate control of the gradient system was essential not only to enable reproducibility of technique as faults of this nature could be perpetuated unknowingly, but conclusions deduced from elution pattern results, then used for elution adjustments would be entirely misleading. This was especially so if a new elution system was introduced to meet certain needs and was based on false findings.

The experimental details for the cation exchange chromatography of the crystalline pancreatic ribonuclease sample by the method described by Taborsky[90] is presented in Experiment 19, page 370 The elution pattern illustrates five separate protein peaks, Figure 18, page 162 . The extent of elution between the peaks and the fact that one of the peaks E. was not eluted within the gradient span illustrated that the gradient had not gone to completion for the reasons already described. The peak E which was a ribonuclease active fraction was eluted from the column after 1M NaCl was passed

into the column. A separation similar to that reported by Taborsky[90] and by Aqvist and Anfinsen[9] was obtained. On assay peaks A and B contained no ribonuclease activity. Peaks C, D and E exhibit the characteristic high ribonuclease reactivity of pancreatic ribonuclease[9]. It was believed the peak E contained two ribonuclease activities in accordance with previous findings[9,90]. The chromatographic scheme, the elution error discounted, exhibited high resolution with an excellent capacity to retain protein and presented a technique with favourable qualities which might be adapted to a purification scheme for the spleen ribonuclease preparation.

Taborsky[90] unlike Kaplan and Heppel[8] or Aqvist and Anfinsen[9] did not use a phosphate buffer at acid pH for elution as there was a tendency for the basic ribonuclease molecule to form complexes with acidic materials, e.g. the phosphate anion, and irregularities could be produced on the column. Taborsky[90] also intimated that chromatography could take place satisfactorily at neutral pH values which had merit since they were clear of the range of the pK of the ion exchanger at pK 3.6 in 0.5M NaCl[88].

The first attempts at chromatography of the spleen ribonuclease active preparation were not altogether of negative value. The greater part of the enzyme sample was not retained on the C.M. cellulose column equilibrated at pH 8. The major portion of the protein band was lightly held and began to elute down the column as the sample was applied. This material was in excess of other components which passed through the column in the dead volume and not

retained. The material lightly retained exhibited ribonuclease activity and was eluted with equilibrating buffer wash only.

Another spleen ribonuclease enzyme sample applied at pH 6, after the column was equilibrated accordingly with respect to this pH, was retained on the column. After a gradient elution extending over 1 litre where the salt content increased from zero NaCl to 0.2M NaCl, ribonuclease activity was not located in any of the fractions collected. Apparently the ribonuclease activity was retained on the column top at this pH.

The crude spleen preparation obtained by the method outlined on page 118 was available in large quantities for further preliminary chromatography investigation work. As already described the intention was to load the column with protein then after the elution development, a concentration of ribonuclease active component should result. For this reason 400-500 mg. by weight of freeze dried crude spleen ribonuclease preparation was applied after dissolving the sample in the appropriate buffer solvent. Figure 19, page 162, depicts the elution pattern of the first particularly satisfactory chromatographic attempt on 420 mg. by weight of the spleen preparation. The experimental details are outlined in Experiment 20, page 371 . By calculation it was shown that the actual protein^{180} applied was 325 mg. which suggested that 22% of the sample was not proteinaceous. Linear gradient elution was over 1 litre at a constant pH 7.5 where the NaCl concentration increased from zero to 4M. The distribution of protein indicated that this salt strength was excessive and not

Fig. 18.

Cation exchange chromatography of (crystalline) Pancreatic ribonuclease on C.M. cellulose.(Exp. 19).

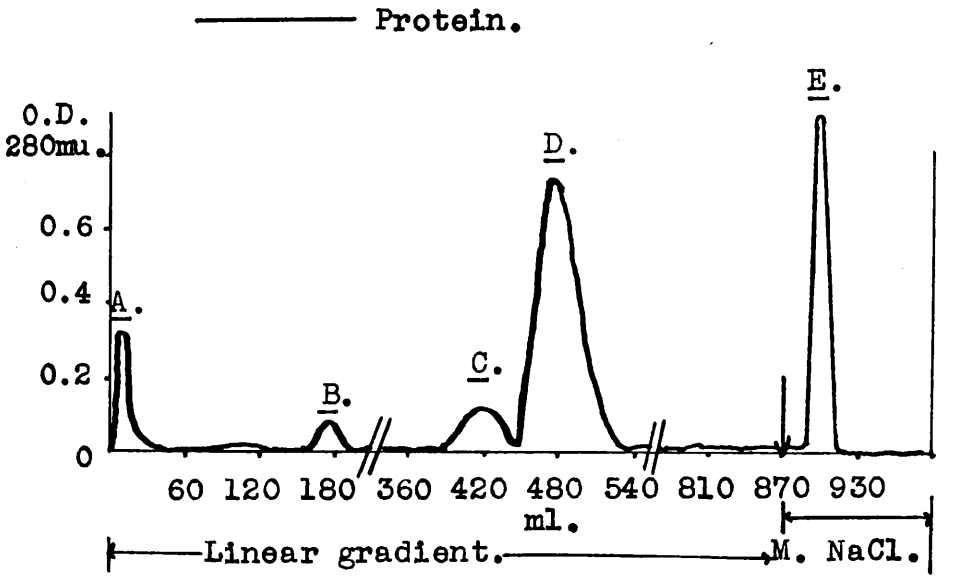
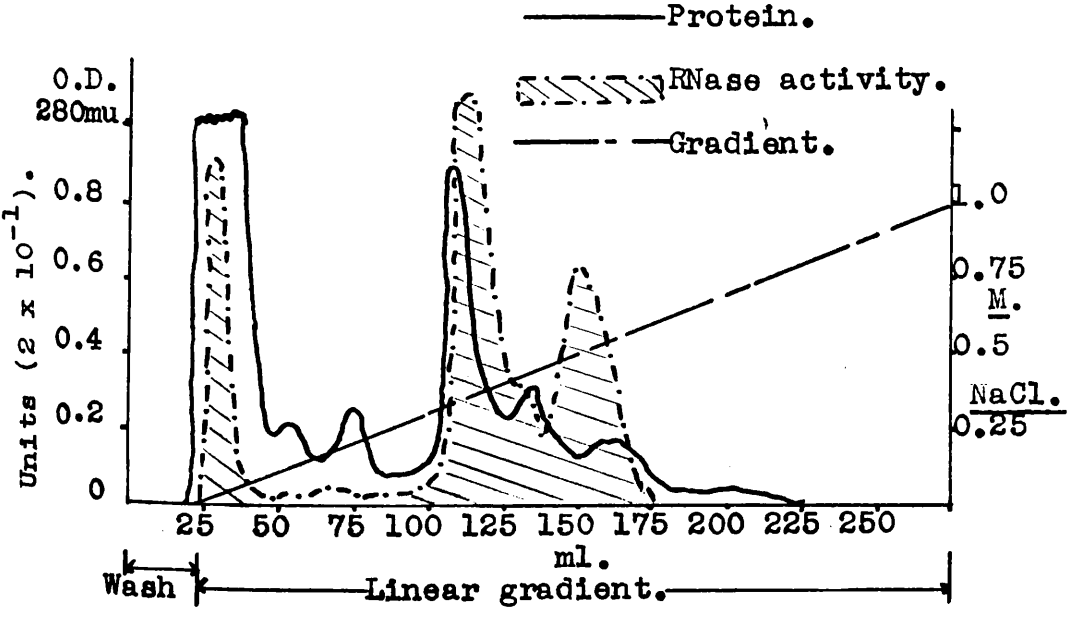


Fig. 19.

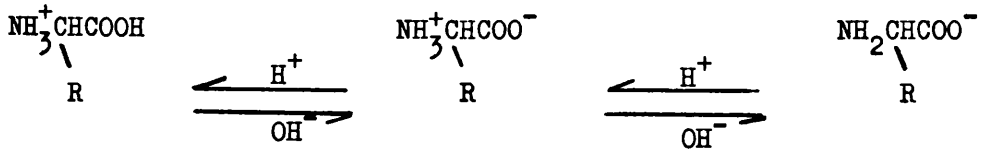
Cation exchange chromatography of the crude spleen ribonuclease sample on C.M. cellulose at pH 7.5 (Exp. 20).



required to elute the protein at this pH. The severe gradient was used to ensure all the protein was displaced and yielded a result on which refinements could be considered. About 20% of the ribonuclease activity was not retained on the column and was eluted in the dead volume. By the Folin[180] method of estimating protein it was shown that 60% of the protein sample was not retained on the column. 77% estimated as U.V. optical density units was not retained on the column. These values suggested that U.V. adsorbing polymeric substances contaminate the sample and were not retained. The protein retained on the column appeared to have chromatographed satisfactorily as several protein peaks could be distinguished though clustered together because of the severity of the gradient. Despite the strong competing ion effect, two ribonuclease activity peaks appeared in the gradient elution pattern in close proximity to each other and contained approximately 54% and 26% of the total ribonuclease activity. In all, three regions of ribonuclease activity were located which presented a picture of some complexity. No good reason could be attributed to the appearance of ribonuclease activity in the dead volume. If many different ribonuclease activities did not exist it was considered possible that the high protein concentration of 35 mg/ml favoured a certain amount of inter component attraction in preference to adsorption on the column at application. Small molecular weight substances with basic properties could adsorb on to the high proportion of acidic polymer not retained and pass through the column.

Because the above experiment had given promising results it was repeated with another portion of the stock enzyme solution under slightly modified conditions. A linear gradient over 1 litre from zero NaCl concentration to 3M NaCl was employed. 500 mg. of spleen preparation was applied to the column under equilibrated conditions of lower pH at pH 6.8. It was considered that under lower pH conditions the proteins exhibited a greater net positive charge and had a greater tendency to adsorb on the exchanger.

Proteins like amino acids are amphoteric and the arrangement can be illustrated simply with an amino acid thus:



net $^{+}$ve charged isoelectric state net $^{-}$ve charged

Basic proteins have a greater proportion of free amino, guanidino and iminazole groups on the chain and the greater the number of net positive charges on any protein molecule the firmer the attachment to the ion exchange adsorbent as a greater number of bonds can be formed.

The experimental details for the chromatography at pH 6.8 are presented on page 372 , Experiment 21, and the elution pattern is illustrated graphically on page 166 , Figure 20. On assay of the fractions two ribonuclease activity peaks were obtained within the gradient span and were not completely fractionated. No ribonuclease activity was located in the dead volume on this occasion. The assays

were not quite optimal but it could be calculated that the first ribonuclease activity peak eluted amounted to 57% of the ribonuclease activity, c.f. 54% in the previous experiment. An estimate by U.V. optical density units (G.M. 15c) showed 72% of the U.V. absorbing material applied was not retained on the column. According to the Folin method[180] of protein estimation (G.M.15b) 41% of the protein passed through the column at application and washing with starting buffer. The Folin[180] estimate for protein gave a true reflection on the distribution of protein. Figure 21, page 166, illustrates the elution pattern for Experiment 21, page 372 . The distribution of the U.V. absorbing material is compared with that for protein as estimated by the Folin method over the elution pattern. Much less protein was rejected by the column than first appreciated and the patterns over a substantial portion of the span of elution were in agreement. Towards the end of the elution pattern more protein appeared to be present as detected by the Folin reagent than represented by optical density measurements.

It was concluded that in Experiment 20, the previous relatively unsuccessful experiment, the conditions were not conducive to strong retention of the ribonuclease active component(s) on the adsorbent. The low content of ribonuclease active component(s) present in large quantities of crude preparation meant that conditions were required to cope adequately with large samples if a fractionation was to be introduced at this stage of low purification. Lowering the pH to 6.8 in Experiment 21 appeared to facilitate the retention of all the

Fig. 20.

Cation exchange chromatography of the crude spleen ribonuclease sample on C.M. cellulose at pH 6.8 (Exp. 21).

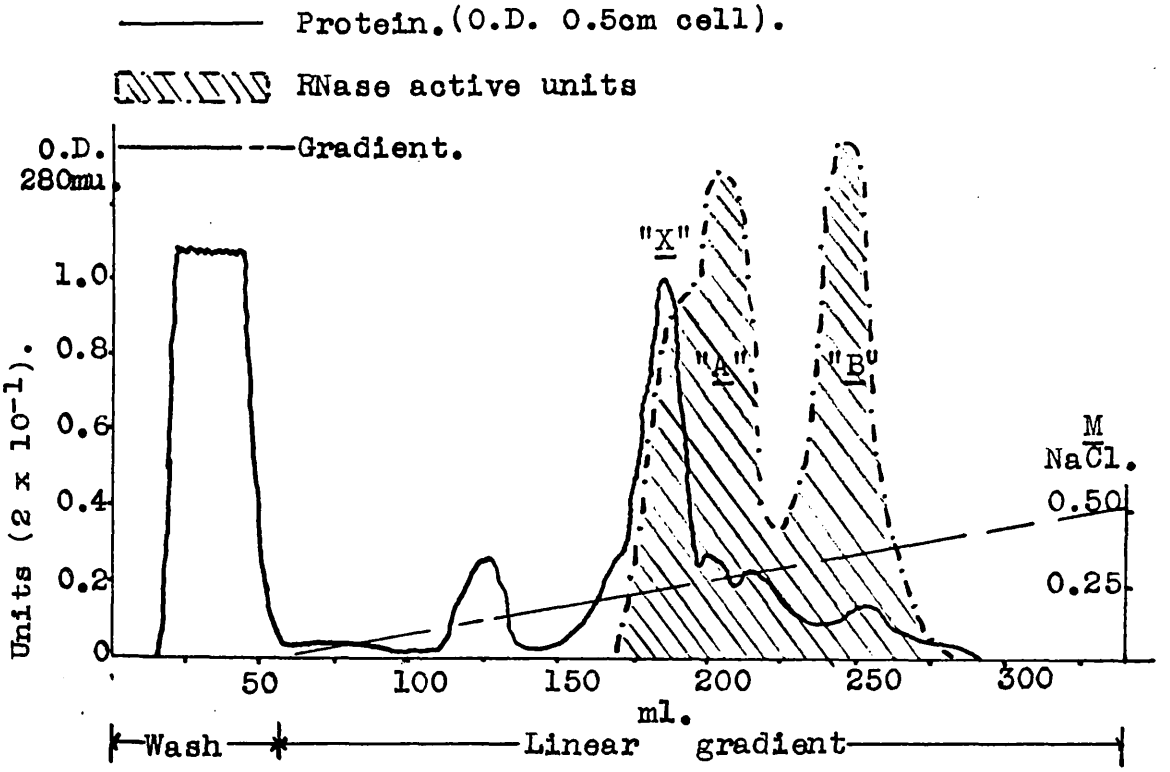
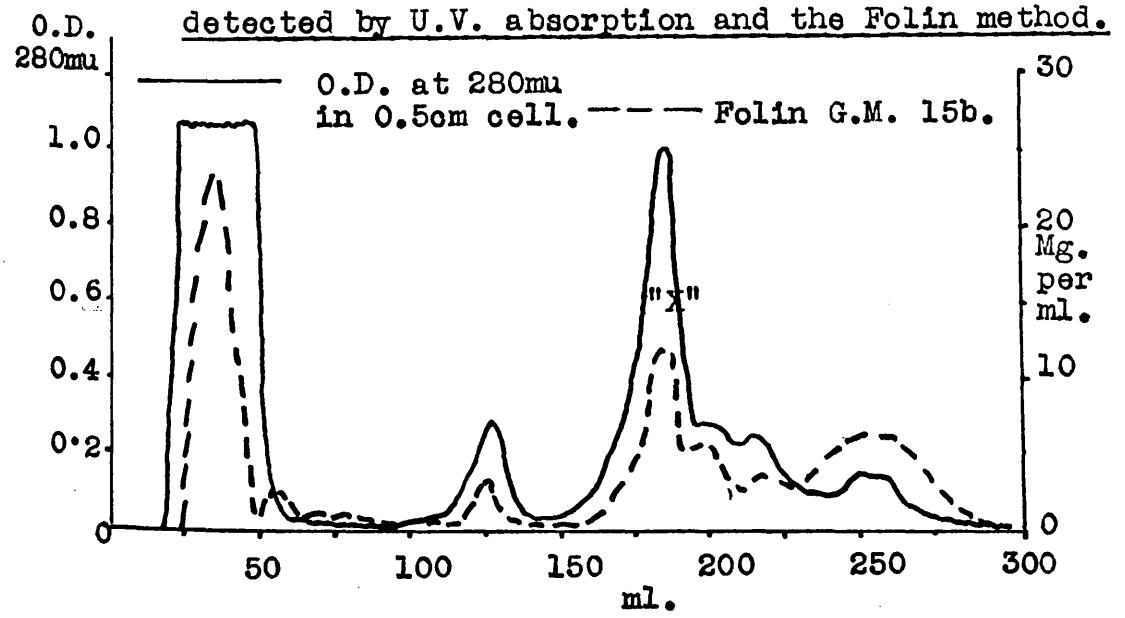


Fig. 21. The protein elution profile of Exp. 21 detected by U.V. absorption and the Folin method.



ribonuclease active components from a 500 mg sample. The protein elution patterns for the two chromatograms were very similar despite the changes introduced. It was not possible to record with certainty which of the two ribonuclease active peaks gained from the retention of all the ribonuclease activity. From the distribution of ribonuclease activity in Experiments 20 and 21 it could be tentatively suggested that the second ribonuclease activity peak eluted from the column in Experiment 21, Figure 20, had gained as it contained 43% of the ribonuclease activity compared to 26% in Figure 19 (page 162), i.e. the more basic component increased in concentration.

These two experiments demonstrated that a lower pH had to be used to attach the ribonuclease activity to the first 2% of the ion exchange adsorbent at the column top. It was considered that the initial buffer strength of 0.02M tris might aid protein desorption, thus it was reduced to 0.005M tris as used at the earlier chromatography by the Taborsky method[90]. Figures 19 and 20 illustrate that the protein peaks eluted cluster together, thus it was decided that the gradient effect should be refined by including some of the considerations of Alm et al[91] and applying the convex system recommended by Cherkin, Martinez and Dunn[93]. In Figure 20 both peaks of ribonuclease activity were not associated directly with protein peaks. This was particularly so with the large protein peak marked "X" and the first ribonuclease activity peak "A". When the columns were finally eluted with 4M NaCl in the buffer solution, there was no evidence of residual U.V. absorbing material retained on the column

after the gradient strength had reached 0.5M NaCl at pH 6.8.

By extrapolation it could be gauged that the second ribonuclease active peak eluted, labelled peak "B" on Figure 20, was desorbed by a salt concentration of 0.3M NaCl. It was appreciated that when new variables were introduced this value might not be in any way reproducible.

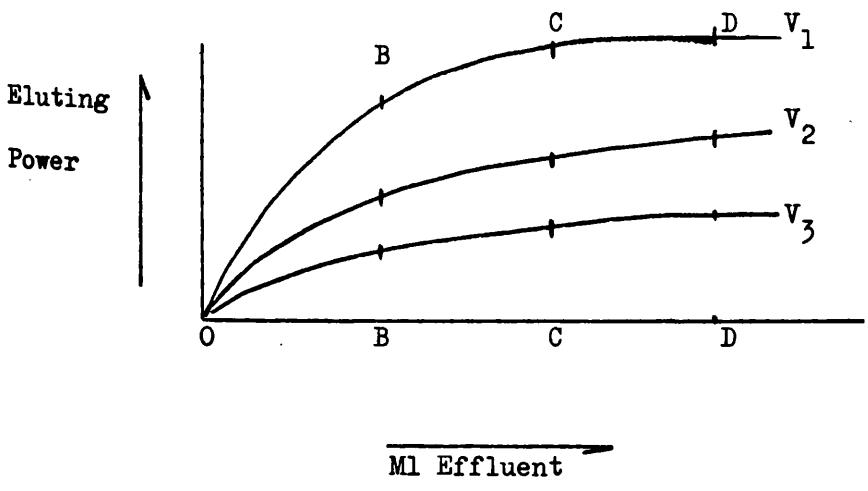
(b) Convex gradient elution

The linear gradient assembly, Figure 35, page 312 , was replaced by a convex gradient system of elution, Figure 36, page 312 , which had a fixed volume mixing device to provide the convex gradient effect. This system was used by Taborsky[90] for chromatography of pancreatic ribonuclease. The extent of the elution pattern is determined by the volume of the fixed volume in the mixing chamber and eluting strength conditions within the gradient assembly. The volume of the fixed volume mixing solution must at least equal the volume of the effluent to be collected and must greatly exceed the dead volume of the column for satisfactory resolution. The effectiveness of the increasing eluting concentration produced by a convex gradient is negligible by that point where twice the volume of the fixed volume solution has eluted the column[93], Figure 38, page 314 Convex gradient elution chromatography begins with a constant volume weakly displacing eluent and finishes with a sufficiently strong eluent to remove the most strongly held substances of importance from the column. After the final eluting strength in the stock reservoir

is settled on it is possible to adjust the volume of the fixed volume solution to decrease or increase the slope of the convex gradient, i.e. the steeper the gradient then the lower the fixed volume under the particular conditions of chosen eluting strength. By adjusting the eluting concentrations in the fixed volume chamber and stock volume chamber, it is possible to choose a gradient of any desired slope or range in any concentration range to meet many requirements. Alm et al[91] discuss the effect of the convex gradient on the chromatography of a mixture of components. The fixed volume in the mixing chamber is represented by V and the convex gradients can be represented in Figure 27 when $V_3 > V_2 > V_1$ where eluting power is plotted against the volume of the solution eluted.

FIGURE 27

Convex gradients when $V_3 > V_2 > V_1$.



The convex gradient of the V_1 system can be divided into three sections. The initial section of the gradient pattern is steep and

falls away as the elution is extended. Components eluted within section O → B will be chromatographed crowded together and a slightly better resolution will be expected in section B → C where the gradient is not so steep. In the section C → D the components eluted may be well separated but possibly more spread out than in the earlier part of the elution pattern.

(c) Purpose of the convex gradient

It was decided to attempt the application of these effects to the situation obtained by the elution pattern illustrated in Figure 20 (page 166) in order to resolve the many contaminants from the ribonuclease active component or components. The plan was to find conditions where the low gradient section C → D Figure 27 would coincide with the region of elution where the two ribonuclease active peaks would be eluted. Under these conditions it was expected that the components eluted prior to the first ribonuclease activity peak "A", particularly the major protein peak "X", would be eluted earlier in a component crowded and ribonuclease activity free O → B section of the convex gradient. Other components not exhibiting ribonuclease activity and more resistant to elution would remain on the column after the second ribonuclease activity peak "B" was eluted near that point the gradient ceased to be effective (section C → D). This residual protein could be removed by eluting with a strong salt solution. If the convex gradient was shallow enough to give good resolution in the elution section C → D Figure 27 where the eluted peaks might be broader in outline than elsewhere,

care had to be taken that very broad bands did not escape detection as they might have merged with the drift of the base line.

Where changes in the conditions of the eluting system are made during a chromatographic run, the possibilities of artifacts appearing are greatly increased. A continuous eluting system which embraces the particular requirements of a chromatogram is much less prone to yield chromatographic artifacts, though the possibility of their appearance cannot be ruled out as other intrinsic factors may yield them[88].

(d) Development of the conditions for column chromatography with convex gradient elution

Under the conditions of convex gradient elution, column length was increased to 60 cm to promote resolution of the components. Two preliminary chromatographic runs were undertaken using 500 mg samples of the crude spleen preparation and trial convex gradients for assessment purposes. The gradient assembly contained a fixed volume of 400 ml. with zero NaCl content and gave a convex gradient over 800 ml. to a final salt content of 0.8M and 0.45M NaCl respectively at constant pH 6.8. Both columns produce a similar elution pattern to that obtained for Experiment 21, Figure 20 (page 166) where all the protein was eluted before 300 ml. had passed. Chromatography had taken place in the steep region of the convex gradient and these conditions were no better than the methods already tried (Figure 20, page 166).

Experiment 22 describes the chromatography of 500 mg of the

crude spleen enzyme preparation by a convex gradient over 800 ml. from zero NaCl content to 0.25M NaCl where the buffer strength and pH were constant at 0.005M tris HCl pH 6.8. Figure 22 (page 173) illustrates the elution pattern obtained. Many protein peaks were clustered in the first 175 ml. of the elution, then no significant quantity of protein appeared from the column up to 1080 ml. when the effluent contained almost 0.25M NaCl and the gradient effect was ineffective. After the gradient was discontinued the column was eluted with 1M NaCl in the buffer solution and a protein peak was obtained. Assay of the effluent fractions prior to the stepwise 1M NaCl wash revealed no ribonuclease activity. All the ribonuclease applied was located in the 1M NaCl desorbed residual protein peak. This column demonstrated that a final salt concentration of 0.25M NaCl did not have sufficient eluting strength to achieve the full gradient effect already discussed, though the initial requirement of condensing unwanted protein in the first section of the gradient was realised. The ribonuclease component(s) was not yet resolved satisfactorily as several contaminating proteins could still be present. It was felt that these components could be eliminated if the ribonuclease activity was eluted in the latter less effective region of the convex gradient.

An alternative scheme considered worthy of investigation was to repeat the conditions of Experiment 22 and after 600-700 ml of the convex gradient from zero NaCl to 0.25M NaCl had eluted the column, the 0.25M NaCl in the stock supply reservoir was replaced by a

Fig. 22.

Cation exchange chromatography of the crude spleen ribonuclease sample on C.M. cellulose at pH 6.8 with a low strength NaCl gradient. (Exp. 22).

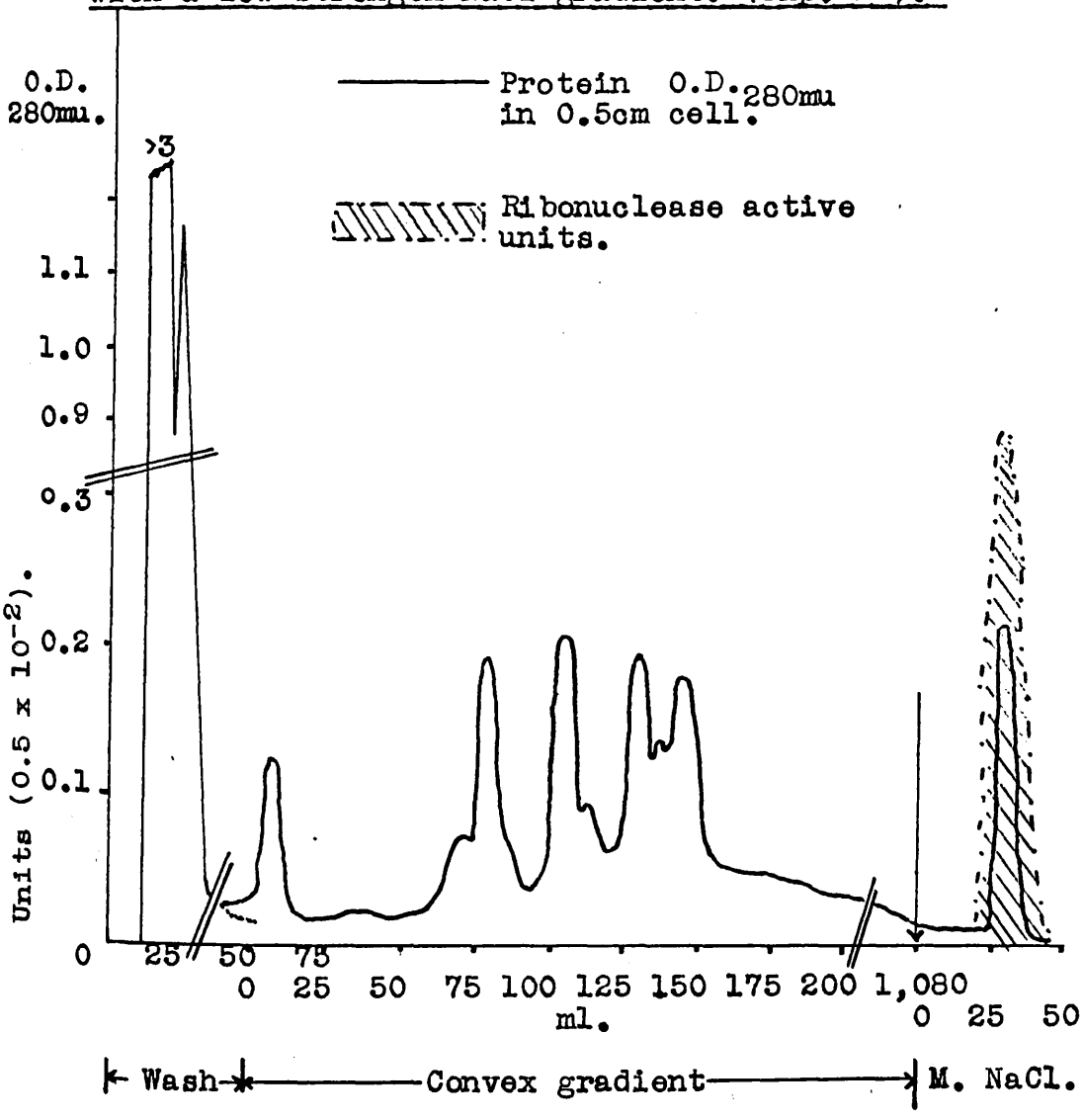
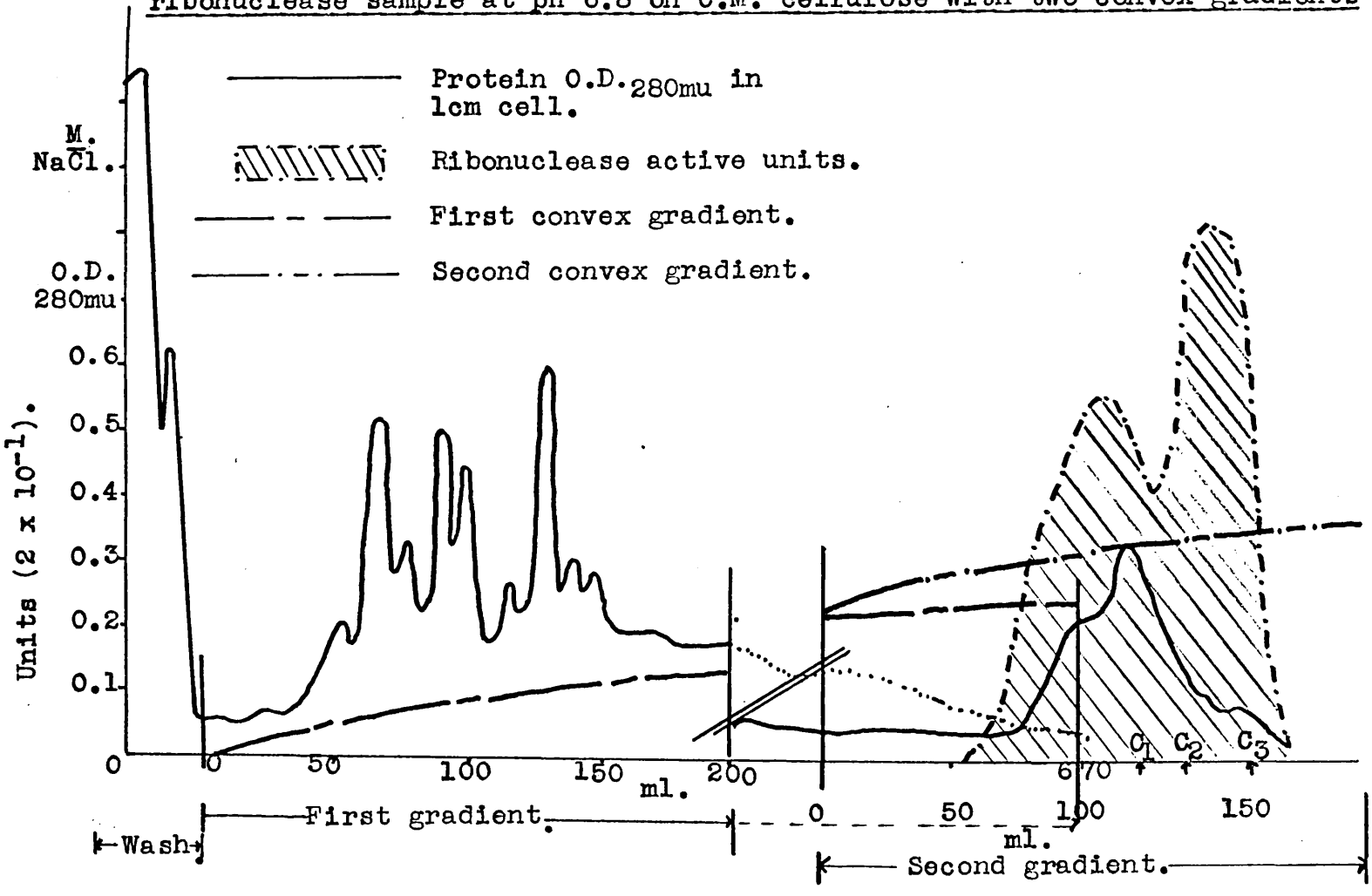


Fig. 23.

Cation exchange chromatography of the crude spleen ribonuclease sample at pH 6.8 on C.M. cellulose with two convex gradients



solution with higher eluting strength. The mixing chamber was charged with solution of the same salt concentration as eluted the column at the part the first gradient was discontinued.

In a subsidiary experiment using exactly the same conditions outlined for Experiment 22, except that after 670 ml of the first convex gradient had passed, i.e. in the regions no more protein peaks appeared, the stock solution in the chamber B' of the convex gradient system, Figure 36, page 312, was replaced by 0.005M tris/HCl pH 6.8 made 0.5M with respect to NaCl and the convex gradient continued. The mixing chamber contained the same buffer made 0.2M W.R.T. NaCl. The elution pattern presented in Figure 23, (page 174), showed a poor resolution of two ribonuclease activities shortly after the second gradient was applied. The result was disappointing, and although 0.25M NaCl at pH 6.8 did not elute the ribonuclease activity, Figure 22, the chromatogram illustrated in Figure 23 indicated that a much more refined separation was required to separate completely the two potential ribonuclease activities. It also demonstrated that a salt concentration not much higher than 0.25M NaCl was required for the final eluting solution.

Alternatively the sudden change of the solvent strength in the first gradient development by the introduction of the second gradient was instrumental in rapidly desorbing the resolving components which were then eluted only partially resolved. It was felt that a considerably smaller increase in the stock solution salt concentration than the two fold enhancement made, might have considerably

improved the elution pattern. A stepwise elution effect, or the initial steep rise of the second convex elution, or a combination of both were considered responsible for the poor result and any attempt to combine gradients was not pursued.

The observation, that after application of the spleen enzyme sample to the C.M. cellulose columns, lightly held material chromatographed rapidly down the column as a brown coloured band in addition to the polymeric components not retained, was viewed with concern as this manifestation persisted. The question of whether the two ribonuclease active peaks were chromatographic artifacts from irregularities at adsorption or elution, or two separate and distinguishable components, added to the difficulty of deciding on the best course of action to pursue. Evidence from the literature[88,163,164] supported the idea that protein samples applied in high concentration, could give rise to a zone of different properties after some of the components were adsorbed and a redistribution of particular components could take place (page 204). In these circumstances partial elution of some components was possible and chromatographic artifacts would be obtained. With this possibility in mind, greater efforts designed to adsorb firmly all protein components which were retained, was introduced by lowering the pH at the application of the enzyme sample.

The column procedure for Experiment 22 (page 373) was repeated at a different initial pH. The Experiment 24 is described on page 375 and the elution pattern illustrated on Figure 25, page 178.

10^{-4} MEDTA was included in the eluting solvent for its protective action against heavy metal contamination as a matter of form (page 47). Before the sample was applied the crude protein preparation was pretreated as described on page 198 , by passing the solution through a D.E.A.E. cellulose column. The solution containing the ribonuclease activity was applied to the same column used for the previous Experiment 22 with C.M. cellulose at the lower pH = 6. The decrease in pH was intended to reduce the anionic character of the proteins and bind all components to the adsorbent much more firmly initially. The convex gradient was over the same range, with a 400 ml. fixed mixing chamber volume and final salt concentration of 0.25M NaCl. A pH effect was introduced, in that over the 800 ml. elution span, the pH could rise to pH 7 with the final eluting solvent. An increase in the pH of the solvent would suppress the ionisation of the basic groups on the protein molecules and selectively reduce the binding forces. At the same time the competing ion effect with Na^+ desorbs and redistributes the proteins on elution. The elution pattern, Figure 25, can be directly compared with Figure 22 (page 173). The effect obtained was much nearer that required. The bulk of the inert protein was eluted in a crowded region near the start of the elution pattern. The initial peak containing protein not retained by the column, was considerably reduced in amplitude which demonstrated the effect of the DEAE cellulose pretreatment (page 198). The first ribonuclease activity peak (peak "A") was eluted associated with a protein peak. The true

Fig. 24.

The pretreatment of the crude spleen RNase sample on D.E.A.E. cellulose at pH 7. (Exp. 23).

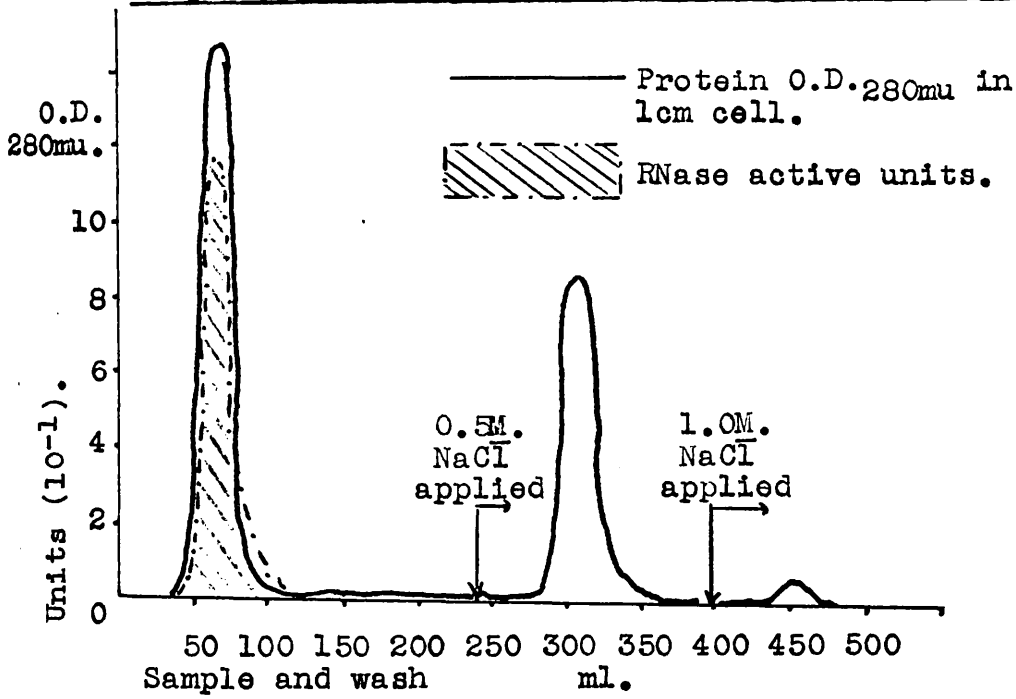
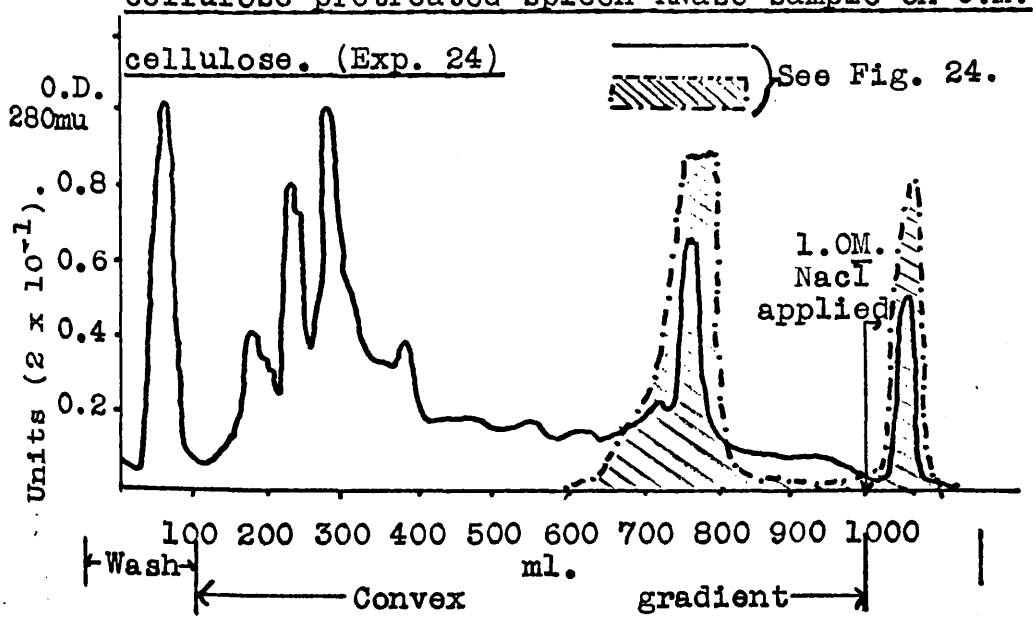


Fig. 25.

The cation exchange chromatography of D.E.A.E. cellulose pretreated spleen RNase sample on C.M. cellulose. (Exp. 24)



dimension of both peaks could not be determined as several fractions were lost at this point when the fraction collector failed. A second peak of ribonuclease activity (peak "B") was obtained when the residual protein was removed from the column by eluting with 1.5M NaCl in the buffer solvent. In this chromatogram, under the final conditions of a stepwise elution, it could not be recorded that two distinct ribonuclease activities were resolved. The application of a second convex gradient after the elution of the first active peak, to obtain a gradual elution of the second by a method similar to that attempted earlier, page 175 , Figure 23, was not undertaken. In the present circumstances, if two ribonuclease activities existed it was not an advantageous policy to have a stepwise change in elution by the introduction of a second convex gradient or substitution gradient.

(e) A preliminary investigation of C.M. Sephadex C 50 as a medium to chromatograph the spleen preparation using the methods already adopted

Introduction

A very recent addition to the list of cation exchange media is C.M. Sephadex in which carboxymethyl groups are attached to the cross linked dextran structure of ordinary Sephadex. The dual effect of the molecular sieve action and cationic multistage desorption are the factors responsible for component resolution at column chromatography. The normal molecular sieve properties of the Sephadex are significantly altered. Molecules which are completely excluded will have a

decreased capacity for adsorption to the exchanger and a comparable situation to that first observed with the synthetic resins and protein molecules results for those molecules. Molecules partially or completely occluded by the gel are subject to the resolving effect of both the molecular sieve action and multistage adsorption process produced by the eluting strength and appears complex[49]. Information supplied by the manufacturers of C.M. Sephadex[94] reveals that marked changes in column volume accompany variations in the pH and ionic strength of the eluting solution. The recommendation is made that columns and the initial eluting solution ought to contain between 0.05M and 0.1M salt to stabilise the gel.

C.M. Sephadex chromatography

A very preliminary investigation of the material as a substitute for C.M. cellulose was made in Experiment 25 (page 376). For comparative purposes this experiment was a repeat of the experimental conditions of Experiment 24. The equilibrating solution contained 0.05M NaCl and the convex gradient ranges from 0.05M NaCl to 0.3M NaCl over 1 litre to pH 7. Column dimensions at the start of the elution were 1 cm. x 38 cms. As the first gradient progressed the column length decreased because of shrinkage to 30 cm. by the time 400 ml. of the gradient had eluted the column. The length decreased to 26 cm. by the end of the first elution. The final dimensions of the column were 1 x 20.5 cm. after the 1.2M NaCl wash.

Over the span of the gradient 0.05M NaCl to 0.3M NaCl where the pH was 6 initially, rising to pH 7 by the end of the gradient, the

column diminished in length by 30% and by as much as 48% at the final elution. This feature of extensive shrinkage was viewed with apprehension and any ideas to include the exchanger in preliminary fractionation of the crude spleen ribonuclease were immediately abandoned. The behaviour was regarded as inconsistent with satisfactory column procedure. The same conditions already implemented with C.M. cellulose were obviously not favourable for chromatography on C.M. Sephadex though the elution pattern compared favourably, Figure 26, page 191 , with both Figure 22 and Figure 25. The protein elution patterns were very similar in outline which tentatively suggests that the same overall effect could be achieved on the more stable cellulose exchanger. At this time the investigation with C.M. Sephadex for preliminary fractionation was not pursued, mainly as a result of the excessive shrinkage experienced.

5. Preparative scale cation exchange chromatography

(a) Conditions for the preparative chromatography of the spleen enzyme preparation on a scaled up C.M. cellulose column

The general way to a single, one stage optimum chromatographic separation appeared very hard to fix upon. It might be that several chromatographic procedures should be used in an extensive system to purify an enzyme and give a much greater overall effect. The DEAE cellulose pretreatment of Experiment 23, page 374 , was introduced to decrease the amount of contamination, particularly with respect to the load applied to the C.M. cellulose columns, and act as a

check on the efficiency of the heat treatment, page 72 . The alternatives to further experimentation to perfect the resolution of components on the C.M. cellulose system so far developed was to rechromatograph the ribonuclease active regions obtained under different conditions from those already used. With a much purified sample, the system which suggested greatest purification potential was one similar to Experiment 19 (page 370), i.e. the prototype column already tested, where a pure crystalline sample of pancreatic ribonuclease was fractionated at constant pH 8. Ion exchange chromatography is suitable for large scale production as well as investigation at the analytical level. The system developed to this stage was regarded as at the analytical level as the large quantity of protein processed yielded a small amount of ribonuclease activity. The requirements for a rechromatography of the ribonuclease active samples indicated that much greater quantities of the crude spleen preparation would have to be processed by the system already developed.

To accommodate this increase in quantity the system already developed was scaled up to a tenfold increase in capacity and the experimental assembly constructed accordingly. The column dimensions arrived at were 3 cm. i.d. x 60 cm. effective length. The resolution of components would be approximately the same, thus no increase in length was considered necessary. A 3 cm. internal diameter column produced a tenfold increase in effective volume of ion exchanger. This column should cope with a tenfold increase in

load without loss of resolution. 50 ml. samples were collected and a tenfold increase in the volume of the gradient elution assembly introduced, since it was required that the elution should take place over the same span, as already described (page 169) The enzyme sample was applied in a larger volume to conform with the increase in the quantity of material which was applied. The column flow rate was not increased tenfold and was slightly less at sixfold. The decrease in flow rate was mainly an attempt to improve the resolution on the same column length at the grander scale. Many of these decisions taken on the process of scaling up the system had no foundation for their adoption other than speculative reasoning based on experience.

(b) Application of the preparative scale cation exchange chromatography on C.M. cellulose

The large scale chromatography was carried out on the system developed and a few alterations were made which did not entirely conform to recommended column procedures. These alterations were introduced to facilitate the chromatography prior to a precise rechromatography. The crude enzyme preparation was applied to the column at a low pH 6 as used in Experiment 24 (page 375) without a change in the solvent system to a buffer designed for stabilising this range of pH. The main reason for this was that the rechromatography was scheduled to take place at constant pH 8 in the tris buffer system[106] already generally used[90]. A buffer change involving many manipulations of either dialysis, freeze drying

followed by redissolving and re-equilibration by dialysis, or concentration by freeze drying them desalting with a buffer change on Sephadex by gel filtration[99,103,194] were considered time consuming and undesirable. The effect of acid buffers (page 160) was also considered undesirable. Peterson and Sober[158] recommended that a low buffer capacity was desirable in gradient elution chromatography, e.g. 0.005M tris HCl used earlier at pH 7. It was considered that if the desired preliminary effect was obtained by unrefined chromatography then the system served its purpose.

The effect of the increase in pH on the elution profile observed in Figure 25 (page 178), where some of the ribonuclease activity (peak "A") chromatographed within the span of the convex gradient, was noted of importance. The pH range effect was increased from pH = 6 at application of the sample to pH = 8 in the final eluting stock solution of the gradient. To assist the multi-stage desorption process, a slight increase was made in the final NaCl concentration to 0.3M to ensure that the ribonuclease activity was eluted within the span of the single convex gradient without loss of the resolution so far obtained. Figure 28, page 187, gives the elution pattern obtained by the scaled up Experiment 26, page

4.5 g of crude spleen preparation (page 118) was chromatographed. An elution pattern with improved features was arrived at on this fractionation attempt, which was considered successful. The ribonuclease activity peaks "A" and "B", as previously labelled in Figure 20, occupied sufficiently well resolved fractions after a

single gradient elution to be treated separately thereafter. An assay conducted on the samples revealed that 97% of the total ribonuclease activity applied was present in the combined total ribonuclease activity of both ribonuclease peaks, Figure 28, page 187 . Activity peak "A" contained 26% of this total ribonuclease activity while the remainder was distributed in ribonuclease active peak "B". Excluding three days to assay the effluent, a minimum of 10-12 days were required to complete the work on a large scale preparative column.

The ribonuclease active peaks were eluted earlier than the original plan intended (page 170). However, the resolution of the more basic ribonuclease activity peak "B", which might have been inconveniently more spread out if eluted at the 8 litre mark, was satisfactory. The lack of a significant protein peak, as measured at 280 mμ on the U.V. spectrophotometer, in the elution pattern of this region was noted for consideration. The low protein content in this region of the elution could have merged with the base line and been obscured (see Figure 20, page 166). The residual protein was removed from the column with 1.3M NaCl and a substantial peak of protein was eluted along with a very small quantity of ribonuclease activity probably from the tail of the second ribonuclease activity peak "B" by the stepwise elution effect[88,161]. The overall effect outlined on page 170 was achieved. Contaminants with greater basic properties were retained until after the second ribonuclease activity peak "B" was eluted, while the more acidic

components were condensed in the initial region of the convex elution pattern prior to the elution of the ribonuclease activity peak "A". The ribonuclease activity peak "A" had considerable protein eluted associated with it. Analysis of these active fractions by disc electrophoresis is reported in Section IV, page 219. The findings are discussed later after the details of the rechromatography (page 193).

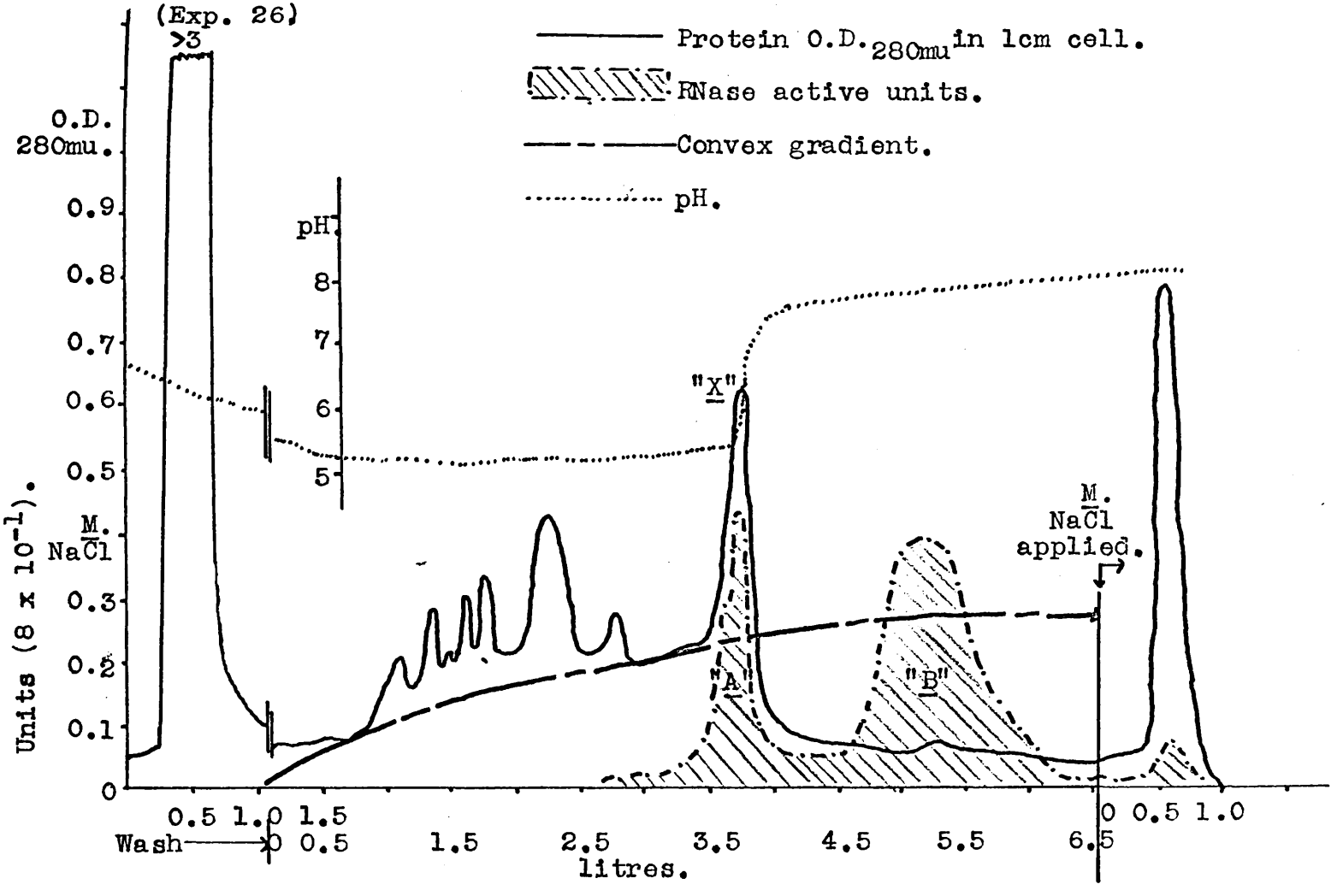
45% of the U.V. absorbing units representing protein as measured at 280 mp on the U.V. spectrophotometer were retained on the ion exchanger and fractionated. Of this quantity 5% remained on the column after the convex gradient was discontinued. This large scale chromatography system was adopted to obtain ribonuclease active samples of the heat stable spleen ribonucleases, particularly the ribonuclease activity peak "B" for further study and G.M.8b, page 316 outlines the procedure adopted from Experiment 26.

6. Rechromatography by cation exchange chromatography

(a) Rechromatography of the ribonuclease active fractions labelled "A" and "B" from the scaled up C.M. cellulose column.

The two ribonuclease activity peaks "A" and "B" were sufficiently resolved in the scaled up column to investigate them separately. The precise refractionation of these peaks was carried out on columns 1.8 cm. i.d. x 26 cm. effective length on separate occasions with C.M. cellulose and C.M. Sephadex exchangers after a separation on the scaled up column with effectively the same elution

Fig. 28. Preparative scale cation exchange chromatography on C.M. cellulose.



patterns, irrespective of the nature of the exchanger. As already described (page 179) C.M. Sephadex C 50 offers certain additional advantageous properties as cation exchanger for chromatography. An important feature not listed earlier is that Sephadex C 50 has a much higher exchange capacity than C.M. cellulose and ought to retain adequately substantial quantities of protein on the first 2-5% of the column top especially when applied in a large volume of solution. The C.M. Sephadex exchanger was reintroduced to act as the exchange medium under the more standard eluting conditions for refined and exacting rechromatography of the two ribonuclease activity peaks produced by Experiment 26 and represented in the elution pattern of Figure 28, page 187.

The ribonuclease active peaks were bulked separately then dialysed for a short period to equilibrate, after they were adjusted to pH 8 (page 286). The samples were applied direct to the columns in the original volumes. The three Experiments 27, 28 and 29 (pages 378 - 380) were carried out on the same column which was repacked on each occasion, after re-equilibration, to the same dimensions and to ensure even distribution of the gel medium. The equilibrating and initial buffer was 0.005M tris HCl 10^{-4} MEDTA pH 8. The eluting strength was produced by the convex gradient system, Figure 36, page 312 , where the fixed volume was 400 ml. of initial buffer and the elution increased from zero NaCl to 0.3M NaCl over 1 litre.

Experiment 27 (page 378) describes the rechromatography of the

ribonuclease active peak "B" on C.M. Sephadex C 50 and the elution pattern is illustrated in Figure 29 (page 190). Considerable U.V. absorbing material was eluted in the dead volume of the column and collected in 50 ml. fractions. No ribonuclease activity was detected in these fractions. Over the convex gradient elution span only one ribonuclease active peak associated with a small protein peak was eluted towards the end of the elution span. No ribonuclease activity remained on the column after the gradient was discontinued. Purity and molecular size estimates of this ribonuclease active fraction "B" are described later in Sections IV and V.

Experiment 28 (page 379) describes the rechromatography of the ribonuclease active peak "A" on C.M. Sephadex C 50 and the elution pattern is illustrated in Figure 30 (page 190). A refractionation of the ribonuclease active peak "A" into two active fractions was obtained and the pattern compared favourably with that observed on the scaled up preparative scale chromatogram, Figure 28, page 187. In Figure 30 ribonuclease active peak "A" fractionated closely associated with the large protein peak "X" of Figure 28. This feature appeared consistent and reproducible in repeated chromatograms and characterised the elution position of ribonuclease active peak "A" on rechromatography. The second ribonuclease active peak eluted, chromatographed towards the end of the elution span and was eluted in an exactly similar position to ribonuclease activity peak "B" of Figure 29. This suggested they were the same ribonuclease activity entity (page 193). Of particular significance was the distribution

Fig. 29.

Rechromatography of the RNase active peak "B" from fig. 28 (Exp. 26) on C.M. Sephadex at pH 8 (Exp. 27).

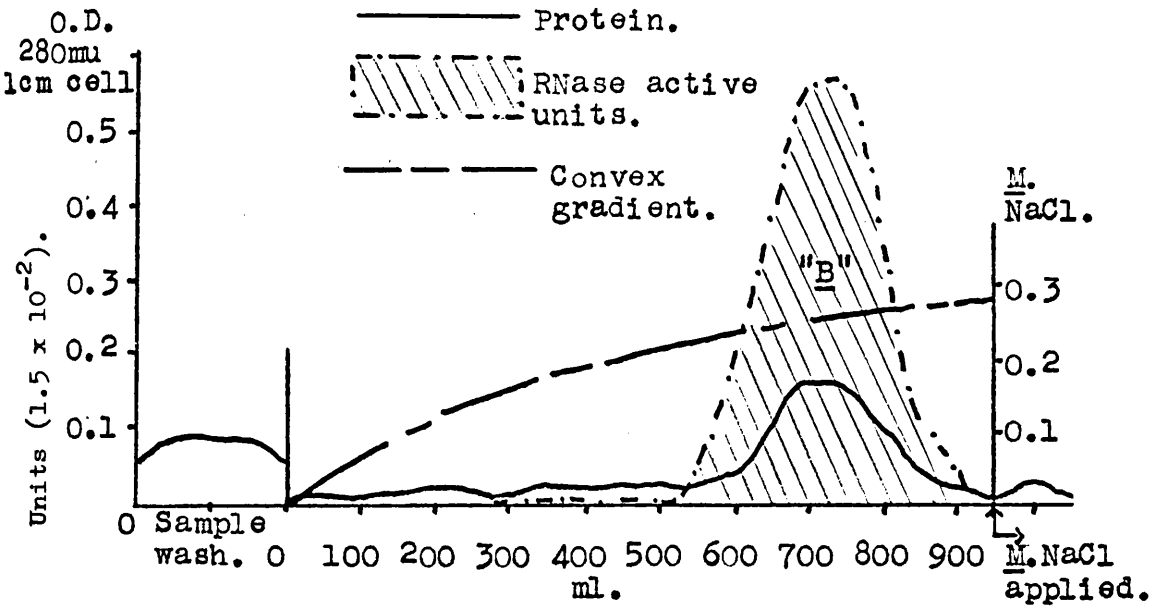


Fig. 30.

Rechromatography of the RNase active peak "A" from fig. 28 (Exp. 26) on C.M. Sephadex at pH 8 (Exp. 28).

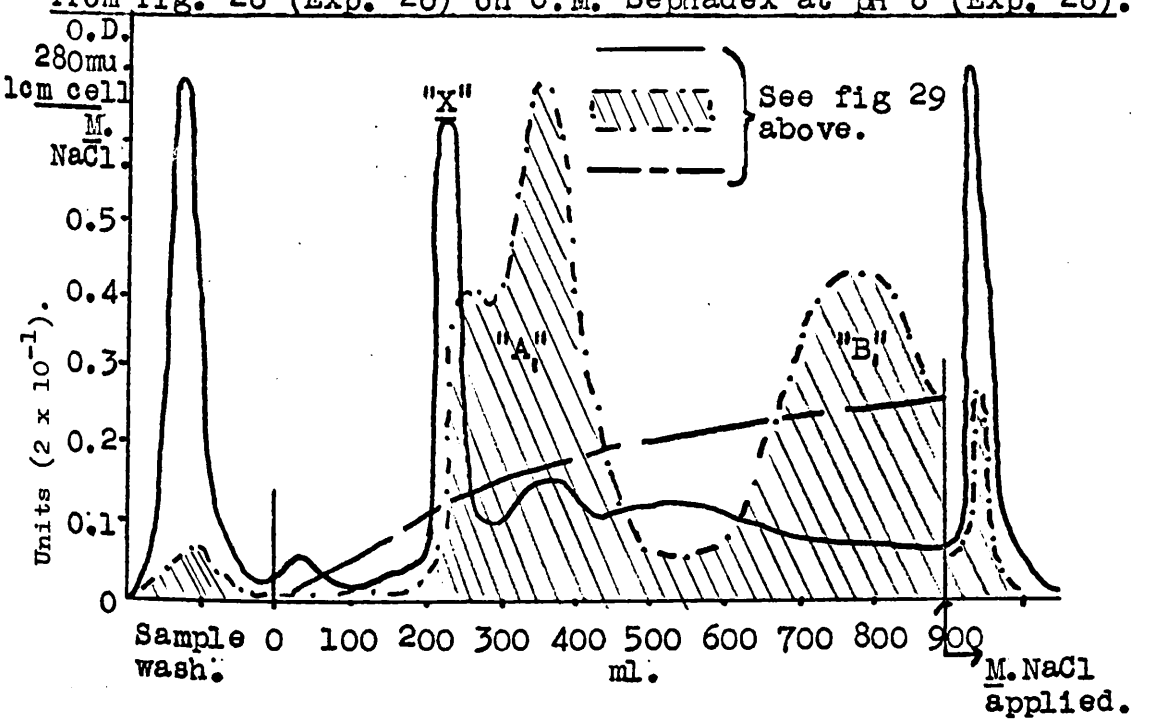


Fig. 31.

Rechromatography of the RNase active peak "A" from fig. 30 (Exp. 28) on C.M. Sephadex at pH 8 (Exp. 29).

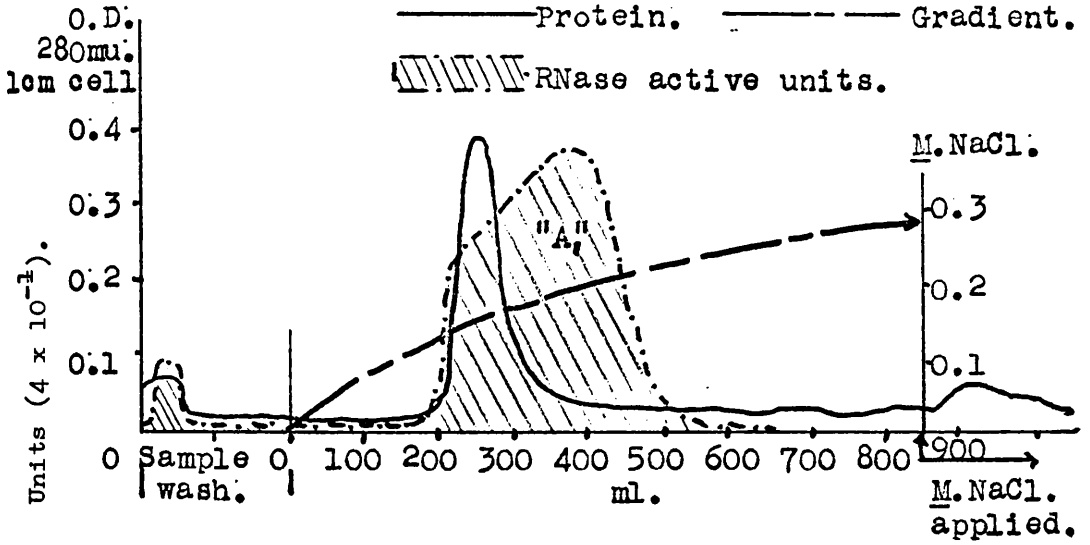
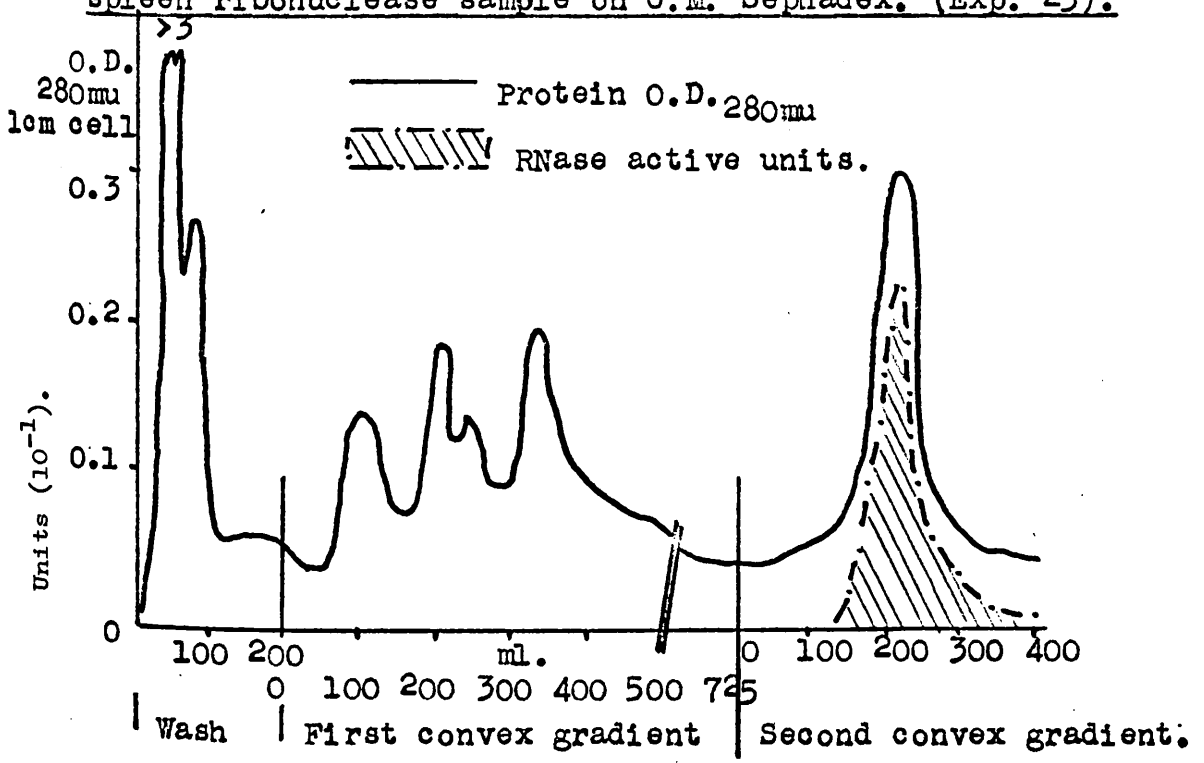


Fig. 26.

Cation exchange chromatography of the crude spleen ribonuclease sample on C.M. Sephadex. (Exp. 25).



of protein on chromatography at this pH. Some protein components were not retained by the column and a considerable proportion were not eluted by the final eluting strength of the gradient at 0.3M NaCl. They were desorbed as residual protein with 1.5M NaCl. It was not expected that protein components could appear with greater basic properties. The significance of this sub-fractionation is discussed on page 194 . Purity and molecular size estimates are determined and described later in Sections IV and V.

The rechromatography of ribonuclease active fraction "A" from Figure 30 on C.M. Sephadex C 50 is outlined in Experiment 29, page 191. This experiment was a repeat of the experimental conditions of Experiment 28 and designed to find out if the ribonuclease activity "A" would refractionate a second time and redistribute into two ribonuclease active peaks. Figure 31 (page 191) illustrates the elution pattern. The rechromatography produced the ribonuclease activity peak "A" only. This activity peak was again closely associated with the protein peak "X".

The gross shrinkage effect in the column experienced earlier in Experiment 25, page 180, was again manifest. The column length initially measured 26 cm. At the end of the gradient elution at 0.3M NaCl the column length was reduced to 16 cm., i.e. a 39% reduction in length. After eluting with 1.5M NaCl to remove residual protein the column measured 10 cm. in length, which was a 61% reduction in length. This feature was regarded as highly disadvantageous. The column had to be dismantled, re-equilibrated and

repacked before each sample could be applied. The shrinkage during the chromatography was observed to be uniform without cavities forming, probably a result of packing the material at mild atmospheric conditions and the elution patterns appeared regular. Experiments 27, 28 and 29 have been repeated in an exactly similar manner using C.M. cellulose which did not exhibit this shrinking property. With these columns the two ribonuclease activity peaks from other scaled up C.M. cellulose columns were rechromatographed and exactly similar elution patterns as deputed in Figure 29, 30 and 31 were obtained for the columns respectively.

(b) The effect of the rechromatography at constant pH 8

These rechromatography experiments clearly show the occurrence of an important effect. The crude spleen preparation fractionated into two ribonuclease active peaks "A" and "B" at preliminary chromatography, Figure 28. Active peak "B" on rechromatography eluted as a single active peak, Figure 29. However, active peak "A" on rechromatography split into two active peaks "A_1" and "B_1", Figure 30. Here the elution pattern is similar to the original pattern at preliminary chromatography, Figure 28. Peak "A_1" chromatographed in proximity to the major protein peak "X" similar to that in Figure 28 suggesting "A_1" $\equiv$ "A". Active peak "B_1" eluted at a similar position to active peak "B" in Figure 29 suggesting "B_1" $\equiv$ "B". When active peak "A_1", Figure 30, was rechromatographed again it did not subsequently refractionate. On rechromatography peak "A" should not have refractionated if the eluting conditions

were normal in Experiment 26, Figure 28. Three alternative explanations were contemplated.

i. Three ribonuclease active components were present recognised as ribonuclease activity "A_1" Figure 31, ribonuclease activity "B_1" Figure 30, and ribonuclease activity peak "B" Figure 29. All on pages 190 and 191. A comparison of the elution patterns of Experiment 27, Figure 29, and Experiment 28, Figure 30, suggested very strongly that ribonuclease activity "B" and ribonuclease activity "B_1" were the same.

ii. In that case two ribonuclease activities were present. It was considered that the situation at rechromatography depicted in Figure 30 was produced as a consequence of partial elution of ribonuclease activity component "B" in the scaled up C.M. cellulose column (page 205). An intrinsic column irregularity could cause some of the ribonuclease active component "B" to elute with the components in the ribonuclease activity peak "A". The following rechromatography of the ribonuclease activity peak "A" at the different pH rectified the situation and the artifact components redistributed to yield the balance of the ribonuclease activity "B" at the correct elution position. This explanation was supported by the observation that other protein components were redistributed from ribonuclease activity peak "A" and exhibited a greater basic property which should not be the case at a higher pH of 8, Figure 30. In Figure 30 redistribution of ribonuclease activity peak "A" exhibited protein components which were not eluted at constant pH 8

after the salt gradient had gone to completion. Protein redistribution could be expected at an alternative pH. However at more alkaline pH values proteins exhibit a more anionic character and should have less tendency to adsorb on the cation exchanger. It was expected that the ribonuclease activity components "A" or "B" would be the last components to desorb on rechromatography, Figure 30, but this was not the case. A considerable quantity of protein exhibited even greater basic properties and was eluted from the column in Experiment 28 after the gradient was complete at 0.3M NaCl by eluting with 1.5M NaCl.

By direct comparison of Figures 29 and 30 (page 190), the evidence from these elution patterns implied that the ribonuclease activity region "A" from the scaled up C.M. cellulose chromatography had within its composition components which were potentially more basic in character than the ribonuclease activity "B" eluted afterwards in the scaled up column, Figure 28, page 187, or rechromatographed ribonuclease activity "B" peaks in Figures 29 and 30.

This observation was contrary to the understanding of the effect an increased environmental pH has in reducing the net positive charge exhibited by protein molecules. Figure 30 illustrated that other interactions than direct ion exchange were favoured within the protein milieu and an anomalous elution resulted in the scaled up column (page 187). Possible explanations for this anomalous condition are presented later (page 201).

iii. It was considered possible that only one ribonuclease activity

was present. This could be possible if the ribonuclease active entity existed in several interchangeable forms. The entity could for example associate intermolecularly with proteins other than itself and an artifact distribution at the ribonuclease activity region "A" might result. Alternatively, the molecule could exist in two equilibrium forms which were interchangeable. The chromatographic effect exhibited in Figure 30 suggested that one form had less basic properties for cationic adsorption and was eluted earlier in the scaled up column. Redistribution as exhibited in Figure 30 and failure to redistribute a second time, Figure 31, added to the problem of justifying only one ribonuclease activity. Also ribonuclease activity "B" did not redistribute to a less basic component in Figure 29. There was little evidence that only one ribonuclease activity was present.

The most favourable situation was that outlined in interpretation ii above. This was supported by the information on page 216, which suggests that spleen ought to contain two heat stable ribonuclease active enzymes.

(c) The results of the purification procedure by cation exchange chromatography and rechromatography

The results presented in Table 24 (page 197) represent the outcome of the purification procedures carried through to completion by the cation exchange chromatography of Experiment 26 (page 377) and the cation exchange rechromatography of Experiments 27, 28 and 29 (pages 378 - 380) on the crude spleen enzyme preparation as

TABLE 24. Purification of the crude spleen preparation by cation exchange chromatography

Experiment and Fraction sample	Total volume	Total R.A. Units	R.A. Units/ml.	Protein mg./ml	S.A.Units/mg Protein
Crude heat stable spleen protein sample the product of heat treatment, dialysis then freeze drying (p.118)	80	14,960	187	55	3.4
Expt. 26 p.377 R.A. Fraction "A" Fig. 28 p.187	350	2,975	8.5	0.24	35.4
Expt. 26 p.377 R.A. Fraction "B" Fig. 29 p. 190	800	11,200	14.0	0.11	127.3
Expt. 27 p.378 R.A. Fraction "B" Fig. 29 p. 190	250	9,875	39.5	0.062	637
Expt. 28 p.379 R.A. Fraction "A" Fig. 30 p. 190	240	1,536	6.4	0.082	78
Expt. 28 p.379 R.A. Fraction "B" Fig. 30 p. 190	250	1,290	5.16	0.013	397
Expt. 29 p.380 R.A. Fraction "A"	230	1,506	6.55	0.079	83

R.A. = Ribonuclease Activity

S.A. = Specific Activity

starting material. The significance of these findings is discussed on page 210 when the full effects of chromatography by column techniques are discussed.

7. A pretreatment of the spleen preparation by simple chromatography on DEAE cellulose

From the experiments on cation exchange chromatography carried out it was known that the ribonuclease active components present in the crude heat stable spleen preparation were retained on the cation exchanger C.M. cellulose at pH 6.8 at a low buffer concentration of 0.005-0.02M tris HCl, e.g. see Experiment 22 of this work and page 167 It was considered that the considerable quantity of polymeric material, page 161 , not retained by the cation exchanger on application of the crude enzyme solution influenced the extent of the zone of attachment of the adsorbed components, e.g. cations present as the salts of the unadsorbed anionic molecules at pH 6.8-7.0 could act as competing ions causing displacement of adsorbed components during the application of the sample. This could have caused partial adsorption or a reduced adsorption of the cationic components. The load applied to the cation exchange column was reduced by passing the enzyme solution through an anion exchange DEAE cellulose column before the sample was applied to the C.M. cellulose column.

Diethyl amino ethyl cellulose, i.e. DEAE cellulose, is a weak anion exchanger which can be used for the chromatography of acid to slightly basic proteins adsorbed at low salt concentration and high pH (pH 6-8)[159]. Some guide to the conditions most suitable for

a pretreatment could be obtained from the paper by Maver et al[40] on the chromatography of spleen ribonuclease on DEAE cellulose. The major portion of the ribonuclease activity of their spleen preparation which was not heat treated was retained on a 1 x 52 cm. DEAE - SF cellulose column equilibrated with 0.005M sodium phosphate at pH 7 or 8. Certain enzymes were not retained at these pH values along with some ribonuclease activity. A more recent paper of Maver and Greco[36] reported that the alkaline ribonuclease activity from spleen was not retained on a DEAE cellulose column 1 x 52 cm. when 400 mg. of their spleen preparation was applied in 4 ml. to the column equilibrated with 0.02M sodium acetate at pH 7. Only 12% of the ribonuclease activity of their spleen preparation which did not undergo a heat treatment, passed through the anion exchanger. These points illustrated that a pretreatment on DEAE cellulose would be of interest, particularly the distribution of the heat treated preparation on a DEAE cellulose column under similar conditions.

A pH of 7 was chosen as suitable since little pH adjustment would be required before the preparation and application of the component reduced ribonuclease active sample to the C.M. cellulose column, Experiment 24, page 375 .

The DEAE cellulose column and spleen preparation were equilibrated with the same buffer solvent as the cation exchanger to be used at the following step (Experiment 23, page 374).

The idea was to pass the protein solution through the anion exchange column which would adsorb some contaminating proteins and

a purified sample would emerge in almost the same volume as applied. The pretreatment on a column was settled on in preference to a batch treatment. The length of column through which the sample passed should be sufficient to adsorb quantitatively certain of the anionic components at the set pH. In this way precise elimination of specific proteins by a simple fractionation would be achieved. The heat stable ribonuclease activities should have the lowest affinity of all the protein components of the mixture for the exchanger.

Experiment 23 (page 374) details the pretreatment of 1.5g of the crude spleen preparation on DEAE cellulose. Figure 24 (page 178) illustrates the distribution of the components after elution by the various solvent strengths described. 63% of the U.V. absorbing material measured as U.V. absorbing units at 280 mμ were not retained by the DEAE cellulose column. All of the ribonuclease activity applied was accounted for in the solution containing components not retained by the column. The protein components adsorbed on the DEAE cellulose were desorbed by eluting with 0.5M NaCl then 1M NaCl and on assay shown to be free of ribonuclease activity. Thus, 37% of the polymeric contaminants were removed with a complete return of ribonuclease activity and a purification of 1.8 fold. The ribonuclease active sample was then applied to the C.M. cellulose column described in Experiment 24 (page 375). The pretreatment should act as a check on the efficiency of the heat treatment of Experiment 3. According to the experimental presented by Maver et al[35] the heat labile acid ribonuclease was retained on DEAE cellulose under these

conditions of application.

This pretreatment was used regularly in the laboratory as GM 8a, page 316 after desalting on Sephadex G-75 (page 303) or dialysis to desalt and equilibrate with buffer before the enzyme samples were applied to the large C.M. cellulose scaled up column for the initial fractionation on this exchanger.

8. Possible explanations for the anomalous elution effect obtained at cation exchange chromatography

The anomalous elution effect observed in the chromatographic patterns for the ribonuclease activity peak "A", Figure 28, page 187, and illustrated by the rechromatography in Figure 30, page 190, initially confused the results with respect to the validity of the existence of two heat stable ribonucleases. The rechromatography of the ribonuclease activities at constant pH 8 Figures 29, 30 and 31 of the purer samples from Figure 28 indicated the existence of two separate ribonuclease activities. Very recent work in this laboratory[109] has demonstrated that the activity peaks "A" and "B" from Figure 28 exhibit different specificities towards the substrate R.N.A. and this indicated beyond doubt that there were two ribonuclease activities and that an elution anomaly existed.

As described earlier (page 154) the initial cation exchange column experiments were modelled on the column chromatography findings of Taborsky[90] who fractionated crystalline pancreatic ribonuclease into several components. In the present work a linear salt gradient was employed which produced an elution pattern

suggesting two ribonuclease activities were present in the spleen preparation, Figure 20. To separate these completely and remove secondary contamination from the sample, a convex salt gradient was devised, where the salt effect levelled off at that point it was considered the ribonuclease activities would be fractionating on the column. At the same time much of the unwanted protein was condensed into the initial part of the elution pattern with reduced separation in this region, while more basic components were not eluted within the span of the gradient. A complete separation of the two ribonuclease active components was apparently effected in this way Figure 28, page 187, until the rechromatography of the ribonuclease active peak "A" under almost the same elution conditions, but with constant pH control, demonstrated fractionation a second time into two ribonuclease active peaks Figure 30, page 190.

Artifacts at column chromatography and rechromatography were reported at the chromatographic examination of mouse and bovine pancreatic ribonuclease by Dickman et al[92], and with the ribonuclease extract from Thiobacillus thioparus by Walczak and Ostrowski[95] where five ribonuclease active peaks were obtained by a series of rechromatography experiments. Among many other reports of artifact separations are those presented by Bjork[163], Shapiro and Parker[149], Clayton and Bushuk[164], Bjork and Svensson[165], Bartos and Uziel[166] and Read[167].

In the present work it was not suspected that the mild treatments of a brief dialysis against the column equilibrating solution or the

slight pH adjustment from pH 6.8 to 8.0, after the preparative scale chromatography on C.M. cellulose, were responsible for the anomalous elution effect at the following rechromatography. In explanation there are several points which could contribute to the anomalous elution situation. They are in some ways related and could all be involved.

It can be suggested that there was insufficient pH control in the column environment, resulting in an artifact separation[164] or double fronting effect[88,163,168] even though the elution was continuous. As described earlier (page 176) it proved difficult, even with completely dialysed protein samples, to retain a large protein concentration on the first 5-10% of the column length at neutral pH values. By lowering the pH of the buffer used in Experiment 26 to 5.5 which was outwith the range for tris-HCl as a buffer[106] (page 183), the protein sample was retained on the scaled up column and could be safely eluted with starting buffers without desorption. Previously it was considered that the resolution into two ribonuclease active fractions was due to a partial desorption effect at the application of the sample (pages 163, 176). However, with the present circumstances, on developing the column with the gradient, the competing ion and pH effect would tend to rise quickly by the convex manner almost immediately. The tendency for desorption and exchange to take place must have been considerably increased with the result that overloading of the exchange sites took place and a multiprotein band formed having little retention

by the column.

Sudden changes of pH or ionic strength fail to subject proteins to the resolving power characteristic of elution analysis and these multiprotein bands are eluted unresolved[88]. Under these conditions an additional effect is possible. Where a local high protein concentration is produced as in a multiprotein band, a portion of the more basically charged components may readsorb. This effect may give rise to a zone of different pH and the band being less adsorptive passes unresolved through the column[88]. These effects could produce the partial elution of certain components particularly the ribonuclease active component "B" in the present work.

Carboxymethyl cellulose is a high capacity exchanger which encourages the high local protein concentration already described. Lack of pH control insitu must result in a failure to control cation exchange[164]. According to formula 2 (page 156) proteins are adsorbed mainly by electrostatic interaction or attraction between oppositely charged ionic groupings.

$$R(-COO^-)n\ nNa^+ + Pr^{\alpha+} \rightleftharpoons R(-COO^-)n\ Pr^{\alpha+} + nNa^+$$

According to Clayton and Bushuk[164] the adsorption process, which involves the displacement of sodium ions from the C.M. cellulose by positively charged basic groups on the protein, may cause a slight rise in the pH, since sodium ions are more basic than any basic protein group. In this case certain lightly held proteins would be less likely to adsorb and more likely to partially desorb.

In the absence of strict pH control the pH would have to be lowered to fully adsorb these proteins. Clayton and Bushuk[164] maintain that when proteins are displaced from C.M. cellulose, the reverse occurs. The more basic sodium ions in solution replace relatively weakly basic positively charged protein groups from the cellulose. The protein basic groups, when not adsorbed onto the cellulose, are probably not in equilibrium with the corresponding free base because the charges are balanced by nearly opposite charges on the cellulose[164]. When the protein is displaced by sodium ion, some of the basic groups of the protein would tend to hydrolyse and establish an equilibrium with the free base form. This process leads to a drop in the observed pH of the solution with the result there ought to be a shift of elution desorption to higher competing ion concentrations as the eluting effectiveness decreases, i.e. the pH charges in a direction which promotes the retention of appropriate protein molecules as they are eluted down the column. The more basic entities may be readsorbed giving an artifact separation. In the present work this possibility is supported by the pH elution pattern of Figure 28 (page 187). After the major protein peak or band "X" which appeared to exert a lower pH control effect or intrinsic buffer effect was eluted, the pH of the column environment rose by one pH unit (pH 7 → 8) before ribonuclease activity "B" was eluted.

As suggested above, the adsorption mechanism is also influenced by dissociated groups contributing to the net charge of the molecule which for steric reasons, may not all participate in the ion

exchange[164]. It should also be mentioned that non ionic adsorption affinities, e.g. hydrogen and lyophobic bondings, influence the total adsorption strength.

The effects of mutual displacement phenomena by proteins, in addition to displacement by salts can enter into the explanation[88]. Certain proteins are apparently able to displace others from the cation exchange positions[88] (page 157).

$$R\begin{pmatrix}(-COO^-)nPr^{\alpha+}\\(-COO^-)nPr^{\alpha+}\end{pmatrix} + Pr^{\beta+} \rightleftharpoons R\begin{pmatrix}(-COO^-)nPr^{\alpha+}\\(-COO^-)nPr^{\beta+}\end{pmatrix} + Pr^{\alpha+}$$

In this simplified formula a partial desorption is suggested. The protein $Pr^{\beta+}$ partially desorbs the protein $Pr^{\alpha+}$ from the exchange sites leaving some $Pr^{\alpha+}$ and $Pr^{\beta+}$ on the exchanger and a partial elution may result especially with lightly adsorbed entities.

In a concentrated protein milieu in addition to attractive forces between the stationary exchanger and the proteins the possibility exists that an association between different and identical protein molecules, between proteins and salts or other low molecular weight organic compounds, e.g. buffer ions, may be favoured[88]. The conditions for complex association may be increased in the reactive adsorbent environment as the steric limitations of the protein molecules prevents equalisation of electrostatic interaction which leaves the molecules suitably primed and charged for intermolecular association by the free bonding forces. Protein-protein association and exchange may predominate with the result that ion exchange by salt displacement takes second place or becomes of secondary importance.

In this work, rechromatography of the ribonuclease active region "A" (Figure 31, page 191) for a second time in the effective buffering range of the tris HCl buffer failed to fractionate out more ribonuclease activity "B". The distinctive protein peak "X" was always closely associated with the ribonuclease activity peak "A". In all the columns this peak moved as a brown band and dominated the pattern. This protein peak contained many protein components (page 236) which were not fractionated even at the second refractionation, Figure 31, page 191. The inherent stability of this protein band suggested irregular elution and it is not certain whether the explanations already presented are suitable interpretations of the observations made.

To alleviate the anomalous condition may require greater pH control of the column environment by an increased buffer capacity[164]. It is apparent that some attempt to increase the exchange load on the cellulose adsorption sites is required and a greater control of the pH may achieve this. A high buffering capacity would be contrary to the suggestion of Peterson and Sober[158] that a low buffering capacity is desirable in gradient elution chromatography.

It may be necessary to disperse competing intermolecular forces such that the major influence lies between the protein molecules and the exchanger, if the former constitute a major diversion to the true ionic exchange effect thereby impeding resolution or separation of individual components. The possibility of dispersing what may be more an intermolecular association rather

than a simple ion exchange arrangement with the cellulose by methods similar to those suggested by Cole[107] and Wilson and Smith[108] may be warranted[109]. In the present work, at the time the investigation of the cation exchange columns was in progress, attempts were made to reduce the load applied to these columns by a desalting and partial purification on Sephadex G-75 followed by a pretreatment on a DEAE cellulose column (pages 146 and 198 respectively). The details of the column purification sequence are described in the next subsection with a summary of the results.

9. The purification procedure for the spleen ribonuclease active fraction "B" based on the successive fractionation of the spleen ribonuclease preparation by the chromatographic procedures developed.

The column chromatography investigations carried out as already described in this section were fitted into a sequence as a purification procedure. Outlined at the end of Section II page 118 are the three key stages by which the crude spleen preparations were obtained before the samples were fractionated by column chromatography. These stages were, the extracting and salting out to concentrate the spleen protein from the spleen homogenate as described in Experiment 1, page 353 , the heat treatment of the spleen protein concentrate according to Experiment 3, page 358 and the concentration of the heat stable spleen protein according to Experiment 5, page 361.

The concentrated heat stable protein sample from Experiment 5 was fractionated successively on four different columns with the

minimum of manipulation of the enzyme sample between each application. These four column techniques were derived from the chromatographic procedures investigated in this section.

Column 1.

The concentrated heat stable spleen ribonuclease active fraction from Experiment 5, page 361 , was desalted by gel filtration on a preparative scale Sephadex G-75 column by a series of applications until the entire sample was processed according to G.M.6, page 303, and Experiment 32, page 382.

Column 11.

The effluent from the G-75 column 1 containing all the ribonuclease active protein was bulked then passed through a DEAE cellulose anion exchange column according to G.M.8a, page 316 , as a pretreatment before the ribonuclease active fraction was applied to the scaled up preparative C.M. cellulose column 111.

Column 111.

The heat stable ribonuclease activity which was not adsorbed on the DEAE cellulose, page 198 , was very briefly dialysed to equilibrate against the tris buffer then applied to the scaled up C.M. cellulose column as described in Experiment 26, page 377, G.M.8b, page 316 . All the ribonuclease activity was retained by the column and the system developed by a convex gradient elution, Figure 36, page 312.

Column 1V

The fractionation on C.M. cellulose column 111 established two ribonuclease active fractions, peaks "A" and "B" which were rechromatographed on C.M. cellulose at a different and constant pH from column 111 according to G.M.8c, page 318 . The G.M. was based on the experimental conditions described in Experiments 27, 28 and 29 where Sephadex C 50 was used. The Sephadex cation exchanger was replaced by C.M. cellulose with an equal resolving effect on the components and greater column stability (pages 180 and 192). Before samples from column 111 could be applied they were briefly dialysed as described on page 286.

Table 25 illustrates the results from the column purification scheme. The gel filtration on Sephadex G-75 achieved a little purification as described on page 150 already, but the main purpose of this step was to eliminate the extensive overnight dialysis which was responsible for a 40% loss of ribonuclease activity (page 96). To compensate for the low volume of solution applied to the Sephadex G-75 column the protein samples were increased in concentration to over 50 mg/ml by experiments. No more than about 10 ml was applied to the 5 cm. i.d. x 75 cm. column at a single application thus several successive runs were repeated on the same column to process the entire sample.

Considerable dilution of the sample was experienced. A 10 ml. sample was diluted at elution to over 300 ml. of ribonuclease active effluent, Figure 34, page 149 , and after 4-5 column runs over 1 litre

TABLE 25. Purification by column chromatography

Stage at column chromatography	Volume of sample ml.	Total R.A. Units	R.A. Units/ml.	Protein mg/ml.	S.A. Units/ mg. protein
Crude Spleen Sample Heat stable ribonuclease fraction from Exp.5.	48	11,136	232	56.6	4.1
Desalting on Sephadex G-75 Column I.	1770	10,850	6.13	1.09	5.62
Pretreatment on D.E.A.E. cellulose Column II	1840	10,928	5.92	0.6	9.87
Fractionation on scaled up C.M. cellulose column III ribonuclease activity "B".	780	8,034	10.3	0.073	141
Refractionation on C.M. cellulose at pH 8 Column IV. Ribonuclease activity "B".	210	7,476	35.6	0.06	593

R.A. = Ribonuclease Activity
S.A. = Specific Activity

of bulked effluent was applied to the following ion exchange columns.

An unfortunate aspect of this column sequence was that a desalting or reduction in the ionic strength of the sample between certain columns was required, e.g. the ribonuclease activity fraction "B" from the scaled up column 111 with C.M. cellulose contained 0.28 M NaCl. Before this sample could be applied and adsorbed to column 1V, the second C.M. cellulose column at pH 8 for refractionation, it was necessary to reduce the NaCl concentration. A very brief dialysis was reintroduced for this purpose. The volume of protein solution was dialysed for $1\frac{1}{2}$ hours against an equal volume of the equilibrating buffer also used to equilibrate the column. The diffusate solution was rejected x 3 within 6 hours. Alternatives to dialysis were extensive dilution sixfold to lower the ionic strength before application, or freeze-drying to concentrate the large volume and desalt again on Sephadex. Neither of these alternatives were favoured as the samples were already large and freeze drying was a dubious step (pages 139 and 265).

A very brief dialysis was undertaken after the solution was adjusted to the pH of the equilibrating buffers before application to columns 111 and 1V. A further brief dialysis was required on those occasions the sample obtained from the final column 1V was concentrated by G.M. 8d. (page 319). No attempt was made to estimate by assay the amount of ribonuclease activity lost at each brief dialysis. From the return of ribonuclease activity after each

column the amount of activity lost by the overall dialysis and column process did not exceed 10% on each occasion.

The results in Table 24 represent the column purification procedures carried through to completion. Analysis by disc electrophoresis on polyacrylamide gels (page 226) showed that the ribonuclease sample "B" contained only one protein component in major proportions suggesting a considerable degree of purity. The refractionation of ribonuclease sample "A" on the other hand did not purify the fraction sufficiently and four major components with other minor contaminants were shown to be present, (page 236). The ribonuclease fraction "A" was not purified further in the present work. Since the ribonuclease active fraction "B" was more basic (page 194) in its elution properties and was obtained in a reasonably pure state, estimates of purity were based on the purest sample obtained, i.e. rechromatographed samples of ribonuclease activity "B", Figure 29.

The reasons for including the pretreatment on DEAE cellulose has already been detailed on page 198.

The original paper by Kaplan and Heppel[8] demonstrated that a purification of 420 fold overall from the homogenate was obtained (page 30). For purposes of comparison it is more reliable to compare the effectiveness of purification procedures using the heat treated product as basic material (see page 117). From this point onwards Kaplan and Heppel obtained a purification of 29 fold overall. This contrasts with the 190 and 170 fold overall purification

obtained by column chromatography in the present work as represented in Tables 24 and 25 respectively. A purification of this order represents a 3000 fold purification based on a nominal value of 0.2 for the specific activity of the heat treated homogenate (page 116).

The purification scheme adopted used very mild fractionation techniques and ribonuclease activity was discarded at the brief dialysis, or rejected when bulking fraction samples after column chromatography. At each fractionation 90% or more of the ribonuclease activity was retained. Compared to the predefined fractionation by the conventional precipitation techniques of salting out etc. (Section II) which had often an unpredictable outcome entire and specific fractions were selected from each column elution pattern after analysis. However, compared to the concentrating effect at each precipitation step by the conventional techniques, successive column applications tended to increase the sample volume at each elution, e.g. the volume increased 30 fold at desalting on Sephadex G-75 and at the next stage, the pretreatment on DEAE cellulose, the volume increased further. Although some concentration took place at the cation exchange adsorption columns III and IV the volume and dilution was considerable, Table 25.

The ribonuclease activity "A" amounted to between 16-20% of the heat stable activity applied to the columns. It must be borne in mind, however, that both ribonuclease activity peaks "A" and "B" were assayed at pH 6.5 only (G.M.14). A true assessment of the relative

proportions cannot be made until assays and total ribonuclease activity measurements are carried out at the optimal requirements of both enzymes. This was not attempted in the present work.

10. A note on the heat stability of the spleen ribonuclease active fraction "A".

The ribonuclease active fraction "A", Figure 31, page 191 amounted to between 16-20% of the total heat stable ribonuclease, Table 25, page 211 , according to the assay conditions adopted, G.M.14, page 341 . It was considered that this low amount might have been the result of an inadequate heat treatment at 60° for 10 minutes at pH 3.5, Experiment 3, page 358 . The possibility existed that this fraction was heat labile ribonuclease which had escaped the heat denaturation effect. It was demonstrated that the ribonuclease active fraction "A" was heat stable. To check this possibility 420 units (50 ml.) from the sample of the spleen ribonuclease active fraction "A_1" (Figure 31) were heat treated for a second time by the procedure outlined in Experiment 3, page 358. A slightly higher temperature was used. The temperature of the 50 ml. sample was raised to 70° by immersing it in a water bath at 85°, then by using a second water bath maintained at 70°, the sample was held at this temperature for 10 minutes at pH 3.5. According to the assay technique, G.M.14, page 341 , for total ribonuclease activity, all of the ribonuclease active sample resisted heat denaturation.

11. Possible explanations for the location of only one heat stable spleen ribonuclease activity by previous purification procedures.

As already described, page 18, according to the findings expressed in the published reports[8,35] there could be two different heat stable ribonuclease active entities in calf spleen. A survey of these reports suggested that on each occasion one of the heat stable ribonuclease activities was ignored. At the first three steps of the work carried out by Kaplan and Heppel[8], page 26 , the existence of two heat stable ribonuclease activities must have been obscured by the much greater quantity of heat labile "acid ribonuclease" and spleen phosphodiesterase extracted. Of the total ribonuclease activity present in the homogenate 17% survived the heat treatment.

In the present work, the crude estimate of each heat stable ribonuclease activity fractionated in a ratio 1:6 ribonuclease active fraction "A" to ribonuclease active fraction "B" would suggest that the lesser component could have been progressively eliminated by the lengthy series of five conventional fractionation steps undertaken by Kaplan and Heppel[8]. Had both ribonucleases survived this successive partition then the column chromatography on Amberlite IRC 50 (X.E.64) used by these workers, page 30 , was not refined enough to reveal the presence of closely fractionating ribonuclease activities. On the Amberlite column a very minor ribonuclease active peak was eluted as a preliminary to the elution of the major ribonuclease active active peak which had a ribonuclease specificity

similar to pancreatic ribonuclease. No mention was made of the minor ribonuclease active peak in the report[8].

Maver and Greco[35] failed to detect the spleen ribonuclease purified by Kaplan and Heppel[8]. It is possible that this low molecular weight ribonuclease was discarded by the extensive dialysis these workers used at an early purification stage. These workers[35] in their purification of the heat stable "alkaline" ribonuclease of calf spleen on C.M. cellulose, halted the progress of their concave salt gradient before the ribonuclease activity was eluted. The preparation of the ribonuclease sample prior to chromatography avoided heat treatment and when the fraction containing the "alkaline ribonuclease" which was not retained by DEAE cellulose (page 72) was applied to the C.M. cellulose column, acid phosphotase and non specific phosphodiesterase contaminants were not retained[35]. Maver and Greco[35] stated that after tests for a cyclic hydrolysing enzyme, (i.e. a phosphodiesterase without ribonuclease activity), and deoxyribonuclease in the effluent of the concave gradient were negative, the concave gradient was discontinued and 1M NaCl used to desorb the "alkaline ribonuclease" by a stepwise elution effect. No attempt was made to fractionate further the heat stable "alkaline ribonuclease" by continuing the gradient elution.

Specificity trials by these workers demonstrated that the "alkaline ribonuclease" fraction was non-specific. All four mononucleotides were released from R.N.A., thus the presence of the more specific ribonucleate pyrimidine nucleotido 2' transferase

(cyclising) spleen ribonuclease purified by Kaplan and Heppel[8] could have been present but obscured. The report by Maver and Greco[35] that pyrimidine to purine mononucleotide release by the action of the "alkaline ribonuclease" was in a ratio of 3.1, was quite significant. The low purine release would signify a greater cleavage of bonds in favour of pyrimidine mononucleotide release and the presence of two ribonuclease active enzymes might be indicated as an alternative to the "alkaline ribonuclease" having a rate specificity for pyrimidine internucleotide cleavage.

The "alkaline ribonuclease" of Maver and Greco[35] and the spleen ribonuclease of Kaplan and Heppel[8] are the only two ribonucleases described as heat stable in calf spleen tissue. In these two reports the presence of two heat stable ribonuclease components was not demonstrated in either spleen preparation, whether heat treatments were included or not. Substantial evidence in the present investigation confirmed that two separate heat stable ribonuclease active components were present as suspected (page 22). In addition to the specificity determinations carried out in this laboratory[109] two vital results demonstrated the existence of two active components. They are:

(a) the fractionation of the heat stable ribonuclease activity on cation exchangers into two active peaks "A" and "B" as described in this section and presented in Figure 28, 29, 30 and 31, and

(b) the molecular size estimations described in Section V where ribonuclease activity "A" (M.W. 24,000) was shown to be much larger than ribonuclease activity "B" (M.W. 10,000).

SECTION IV

Examination of the spleen ribonuclease active fractions obtained by chromatography on the cation exchangers C.M. cellulose and C.M. Sephadex for heterogeniety

The main findings detailed in this section are summarised in subsection 4, page 252 , where a broad picture of the results is presented. The extent of purification of the ribonuclease active fractions obtained by cation exchange chromatography on C.M. cellulose and C.M. Sephadex was examined by disc electrophoresis on polyacrylamide gels. The figures which present the electrophoretograms of importance are as follows:-

Figure 42, page 237 , displays the extent of purification of the ribonuclease active peak "A" (elution pattern Figure 31, page 191)

Figures 44, 46 and 47, pages 242, 244 and 245 respectively illustrate the high purification achieved with ribonuclease active fraction "B" (elution pattern Figure 29, page 190)

Figure 49, page 251 , displays the extent of purification of the ribonuclease active fraction "B_1" (elution pattern Figure 30, page 190). Evidence is presented which suggests ribonuclease activity "B" and "B_1" are the same. Reference to the text will illustrate the subsidiary importance of the other figures.

1. Introduction

Tests for the purity of protein preparations have depended largely on physical techniques. These techniques include electrophoresis, ultra-centrifugation and solubility studies.[49,198] Chemical techniques are N and C terminal amino acid determinations and the evidence of constancy of biological properties which can be assayed and estimated. All these methods demonstrate lack of heterogeniety in the test sample. Starch gel electrophoresis[115] has been added to this list recently and has been supplemented by the more exacting technique of disc electrophoresis on polyacrylamide gels[111,112,121].

As an estimate of purity in enzyme studies constancy of biological property leaves much to be desired. Biological activity may be constant only because the limits of the purification systems available, including crystallisation, have been exhausted without an improvement to the purification[138]. Solubility studies require large amounts of material which may not become available from a source of meagre content.

The most recent addition to the techniques available for determining the extent of heterogeniety of protein samples is disc electrophoresis[111]. The technique in addition to other favourable qualities offers high sensitivity in addition to high resolving power. The compactness of each resolved component makes it possible to detect any trace components that would not be detected either at conventional electrophoresis where the components were well separated

or by the other methods mentioned above. With disc electrophoresis a combination of properties, e.g. shape, size and charge, are exploited and influence the protein separation. These properties can be put to use under many alternative conditions and the process is in effect a molecular sieve electrophoresis where the degree of resolution is much higher than that found by any previous method[111].

Disc electrophoresis in small gel cylinders 0.4 cm. x 7 cm. was introduced by Ornstein and Davis[111] for the examination of serum proteins. The technique was adapted by Reisfeld et al[112] for the electrophoresis of neutral and basic proteins. The method of Reisfeld et al was adopted here for the estimation of purity of the spleen ribonuclease active fractions prepared by the chromatographic techniques.

2. Disc electrophoresis as a technique for determining the degree of heterogeniety of protein samples

Electrophoretic mobility in free solution under fixed conditions of pH and buffer type is principally a function of the net electrostatic charge density on each protein molecule. In free solution the size and shape of the molecule affect resolution to a much lesser degree and resolution is limited by not making use of these properties. Many molecules of different shape composition and size may have similar electrophoretic mobility. By contrast, in disc electrophoresis the pore size of the polyacrylamide gel which acts as the electrophoretic solid phase can be controlled. When the pore size is sufficiently small a much greater degree of resistance to the

movement of larger molecules than small ones is manifest[111]. This is in direct contrast to the molecular sieve effect with Sephadex gel which allows large molecules to pass. Large molecules of high electrophoretic mobility may fall far behind smaller molecules of lesser electrophoretic mobility if the pore size has been designed to discriminate strongly between them on the basis of molecular size. Small highly charged molecules should move well in advance of the molecules with larger dimensions and lower electrophoretic mobility, but this depends on the pore size of the gel medium. The system is complex. In disc electrophoresis the system is made discontinuous with respect to pH, buffer species, acrylamide concentration and gel porosity[111]. The conditions are described on pages 320 - 336.

In contrast to starch gel electrophoresis[113,115] which also sieves at the molecular level, polyacrylamide gels are thermostable transparent, tensile, relatively chemically inert, non ionic and can be prepared in a large range of average pore size. The average pore size depends on the concentration of polymer[111] and if complete polymerisation takes place, is directly related to the concentration of acrylamide monomer. The pores size can be adjusted to suit the dimensions of the molecules to be separated. 7.5% acrylamide polymerised gives an average pore size of 50A° suitable for fairly large proteins of 60,000 and 80,000 molecular weight, while 30% acrylamide polymerised gives an average pore size of 20A°, suitable for proteins of a much lower molecular weight[111]. The small pore

gel, Figure 51, page 322, is formed in a glass tube by polymerising an aqueous solution of between 7.5% to 30% acrylamide, depending on the proteins to be separated, and stationary buffer components appropriate for the intended electrophoresis. Above this the large pore spacer gel has a 2.5% acrylamide content. No molecular sieving takes place in this gel which prevents convection in the initial stages of electrophoresis. The protein sample is added in another portion of 2.5% acrylamide which is polymerised above the spacer gel. The position of the electrode buffers are illustrated in Figure 52, page 323 . The electrode buffer is different in composition and pH from the spacer buffer and small pore buffer and it establishes the conditions under which electrophoresis of the proteins will occur in the small pore gel.

The very important feature of zone sharpening occurs in the spacer gel due to the Kohlrausch effect[111]. This phenomenon occurs if a mixture of ions of high and low mobility are subjected to electrophoresis. The faster ions move ahead while the slower ions follow immediately to form a very sharp boundary. If a protein solution is introduced into such a system under the most suitable conditions they will be concentrated at this boundary into very thin layers in order of decreasing mobilities, with the K^+ ion boundary at the front and the β alanine ion boundary taking up the rear[116]. The initially dilute sample then enters the molecular sieving gel as a very thin layer. Thus in the spacer gel separation and concentration of components to a starting zone of as little as

10 microns thick from as much as 1 cm. can be achieved[111]. No other analytical technique exhibits this concentrating effect. By contrast resolution on paper chromatography, starch gel electrophoresis etc. is limited by the volume of the sample when first applied. Without taking into account volume increase by diffusion, the system requires that the chromatographic run must be of sufficient duration to allow the components to resolve at least one length of the volume originally occupied farther than the other for a satisfactory separation. The combination of a very thin starting zone, adequate pore size control and suitable voltage gradient achieves a very high resolution of even the trace components on polyacrylamide gels in a short running time.

On entering the small pore gel the proteins are already stacked in order of electrophoretic mobility and the buffer ions electrophorete ahead when the mobility of the protein components is decreased by the fractional properties of the gel. The electrophoresis continues at a relatively constant pH which is slightly higher than the original pH of the small pore gel, and effected by a linear voltage gradient[111]. The protein components separate further in accordance with their electrophoretic mobility at this pH and their molecular size as reflected by their frictional interaction with the polyacrylamide gel. After a suitable electrophoresis time the gels are displaced and the protein bands located and fixed by establishing a protein-dye complex within the gel. Excess dye is removed by electrophoresis and an electrophoretic pattern 3-5 cm. in length reveals the proteins as a

number of very narrow bands.

This very sophisticated and highly sensitive technique was introduced to examine the considerably purified fractions of ribonuclease activity obtained at column chromatography. The system was adopted as it could be inexpensively introduced as a method for detecting heterogeniety more distinctly and much quicker than the expensive equipment required by ultracentrifugation or lengthy procedures for N. and C. terminal amino acid investigations which were not established in this laboratory. Eight electrophoretic patterns can be conveniently obtained in a period of 30 minutes for a single run. The transparent resilient gels offered conditions to compare the migration of proteins under set conditions against a standard protein of known molecular dimension for comparative studies. The technique was chosen in preference to the less sensitive and standard pore starch gel electrophoresis[113,123] for the many reasons already outlined, but mainly because of the higher resolution instituted by the initial concentrating of sample and the fact that the polyacrylamide gels can be prepared in a wide range of concentrations.

The system can adequately cope with as little as 50 µg. of protein and over a range of protein concentration of 50-200 µg.[111]. This feature has considerable advantage over all systems which require milligram or larger quantities of scarce or difficulty acquired material before testing can commence. A superior asset is that the system can be varied on two highly discriminating properties. The

protein molecules can be altered with respect to charge properties by adjusting the conditions of pH while the pore size of the electrophoretic medium can be varied to discriminate against them on the basis of shape and size. Quite recently a simple relationship relating molecular weight to gel concentration and mobility has been found by Tombs[113] which demonstrates the importance of the molecular sieve action in relation to size and charge composition of the electrophoreted molecule.

3. Disc electrophoresis of spleen ribonuclease samples

The methods for setting up and operating the disc electrophoresis analysis technique are set out in the procedures under G.M.9, page 320 and G.M.10, page 332 . Experimental conditions are briefly outlined on page 382 as Experiment 33. Photographic evidence of the electrophoretograms from disc electrophoresis analysis of important samples is presented. Many of the photographs have not reproduced the resolution of protein components as clearly as the gels depict them visually. This was considered due to shadow effects, especially from very dark areas on the gel at the film exposure. In each figure an arrow represents the direction of electrophoresis and the components with greatest mobility are located towards the "lower" end of the gels.

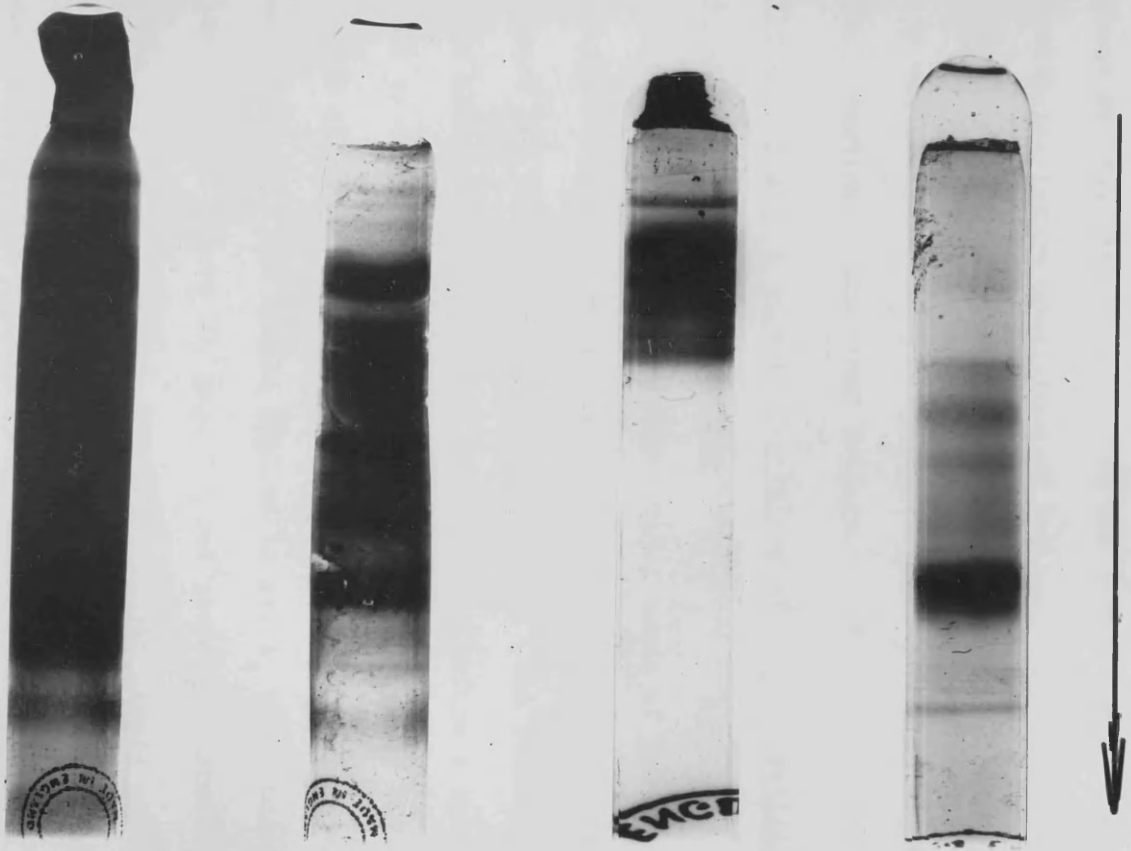
It was found that quite frequently comparisons between gels could not be satisfactorily made, especially when very many protein bands appeared on the gel. The extent to which components were

removed from a sample could be assessed by counting the number of bands on each gel, but to state categorically that a particular component, or which of several bands had been eliminated, was frequently impossible. On occasions reproducibility of pattern was not satisfactorily achieved. It was considered that this was a reflection of the conditions within the starting material. Although proteins samples were mixed with an equal volume of solution A at pH 6.8 (page 327) before photopolymerisation, the conditions of salt concentration and pH within the samples could be different and affect the electrophoretogram accordingly. The amount of protein applied sometimes had to be adjusted until a satisfactory level was obtained. Solutions could be diluted when the concentration was high but if over dilute more solution had to be applied direct to the gels. The volume of sample applied would have to be restricted and some measures taken to standardise the protein solution if absolute reproducibility is required.

(a) The spleen preparation before column chromatography

The highly discriminating conditions at disc electrophoresis has demonstrated the very many components in the enzyme preparation prior to column chromatography. Figure 39, page 228 depicts gels A_1 and A_2 which partially illustrate the very impure nature of the crude heat stable ribonuclease active sample applied both to the investigation columns Experiments 21 and 22, page 372 and to the scaled up C.M. cellulose column described in Experiment 26, page 377 and reported on page 184 . Gel A_2 was a duplicate gel of A_1 and was

FIGURE 39.



A1. A2. A3. A4.

sliced longitudinally after staining to reveal the distribution of components in the overloaded central region. In all 15 major protein bands were detected and this might not be the full complement as less conspicuous bands could be masked. These gels demonstrate the impure conditions of the samples prior to chromatography, page 118, and illustrate that even if the ribonuclease activity was one or more of these major bands it would represent a very small portion of the total protein.

(b) The purity of the spleen preparation after the preliminary cation exchange chromatography

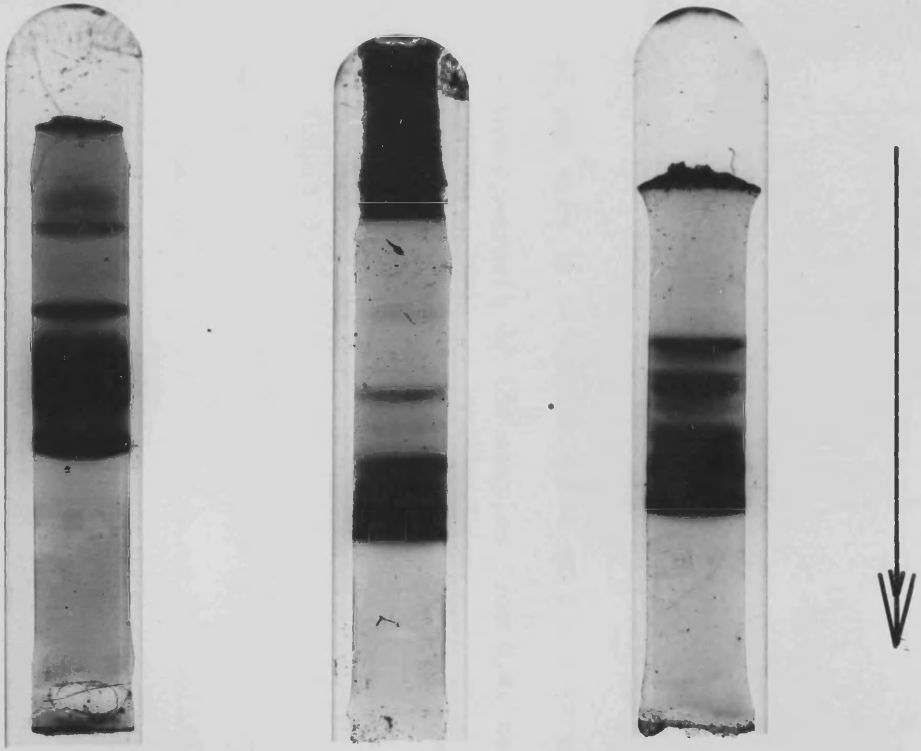
Gel A_3 of Figure 39 shows eight major bands of protein. The sample applied to this gel was the ribonuclease activity "A" peak from Figure 20, page 166 , and Experiment 21. The peak was bulked after the elution, dialysed then freeze-dried before a sample was submitted to disc electrophoresis. Gel A_4 of the same figure also illustrates eight bands, two of which are major and migrated the greatest distance. The sample applied to gel A_4 was ribonuclease activity "B" from Figure 20, page 116, and pretreated similarly to the sample applied to gel A_3. The crude spleen sample applied to the C.M. cellulose investigation column of Experiment 21 was from the same stock as that applied to the scaled up column of Experiment 26. Clearly the effect of adsorbtion on the cation exchange column was to eliminate more than half of the components in the sample applied (page 163). The results of two gels each displaying eight protein components all retained on the column,

suggested the resolution of the ribonuclease activity peaks "A" and "B" Figure 20 was not sufficient in Figure 40.

Figure 40 presents gels C_1, C_2, C_3. The protein samples applied to these gels were from individual fractions of a C.M. cellulose column elution as depicted in Figure 23, page 174 . The column was a subsidiary investigation of Experiment 22 described on page 172 where two ribonuclease activity peaks were eluted very close together on a second convex gradient. The crude spleen sample applied to this column was from the same bulk sample electrophoreted in A_1, A_2 gels, Figure 39. The number of protein bands located in each gel of Figure 40 is presented in Table 26.

These protein samples were similar to those applied to the gels A_3 and A_4 and demonstrated that very many protein components had been adsorbed on the cellulose column. After the first gradient eluted the column, elution pattern Figure 23, page 174 , a stepwise elution condition existed and many components were common to all three gels as the ribonuclease activity eluted almost unresolved. Nevertheless 11 protein components at a minimum were present. Prior to the elution of the ribonuclease active peaks the elution pattern shows 10 protein peaks were resolved on the column by the first gradient. This evidence suggested that in all 21 components at a minimum were adsorbed by the column. Many more components were eluted in the bulk of the protein sample not adsorbed. From this analysis it was evident gels A_1, A_2 of Figure 39 exhibit only the very major protein components of the crude mixture prior to chromatography and the

FIGURE 40



ribonuclease components were a very minor amount of the total.

TABLE 26

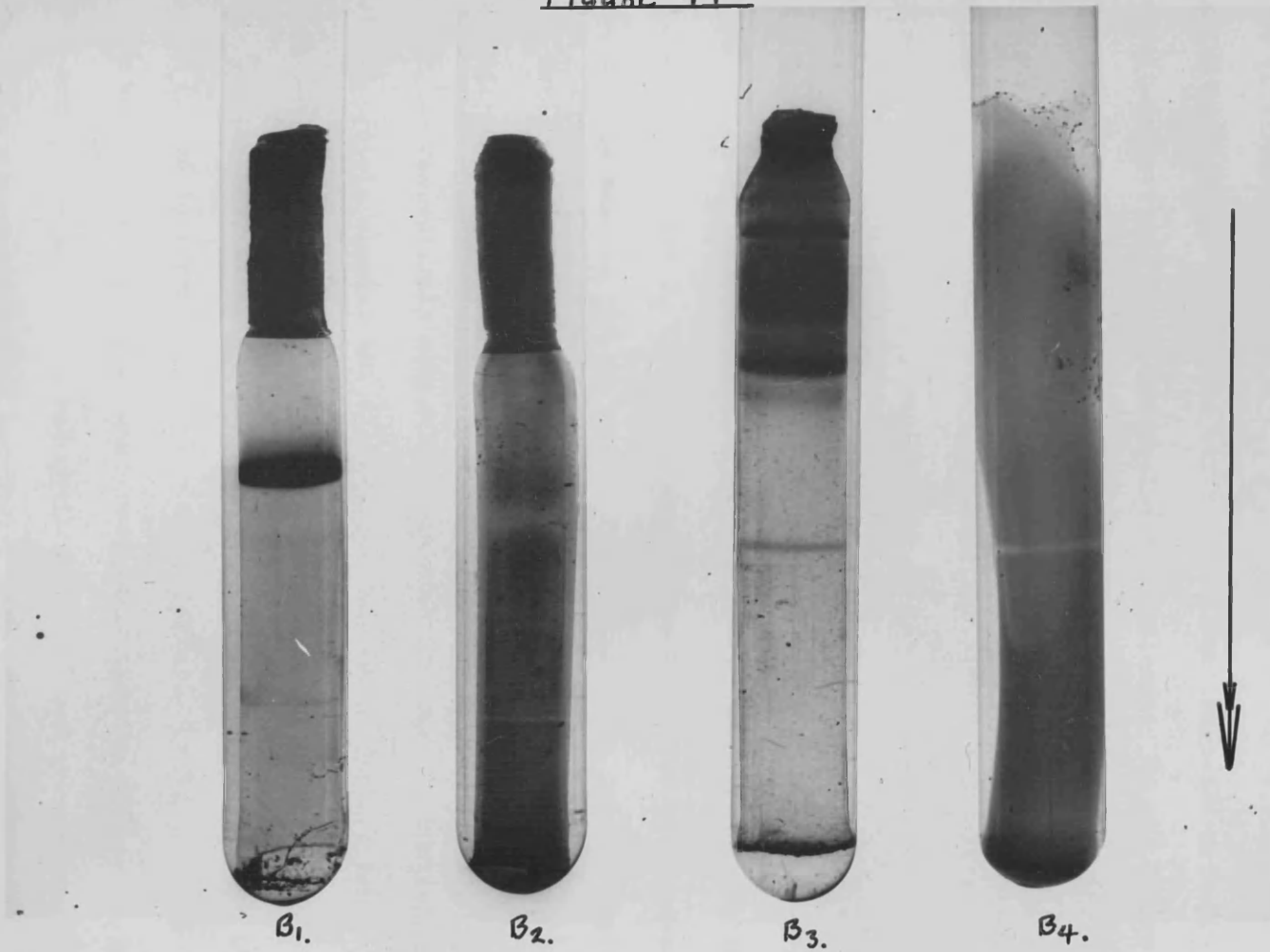
Gel	Bands determined usually as:			Total No. of Bands
	Major	Minor	Trace Amounts	
C_1	5	1	3	9
C_2	4	2	5	11
C_3	7	1	3	11

(c) An attempt to detect ribonuclease activity on gel electrophoretograms

An attempt was made to determine which of the very many bands present after electrophoresis of the samples from C.M. cellulose columns was responsible for ribonuclease activity. The method adopted is described in Experiment 33 in detail and was successful when the electrophoreted sample was pancreatic ribonuclease. Spleen ribonuclease could not be detected. After gel electrophoresis polyacrylamide gels containing enzyme samples were incubated in an RNA solution. After a period of digestion the gels were washed to remove superficial RNA then placed in MacFadyen's reagent[114] to precipitate RNA which had soaked into the gel. It was expected that sections of the gel, where ribonuclease activity was located would be transparent as the enzyme depolymerised the RNA.

A sample of pancreatic ribonuclease was disc electrophoreted on gels B_1, B_2 Figure 41 (page 233). Gel B_1 was stained in the usual manner to locate the protein components. The gel B_2 was digested as

FIGURE 41



described in Experiment 33 (page 382) and outlined above. Similarly a sample of spleen ribonuclease "B" activity as applied in A_4 gel was disc electrophoreted in gels B_3, B_4. The gel B_3 was stained to locate the protein components while gel B_4 was incubated with RNA as described previously with pancreatic ribonuclease. On gel B_2 two transparent bands are visible. The broad diffuse band near the upper half of the gel was sufficiently well positioned to suggest the pancreatic ribonuclease band, presented for direct contrast in the adjacent duplicate gel B_1, was located at the transparent section of gel B_2. A similar transparent section was not found in gel B_4. A much more well defined band of unprecipitated RNA was evident at the lower region of both gels B_2 and B_4. By direct comparison with the stained gels B_1 and B_3 this transparent line was found to correspond with a false band on the protein stained gels. This false protein band was found later on blank gels where no protein samples were applied. Gels D_1, E_1, F_1, G_1, H_1, I_3, K_2 and Figures 42, 43, 44, 45, 46, 47, 49 respectively are gels which were included as controls without protein samples and all exhibit this false protein band (page 330).

The presence of this false band in the spleen ribonuclease gels B_3 and B_4 led to erroneous conclusions at first. It was thought that a very small basic component was electrophoreting in advance of the other components and it was not until the blank and gels with pancreatic ribonuclease were introduced that the situation was rectified. The situation of a false enzyme action in B_4 gel was

finally clinched when a blank gel after electrophoresis was incubated with the RNA solution in a similar manner to the gels containing the enzymes. The blank gel was shown to exhibit a well defined transparent zone exactly similar to that on gel B_4. This effect was attributed to the electrophoresis front which was a boundary of cationic material in high concentration. The RNA molecules were either excluded from this region on diffusion into the gel or not precipitated by MacFadyen's reagent and remained in solution because of a high buffering effect.

No trace of ribonuclease activity in spleen samples could be demonstrated on polyacrylamide gels under the conditions used in Experiment 33, page 384, where the incubation time was raised from 4 hours to 16 hours and the concentration of RNA in the substrate solution increased from 6 mg. per ml. to 12 mg. per ml. as alternative conditions.

At this time an attempt was made to diffuse the proteins from the gel. Each gel was cut transversely into 2mm cylindrical sections and each section immersed in 1ml. of 0.1M succinic acid/sodium succinate buffer and 0.05 M.$MgCl_2$, pH 6.5 for 6 hours to allow time for the protein components to diffuse from the relevant sections. The buffer solutions in each case were assayed for ribonuclease activity (G.M.14). Where spleen ribonuclease samples were subjected to disc electrophoresis and the gels cross sectioned, ribonuclease activity was not detected by the assay of the 1 ml. buffer solutions. On the other hand the gel on which pancreatic ribonuclease was

electrophoreted then cross sectioned into sections yielded ribonuclease activity from certain of the sections. This region corresponded to that occupied by genuine pancreatic ribonuclease in a control duplicate gel which was stained.

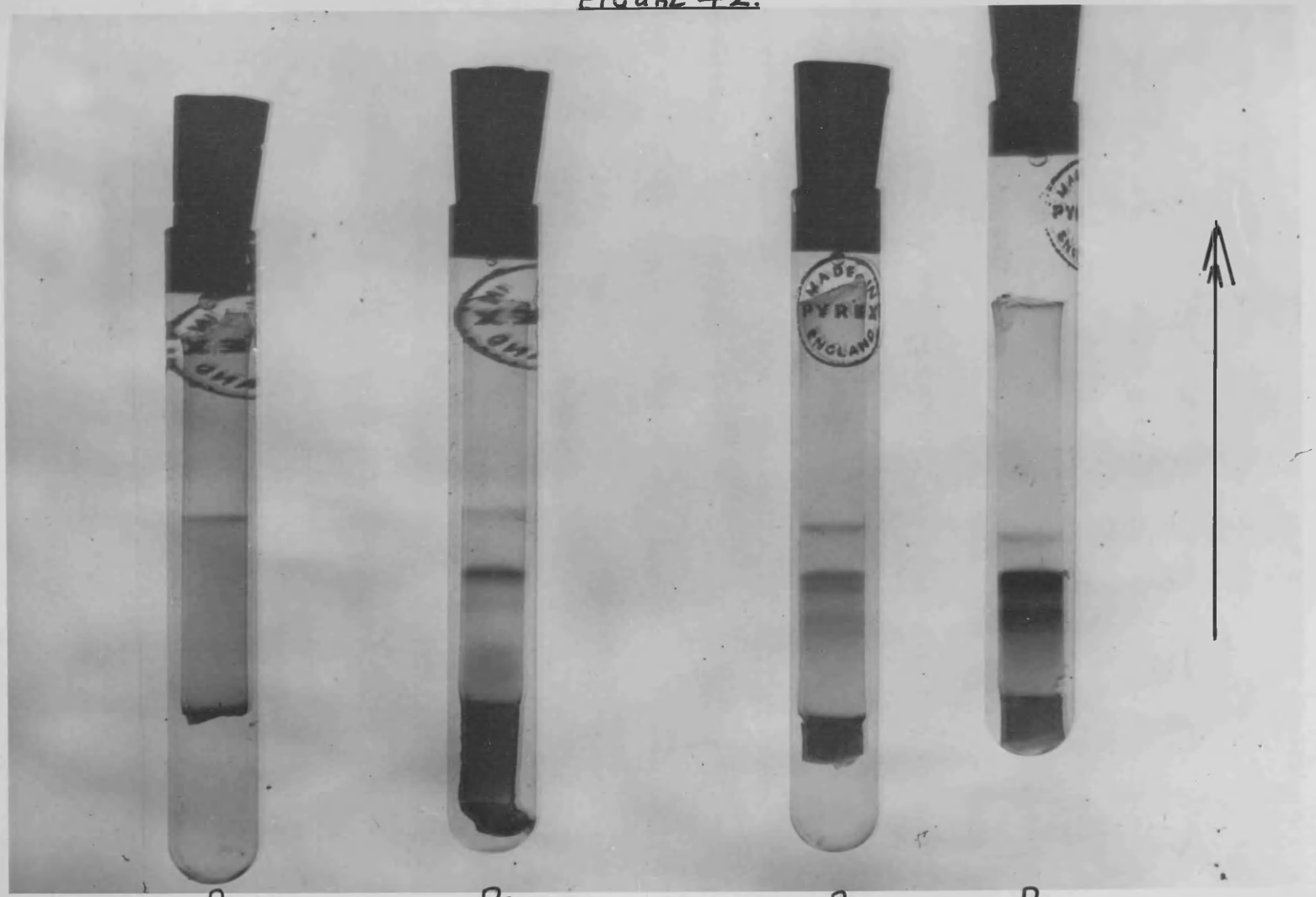
(d) Disc electrophoresis of the ribonuclease active fractions from the rechromatography columns represented by the elution patterns on pages 187, 190 and 191.

The gels presented in Figure 42, page 237, demonstrate the considerable purification achieved by chromatography on the scaled up cation exchange column of Experiment 26 followed by the rechromatography at constant pH 8 of the ribonuclease fractions "A" and "B" obtained.

The gel D_1 was a blank gel electrophoreted with the other samples presented in this figure and contained no protein. A single stained band was evident and believed to be the electrophoretic front as already outlined, page 234. This band appeared in all four gels. The gel D_2 contained a sample from the rechromatographed ribonuclease activity peak "B" of Experiment 27 as depicted in Figure 29, page 190. The gel showed one major protein component present. The presence of a trace component of lower motility might be suggested by the diffused stained region above the distinct protein band which was thought to be the ribonuclease activity "B" component. Further evidence is presented to demonstrate that this component has been highly purified, Figure 47, page 245.

The gel D_3 contained a sample from the rechromatographed ribonuclease activity peak "A_1" from Experiment 29 as depicted by the

FIGURE 42.



D_1. D_2. D_3 D_4

elution pattern in Figure 31, page 191 . Four major protein components were shown to be present in the sample. The gel D_4 was similar to gel D_3 and contained twice as much sample. Four protein components were again present which demonstrated a lack of trace components which could have been missed in gel D_3. It was very tempting to suggest that the major protein band in gel D_2 corresponded favourably with the third fastest component in gel D_3 on measuring the distance migrated by each component. Both have migrated exactly the same distance. No attempt was made to elucidate which component might be ribonuclease activity "A_1". To be consistent with the basic idea expressed on page 21 further purification is required first. Attention was concentrated on the ribonuclease active fraction "B" since it was now in a considerably pure state and had exhibited the heat stable basic properties ascribed to the spleen ribonuclease purified by Kaplan and Heppel[8].

(e) <u>The comparative study of the spleen ribonuclease active fraction "B", with pancreatic ribonuclease as a standard by disc electrophoresis, coupled with an attempt to exhibit heterogeniety by decreasing the pore size of the polyacrylamide gel.</u>

An attempt to find secondary components in the ribonuclease activity "B" fraction, Figure 29, page 190 , was made by preparing gels with average pore sizes ranging from 50Å to 20Å as electrophoretic medium for this sample. This was accomplished by varying the concentration of acrylamide and cross linking agent in each solution required to produce the different small pore gels, Table 11, page 328 . In

addition pancreatic ribonuclease was submitted to disc electrophoresis on duplicate gels as a standard for comparative purposes mainly because of its low molecular weight and alleged similarity to the spleen ribonuclease described by Kaplan and Heppel[8]. For each pore size used the four gels in each series were submitted to electrophoresis together and were directly comparable. Blank gels, represented by the subscript 1 which were submitted to electrophoresis without a protein sample, acted as a control in each case. The falsely stained band was located and found to be present in the gels containing the protein samples also. Gels represented by the subscript 2 contained pancreatic ribonuclease, gels represented by the subscript 3 contained a mixture of pancreatic ribonuclease and spleen ribonuclease "B" and gels represented by the subscript 4 contained only the spleen ribonuclease activity "B". Table 27 summarises some of the details of the groups of gels presented in the figures listed.

TABLE 27

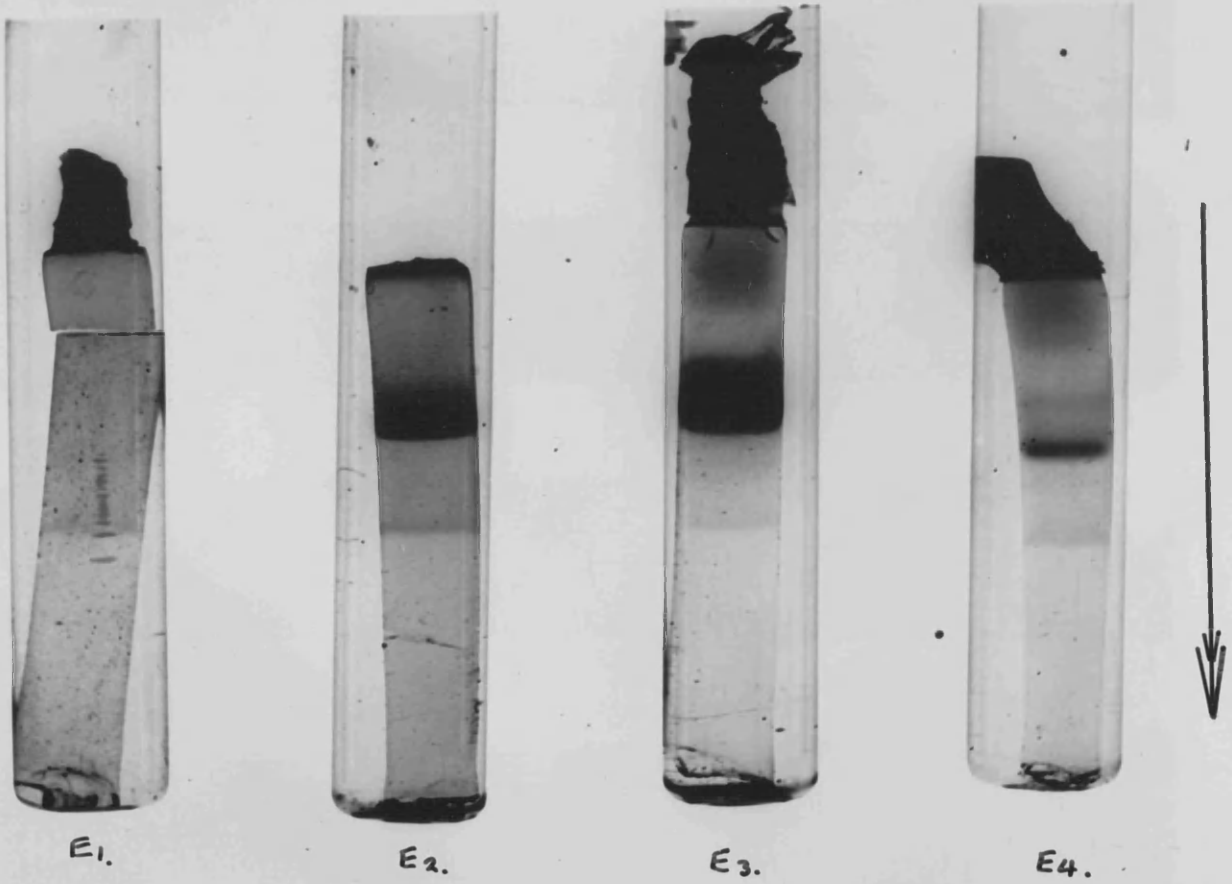
Fig.	Page	% Acrylamide	Average Pore size	PROTEIN SAMPLE			
				Blank	Pancreatic ribonuclease	Pancreatic + spleen R.A. "B"	Spleen R.A."B"
43	241	7.5	+50Å-*60Å	E_1	E_2	E_3	E_4
44	242	15	- *40Å	F_1	F_2	F_3	F_4
45	243	22.5	- *30Å	G_1	G_2	G_3	G_4
46	244	30	+20Å	H_1	H_2	H_3	H_4

+ = Ref. 111
* = Ref. 113
R.A. = Ribonuclease Activity

According to the evidence from the electrophoretic patterns of these gels the major protein component, considered to be ribonuclease activity "B" and pancreatic ribonuclease electrophoreted practically together at each pore size used. It could be observed that the major spleen protein band present in all the gels subscripted with the number 4 was electrophoreted not more than 1 m.m. further than the pancreatic ribonuclease in the gels subscripted 2. This was confirmed in those gels where the mixture of pancreatic and spleen ribonuclease was submitted to electrophoresis. The spleen band on staining appeared blue while the pancreatic band was black, thus in the gels subscripted with the number 3 the black band on top of the blue band could be distinguished. If anything the spleen ribonuclease sample "B" had slightly greater mobility than pancreatic ribonuclease.

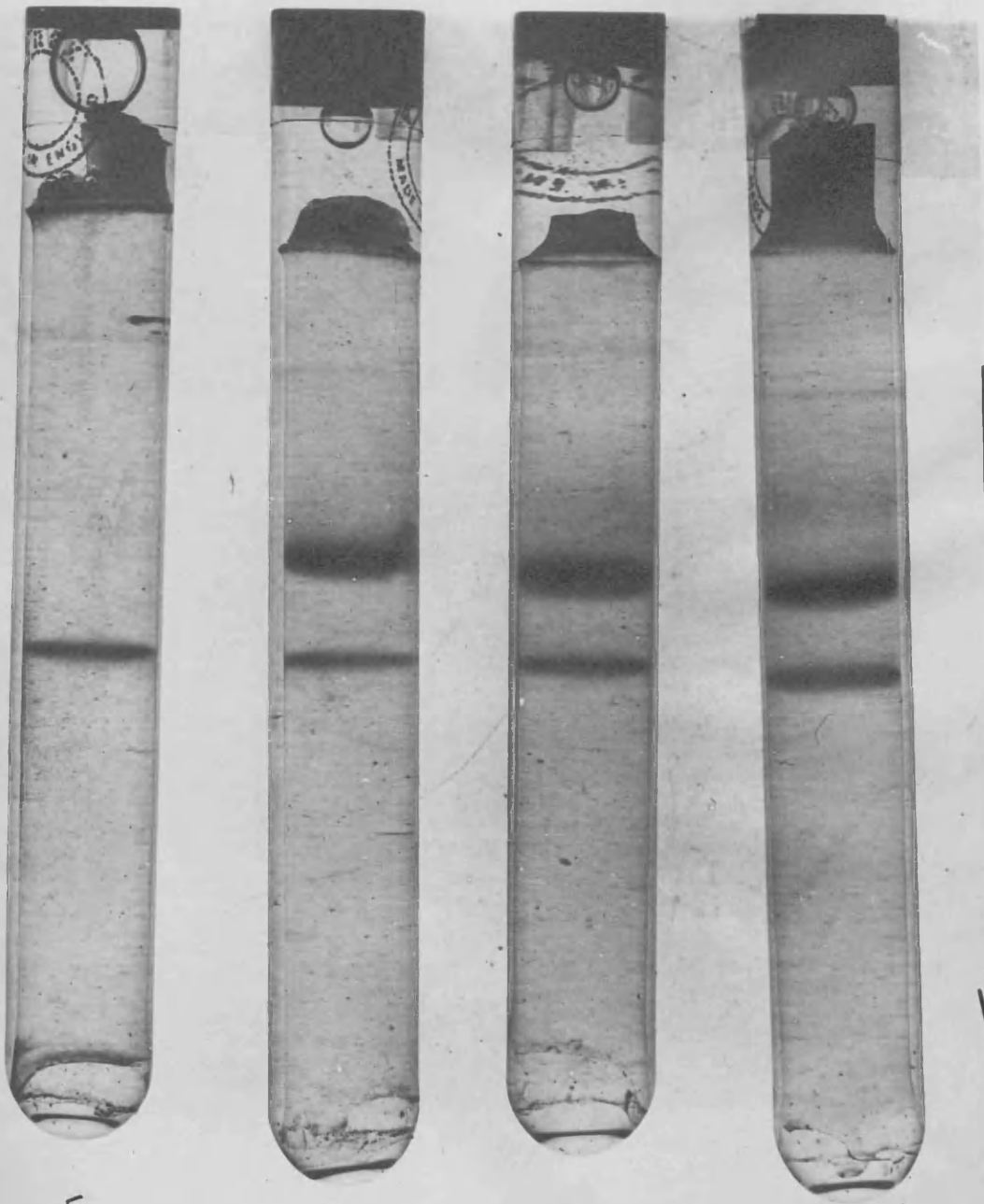
The gel G_5, Figure 45, was made from 7.5% acrylamide whereas other G gels were 22.5% acrylamide. This gel contained the same spleen ribonuclease "B" sample as the gel G_4 and all the G gels were submitted to electrophoresis together. Under the same conditions of electrophoresis the difference in mobility of the component as a result of difference in pore size was demonstrated. Gel G_4 is presented again in Figure 47, page 245 , where the photographic reproduction is superior. The darkening effect is less and one protein band only can be discerned. Figure 47 brings together for comparison the gels subscripted with the number 4. These gels contained the spleen ribonuclease activity "B" sample. Gel F_1 is included as the control for gel F_4 and the presence of the false protein band was evident in

FIGURE 43



E1. E2. E3. E4.

FIGURE 44



F1. F2. F3. F4.

FIGURE 45

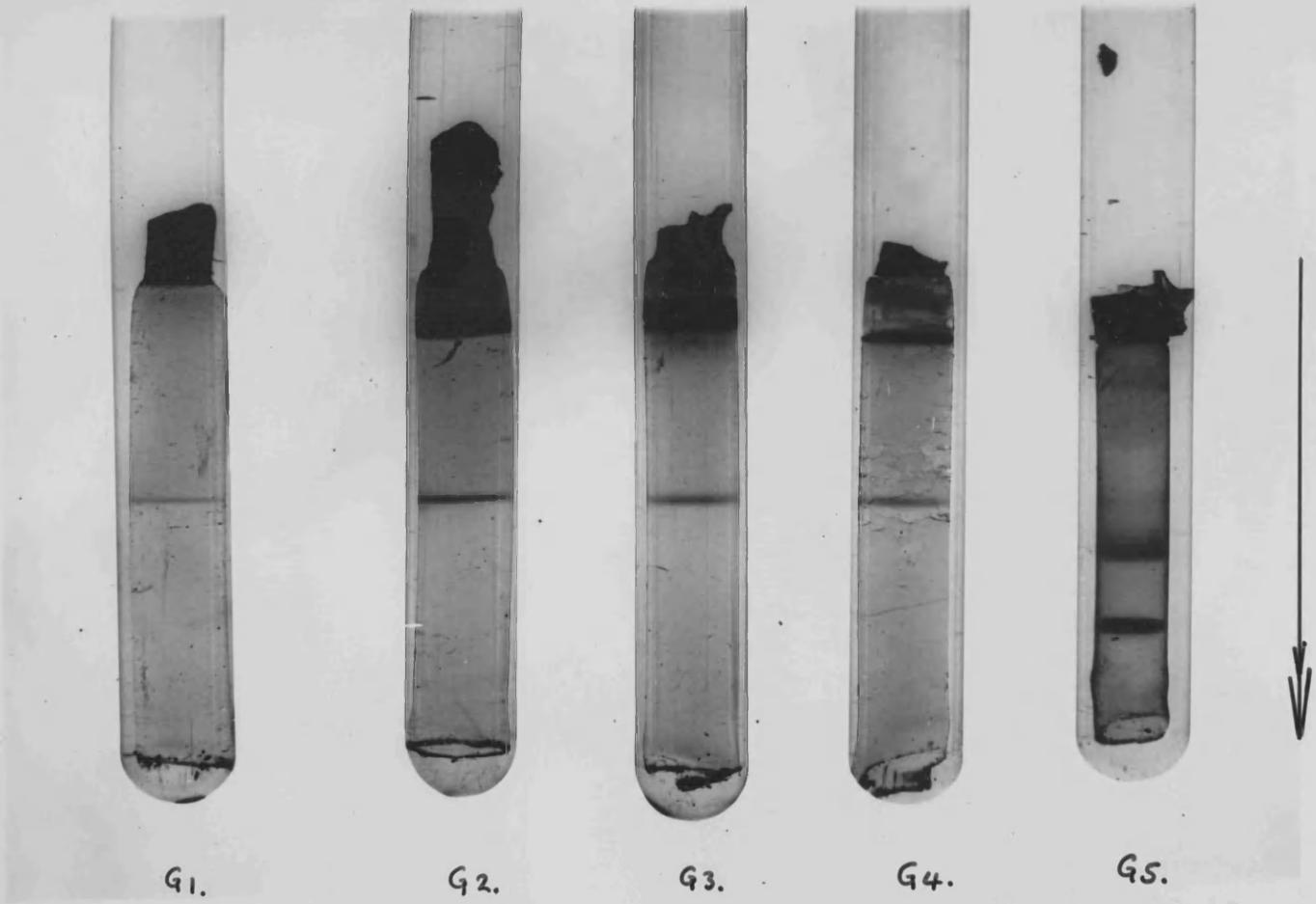


FIGURE 46

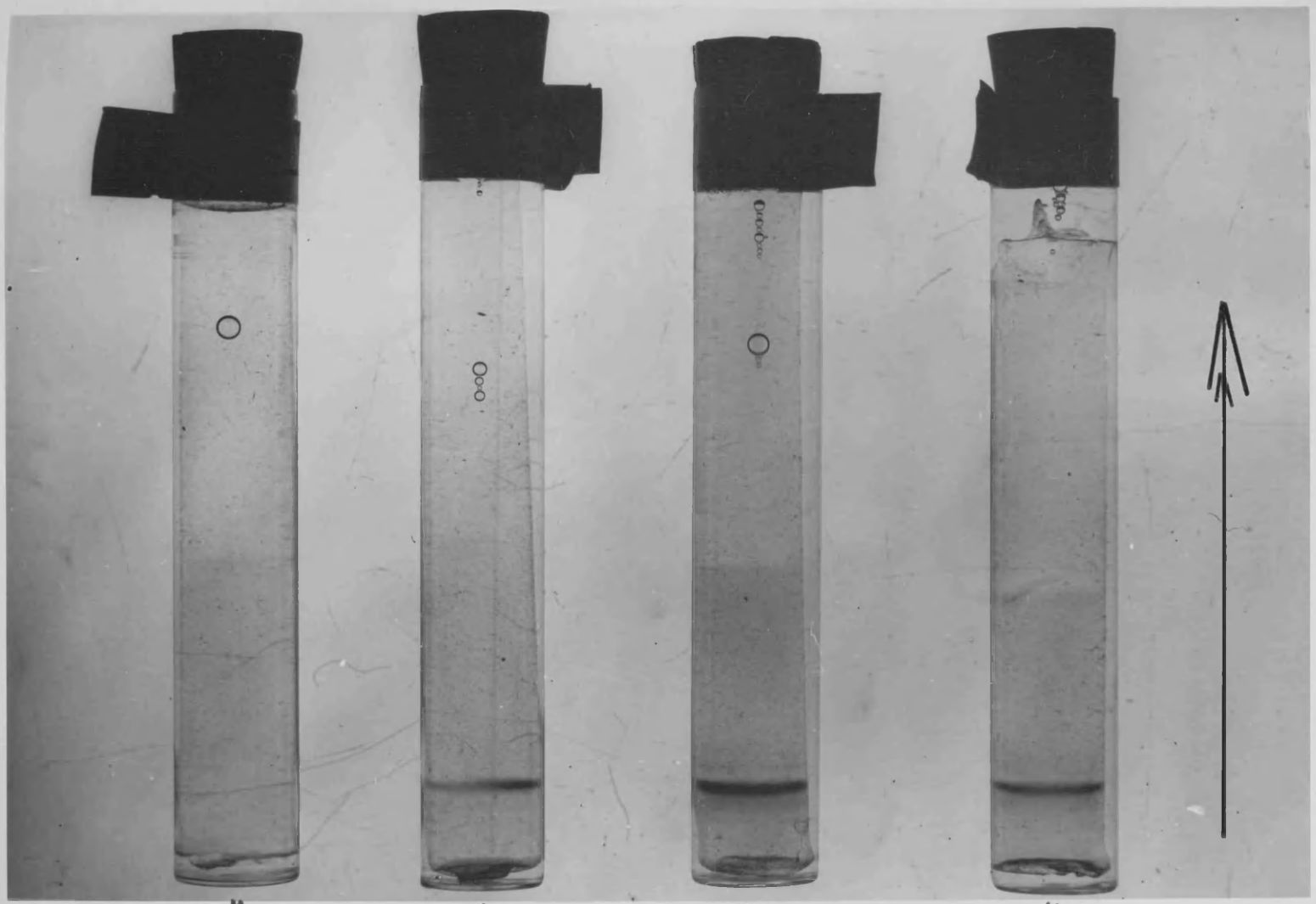
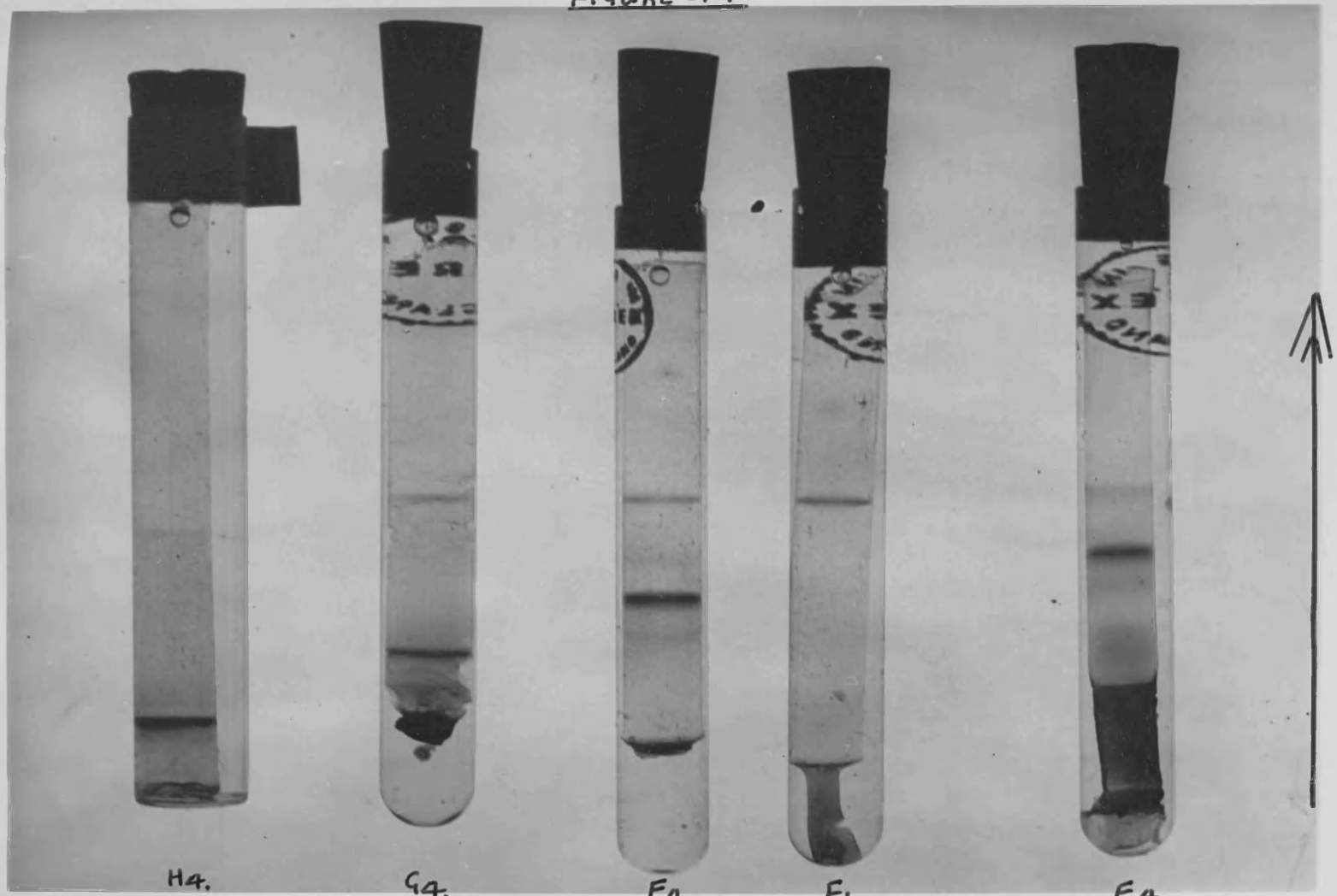


FIGURE 47



all the gels containing the protein samples. The percentage of acrylamide forming the gels E_4, F_4, G_4 and H_4 was 7.5, 15, 22.5 and 30% respectively. These gels illustrate that with decreasing pore size in the polyacrylamide gel at electrophoresis, where molecular sieve action should have discriminated against different sized proteins, heterogeniety in the sample was not demonstrated.

These gels suggested the major protein band of the spleen ribonuclease active sample was homogeneous and that this component had similar molecular size and mobility to pancreatic ribonuclease.

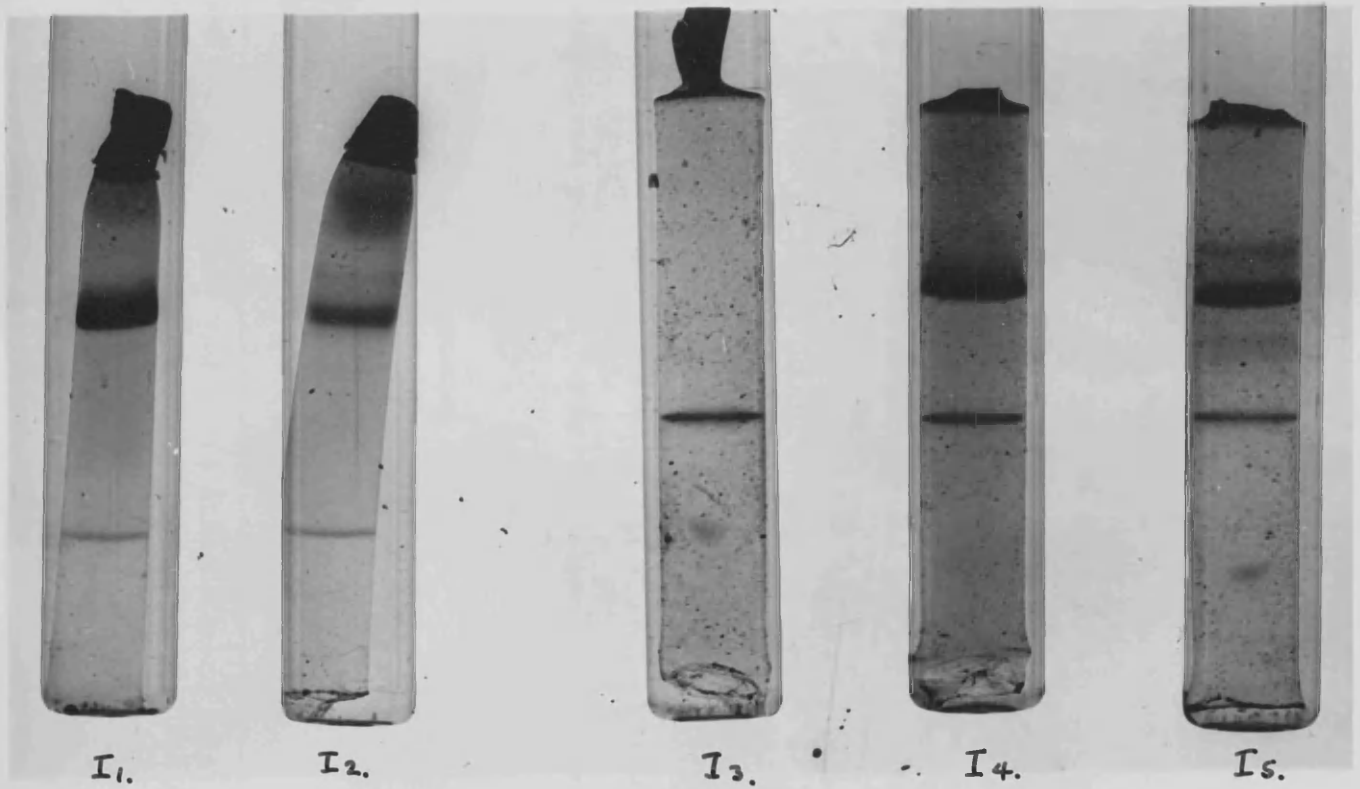
(f) Disc electrophoresis of the purified spleen ribonuclease sample at selected pH values for the small pore gel

An alternative to decreasing the pore size of the acrylamide gel in order to influence the molecular sieve action by increasing the frictional resistance to larger molecules, was to alter the pH of the environment of the small pore gel. The 15% small pore gel solution described on page 331 was made up especially for this purpose. The pH of the solution was adjusted with appropriate quantities of acetic acid or KOH before standard volumes were made up to give the desired acrylamide concentration at 15% and the solution was polymerised at this pH. The buffer solutions in the electrode vessels was retained at pH 4.3 on each occasion to ensure that all the components would migrate towards the cathode at electrophoresis. On reaching the small pore gel set at the various pH values it was assumed that the components electrophoreted at a pH slightly less than that of the original pH of the small pore when polymerised[111]. With the 15%

acrylamide gel which gives a suitable pore size for disc electrophoresis of ribonuclease[112,132] four small pore pH environments were tried out at pH 4.5, 5.5, 6.5 and 7.5. It was expected that if several protein components were present then different mobilities would be expressed by changes in the net charge of the different components at the different pH conditions to produce a separation and heterogeniety could be demonstrated.

In Figure 48, page 248, gels I_1 and I_2 compare the electrophoresis pattern of pancreatic ribonuclease and spleen ribonuclease activity peak "B" from Figure 29, page 190, on 15% acrylamide gels at pH 4.5. As in the gels presented in Figure 44, the two components moved almost the same distance. The gels I_3, I_4 and I_5 were made by polymerising 15% acrylamide at pH 5.5. On this occasion the ribonuclease activity peak "B_1", from Figure 30, page 191, was submitted to electrophoresis. The gel I_3 illustrates the pattern of electrophoresis of the blank. The spurious protein band shown to be present can also be observed in gels I_4 and I_5. The major protein band of the spleen ribonuclease B sample in gel I_5 was again evident, and characteristically blue in colour after staining. The band has been electrophoreted a comparable distance to the pancreatic ribonuclease electrophoreted in conjunction on gel I_4. On this occasion c.f. gel D_4, Figure 42, trace protein contaminants were present and migrated behind and in advance of the major protein component in trace amounts. This is much more evident in Gel J_5, Figure 49. Gel I_5 electrophoretogram suggested that the ribonuclease

FIGURE 48



activity "B_1" peak depicted in the column elution pattern of Figure 30, page 190, was not as pure as that obtained in the column elution pattern of Figure 29. However, of greater significance was the fact that the ribonuclease activity "B_1" from the column effluent pattern of Figure 30 and ribonuclease activity "B" from column elution pattern, Figure 29, page 190 both contain the major protein component and demonstrated to be of almost equivalent mobility and size to pancreatic ribonuclease.

All the gels represented in Figure 49, page 251, were polymerised from 15% acrylamide. 15% was chosen as the gel strength most likely to effect a separation as Reisfeld et al[112] had demonstrated that pancreatic ribonuclease, Worthington, crystallised x 3 was a mixture of one major and three minor components. Gels J_1, J_4, J_6, J_8 contained pancreatic ribonuclease while J_2, J_5, J_7, J_9 contained the spleen ribonuclease sample "B_1" from the column elution pattern depicted in Figure 30, page 190 . Table 28 summarises the conditions in the gels which could be compared directly.

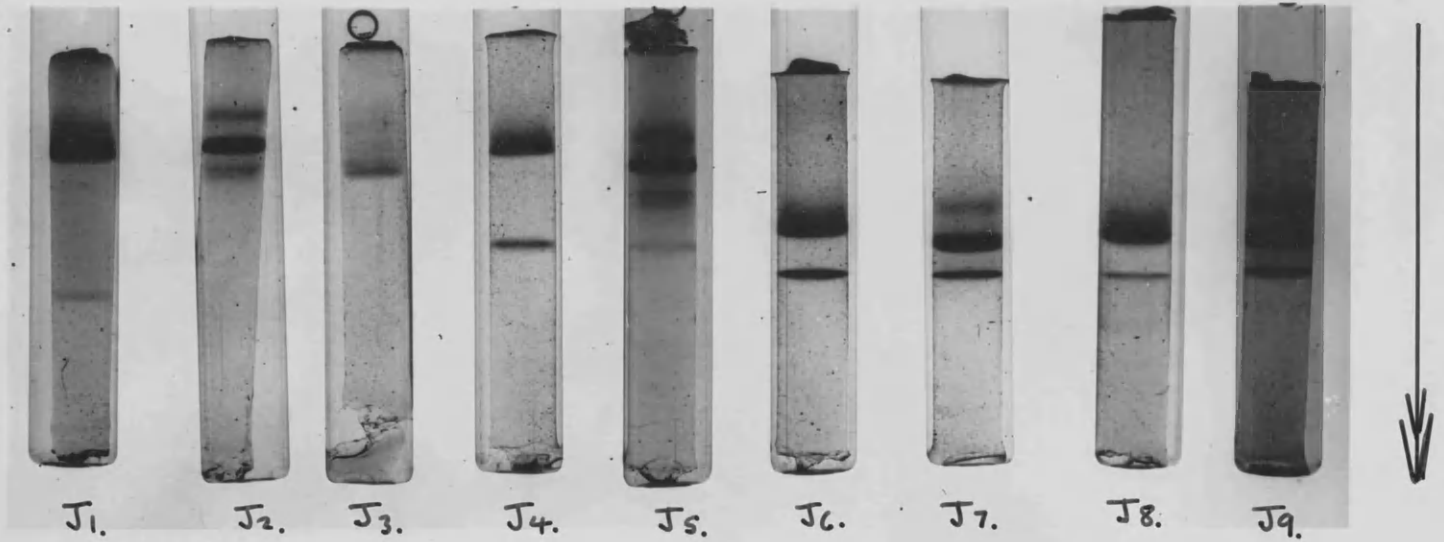
TABLE 28

Figure 49 gels

Small Pore 15% Acrylamide Polymerised at pH	Pancreatic Ribonuclease Electrophoreted in Gels	Spleen Ribonuclease "B_1" Electrophoreted in Gels
4.5	J_1	J_2
5.5	J_4	J_5
6.5	J_6	J_7
7.5	J_8	J_9

In all the gels presented, with the exception of J_2 and J_3 the spurious band is represented by the stained region which has been electrophoreted the furthest. Gels J_2 and J_3 from a different run did not produce the false band. The major protein component in the spleen ribonuclease active fraction "B_1" had electrophoreted in a similar manner to pancreatic ribonuclease under all the different conditions of pH. The results were similar to those already demonstrated in Figures 43, 44, 45 and 46 where the gel pore size decreased. Both pancreatic ribonuclease and the major component of the spleen ribonuclease "B_1" fraction migrated much the same distance on each occasion with respect to each other, but were electrophoreted much further as the pH was increased in the small pore environment. A superior resolution of components was obtained for spleen ribonuclease "B_1", in Gel J_5 which was electrophoreted at pH 5.5, c.f. gel I_5, Figure 48, and J_2, Figure 49. Four protein components are illustrated. The major protein component stained blue and migrated an equivalent distance to the pancreatic ribonuclease standard, while the trace band migrated furthest in other gels I_5 and J_2 containing this sample, was shown to contain two trace components electrophoreting very close together. A fourth trace component is also present to the rear of the major protein component. At pH value of 6.5 and 7.5 the small pore gels did not exhibit the trace impurities of greater mobility than the major protein component, though the trace impurity of slower mobility was present and demonstrated that some resolution had been effected at these pH values.[116]

FIGURE 49



The gel J_3 which contained the spleen ribonuclease active sample "A" from the column elution pattern exhibited in Figure 30, page 190 , was electrophoreted under the same conditions as spleen ribonuclease activity sample "B_1" from the same elution pattern. In this gel the two components in greatest concentration were electrophoreted similar to the two trace components of greater mobility than the major protein component present in the spleen ribonuclease active sample "B_1" as depicted in gel J_2 electrophoretogram. Gels J_2 and J_3 are directly comparable. It can be suggested from this observation that there was considerable cross contamination during the elution of the column outlined in Experiment 28 to give the elution pattern depicted in Figure 30, page 190 . This finding illustrated that the desorption of protein was not clear cut.

Poor resolutions of protein components at column chromatography on cellulose ion exchangers were reported by Tombs et al[160] and Cooke et al[169] who made a comprehensive survey of column effluent by immunoelectrophoresis, e.g. Albumin was located in several fractions throughout the elution patterns[160].

4. Summary of the main findings from the analysis by disc electrophoresis of the spleen ribonuclease active fractions

The main findings to emerge from the use of the disc electrophoresis technique are as follows :-

(i) It was shown that the ribonuclease active peak "A_1", Figure 31, page 191, contained four protein components in major amounts. This fraction requires further purification.

(ii) The ribonuclease active fraction "B", Figure 29, page 190, which was obtained by the rechromatography of the ribonuclease active protein "B" from the scaled up C.M. cellulose column, Figure 28, was shown to have only one component present. This was demonstrated after electrophoresis on four gels of different pore size, Figure 47, page 245. Ribonuclease active fraction "B" was considered pure. On comparable electrophoretograms this component migrated slightly further than the pancreatic ribonuclease standard.

(iii) The major protein component of the ribonuclease active fraction "B_1", Figure 30, page 190 , migrated at disc electrophoresis to exactly the same position as the ribonuclease active component "B" described in (ii) above and slightly further than the pancreatic ribonuclease standard. This ribonuclease active "B_1" fraction was shown not to be as pure as the "B" fraction of elution pattern, Figure 29 (ii above). Three other components were shown to be present in trace amounts.

The fact that the major component of ribonuclease active fraction "B_1", Figure 30, and the only component in ribonuclease active fraction "B" Figure 29

(a) migrated to the same point at disc electrophoresis

(b) both stained a distinctive blue colour with Amido Schwarz

(c) were eluted from a C.M. Sephadex C-50 column at the same point, Section III, Figures 29 and 30, page 190 , indicated that they were of the same ribonuclease activity.

The disc electrophoretograms indicated that the cation exchange

column procedures developed as described in Section III were successful by achieving a considerable purification. The ribonuclease active fraction "B" prepared according to Experiment 26, page 377 , and then rechromatographed at constant pH8 according to Experiment 27, page 378 , Figures 28 and 29, pages 187 and 190 respectively yielded a single component when analysed by the disc electrophoresis technique for heterogeniety. As intimated in Section III, subsection 9, the column techniques described in these experiments were incorporated in the purification procedure to obtain pure samples of ribonuclease active fraction "B".

SECTION V

The molecular weight estimation of the spleen ribonuclease active components by gel filtration

1. Introduction

Columns of starch grains were first used in the estimation of relative molecular sizes of carbohydrate and proteinaceous substances by Lathe and Ruthven[117] who showed that neutral molecules could be eluted in descending order of molecular weight. In this work it was shown that NaCl, included in the eluting solution, prevented interaction between charged solute and the starch grains. Andrews[118,119] established a direct relationship between the molecular weights of proteins and their gel filtration behaviour. Details have been published[119,122,128] which illustrated that an excellent correlation exists between the behaviour of carbohydrate free globular proteins during molecular sieving on Sephadex and their molecular weights under very stringent conditions of technical accuracy. Very recently by utilising gels with a very low cross linkage, the range for molecular weight estimation has been extended[120].

Molecular weight estimation by gel filtration involved a comparison between the gel filtration behaviour of the entities under investigation with that of related compounds. All of the compounds should conform to an appropriate relationship between molecular weight and gel filtration behaviour. The size and shape of molecules[120,124]

influence their behaviour on gel filtration, thus discretion was exercised in choosing standards which conformed to the most likely form of the entities under scrutiny. Since most enzymes are globular it would be expected that the spleen ribonuclease activities would not be atypical and established standards very chosen with significant differences of between 10,000 and 70,000 in molecular weight to calibrate the gel column.

The molecular sieve process has already been described in Section III, part A. The resolving properties of each Sephadex type, page 126 , has been defined in the range of molecular weight, in which optimum separation of macromolecules takes place. Andrews[119] has demonstrated that the effective fractionation range was greater for proteins than for dextrans. Effective fractionation of proteins in the molecular weight range 3,000 to 70,000 has been established with Sephadex G-75. This molecular weight range includes the low molecular weight region in which the ribonuclease activity from spleen could be resolved (page 136) and was adopted for the estimation. Other Sephadex types G-100, G-200 are more favourable to molecular weight estimations of entities exhibiting molecular weights in the range 10,000 → 820,000[120].

2. Calibration of the Sephadex G-75 column and conditions for the chromatography.

Pancreatic ribonuclease was included as a standard of importance since a direct comparison could be observed between the relative elution positions for pancreatic ribonuclease and the two ribonuclease

active fractions from spleen. Andrews[119] has already demonstrated that the proteins selected as standards, i.e. glucagon, pancreatic ribonuclease, trypsin inhibitor from soya bean, ovalbumin and bovine serum albumin produced a direct relationship between their elution volumes and molecular weight. Glucagon was included as an excellent standard of low molecular weight 3,500 which would suitably mark elution in the range of molecular weight lower than pancreatic ribonuclease. These standards were from crystalline sources and used to calibrate the 2-3 cm. i.d. x 55 cm. effective length gel column described in Experiment 34, page 386 . The inclusion of "blue dextran 2,000" and sucrose when they would not interfere with the direct interpretation of elution patterns with both the standards and unknowns, eliminated uncertainties about variation in elution volume, or unsatisfactory elution spans, from run to run. Blue dextran was used to check the void volume while sucrose marked the end of the elution patterns. The range of elution pattern, its reproducibility and gel stability were gauged by the values obtained for Vo and Vt, the void volume and total volume respectively, from one chromatogram to the next (Table 30, page 271).

As will be clear from what was described in Sections III and IV of the present study, two similar activities had already been completely fractionated on the cation exchangers and were available for scrutiny on the molecular sieve medium. One of the activities, that designated spleen ribonuclease activity "B", Figure 29, page 190, has been shown to be free of contaminants while the other spleen

ribonuclease activity "A", Figure 31, page 191 , has considerable quantities of other components in the sample (Section IV, page 236)

Since the molecular weight estimation technique lends itself equally well to the study of impure enzyme samples the molecular weight of both ribonuclease activities were examined. With enzymes only catalytic amounts are necessary[119] and molecular weights were estimated at concentrations similar to those employed for their assay. In addition low protein concentrations were used in the standard solutions to calibrate the gel column.

It has been demonstrated that the advancing edge of a zone containing 5 mg of ovalbumin would be eluted 3-4 ml. later than that of a similar zone containing 1 mg. ovalbumin[125,126]. The corresponding difference in molecular weight would be 4,000. This effect was not detected by Andrews[119,120] and others[127,128] when small sample volumes were used and the migration of protein through the columns was measured by the elution values (Ve) at which the solutes were at a maximum concentration as used in the compilation of Table 30, page 271, for the present work. Since catalytic concentrations only of unknowns were used at the investigation of their molecular weight the extensively pure protein standards were used at low concentrations, Table 29, page 389 , and detected at the more sensitive wavelength of 230 mμ , c.f. 280 mμ on the U.V. spectrophotometer in an attempt to satisfactorily equate the inherent conditions and eliminate the uncertainty of secondary concentration effects.

The same buffer eluting conditions (0.05M tris HCl pH 7.5) as

that employed by Andrews[119], except that the content of NaCl was 0.25M, was used to elute the system. The higher salt content was introduced for two reasons. The spleen ribonuclease activities particularly that designated fraction "B" was eluted from the cation exchange column at a high salt strength, i.e. 0.25M NaCl at pH values near neutral, Figures 28 and 29, pages 187 and 190. It was considered that secondary interactions with the Sephadex gel particles other than a true steric exclusion from the gel phase at molecular sieving[124] ought to be eliminated. Sephadex is known to possess a small capacity for cationic adsorption[103,129,130,131] and this could be important with catalytic quantities of charged protein at gel filtration. Andrews[118] has demonstrated that there was no further elution effects with charged entities at gel filtration on agar gel when concentrations of KCl above 0.24M were included in the eluting buffer.

The active fractions from spleen were obtained directly after column chromatography on C.M. cellulose and contained NaCl at a concentration $\simeq$ 0.25M. To accommodate their immediate utilisation, without further processing (page 388), the molecular weight estimations were carried out with 0.25M NaCl in the equilibrating and eluting buffer and the column was calibrated with the standard proteins in this environment.

There was the possibility that proteins exhibiting high net positive or negative charges and subjected to alternative pH values and salt concentrations would exhibit molecular conformation changes

similar to those exhibited by oligonucleotides[193] and glycoproteins[120]. Andrews[120] suggested that gel filtration indicated that some glycoproteins had more expanded structures than typical globular proteins. In addition to problems of molecular conformation, the observation by Gelotte[129], that molecules containing a higher proportion of aromatic ring structures were retarded to a greater extent on Sephadex by an adsorptive interaction with the dextran matrix, served to illustrate uncertainties of a decisive nature could exist. Anomalous elution patterns could be produced by parallel effects involving aromatic amino acid units at the molecular sieving of proteins. Accordingly elution results and conclusions based on them must be interpreted and presented with caution.

3. Some irregular elution patterns obtained during molecular sieve chromatography of certain spleen ribonuclease active samples

An irregular chromatography effect was observed with certain spleen ribonuclease active fractions during the molecular weight estimations by gel filtration, Experiment 34, page 386 , and warrants mention. Figures 57, 58, 59 and 60 illustrate the elution patterns for four spleen ribonuclease preparations applied to the 2.3 cm. x 55 cm. Sephadex column used in Experiment 34. It was considered that the different ways by which the ribonuclease samples were prepared had an effect on the elution pattern at molecular sieving. The method of preparation for all the samples was that outlined in Section III, pages 208 - 210.

Figure 57 shows a symmetrical elution peak for a ribonuclease

active sample "A". This spleen ribonuclease sample "A" was obtained from Experiment 29, page 380, Figure 31, page 191, and was chromatographed free of the ribonuclease active component "B" then stored in the deep freeze at -12° after freeze drying. The ribonuclease activity was eluted over a span of 52 ml.

Figure 58 illustrates a ribonuclease activity peak with a distinct discontinuity in the pattern and the span of ribonuclease activity extends over 75 ml. This gel filtration was carried out on spleen ribonuclease active fraction "A" which was prepared in exactly the same manner as that sample molecular sieved in Figure 57, except that after the fractionation on C.M. cellulose it was heat treated at 70° for 10 minutes at pH 3.5 as described in Section III, subsection 10, page 215 . The sample was not stored or freeze dried at any time.

Figure 59 illustrates the elution pattern for the ribonuclease active fraction "B" which was gel filtered directly after the sample was eluted from the C.M. cellulose columns after fractionation, i.e. column IV, page 210 . A symmetrical peak of ribonuclease activity which spanned 45 ml., was obtained. The sample was not freeze dried or stored during the purification.

Figure 60 illustrates the elution pattern of spleen ribonuclease active fraction "B" which had been stored at -12° after freeze drying. A distinct discontinuity was obtained in the elution pattern and a ribonuclease activity, spanning just over 75 ml. was eluted.

In contrast to Figures 57, 58 and 60 other components molecular

The elution profiles of spleen ribonuclease active samples molecular sieved on the sephadex G-75 column 2.3 x 55cm.

Fig. 57.

Elution profile of spleen RNase active sample "A" which had been freeze dried then stored at -12°.

V_e= 123ml.
Elution span 52ml.

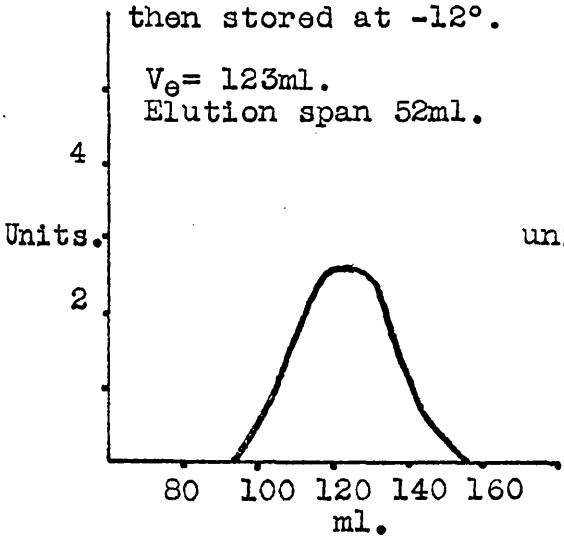


Fig. 58.

Elution profile of spleen RNase active sample "A" which had been heat treated at 70° for 10 min. at pH 3.5

Elution span 75ml.

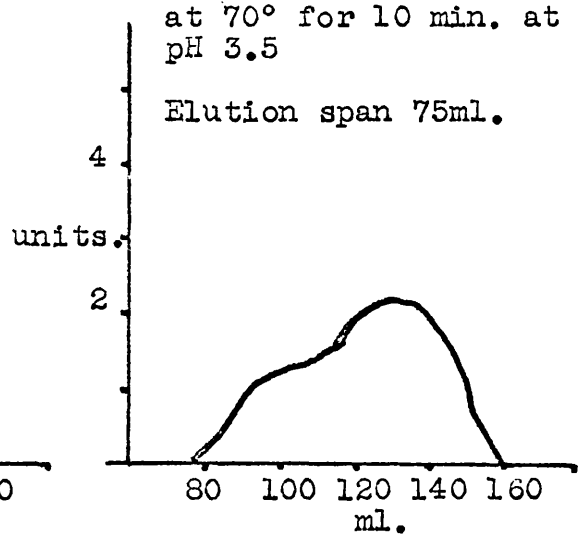


Fig. 59.

Elution profile of spleen RNase active sample "B" directly after ion exchange chromatography.

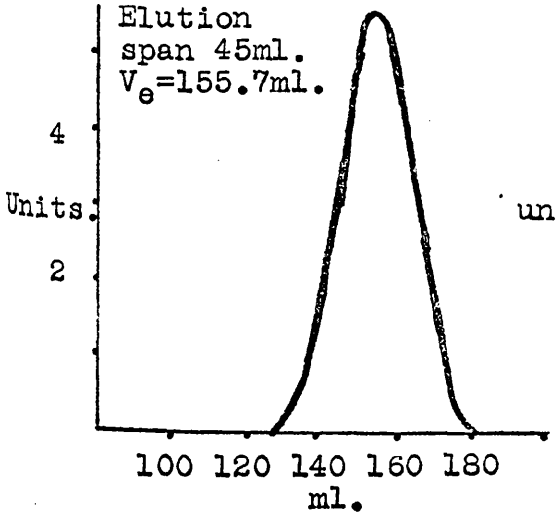
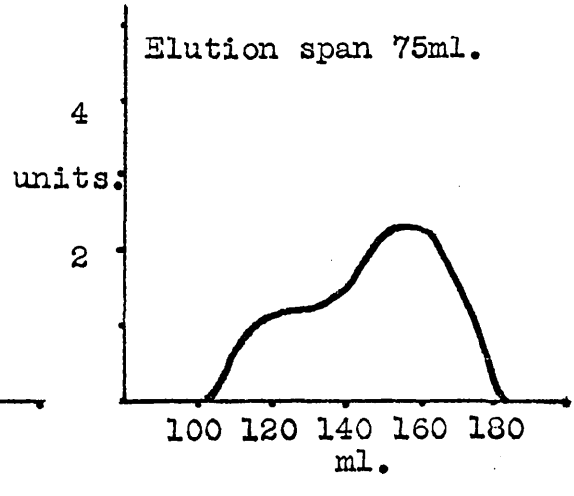


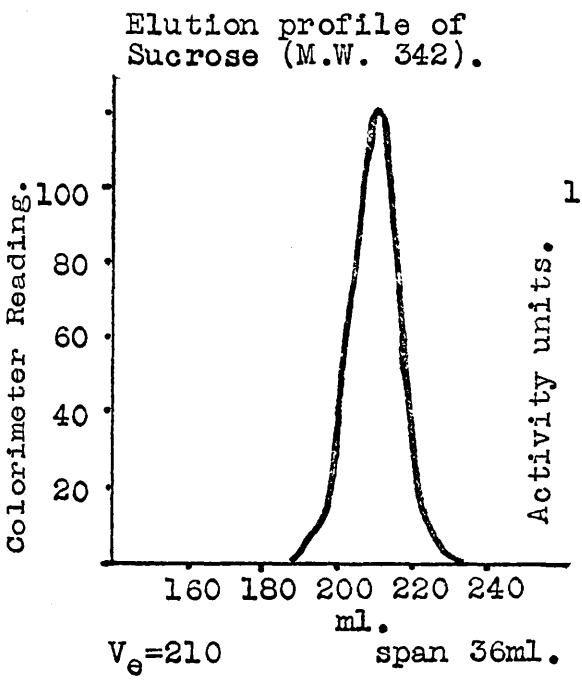
Fig. 60.

Elution profile of spleen RNase active sample "B" which had been freeze dried to concentrate then stored at -12°.



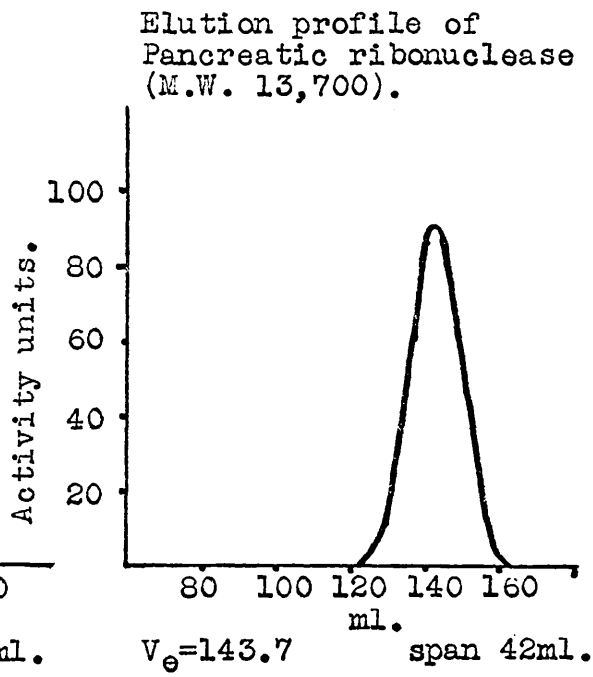
Typical elution profiles of standard substances molecular sieved on the sephadex G-75 column 2.3 x 55cm.

Fig. 61.



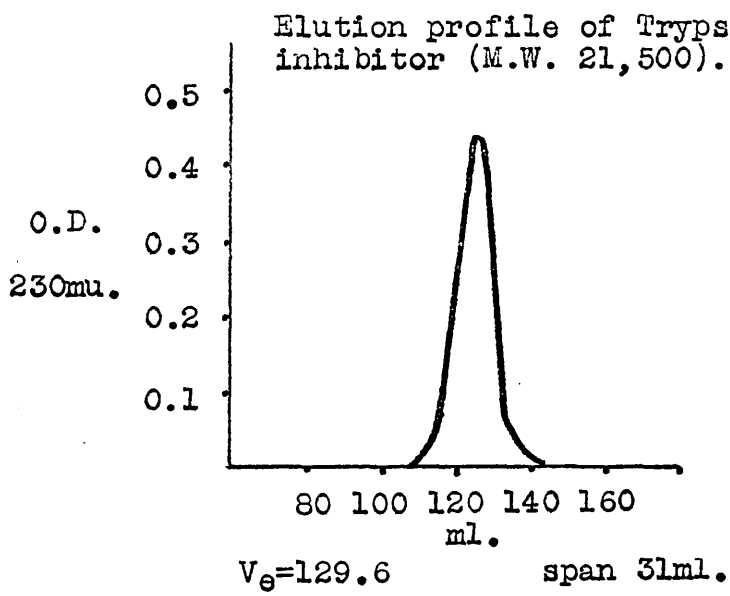
V_e=210 span 36ml.

Fig. 62.



V_e=143.7 span 42ml.

Fig. 63.



V_e=129.6 span 31ml.

sieved on the same column exhibited typical elution patterns. At the termination of a Sephadex column elution pattern, sucrose exhibited an elution span of only 36 ml. which is the widest span expected for a single entity under these conditions (Figure 61, page 263). In Experiment 34, page 386 , pancreatic ribonuclease was eluted as a single symmetrical ribonuclease active peak over a span of about 42 ml., Figure 62, page 263 . This compared very favourably with the elution pattern of the spleen ribonuclease active fraction "B" depicted in Figure 59, page 262, and suggested these elution profiles were typical of enzymes which were singular molecular entities. It was expected that enzymic activity would be more readily detected than protein components detected by a less sensitive protein estimation. Consequently the enzymic active components molecular sieved should exhibit a greater elution span than ordinary proteins detected by optical density measurements, e.g. in Figure 63, page 263 , trypsin inhibitor M.W. 21,500 on molecular sieving spanned 31 ml. at elution. From these elution span volumes and elution pattern profiles it was observed that those presented in Figures 58, 60 and possibly Figure 57 exhibited irregular features. A 75 ml. elution span and pattern profile exhibiting a distinct discontinuity were not typical of the symmetrical elution peak and maximum elution span expected when a singular molecular entity was molecular sieved. As illustrated in Figure 57 an elution span of 52 ml. for spleen ribonuclease active component "A" M.W. 24,000 (page 272) did not compare favourably with the 30 ml. elution span of trypsin inhibitor M.W. 21,500, Figure 63, page 263.

4. Possible explanations for the irregular elution effects observed and their influence on the validity of the molecular weight estimations made in subsection 5.

Variation in protein concentration from one sample to another at gel filtration has been examined by Andrews[120]. Several workers[120,127,128] have demonstrated that elution patterns were not influenced by reasonable protein concentrations (page 258). The experimental details presented in Table 29, page 389 , and Experiment 35, page 390, list the protein concentrations applied in each sample. At no time were samples applied which were atypical in concentration or viscosity and it was unlikely that the elution irregularities in the present work were caused by a simple concentration effect.

In earlier Sephadex column experiments (Section III, pages 134 and 139) where very impure spleen ribonuclease active fractions were molecular sieved, anomalous elution patterns were obtained. At this time the irregularities were attributed to the fact the enzyme samples were freeze dried to concentrate, then stored at -12° prior to molecular sieving. It was considered quite significant that the samples applied to the column to produce the elution profiles in Figures 57 and 60 (pages 262 and 263) were in a freeze dried state, stored at -12° in the deep freeze, prior to molecular sieving. It is now well established that freeze drying pancreatic ribonuclease in the presence of acetic acid produces molecular dimers and higher aggregates[170,177] which can be detected at gel filtration. Andrews[120]

has demonstrated that thyroglobulin, which had been freeze dried, was eluted both at the void volume and some 10 ml. later on the elution pattern at gel filtration. Andrews maintained that as thyroglobin was known to aggregate on freeze drying[189] the anomalous elution pattern was caused by aggregated molecules. In the present work some of the samples examined were freeze dried in the presence of 0.005M tris HCl 10^{-4} MEDTA pH 7, G.M.17.

The ribonuclease active component "B" MW 10,000, page 272, if dimerised would exhibit a molecular weight of 20,000. According to the elution pattern, Figure 60, page 262, no ribonuclease activity has been eluted in the appropriate section to indicate this. A ribonuclease activity of MW 20,000 would elute at a similar position to the trypsin inhibitor MW 21,500 and this was not observed. From the standard molecular weight calibration plot presented in Figure 50, the elution volume Ve from Figure 60 indicated that the molecular weight of the ribonuclease activity "B" had increased by 4,000-5,000 to M.W. 15,000. It was difficult to estimate the elution volume as the discontinuity in the elution profile suggested that two ribonuclease peaks had eluted almost together. The wide span in the elution of ribonuclease activity "B" was not consistent with the presence of a single entity as demonstrated by the same active component in Figure 59, page 262.

The possibility was contemplated that this elution irregularity indicated a fluctuation in the molecular dimensions of the molecule.[197] Some of the ribonuclease molecules after dehydration by the freeze

drying action might have assumed an alternative structural form when brought into solution prior to molecular sieving and the discriminating action of the Sephadex gel particles at gel filtration was sufficient to differentiate between them to give a wide elution pattern with a distinct discontinuity in the profile.

Another possibility concerning the freeze drying technique is the fact that the ultracentrifugal studies by Carroll[8] indicated a very low molecular weight for the calf spleen ribonuclease at 2,000-5,000. It would not be unreasonable to consider the spleen ribonuclease active component "B" as a strongly bound dimer of two units of MW 5,000 and that the removal of water at freeze drying was sufficient to reorganise this arrangement when the sample was redissolved. Fisher[171] has published a treatise on a limiting law relating the size and shape of molecules to their composition. Fisher maintains that if there are structural or functional requirements for non polar residues in the protein molecule the practical lower limit for any molecular weight must be about 7,000. This consideration excludes the existence of monomer entities of molecular weight 5,000 under normal circumstances, thus any molecular rearrangement of ribonuclease activity "B" would involve dimers, trimers or higher associations only.

With the spleen ribonuclease "A" sample, molecular sieved as depicted in Figure 58, it is not difficult to envisage that structural alterations on heating at 70° for 10 minutes might take place although it has already been shown that this ribonuclease component is heat stable. The spleen ribonuclease "A" samples applied to the columns

to give Figures 57 and 58 (pages 262 and 263) were both heat treated at an early stage in the purification procedure, Experiment 3, page 358 when the ribonuclease component "B" and many protein impurities were present. After some purification to obtain a pure sample of ribonuclease component "B", page 236 , the ribonuclease active sample A_1 was shown to contain four protein components in major proportions. After freeze drying the sample exhibited the irregularly wide elution pattern depicted in Figure 57. Furthermore, the second heat treatment at 70° without activity loss caused this spleen ribonuclease active "A" sample to exhibit an even wider ribonuclease active elution span inconsistent with the elution of a single entity. It is possible that the spleen ribonuclease component "A" formed protein-protein associations with the impurities present to produce the irregular elution phenomena witnessed.

The experiences encountered at the cation exchange chromatography Section III, page 193 , when this same active fraction "A" produced irregular chromatographic effects suggested that the protein milieu of this fraction was intrinsically reactive. It appears that this fact has been further demonstrated by the irregular molecular sieve chromatograms from this sample after heat treatment and freeze drying techniques and these processes may have some bearing on the elution anomaly encountered at cation exchange chromatography.

The irregularities observed after the discriminating molecular sieve action of gel filtration indicated the danger of incorporating apparently harmless well established techniques to the manipulation

of proteins which might be structurally sensitive or reactive. In the present study no attempt was made to obtain the heat stable spleen ribonuclease active fractions without the heat treatment described in Experiment 3, page 358. The molecular weight estimation of 10,000 for the ribonuclease active component "B" was determined with a chromatographically pure sample as determined by disc electrophoresis. The sample was at no time freeze dried. This estimate was considered reliable. On the other hand the disc electrophoresis of the chromatographed spleen ribonuclease active sample "A" indicated at least three contaminants in major proportions and since heat treatment as used at the preparation stages influences its gel filtration behaviour the molecular weight estimate of 24,000 must be viewed with caution. The sample used for this estimate was freeze dried during the preparation.

5. The results from the series of molecular sieve columns carried out in Experiment 34 to estimate the molecular weight of the two heat stable spleen ribonuclease active components

The experimental procedure as described on page 386 for the calibration of the gel column and molecular weight estimates were undertaken at the same time. This measure ensured that no variations would occur from calibration to determination. The amount of experimental procedure required to produce sufficient results with the standards and unknowns to carry out a statistical analysis with appropriate estimates of error would have been very considerable. The number of column runs required to accomplish this would not have

been possible on a single column. An awareness for strict procedure control, efficient column technique with particular care exercised at sample application were grounds for performing two or three runs only with each compound. A total of twenty runs altogether, Table 30, page 271, were completed without considering a restabilisation of the column by repacking and re-equilibrating procedures essential. Accordingly the most suitable value for Ve the elution volume for each substance presented in Table 31, page 272 , was obtained from the results of the column runs on the summary, Table 30, page 271. These values were plotted against the known molecular weights of the five protein standards to produce the linear correlation depicted on Figure 50, page 273 . It was considered reasonable to adopt the error value of 10% in estimating the error for the unknowns. This value was first recommended by Andrews[119] and subsequently reaffirmed as an appropriate value[120] for the uncertainty in molecular weight estimations by gel filtration. The satisfactory linear plot obtained suggested that the calibration could be used to ascertain estimates of the molecular weights exhibited by the spleen fractions.

Under the conditions of the experiment the elution volume Ve for the spleen ribonuclease active fraction "B" clearly indicated that the entity had a smaller molecular dimension than bovine pancreatic ribonuclease (Tables 31, 32, page 272) while the elution volume for the spleen ribonuclease active fraction "A" suggested a much greater molecular dimension. From the standard curve, Figure 50, page 273 , it was ascertained that the ribonuclease active fraction "A"

TABLE 30. Programme of experiments with elution volume for blue dextran and sucrose markers, protein standards and unknown ribonuclease activities tested.

Expt.No.	ELUTION VOLUME Ve/Ml								
Solute	Blue Dextran	Bovine Serum Albumin	Oval-bumin	Trypsin Inhibitor	Pancreatic Ribonuclease	Glucagon	Sucrose	Spleen R.A."A".	Spleen R.A."B".
1	68.1								
2	68.4	83.4							
3							209.7		
4	68.4			129.6			210		
5		83.7					209.7		
6			99.9				209.7		
7	67.8					192	-		
8	67.5						210		
9	67.5			129.6			210		
10	67.9						210	120	
11	67.5						209.1		155.1
12			100.2				210		
13	67.8				143.5		210		
14	67.65				143.7		210		
15	67.3					193			
16							209.4		155.7
17	66.9			128.1			210		
18	66.9						208.8		156
19		83.7					210		
20	67.0						209.4	125	

R.A. = Ribonuclease Activity

TABLE 31. Values for void volume, and elution volumes of the protein standards.

Solute	Molecular weight	Range of elution Ve in ml.	Mean Value taken for Ve in ml.
Glucagon	3,500	192-193	192.5
Pancreatic Ribonuclease	13,700	143.5-143.7	143.6
Trypsin Inhibitor	21,500	128.1-129.6	129
Ovalbumin	45,000	99.9-100.2	100
Bovine serum albumin	67,000	83.4-83.7	83.6
Blue Dextran	2 million	67-68.4	67.8
Sucrose	342	208-210	209.7

Vo the void volume = 67.8 ml.

V_t the final volume = 209.7 ml.

TABLE 32. Molecular weight estimation of the spleen ribonuclease active samples.

Spleen ribonuclease active fractions from Fig.29, Fig.31 page 190. 191.	Range of elution Ve in ml.	Value taken for Ve in ml.	Estimation of molecular wt.from the plot Fig.50.
"A"	120 - 125	123	24,000 $\pm$ 2,400
"B"	155 - 156	155.5	10,000 $\pm$ 1,000

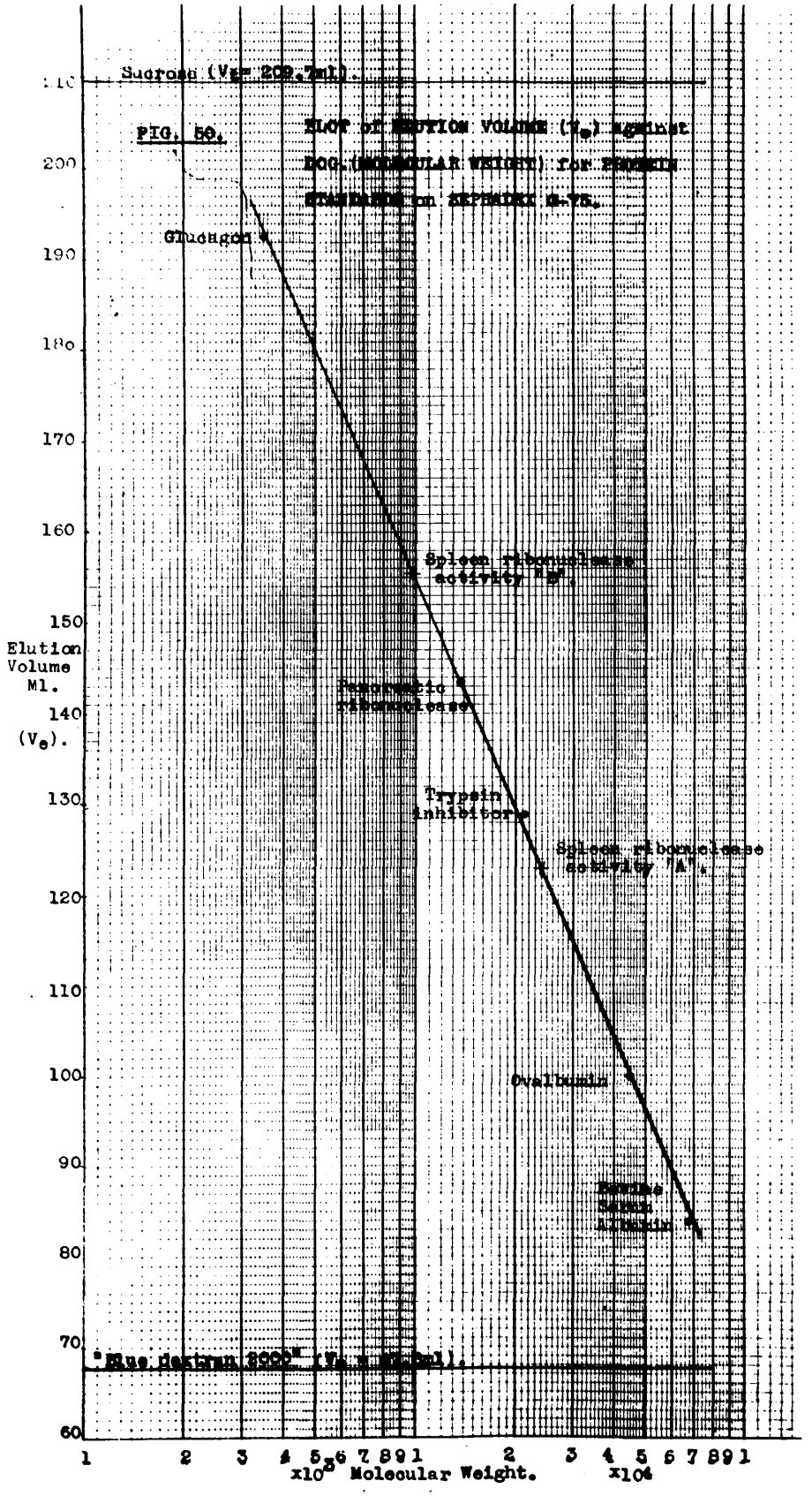


FIG. 50. PLOT OF ELUTION VOLUME (V_e) AGAINST LOG. (MOLECULAR WEIGHT) FOR PROTEIN STANDARDS ON SEPHADEX G-75.

has a molecular weight in the region of 24,000 and ribonuclease active fraction "B" has a molecular weight in the region 10,000, see Table 32, page 272.

6. The significance of the molecular weight estimations

The spleen ribonuclease active fractions "A" and "B" according to these molecular size estimations are different entities. The ribonuclease active component "A" is more than twice the molecular size of the ribonuclease active component "B". Since this estimation was carried out, recent investigations on the specificity of these components have substantiated the fact that there are two distinguishable heat stable ribonuclease active entities in calf spleen[109].

The ribonuclease active fraction "B" with a molecular weight of 10,000 and slightly smaller than pancreatic ribonuclease (13,700) would be expected to electrophorete at disc electrophoresis on polyacrylamide gel in very close proximity to pancreatic ribonuclease as was demonstrated in Section IV, subsection 3(e). However, the spleen ribonuclease described by Kaplan and Heppel[8] was considered to have a particle size of M = 2,000-5,000. This spleen ribonuclease exhibited considerably greater basic properties than pancreatic ribonuclease on the cation exchange resin Amberlite IRC 50 (X.E.64)[8], thus in the present study it was considered that this component would have disc electrophoreted far in advance of pancreatic ribonuclease. It was demonstrated at electrophoresis on polyacrylamide gels of decreasing pore size, (Section IV, page 246), that the heat stable ribonuclease active component "B", the smaller of the two heat stable

ribonuclease active components from calf spleen, exhibited only one component which migrated in close proximity to pancreatic ribonuclease.

The molecular weight at 10,000 for the spleen ribonuclease component "B" suggested that it was this component which was partly discarded when extensive dialysis was used at the early stages of the purification study. Since the molecular weight is higher than reported[8] the brief dialysis used (page 286) with 18/32" visking tubing, which, according to the findings of Craig et al[80], does not pass pancreatic ribonuclease, appears to be less of a hazard. Beard and Razzell[188], however, made strong representation of the fact that their alkaline ribonuclease II from liver exhibited a tendency to readily diffuse through ordinary visking tubing at dialysis. Alkaline ribonuclease II was found by these workers to have a particle size smaller than pancreatic ribonuclease.

7. Conclusion.

In the present study no evidence could be found for the existence of the heat stable spleen ribonuclease enzyme of particle size M = 2000 - 5000 described previously in the paper by Kaplan and Heppel[8]. Instead two heat stable ribonuclease active enzymes were isolated from the calf spleen tissue. They are

(a) the partially purified ribonuclease active component "A" which exhibited gel filtration properties indicating a molecular weight of about 24,000 for this enzyme

(b) the purified ribonuclease active component "B" which exhibited gel filtration properties consistent with a single entity of molecular weight = 10,000.

INDEX TO THE EXPERIMENTAL SECTION

G.M.1. Procedure for the acquisition of animal tissue

Calf spleen were collected fresh from the abattoir and obtained from animals no more than one week old. The spleen were excised immediately from the newly slaughtered animals, placed in ice then transported without delay to the cold room for processing. The spleen were obtained from exsanguinated calves in the following way. The animals were suspended by the rear legs, stunned, then bled by severing the arteries and veins at the throat. The spleen were excised at the next stage when the animals were gutted before skinning. Twelve animals were dealt with in this way within 15 minutes.

The most efficient ice packing was crystal tips from a flaked ice machine (M. C. Bignell Ltd., Middlesex, England). The ice crystals were packed tightly about each spleen and after transportation, the ice persisted as a frozen block effectively encasing the material. This measure was essential as the greatest supply of spleen (100-200 per day) was at a calf market 54 miles from the laboratory. Travel and excision time extended over some six hours. The spleen were in ice for three hours thus an effective ice packing had to be obtained. Local suppliers provided sufficient spleen only for pilot programmes at 10-15 spleen per occasion. These spleen were packed in the same way and processed expeditiously.

G.M.2. Preparation for extracting and salting out of spleen ribonuclease activity at pH 3.5

Before extractions were carried out essential apparatus, reagents and buffers were at hand and functional.

Extracting buffer

0.05M sodium acetate in
10^{-3}M E.D.T.A. at
pH 7.2

The buffer was prepared by dissolving 34 g of $CH_3COONa3H_2O$ (Analar grade reagent) and 1.86 g E.D.T.A. (reagent grade) in 5 litres of deionised water. The pH of this solution was about 7 normally and required only a few drops of N NaOH to adjust the pH to 7.2. A suitable buffer stock was between 20 and 50 litres depending on the number of spleen.

Acid

1N HCl Analar grade reagent

Stock 2 litres

Re-extracting solution

0.05M sodium acetate in
10^{-3}M E.D.T.A. at
pH 3.5 made 30% saturated by the addition of
160 g ammonium sulphate per litre

The re-extracting buffer was prepared by dissolving 34g sodium acetate $3H_2O$ and 1.86 g E.D.T.A. in deionised water to a volume of almost five litres. The pH was then adjusted to 3.5 by the addition

of 5N acetic acid and the total volume taken to 5 litres with deionised water. The solution was then made 30% saturated by the addition of 800 g ammonium sulphate (Analar grade).

Stock solution 5 litres.

Dissolving buffer

0.05M sodium acetate in

10^{-4}M E.D.T.A. at

pH 7.2

A stock solution of 5 litres was prepared by dissolving 34 g Analar reagent sodium acetate $3H_2O$ and 0.186 g E.D.T.A. in 5 litres deionised water.

Stock reagent

50 Kg Analar grade ammonium sulphate.

The buffers and reagents were held at 3^{o} in the cold room at least 24 hours before the arrival of the spleen tissue.

Experiment 1 describes the general method adopted for the extraction and salting out of ribonuclease activity at pH 3.5. Experiment 2 lists the additional information which was used at preliminary extraction and salting out trails before Experiment 1 came into effect as a standard procedure.

G.M.3. Procedure at Dialysis

Cellophane tubing 32/32" 18/32" HMC from the Scientific Instrument Company were available for dialysis. The 18/32" size was used most frequently[80]. If the volume of sample was large several

sacs were made and a greater surface area was presented for diffusion purposes. According to Craig and King[61,80] the 18/32" size is less porous than 32/32" though both types have the thinnest walls of all cellophane tubing types thus the 32/32" type tubing was no longer used at the dialysis of large volumes.

Suitable lengths (60-70 cm.) of 18/32" visking tubing were soaked in 10^{-4}M E.D.T.A. pH 7 until pliable. A knot was tied at one end to seal the tube and as a safety measure a second knot was tied about 1 cm. from the first to secure against leakage. On pulling the knots tight care was taken not to stretch the cellophane on the sac side of the proposed knots. The sac was filled with 10^{-4}M E.D.T.A. pH 7.2(x2) to wash out the inside and check for flaws or pinholes in the membrane. After emptying, sufficient enzyme solution to fill sacs to 1/3rd their length, was added using a filter funnel to overcome the problem of adding solution without contaminating that region of the tubing on which the knots to complete the sac would be situated. Any air in the section above the solution was excluded and two knots tied at the open end to complete the sac. The collapsed 2/3rds of the sac was adequate space to accommodate any increase in volume by osmotic flow.

Extensive dialysis

In the extensive dialysis first used, the sacs were placed in 4-6 litres of 10^{-4}M E.D.T.A. pH 7 for 4-6 hours over a 24 hour period with many changes of equilibrating medium. This technique was altered and the dialysis made much less exhaustive. The sacs were

dialysed for a period of 6-8 hours with three charges of diffusate medium where the volume was x 5 the volume to be dialysed.

Mild agitation of the retentate and diffusate volumes during dialysis facilitates equilibration[61]. One method which was less tedious to set up, made use of a magnetic stirrer in the diffusate medium, turning at sufficient speed only to cause slight movement. The other method was to agitate the sacs using elastic bands which were sufficiently stretched to cause a gentle rocking motion. One end of an elastic band was attached to the long knot tails of both ends of the dialysis sac while the other was fitted to an eccentrically fitted ballrace arrangement which was rotated by an electric motor. The system was balanced such that sufficient tension on the elastic bands produced a slight rocking motion by the eccentric motion of the ballrace wheel. It could be argued that the tension might have been sufficient to stretch the dialysis membrane. According to Craig and King[61] stretching the membrane in a lengthwise direction reduces the porosity of the membrane quite considerably.

In all cases of dialysis of the spleen ribonuclease fractions against 10^{-4}M. E.D.T.A, either after the heat treatment or after the salting out with ammonium sulphate at pH 7.0, a brown coloured precipitate of considerable quantity developed and was removed by centrifugation at 6,000 x g for 10 minutes at 0° on the M.S.E. "Magnum" centrifuge.

Brief dialysis

A short duration dialysis was particularly useful when an

enzyme preparation had to be equilibrated against a particular buffer before its application to a column equilibrated with the same buffer. The enzyme preparation was equilibrated by a 6-8 hour dialysis involving three changes of the buffer diffusate volume which was equal to the volume of the enzyme sample on each occasion.

G.M.4. The general procedure for the acetone fractionation of the spleen ribonuclease sample

The ribonuclease active fraction from the salting out with ammonium sulphate at pH 7, i.e. the fraction precipitated when the saturation was raised from 50% to 85%, was taken up in 0.05M sodium acetate 10^{-4}M EDTA pH 7.2 then dialysed in 18/32" visking tubing sacs for 24 hours against 4 litre portions of 10^{-4}M EDTA pH 7.2 at 4° (G.M.3). Several 4 litre portions, cooled to 4° before use were available to transfer the sacs into at 4-6 hour intervals. Protein estimations were carried out on the enzyme sample after centrifugation of the dialysed solution to remove insoluble material. In the case at hand, c.f. Heppel[8], page 27 , the samples had to be freeze dried on the Edward's High vacuum freeze drying assembly to concentrate them to a suitable protein concentration (G.M.17). The lyophilised powder was dissolved in an appropriate amount of distilled water and a small fraction retained for assay.

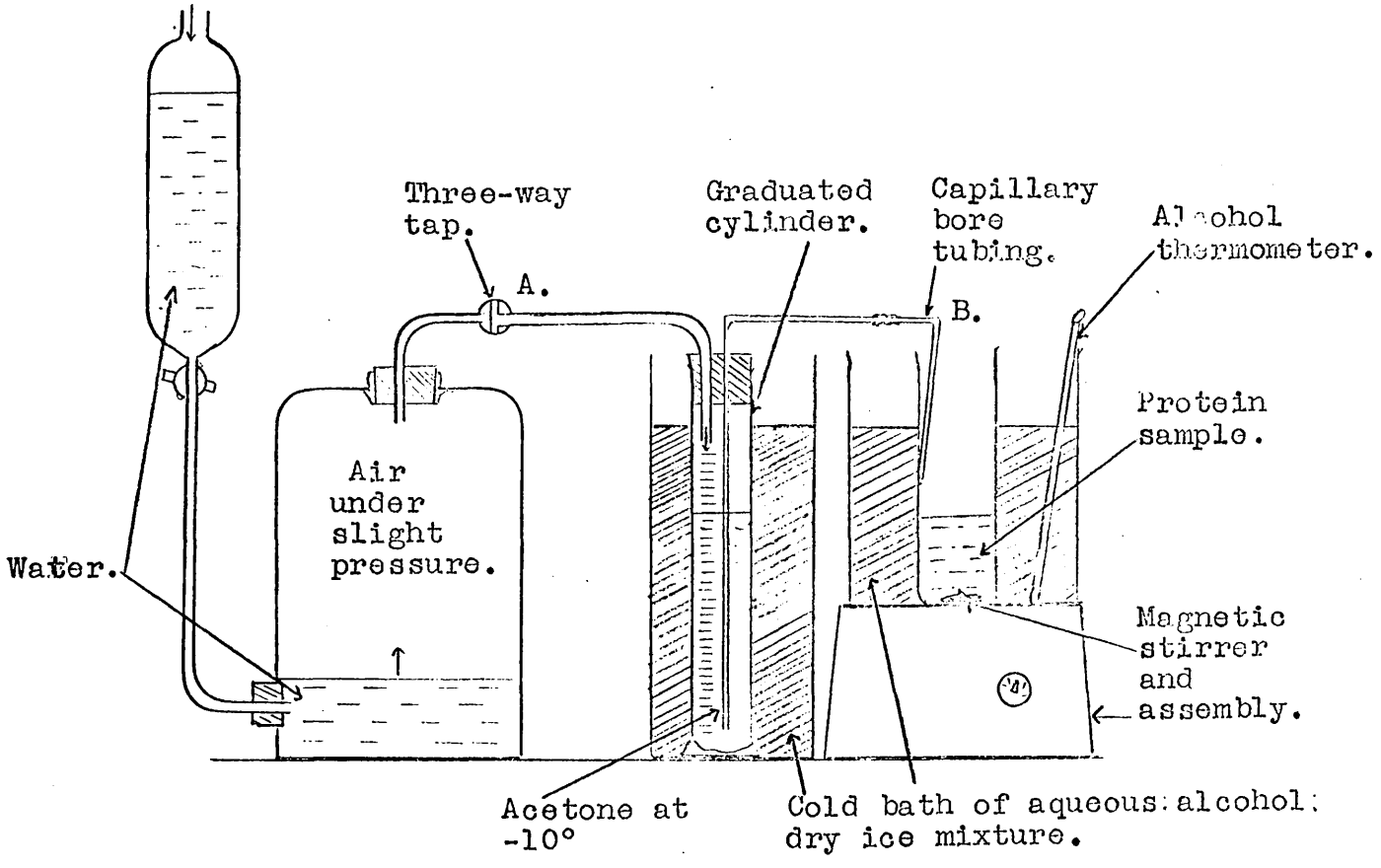
Exactly the same dilution of the protein solution with 1M sodium acetate at a 4:1 ratio, e.g. 72 ml. enzyme solution with 18 ml. 1M sodium acetate as used by Kaplan and Heppel[8] was made before the addition of cold acetone. Analar acetone held at -10° in an aqueous

ethanol-dry ice mixture was introduced very slowly at first to the protein solution which was efficiently and rapidly stirred with a magnetic stirring rod. Previous to the addition, the protein solution was cooled in a thin walled glass beaker by immersion in an aqueous ethanol-dry ice cold bath mixture at $0^{\circ} \rightarrow +3^{\circ}$. The addition of acetone at -10° began as soon as the protein solution had reached this temperature which was then brought to -15° by the addition of more dry ice to the ice bath mixture over the period of time required to add to the enzyme solution 44.4% acetone by volume. After continuing the stirring for 5 minutes to equilibrate, the mixture was transferred to stainless steel cups at -15° and the precipitate removed by centrifugation at about 13,000 x g on the M.S.E. Magnum centrifuge for 3 minutes at -15°. The amount of acetone added could be conveniently measured from the graduations on a measuring cylinder by difference and was introduced into the sample through a small capillary tube B (Figure 6) from which it was allowed to flow down the cold surface of the beaker in a thin stream before mixing in with the protein solution. The acetone in the measuring cylinder was slowly expelled by gentle air pressure induced by a head of water over a pressure reservoir and controlled at the stopcock A.

After centrifugation the supernatant from the first precipitate fraction A was transferred to the clean precipitation beaker at -15°. The precipitate, fraction A was drained at -15° for 5 minutes by inverting the centrifuge tubes, then dissolved in cold 0.05M sodium acetate 10^{-4}M EDTA pH 7 and stored in the deep-freeze until assayed.

Fig. 6.

The apparatus for fractionating protein samples with acetone.



To the supernatant at -15° sufficient acetone at -10° was slowly added with stirring until the acetone content had reached 66.6% by volume. The second precipitate, fraction B was collected by centrifugation, drained for 30 minutes at -15%, redissolved in 0.05M sodium acetate then stored in the deep freeze as described for fraction A.

The supernatant was again returned to the precipitation chamber and a third precipitate obtained by adding acetone at -10° to the solution at -15° and allowing the mixture to cool down to -20° when the acetone addition was complete at 73-75% acetone v/v. After centrifugation at -20% to remove the third precipitate fraction C, the supernatant was discarded and the precipitate after draining at -15° for 5 minutes was dissolved in cold 0.05M sodium acetate 10^{-4}M EDTA pH 7 then stored in the deep-freeze at -12° with the other fractions until required for assay determinations. The untreated sample, before 1M sodium acetate was added, fraction A precipitated with acetone 0 → 44.4% v/v at -15°, fraction B precipitated with acetone 44.4 → 66.6% v/v at -15°, fraction C precipitated with acetone 66.6 → 75% v/v at -20° were examined for protein concentration and assayed for ribonuclease activity, G.M.s 15 and 14 respectively.

A tall thin-walled glass beaker was used as the container in which to carry out the precipitation. At the slow rate of addition of acetone used, adequate control of the temperature using thin glass seemed to result. Certainly no great fluctuations in the

temperature either way were observed in the protein solution during fractionation. The maintenance of the acetone at -10° was carried out by adding dry ice (solid CO_2) to an aqueous ethanol solution (25% ethanol by volume at R.T.) which had a freezing point at -10°/-12°. This cold bath was dealt with separately from the precipitation cold bath after it proved difficult to prevent freezing in the protein solution at the early stages of acetone addition. A small quantity of acetone at -10° had to be added to the protein solution which was slightly above freezing point before the temperature could be taken below 0°. This part of the procedure proved difficult to control.

The precipitation cold bath contained 30% ethanol by volume. This aqueous ethanol solution had a freezing point $\simeq$ -15° [190] and the temperature was lowered by adding powdered dry ice to the aqueous ethanol solution. On approaching 0°C in the protein solution, cold acetone at -10° was slowly introduced and further careful additions of dry ice were made to the aqueous ethanol-dry ice mixture to slowly lower the temperature without ice forming in the protein solution. It was found in a preliminary run that ice formed on the beaker wall if the temperature was lowered too quickly initially. This effect could alter the precipitation conditions entirely and care was taken to prevent ice formation.

The precipitate, fraction B was obtained at a constant temperature of -15° and presented no problem in controlling the temperature. The precipitation of fraction A_1 where the rate of addition of acetone

and the lowering of the temperature coincide, posed a problem on accuracy and reproducibility. By the time -15° has been reached all the acetone required to precipitate this fraction had been added. No real control was possible at the initial addition and subsequent rate of addition of acetone in relation to the fall in temperature.

A further volume of absolute alcohol was added to the aqueous ethanol-dry ice mixture to raise the ethanol content to 40% (this gives a freezing mixture of -23° 190) while the second fraction was in the centrifuge. The temperature was taken to -15° by the addition of dry ice and in a similar manner to the first fractionation the temperature was allowed to fall to -20° during the addition of sufficient cold acetone (-10°) to bring the acetone concentration of the protein solution to about 75% v/v.

The addition of acetone at effectively a dropwise rate required 1-1½ hours for each fractionation. All manipulations at centrifugation and in dissolving the precipitates were hastily carried out to minimise risk of denaturation of the protein samples by inadvertent temperature rise. Three pilot trial experiments were carried out by the procedure described and are reported in Experiments 6, 7 and 8.

G.M.5. Procedure for the preparation of Sephadex and molecular sieve chromatography

i. Preparation of the Sephadex gel

The dry powder of the Sephadex type required, was suspended in a x 10 excess of the equilibrating solution. The Sephadex powder was added to this solution with stirring to prevent clumping. Continuous

stirring over three days permitted the gel to swell but the time depended on the hydrophilic properties of the gel. The greater this was then the longer the time necessary to swell the grains. To equilibrate, wash and remove fine particles, repeated sedimentation and decantation were carried out with a x 4 excess of the equilibrating buffer. At each decantation any persistent fines were carefully removed. It proved beneficial to deaerate the suspension under reduced pressure before use. These operations were always carried out at the temperature of the chromatographic operation.

ii. Storage and recovery of the gel

Initially Sephadex was stored in suspension in screw cap bottles to which sufficient chloroform was added to more than saturate the aqueous solution. Before use the suspension was treated as described under preparation of the Sephadex gel above. The preservation method with chloroform was replaced by storing the gel suspension in the buffer solution made 0.2% with sodium Azide[191].

Dry Sephadex powder was recovered by suspending the gel in a x 10 volume of deionised water x 6 allowing time to settle and decant on each occasion. This wash was followed by suspending the gel x 3 in a x 3 excess of absolute alcohol with constant stirring on each occasion before decanting the supernatant. The dehydrated Sephadex was spread out on a clean hard filter pad (Whatman No.50) on a large watch glass, covered to prevent contamination by dust particles, then placed in an oven at no more than 80°[59] to dry over several days.

iii. Preparation of the Sephadex columns

A. Early columns

Some indication of suitable column dimensions could be ascertained from the recommendations published in the Sephadex manufacturer's literature[110] and published reports[101,129,130,199]. Parallel walled glass columns which were longer than the effective gel length required and scrupulously clean, had 1mm. or 2mm. bore interkey stopcocks at one end and quick-fit female joints at the other. Prior to packing with buffer, equilibrated Sephadex columns were placed in a vertical position using a spirit level in two planes at right angles to each other. With the stopcock closed equilibrating solution was added to half fill the column and a small plug of glass wool inserted above the stopcock at the point the tube narrows. Air bubbles were excluded. A large filter funnel was attached at the uppermost end, Figure 15A, page 296 , and the entire column and to half the depth of the funnel filled with equilibrating solution. An electric stirring device was fitted above the funnel and regulated to stir the funnel contents smoothly. The vertical alignment of the column was rechecked and portions of the prepared gel added at intervals to the funnel where the gel became freely suspended by the stirring action. Particle sedimentation was slow and even, because of the stirring action and narrow bore of the funnel stalk. When the height of the gel column reached 2-3 cm. the stopcock was opened slightly at first then increased with increase in gel length to permit an even buffer flow. During the packing process a rising horizontal gel surface indicated

a uniformity in packing. When the column of gel had reached 1-2 cm. above the required length the unused gel and buffer were removed by a siphon arrangement from the funnel which could then be disconnected. The solvent buffer reservoir supply tube was then connected and the reservoir placed at a position on the same level as the column. A buffer head of 4 cm. was allowed on the gel surface and care was taken to ensure the supply tube was charged with solvent. Generally the column tap was opened fully and the flow rate controlled by raising or lowering the reservoir until a suitable rate was obtained. If the flow rate was too fast then the tap was adjusted. After the gel had settled with washing a disc of Whatman No.54 filter paper cut to the internal diameter of the column was placed on top of the gel to protect the surface.

B. Later columns

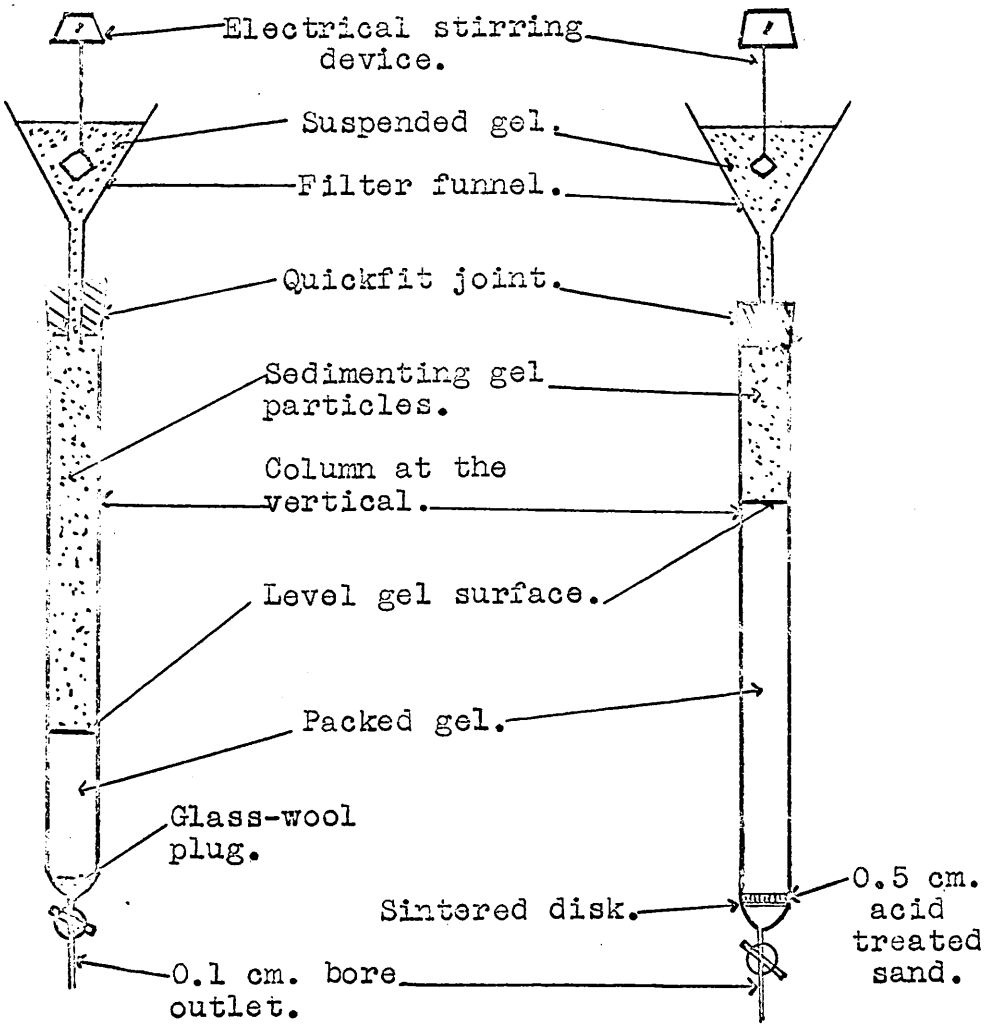
In later columns the glass wool plug was replaced by a sintered glass disc of porosity 1 fused inside the tube just above the stopcock[119], Figure 15B, page 296 . To prevent the gel particles blocking the sinter pores a 0.5-1 cm. layer of acid treated sand was added prior to the gel suspension. The presence of the sintered disc caused a small reservoir to accumulate above the stopcock by the end of each run. Before each new run this reservoir was displaced by air from a polythene syringe by removing the stopcock tap. Acid treated sand was prepared by digesting white quartz sand for 1 hour with concentrated sulphuric acid on a Kjeldahl digestion unit to remove organic contaminants. The mixture was diluted by emptying into a

Fig. 15

Sephadex columns and the method of packing with gel.

Column A.

Column B.



large excess of deionised water then the sand was washed free of acid and salts by repeated suspending and decanting before washing on a buchner with deionised water. The sand was oven dried at 100° then stored in a sealed glass bottle.

Columns were eluted with equilibrating solvent for several hours when the gel was Sephadex G-25 and for much larger periods of time with the other Sephadex types until the effluent solution had the same pH and UV absorbency as the eluent solution.

iv. Application of the sample

Every attempt was made to ensure the sample did not contain insoluble material which would contaminate the Sephadex gel.

Method a

The early column runs were dealt with by this method. The solvent reservoir supply tube was disconnected at the column quickfit joint and the buffer head allowed to drain from the column until the solvent level reached the filter disc on the gel. The protein solution was then placed on the filter pad at 1.0-1.5 ml. taking care not to contaminate the inside wall of the column. The column tap was opened and the sample percolated into the gel. The tap was again turned off and 1 ml. solvent was applied to wash residual protein completely into the gel. The washing on process was now repeated x 2 with 2 ml. samples of solvent, then the column head filled with buffer to 4 cm. before the reservoir supply tube was attached. Care was taken to ensure air was excluded from the tube and the buffer head remained at 4 cm. The entire column assembly, with fraction

collector was fully operational before the sample was applied. This prevented disturbance of the gel or spreading of the sample by diffusion which could be caused by the delay in sieving.

Method b

In later column work the sample application technique was considerably improved. The protein solution was layered on top of the gel between the gel surface and solvent head by displacement of the solvent. The sample layer was introduced by means of a specially made applicator device obtained by converting a pipette at the blow out end which was elongated to a capillary of 6 cm. length. The extremity of this capillary region was turned at right angles and constricted at the bend to help retard the outflow of sample solution. The extremity was funnelled to prevent turbulence as occurs with the rush of solution from a small bore nozzle. The protein solution, which was made sufficiently viscous by adding sucrose at 5 mg. per ml. if needed, was drawn into the applicator and the capillary end cleaned of excess sample with an absorbent tissue. After the reservoir supply tube was disconnected the pipette was introduced into the solvent head of the column until the funnelled capillary almost touched the gel particles, Figure 16, page 299, and the solution was slowly pipetted on to the column bed to form a distinct band 1 cm. deep. The amount of sample applied could be measured exactly on the pipette which was removed with the minimum of mixing or contamination of solvent with sample solution. The charged reservoir supply tube was reconnected and the column outlet tap opened. The filter disc used to protect

Fig. 16.

The method of applying samples to sephadex columns

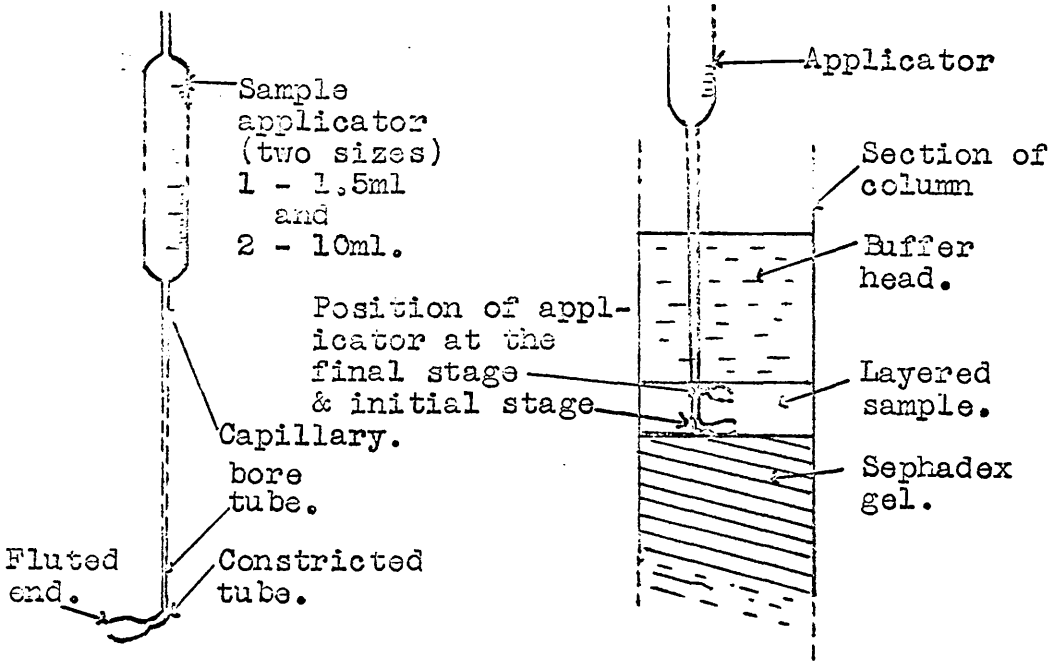
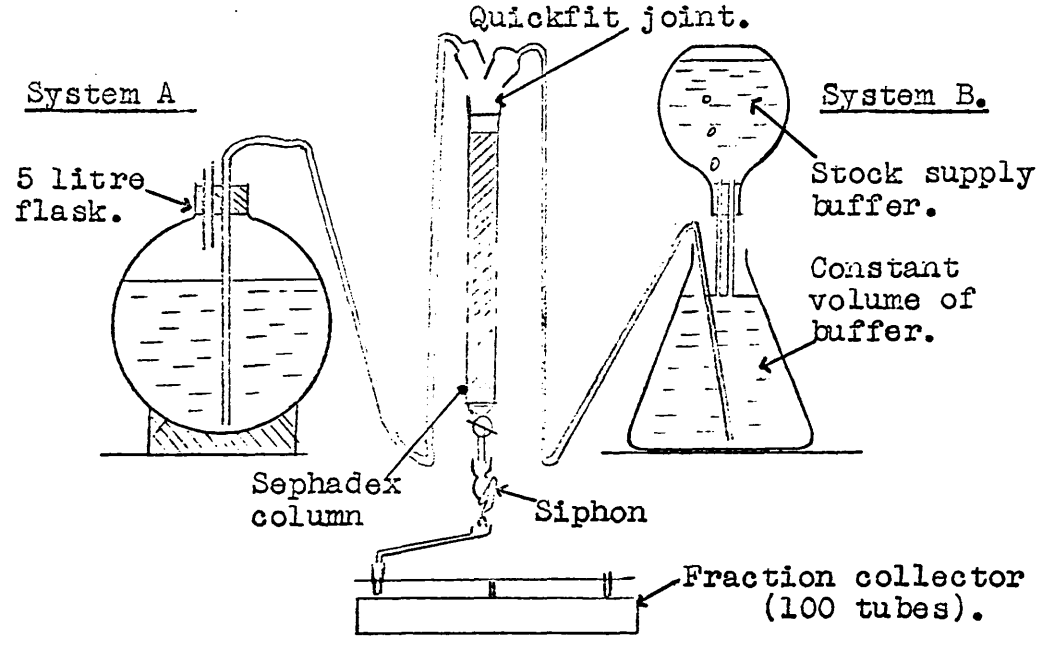


Fig. 17.

The column assembly for eluting sephadex at gel filtration.



the gel of earlier columns was excluded in later columns where the sample solution was layered under the solvent.

Some important points of procedure not stressed already must be mentioned. Precautions were taken to avoid an unnecessary positive hydrostatic pressure at all times. This prevented slow flow rates as the result of over compressed gels. Column medium, test samples, buffer solvents and apparatus were prepared at the operational temperature of the exercise. This prevented the formation of air bubbles in the column and variation in the flow rate. It was found that after a column was packed and a suitable hydrostatic pressure attained by adjusting the reservoir level, a period of preliminary elution was necessary to stabilise the gel. This was necessary, particularly after the top 1-2 cm. of a column was replaced by fresh gel when this region became discoloured or the surface required levelling, i.e. after levelling the surface by stirring the top 1-2 cm. into suspension it was necessary to elute with solvent to settle the surface firmly before the next sample solution could be satisfactorily layered on.

v. Column technique

To obtain comparable elution patterns column effluent was collected in the first tube, or empty siphon over the first tube in the fraction collector at the point the sample solution layered on the gel surface just entered the gel column. When the intention was to compare elution patterns every attempt was made to maintain the same elution rate from trial to trial. The volume of the eluting solvent in the single reservoir vessel diminished as the elution

progressed and the flow rate slowed up slightly as the pressure dropped. In several column experiments no attempt was made to rectify this irregularity except that the flow rate conditions were standard between runs, i.e. when a total standard volume of three litres was available in the solvent reservoir for a 250 ml. elution, at the end of each comparable elution the 250 ml. or sufficient solvent to return the total volume to three litres was added. With a reservoir vessel of appropriate shape the buffer level did not fall by more than 2 cm. when the volume was reduced by 250 ml., Figure 17A, page 299.

It was appreciated that over a longer period and larger elution volume of e.g. 2 litre quantity the rate of flow would decrease appreciably towards the end of the run. It was possible to overcome this and hold the volume constant, by clamping an inverted supply vessel of suitable volume over the reservoir at a convenient level to the reservoir buffer surface into which the only two outlet tubes from the buffer supply vessel were fractionally dipped. As the reservoir buffer level fell at elution by a fraction sufficient to break contact between the buffer solvent surface and one tube, the buffer level was automatically readjusted to the original level by the entry of sufficient air to the supply vessel to replace only the solvent displaced, Figure 17B, page 299.

Any local accumulation of solvent, e.g. that caused by an excessively large glass wool plug or large bore tubing at the stopper end of the column, were eliminated or reduced to a minimum. With the

sintered glass disc of gel support a small reservoir of buffer collected between the disc and the stopper and could contain as much as 7 ml. with the larger Sephadex columns. This dead volume was removed before each run to prevent excessive mixing and to maintain as precise a fraction content as possible.

Fractions of 1-2 ml. were collected on the Locarte automatic drop counting fraction collector while 3 ml., 10 ml. and larger volumes were secured by the Towers Gallenkamp automatic siphon balance fraction collector. To obtain an efficient siphon action the glass siphon had to be scrupulously clean. It was essential to guard against siphon inaction when the protein concentration of the effluent was high, especially if the siphon had a small bore outlet tube. With a 3 ml. siphon in particular a continuous flow could result over several fractions. The 3 ml. siphon was delicately balanced against a counter weight on the turntable switch device. With this small scale siphon assembly ordinary sized drops rocked the balance and on occasions could repeatedly activate the switch mechanism out of turn. It was essential to reduce the drop size by fitting a glass capillary point to the end of the column.

vi. Column preservation

Between each run, or at longer intervals a layer of buffer solvent made 0.2% with sodium Azide w/v and 4M with NaCl was applied to the column similar to a protein sample and eluted with buffer. This measure was intended to curb microbial contamination and remove any protein which was adsorbed. Columns not in use were stored equilibrated

with buffer containing 0.02% sodium Azide which was removed by eluting with equilibrating buffer before the columns were reused.

G.M.6. Large scale gel filtration on Sephadex G-75

100 g Sephadex G-75 Lot No. To 5079 in bead from, particle size 40-120μ, water regain 7.5±0.5 g/g dry gel, bed volume 12-15 ml per g dry gel and supplied by Pharmacia was used for column chromatography by gel filtration. The gel was prepared by the method described as G.M.5, page 292 , where the dry powder was allowed to swell over a period of two days in 0.05M tris HCl 10^{-4}M EDTA pH 7.5 as equilibrating buffer. A gel column of effective length 5 cm. i.d. x 75 cm. was packed by the procedure G.M.5 part iii and was supported on an acid washed sand covered disc of porosity 1. The flow rate was controlled adequately by adjusting the height of the buffer reservoir containing 5 litres 0.05M tris HCl 10^{-4}M EDTA to a position where the top of this buffer solution was 36-38 cm. below the level of the gel column top surface. The column was eluted with the equilibrating buffer for two days until the column stabilised and the pH and optical density of the effluent and eluent were the same. The samples were applied, the column developed and controlled as described in the procedures G.M.5, iii, iv, v.

G.M.7. Ion exchange column chromatography

(a) Preparation of the cellulose ion exchanges for column chromatography

i. DEAE cellulose

D.E.A.E. cellulose (Whatman powder DE50) was cyclised according

to a procedure described by Peterson and Sober[158]. The powder was allowed to sink into 1 litre 1N NaOH and the mixture stirred to suspend the exchanger. The suspension was centrifuged at 400-500 x g for 5 minutes at R.T. and the supernatant discarded. The wet pad was suspended x 2 in deionised water and the supernatant after centrifugation discarded on each occasion. The D.E.A.E. cellulose was resuspended in 1N HCl, centrifuged then washed x 2 with deionised water by the method described for 1N NaOH treatment. The process was repeated again, then the ion exchanger was finally suspended in N NaOH, centrifuged then washed x 4 with deionised water until almost neutral by resuspending and centrifuging to discard the washings. The ion exchanger was then suspended in x 10 volume of deionised water and after permitting a 30 minute settling time the cellulose fines in suspension were decanted with the supernatant. This process of eliminating fires was repeated x 2 before the ion exchanger was equilibrated with the required buffer. Equilibration was carried out by suspending the ion exchanger in x 10 volume of the required buffer, stirring for 30 minutes then allowing 30 minutes for the ion exchanger to settle before decanting the supernatant. The equilibration procedure was repeated x 2 before the slurry could be filled into a column by G.M.7e. D.E.A.E. columns were eluted with 200 ml. of equilibrating buffer made 2M with NaCl to remove any residual coloured matter from the medium. The columns were then eluted copiously with equilibrating buffer until the pH and OD of the effluent was similar to that of the elutant and the column was free of NaCl. Columns

repeatedly used for a single purpose could be regenerated by washing with 2M NaCl in the equilibrating buffer to remove residual protein. Where a new procedure was the intention the column was dismantled and the ion exchanger resuspended in N NaOH only, then the deionised water and equilibrating buffer washing process repeated before the ion exchanger was filled into the column again.

ii. C.M. cellulose

Carboxy methyl cellulose (Whatman powder C.M.70) was cyclised according to a procedure described by Peterson and Sober[158]. An appropriate quantity of C.M. cellulose was allowed to sink into a x 3 excess 0.5N NaOH-0.5M NaCl solution and the particles suspended by stirring for 30 minutes. The supernatant was decanted after centrifugation at 400-500 x g for 5 minutes. The ion exchanger was resuspended x 2 in a x 3 excess of deionised water by volume to wash the exchanger and the centrifugation repeated on each occasion. The ion exchanger was now treated with a x 3 volume of 0.5N HCl-0.5M NaCl, the mixture stirred for a very short interval then centrifuged and the acid supernatant discarded. The ion exchanger was washed with deionised water as already described. The treatment with 0.5N NaOH 0.5M NaCl was repeated x 2 with a deionised water wash between, then the ion exchanger suspended in a large excess of deionised water, allowed to settle then the supernatant discarded by decantation. Suspending and decanting in large volumes of deionised water was repeated until the solution was almost at neutral pH. A short centrifugation facilitated the speed at which washing could be carried out through-

out the treatment. The C.M. cellulose was finally suspended in x 5 volume of equilibrating buffer made 1M with respect to NaCl and several decantings and suspendings made with this solution to equilibrate the exchanger and remove any persistent "fines" suspended after a 30 minute settling time. The ion exchanger was filled into the columns then eluted with the equilibrating buffer containing 1M NaCl, then equilibrating buffer without NaCl until the pH and optical density of the effluent was the same as the eluant and the column had been washed free of NaCl. After the exchanger had been used the column was regenerated by washing with 1M NaCl in the starting buffer, then with starting buffer without NaCl until the column was free of NaCl.

Any change in the use of the ion exchanger meant that the column had to be dismantled and the ion exchanger treated with 0.5N NaOH-0.5M NaCl, washed with deionised water then re-equilibrated with the required starting buffer made 1M with respect to NaCl as described for the latter part of the cyclising procedure before the column was refilled.

b. Preparation of C.M. Sephadex for ion exchange chromatography

C.M. Sephadex C.50, medium grade, lot No. To 160M capacity 4.7 m.eq./g dry Sephadex was cyclised in a manner very similar to that for C.M. cellulose above. The dry powder was allowed to swell in an excess of deionised water for two hours with mechanical stirring to facilitate dispersion then the same cyclising treatment followed. Suspensions were centrifuged slowly at 400-500 x g at R.T.

for 5 minutes to speed up the treatment as vacuum filtration proved difficult to perform satisfactorily. Equilibration with the appropriate starting buffers were made as described for C.M. cellulose except that 1M NaCl was excluded. To recyclise the ion exchanger the columns were dismantled and the exchanger treated with 0.5N NaOH-05M NaCl, washed with deionised water then re-equilibrated as already described for the same purpose with C.M. cellulose.

c. Preparation of the ion exchange column

The column procedure for molecular sieve chromatography has already been presented, G.M.5, iii, and many of the techniques for the preparation of ion exchange columns are similar in detail to the methods described in this procedure. All the columns were parallel walled glass tubes with a tap at the lower end, a quickfit B24 joint at the uppermost end. The columns were plugged with glass wool at the tap end to support the exchanger while filter discs of Whatman No.17 filter paper cut exactly to the internal diameter of the columns were placed at the uppermost end to top the exchanger surface. This latter measure prevented disturbances in the top 2cm. of ion exchanger at sample application or solvent elution. The filter funnel technique, Figure 15, page 296, was used to pack the equilibrated ion exchange medium into the columns to the required dimension. This method entailed the natural sedimentation of ion exchange particles by gravity while a thin air free slurry was dispersed by stirring in the funnel. The C.M. cellulose slurry was packed in equilibrating buffer when a suitable hydrostatic pressure was applied at elution with starting

buffer and gradient. NaCl causes cellulose particles to shrink[86] and have less of a gel like property thus the columns were packed in the presence of M NaCl. In this way the columns were uniformly packed with preshrunk cellulose and after the NaCl had been removed by eluting with the equilibrating buffer there was little change in the overall dimension of the columns with only a slight drop in the flow rate.

The buffer volume above the filter pad and any void spaces where excessive solvent could collect and mix at the end of the column in the region of the glass wool and tap were kept to a minimum to prevent unnecessary mixing spaces. Column dimensions ranged from 1 x 30 cm. to 1 x 60 cm. depending on the purpose of the experiment. Adjustment to diameter and length were made when the amount of sample applied varied or increase in resolution was required. The narrower the column the greater the possibilities of efficient resolution with less tendency to produce skewed bands on elution[86].

d. Preparation and application of the enzyme sample

If not already in solution the enzyme samples were dissolved in the required volume of starting buffer then adjusted to the pH of the starting buffer, The solutions were then equilibrated against an equal volume of starting buffer by dialysis for short periods with three changes of equilibrating solution, G.M.3. It was considered that equilibration with the starting buffer was essential when any of the components of the sample were only loosely adsorbed under the starting buffer conditions. When fairly large volumes had to be

applied it proved essential to have conditions where the components were tightly held. Before the solutions were applied insoluble material was removed by centrifugation.

Small sample volumes were applied, after the head of starting buffer at the column top had sank to the level of the filter pad, by carefully pipetting the solution on to the column top and allowing it to sink in evenly to ensure an even distribution at adsorption. On reaching the filter pad level several small portions of starting buffer were then pipetted on in turn to wash the residual solution into the exchanger. Larger volumes of several hundred ml. of protein solution were applied from a small reservoir over the column then washed on with starting buffer. All samples were applied by gravity flow and care was taken to ensure the columns never ran dry. Sufficient starting buffer was allowed to wash through the column to remove components which were not adsorbed.

e. Column procedure

Starting buffer from the wash reservoir or from the gradient assembly systems were fed into the column top from a suitable length of tubing precharged with starting buffer to prevent air locks or the column top going dry. On overnight runs care was taken to ensure the loop of the feed tube passed below the level of the column outlet as a further precaution against the column going dry from lack of solvent. Samples from 2.5 ml. to 50 ml. of effluent were collected on the Towers automatic siphon fraction collector. Efforts to prevent the column altering in dimension, especially by compaction

from too high a positive hydrostatic pressure with the buffer head in the reservoir chambers, are explained at the Sephadex column procedure G.M.5, and applied equally to the cellulose ion exchange columns.

Flow rates varied with the column dimensions used, type of elution system, and the hydrostatic pressure produced by adjusting the height of the buffer reservoirs relative to the column outlet. Minor variations were observed depending on the salt concentration as this regulated shrinking and swelling of the medium and the efficiency of packing. Flow rates are recorded in the experimental for each run and were adjusted at the column tap finally to a flow rate considered suitable for the resolution required. At the same time flow rates were not slow making the elution period excessive.

f. Gradient elution

i. Linear system[104, 105]

A linear gradient of progressively increasing NaCl concentration over 1 litre was obtained by the system depicted in Figure 35, page 312. Reservoirs A and B were identical shaped conical flasks mounted at the same level. A hydrostatic equilibrium was maintained between the two reservoirs by a prudent choice of flow rate consistent with an equal fall in the contents of both chambers. The gradient could be traced linearly over 1 litre from zero NaCl concentration to the final salt concentration.

Mixing Reservoir A:- Initially contained 500 ml. of starting buffer with zero NaCl.

Stock Reservoir B:- Initially contained 500 ml. of the same buffer as A made the required final salt concentration for the gradient.

Practical points

A screw clip was fitted to the polythene connecting tube and was not released until the gradient was started or the solvent level in reservoir A had fallen to exactly that of reservoir B. When setting up the gradient care had to be taken to ensure air bubbles in the reservoir interconnecting tube were removed. This prevented any tendency to have an air-lock. A stirring motor which would stir at a slow and even rate, especially over prolonged periods. This measure prevented the possibility of solvent mixing between the reservoirs as a result of variations in the miniscus and level caused by large fluctuations in the stirring speed.

ii. Convex system

Convex gradient elution as first described by Alm et al[91] was obtained by the system in Figure 36, page 312. The mixing chamber A' contained a fixed volume 400 ml or, with the x 10 increase in dimensions, 4 litres of the initial eluting solvent. The stock solution chamber B' contained a solution of the solvent at that concentration or eluting strength finally required by the gradient at the end of elution on 900 ml. or 9 litres. Eluent from chamber A', the contents of which were stirred continuously with a magnetic stirrer, was automatically replaced by the stock solution from chamber B' and in this way caused the concentration to the eluent to

Fig. 35.

The linear gradient elution assembly.

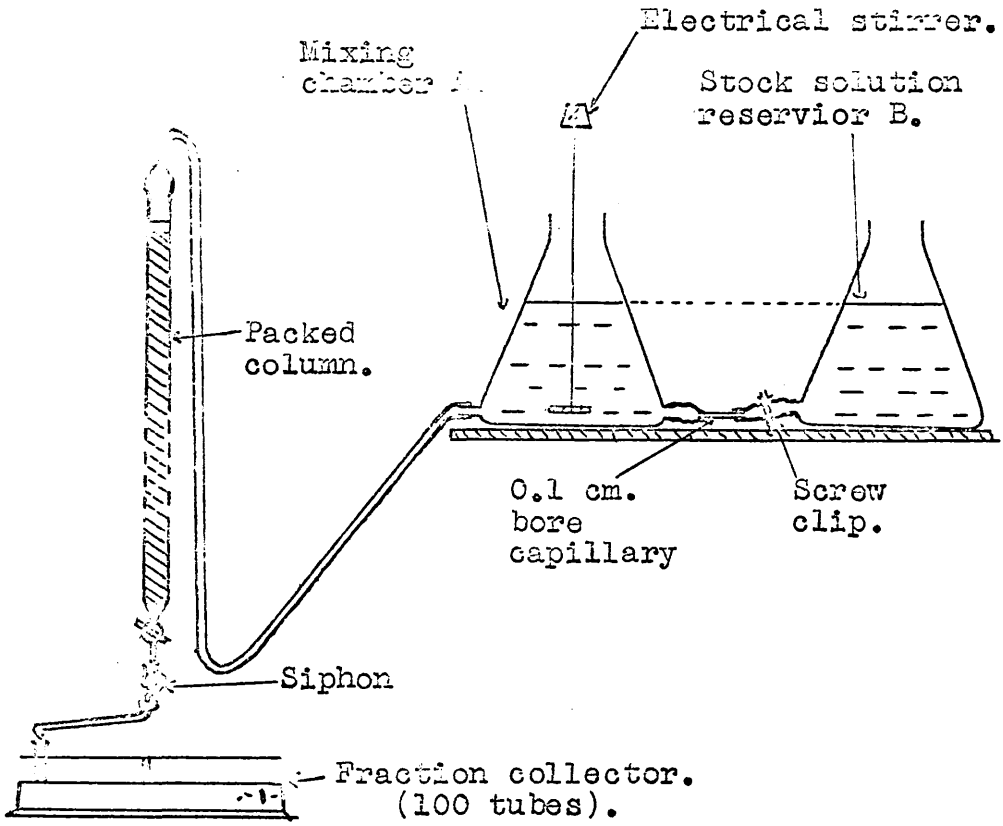
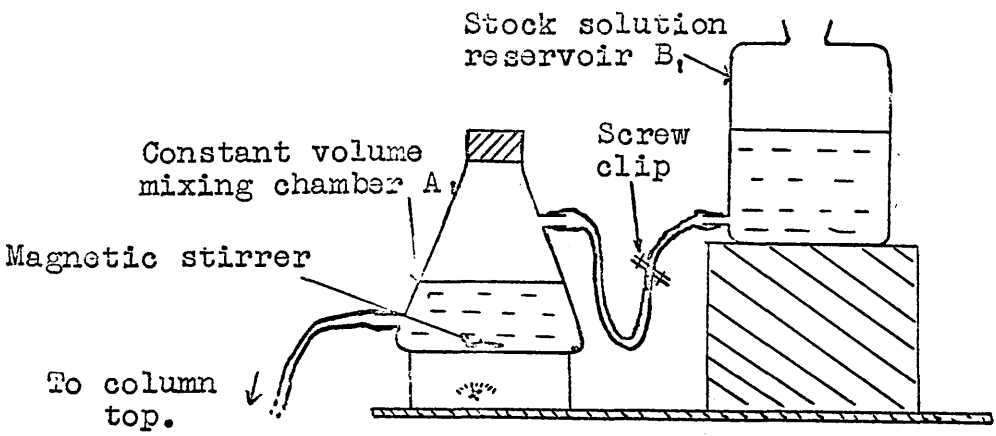


Fig. 36.

The convex gradient elution assembly.



increase in a "convex" manner. Chamber A' must be airtight. Care was taken to site the chamber B' at an optimum level to chamber A' as shown in the figure. The volume of the connecting tube was left free and filled up with stock solution when the screw clip was opened at the start of the gradient. When the small air space in the sealed mixing chamber was compressed the volume of the tube was filled first thus the mixing chamber did not receive a sudden increase in salt from stock solution in-flow.

The convex gradient could be calculated for a particular situation and the relationship of the variables have been expressed as a differential equation by Cherkin, Martinez and Dunn[93]. The concentration C of the eluant leaving the fixed volume mixing chamber could be calculated from the formula:

$$C/Co = \frac{e^k - 1}{e^k} \quad \text{or} \quad C = Co\left(1 - \frac{1}{e^k}\right)$$

Co = concentration of the stock solution entering the fixed volume mixing chamber

k = ratio of the volume of effluent collected to the fixed volume in the mixing chamber

The ratio C/Co for the different values of k have been plotted by Cherkin et al Figure 38. A slight inaccuracy existed in that the dead volume of the column should be taken into consideration when the volume of effluent collected was related to the concentration of the effluent leaving the mixing chamber.

A convex gradient is illustrated, Figure 37, page 314, and the

Fig. 37.

Comparison of the elution profiles for the linear and convex gradients from zero to 0.3M NaCl over 1.0 litre.

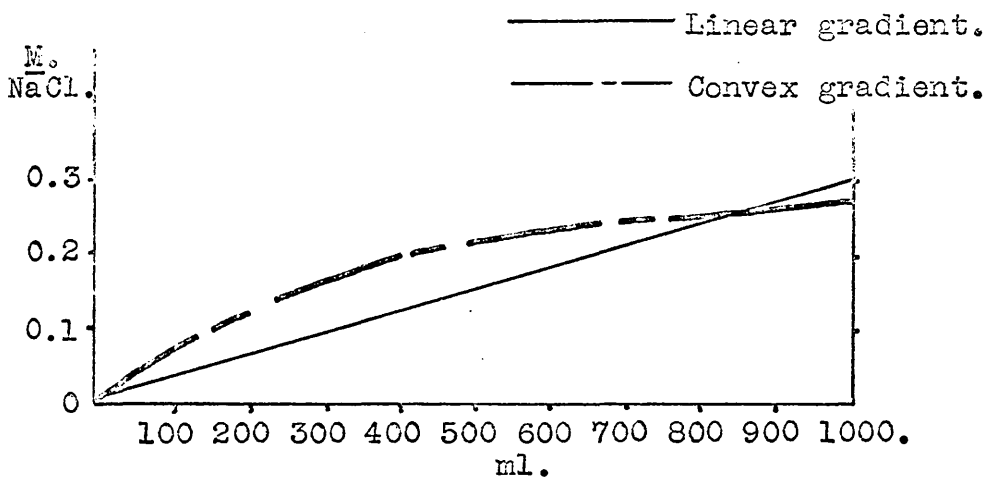
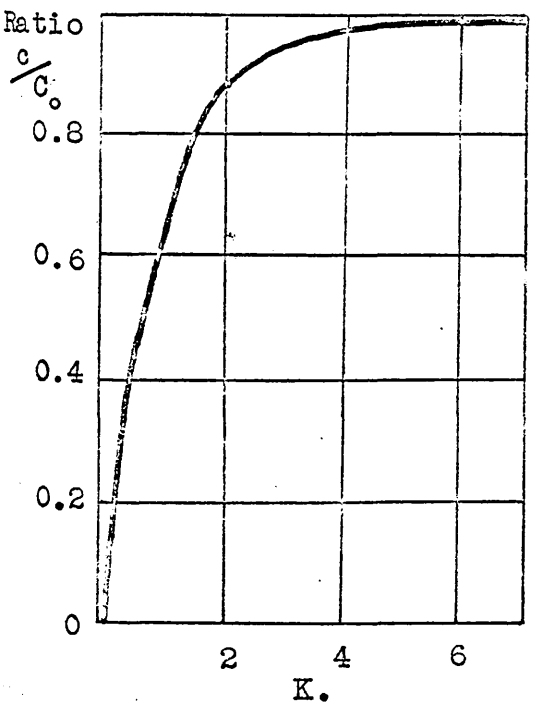


Fig. 38.

The graphic expression for convex gradient elution as devised by Cherkin, Martinez and Dunn.[93]



c = Conc. of elutant leaving the constant volume mixing chamber.

C_o = Conc. of the stock solution entering the constant volume mixing chamber.

K = ratio $\frac{v}{V}$ where

v = Volume of solution which entered the constant volume mixing chamber.

V = Volume of the constant volume mixing solution.

corresponding linear gradient drawn for comparison over the same span of zero NaCl to 0.3M NaCl. Where the final salt concentration Co was 0.3M NaCl, C was zero at nil elution and the fixed volume 400 ml, k could be calculated for the various stages of elution, then these values substituted in the formula above to give values for the salt concentration at each stage. If the initial salt concentration was not nil in the fixed volume mixing chamber at zero elution of the gradient then the modified formula: $C = Co - De^{-k}$ was used where $D = (C_o - C_1)$ and C_1 was the starting salt concentration in the mixing chamber[86]. All columns were run in the cold room at 0-4°. Equilibration of the ion exchangers, packing of the columns, preparation of the enzyme samples for chromatography and application of the sample were carried out at 0-4°.[162]

g. Treatment and storage of fractions

Low volume ribonuclease active fractions were bulked then stored in the deep freeze at -12° in sealed containers. Others were briefly dialysed, freeze dried on the Edwards High Vacuum freeze drier system, G.M.17, then stored as a white powder in sealed bottles at -12°. Bulky volumes were concentrated on small C.M. cellulose columns after a brief dialysis, G.M.8d. The ribonuclease active components were desorbed in the minimum volume possible and stored in solution at -12° in the deep freeze.

G.M.8. Ion exchange column system

a. Column II. The D.E.A.E. cellulose column pretreatment of the spleen enzyme preparation

A D.E.A.E. cellulose column 1.8 cm. i.d. and 24 cm. effective length, equilibrated with 0.005M tris HCl 10^{-4}M EDTA pH 7 was prepared by the procedures set out in G.M.7 (a)i. Spleen ribonuclease active samples bulked from the effluent of the G-75 Sephadex column G.M.6 (preparative column I) were passed through the D.E.A.E. cellulose column at a flow rate of 60-70 ml. per hour, after the bulked fractions were adjusted to pH 7 with 1N HCl. The effluent was collected in 25 ml. fractions and after the D.E.A.E. cellulose was washed with 80-100 ml. of equilibrating buffer, the fractions containing the spleen ribonuclease activity were bulked then taken to pH 6 in preparation for column III.

Inactive protein and other polymeric substances retained on the column were removed by eluting with 2M NaCl in 0.005M tris HCl 10^{-4}M EDTA pH 7 and rejected. After a period of washing, with equilibrating buffer, until free of NaCl the column was primed for a further pretreatment.

b. Column III. The preliminary fractionation of the spleen enzyme preparative scale C.M. cellulose column

The C.M. cellulose column 3 C.M. i.d. x 64 cm. effective length, equilibrated with 0.005M tris HCl, 10^{-4}M EDTA pH 6 was prepared and made operational by the procedures set out in G.M.7(a) iii, (c). The column was eluted for several days with 8-10 litres of equilibrating

solution until the pH and O.D. of the effluent and eluent were identical. Before the spleen enzyme preparation was applied to the column it was briefly dialysed, G.M.3 and page 286 , against the equilibrating solution 0.005M tris HCl 10^{-4}M EDTA pH 6. After dialysis the enzyme solution was applied to the column from a special reservoir at a flow rate of 125-150 ml. per hour. The column was eluted copiously with 1.5 litres of equilibrating solution to wash on the sample completely and remove any material not adsorbed by the exchanger. When the effluent had returned to and maintained the original pH and O.D. the convex gradient system was applied, Figure 36, page 312.

The constant volume mixing chamber A' contained 4 litres of 0.005M tris HCl 10^{-4}M EDTA pH 6. The supply reservoir B' contained the stock solution 0.005M tris HCl 10^{-4}M EDTA 0.3M NaCl pH 8.2. The supply reservoir contained 5 litres of stock solution at the start of the elution and was replenished at regular intervals to the 5 litre mark. At the completion of the gradient, when 7-7.5 litres had left the supply reservoir, the column was eluted with 2 litres 0.005M tris HCl 10^{-4}M EDTA pH 8.2 1.3M NaCl to remove residual protein not displaced by the gradient.

50 ml. fractions were collected at a flow rate of 150 ml. per hour on the Towers automatic siphon balance fraction collector. The elution pattern was constructed by examining the fractions collected according to G.M.15 (c) iii. Spleen ribonuclease active fractions were located by the assay technique described in G.M.14 and estimated

as O.D. active units.

The large C.M. cellulose column was used several times provided no air bubbles appeared as a result of temperature variation when the cold room failed to function. After a wash with 2 litres of equilibrating solution made 1.3M with NaCl to remove residual protein, the column was eluted with 8 litres of equilibrating solution ready for the next application of enzyme solution. On occasions the top 2-3 cm of cellulose exchanger was removed and replaced by fresh exchanger when this region became discoloured. This was carried out midway through the 1.3M NaCl wash.

c. Column IV. Rechromatography of the ribonuclease active fractions obtained by the preparative scale column III on C.M. cellulose at pH 8.

A C.M. cellulose column 2 cm. i.d. x 24 cm. effective length, equilibrated with 0.005M tris HCl 10^{-4}M EDTA pH 8, was prepared and made operational according to G.M.7(a) ii, (c). The column was eluted first with 500 ml. equilibrating buffer made 1M NaCl then washed with 2 litres equilibrating buffer.

The ribonuclease active fractions from column III pertaining to particular peaks were bulked, adjusted to pH 8 with 1N NaOH, briefly dialysed against the equilibrating buffer to reduce the NaCl concentration G.M.3 then applied slowly to the column at 30 ml. per hour. Since the volume applied was large ($\simeq$ 800 ml.), 50 ml. fractions were collected at this stage and the sample washed on finally with $\simeq$ 500 ml. equilibrating buffer until the effluent

recorded a pH and O.D. similar to the eluent solution.

The convex gradient applied to develop the column consisted of 400 ml. 0.005M tris HCl 10^{-4}MEDTA in the constant volume mixing chamber A' and 1 litre of 0.005M tris HCl 10^{-4}MEDTA 0.3M NaCl pH 8.0 in the stock solution reservoir B', Figure 36. After 800 ml. had eluted the column the gradient was discontinued and replaced by 500 ml. equilibrating buffer containing 1.3M NaCl to remove residual protein. The column was then eluted copiously with equilibrating buffer before the next protein sample was applied.

10 ml. sample fractions were collected on the Towers automatic siphon balance fraction collector at a flow rate of 30-35 ml. per hour. The elution pattern was established by locating the distribution of protein according to G.M.15(c) iii, and ribonuclease active fractions were accounted for as O.D. active units according to G.M.14.

d. Concentration of spleen ribonuclease samples on a small C.M. cellulose column

A short C.M. cellulose column 1 cm. i.d. x 3 cm. effective length, equilibrated with 0.005M tris HCl 10^{-4}MEDTA pH 8 was used to concentrate spleen ribonuclease active fractions, especially those from the rechromatography column IV, prior to storage. The C.M. cellulose medium, prepared according to G.M.7(a) ii, and usually a portion of a batch already cyclised, was filled into the column as a slurry, washed with 50 ml. equilibrating buffer made 1M with NaCl, then eluted copiously with 250 ml. equilibrating buffer.

The spleen ribonuclease enzyme fractions, usually ribonuclease

active peaks "A" or "B" (page 193) were bulked, briefly dialysed separately against the column equilibrating buffer G.M.3, then applied very slowly to the concentrating columns. The last of the preparation was washed on with 25 ml. equilibrating buffer. The ribonuclease active sample retained on the exchanger was desorbed with 1M NaCl in equilibrating buffer as a protein rich band which was collected in the minimum volume possible at 6-8 ml. Samples were stored in the deep freeze at -12° in sealed screw cap phials.

G.M.9. The apparatus and stock solutions required for disc electrophoresis

a. Apparatus

Glass tubes 0.4 i.d. x 7 cm., cut from one sample of uniform glass tube and ground at both ends to remove sharp edges, were filled with a polyacrylamide gel column, Figure 51, page 322 . Gel section A contained a 2.5% sample gel where electrophoretic concentration of the protein components was initiated. Gel section B contained a 2.5% acrylamide spacer gel where electrophoretic concentration and orderly stacking of the sample components was completed. Gel section C contained the small pore gel where molecular sieving at electrophoresis produced a separation.

Electrophoresis was performed in a vertical position where the glass tubes were attached to an upper buffer reservoir and the lower ends dipped into the buffer solution of the lower buffer reservoir, Figure 52. Electrodes were placed centrally in each reservoir and the polarity set so that the sample ions migrated towards the small pore

gel. All the apparatus was constructed in the laboratory from inexpensive material. The buffer reservoirs were plastic kitchenware bowls 12 cm. diameter and 4 cm. deep. Cylindrical graphite electrodes cut from clean graphite rods were held centrally in a four legged glass stand designed to hold the graphite rods vertical and in a central position by just fitting into the bowls, Figure 53. Crocodile clips on the power pack leads made contact with the electrodes through a spring clip electrical contact.

Each gel tube was fixed into the uppermost buffer reservoir using rubber grommets to seal the junction and hold the tube in the orifice. With the electrode situated centrally and equidistant from all grommets which were also placed equidistant from each other, the voltage drop across the gels was the same[111]. Two sets of electrodes were available, one for gel electrophoresis the other for destaining. The sets were retained separately for their specific functions and in each case the polarity never reversed. A D.C. power pack, which could operate on two ranges within 100 volts and within 200 volts, was constructed in the laboratory[111].

During the filling and polymerisation of the gels the tubes were fixed vertically in plasticene moulds, Figure 54, by making a pocket with a glass rod of gel tube diameter, then sealing the end of the gel tube in the impression. The plasticene could be easily cut away without causing disturbance to the gel by suction effects. The two important steps in the preparation of the gel column consisted of layering water over small pore gel solution and spacer gel solution

Fig. 51.

The polyacrylamide gel column.

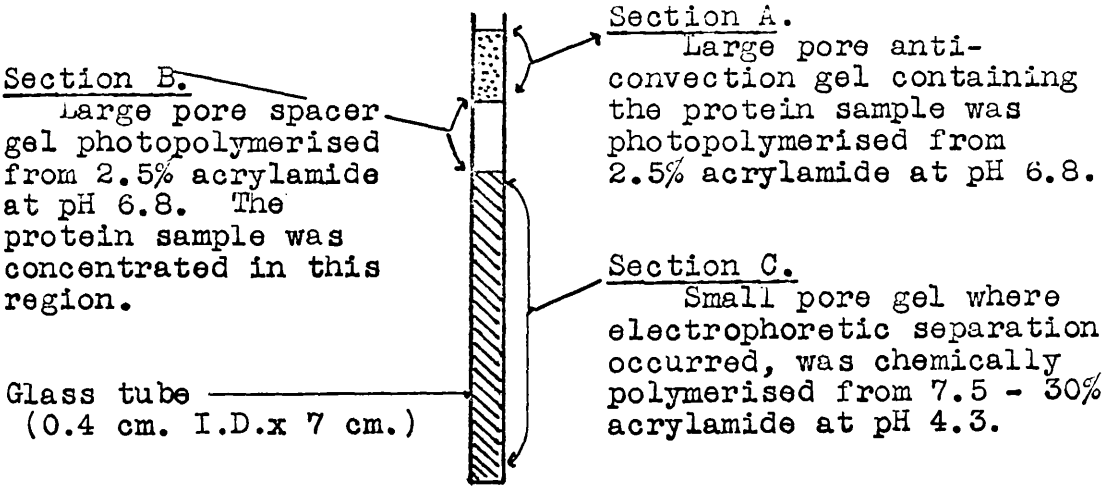


Fig. 54.

Construction of the gel column

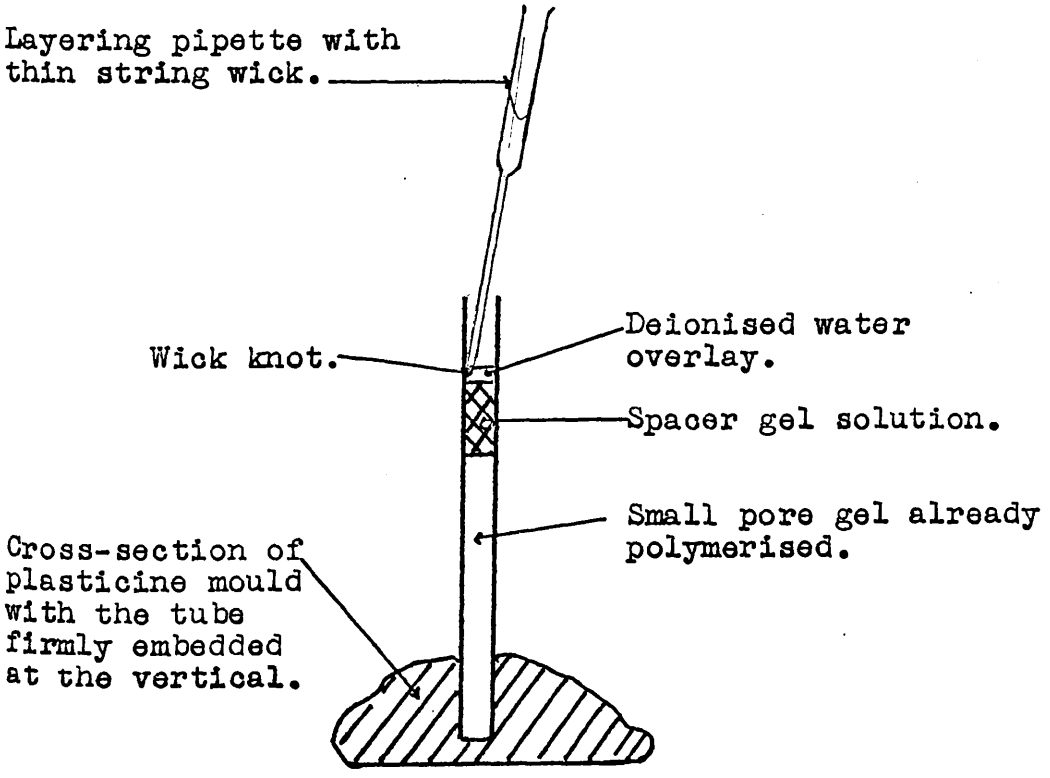


Fig. 52.

Vertical section of the apparatus assembly at Disc Electrophoresis.

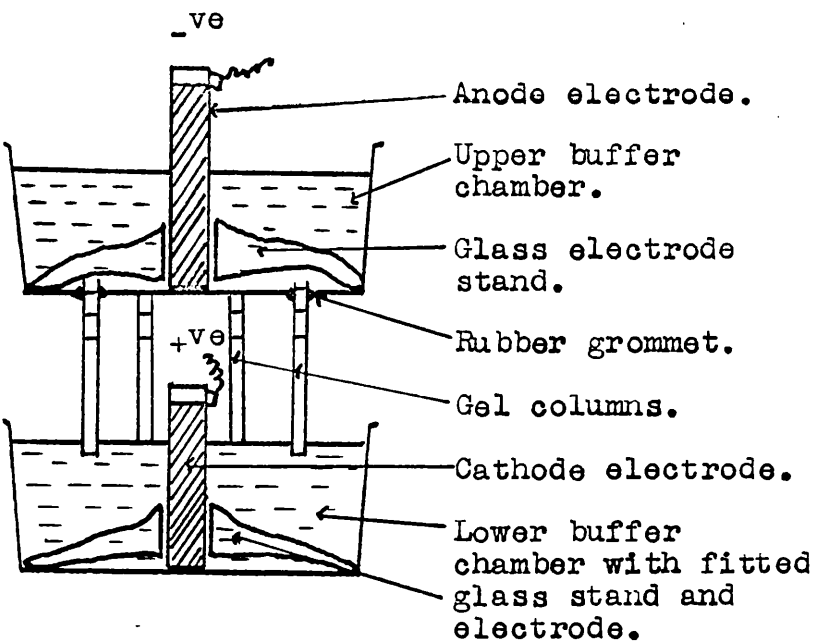


Fig. 53.

The top view of the apparatus assembly at Disc Electrophoresis.

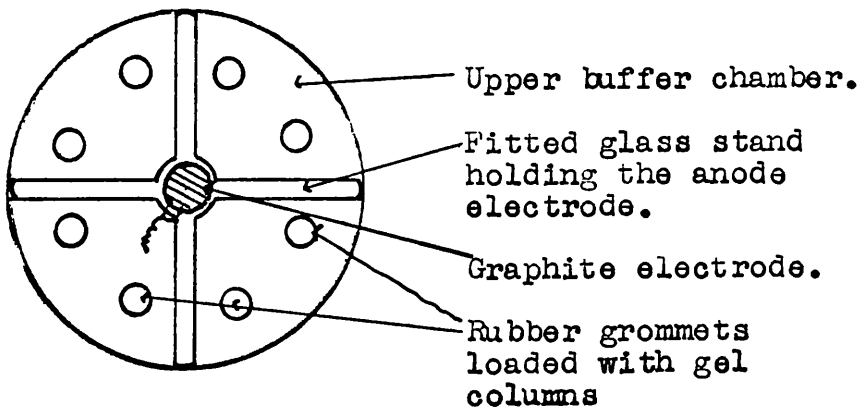


Fig. 55.

The destaining tube.

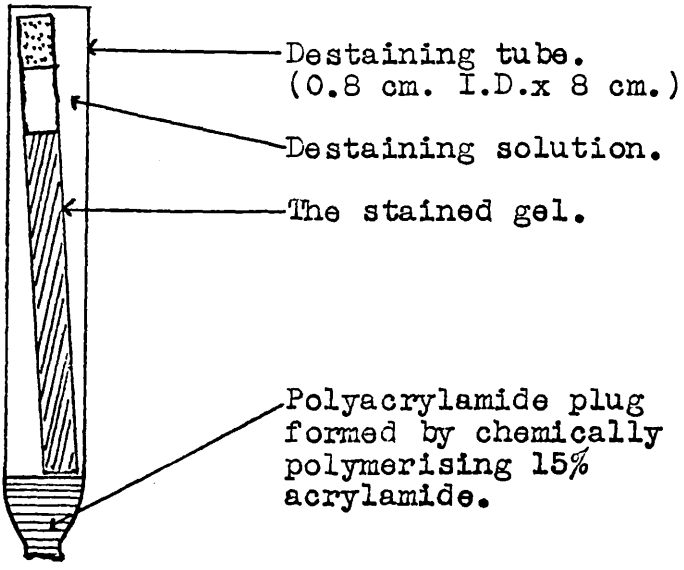
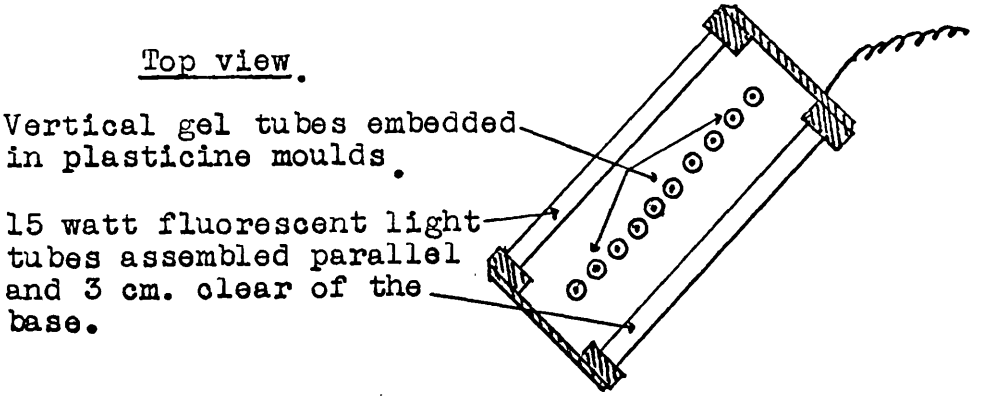


Fig. 56.

The assembly for photopolymerising gel sections A and B.



to eliminate the miniscus and exclude air from the monomer. The overlaying pipette, Figure 54, consisted of a glass dropping tube drawn to a capillary at one end. The tube contained a fine string wick and the end protruding from the capillary end was knotted. The wick permitted the slow overlaying of the more viscous polymer solutions with distilled water, without spurting to disturb the monomer solutions.

After electrophoresis the gels were dislodged from the glass tubes by rimming carefully with a syringe needle connected to the water supply by a polythene tube. Unbound dye after staining was removed from the gels by electrophoresis. The dye-protein complex was fixed and relatively stable[111]. The destaining apparatus was exactly similar to that for gel electrophoresis, Figure 52 and Figure 53. The anode lead was connected to the lower buffer reservoir electrode. The destaining tubes 1 cm. diameter x 8 cm. and constricted at one end, Figure 55, could be fitted into an upper buffer reservoir in a similar manner to the gel tubes. A gel plug polymerised from 15% acrylamide acted as a support for the destaining gel. The plug was formed by setting the destaining tube constricted end downwards in a plastecene mould, pipetting in a 1 cm. column of small pore gel solution then overlaying with distilled water to exclude air and allowing the acrylamide to polymerise. The destaining tubes with the polyacrylamide plugs intact were stored between runs immersed in 7% aqueous acetic acid.

b. Stock solutions

The ingredients required for the various types of gels were made available ready for use as stock solutions which were stored in brown screw cap bottles in the refrigerator between experiments. These solutions were left to warm up to room temperature several hours before the proportions required for gel formation were taken and mixed. Three stock solutions were required for the 2.5% acrylamide large pore gel solution.

Solution I.

	N potassium hydroxide	48 ml.
	Glacial acetic acid	2.87 ml.
N,N,N^1,N^1	Tetramethylethylene diamide	0.46 ml.

and distilled water to a total volume of 100 ml.

Solution II

	Acrylamide	10 g
N,N^1 -	Methylenebisacrylamide	2.5 g

and distilled water to a total of 100 ml.

Solution III

Riboflavin 4.0 mgms dissolved in a

Total volume of 100 ml.

The spacer gel solution B was made up at the time it was required. The pH was 6.8 and this was checked with the pH meter and adjusted with KOH or acetic acid.

Solution B

1 part solution I

2 parts solution II

1 part solution III

4 parts distilled water

The protein sample gel solution which was also called the anti-convection gel solution[111] was prepared from the stock solutions exactly as for solution B, except that the 4 parts of distilled water were replaced by enzyme solution of appropriate concentration to give sample solution A.

Solution A

1 part solution I

2 parts solution II

1 part solution III

Appropriate portions of solution A were mixed with an equal volume of the different protein solutions to be tested. The combined volume was sufficient to fill the number of columns required. The 2.5% acrylamide solution obtained was adjusted to pH 6.8 before photopolymerisation.

Stock solutions to formulate the small pore solution varied with the purpose of the gel.

Solution IV

N potassium hydroxide	48 ml.
Glacial acetic acid	17.2 ml.
N,N,N^1,N^1 tetramethylethylenediamine	4.0 ml.
distilled water was added	
to a total volume of	100 ml.

Solution V varied depending on the pore size required in the gel. Table 27, page 239.

Table 11

PORE SIZE	50°A	40°A	30°A	20°A
% acrylamide	7.5	15	22.5	30
Acrylamide	30g	60g	90g	120g
N,N^1 Methylenebisacrylamide	0.8g	0.4g	0.3g	0.2g

Depending on the selection of % acrylamide the appropriate amount of each component was dissolved in distilled water to a volume of 200 ml.

The preliminary small pore solution C_p which could be effective up to four weeks[111] was obtained by mixing:

1 part solution IV

3 parts solution V

Solution C_p should have a pH = 4.3 which was checked then adjusted with KOH or acetic acid if required. Directly before use solution C_p was carefully mixed with an equal volume of freshly prepared ammonium persulphate solution D to give the small pore solution C which

was pipetted into the gel tubes.

Solutions A, B, and C_p were made up ready for use and stored in brown glass bottles in the refrigerator. They were replaced at the latest every three months.

Solution D

The ammonium persulphate solution was made up fresh on each occasion by dissolving 0.28 g in 100 ml. distilled water. Ammonium persulphate solutions and small pore solution C were discarded immediately the volume of gel tubes had been dealt with at a particular electrophoresis experiment.

Buffer solution

The buffer solution for the reservoirs contained 31.2 g βalanine, 8 ml. glacial acetic acid dissolved in distilled water to 1 litre then the pH checked at 4.5. The buffer solution from the upper and lower reservoir were stored separately from each other and the stock buffer solutions were used for three runs at a maximum then rejected. The polarity of the buffers was maintained on each occasion.

Fixative-Stain solution

This solution was made up by dissolving 1g Amido Schwarz in 100 ml. 7% aqueous acetic acid. The solution was filtered and conserved by retaining the excess dye solution after staining the gels overnight.

Polyacrylamide solution for destaining electrophoresis

Acrylamide 6 g
Riboflavin 0.5 mg
Deionised water to 100 ml.

This solution was exposed to the double row fluorescent light, Figure 56. The light was set vertically and the solution placed centrally between the tubes in a 100 ml. measuring cylinder. Photopolymerisation sometimes proceeded after 1½ hours. Polymerisation could be accelerated by adding a pinch of ammonium persulphate. Before the solution became excessively viscous it was diluted with an equal volume of 14% aqueous acetic acid then stored in a brown glass bottle.

Technical points

If need be protein samples were clarified by centrifugation before solution A was added. When any of the stock solutions appeared cloudy on preparation they were filtered before storage. It became standard practice to set up a gel column without a protein sample in every electrophoretic run to act as a blank. Solution B replaced the protein sample gel solution on these occasions. It proved essential to run a blank gel as on several runs a spurious band appeared on all the gels including the blank gel. This spurious band could be associated with the fastest moving refractile band observed during electrophoresis. Most electrophoretic runs were halted after this refractile band had reached half way down the gel columns. After staining the spurious protein stain appeared at a similar position.

Since separation of proteins takes place in the small pore gel at a slightly higher pH than the original pH of the small pore gel

(page 246) it should be possible to examine protein samples under different conditions of pH by varying the pH of the small pore gel. The small pore gel solution C_p was prepared at different pH values while retaining the same acrylamide concentration. These alternative conditions were used in an attempt to detect heterogeniety in spleen ribonuclease samples. A limited number of trials were carried out where the small pore solution C_p of a 15% acrylamide gel solution was made up at pH 4.5, 5.5, 6.5 and 7.5 then polymerised in the usual manner to form gel columns.

These solutions were prepared from a modified solution IV c.f. page 328.

Modified solution IV

N KOH	48 ml.
Glacial acetic acid	8 ml.
N,N,N^1,N^1 tetramethylethylenediamine	4 ml.
total volume	60 ml.

This solution was then used to make up the small pore solution C_p at each pH value directly. The small pore solutions C_p contained:

Modified solution IV	12 ml.
Acrylamide	24 g.
Methylenebisacrylamide	0.16 g.
Deionised water	28 ml.

The volume of this solution was 64 ml. at pH 5.6. Four proteins of 16 ml. were obtained and for each pH value required the following

quantities of N KOH or glacial acetic acid added to each.

pH 4.5.	0.2 ml.	glacial acetic acid
pH 5.5	0.04 ml.	" " "
pH 6.5	3.2 ml.	N KOH
pH 7.5	4 ml.	N KOH

Each C_p solution was made up to a total volume of 20 ml. with deionised water then stored in brown bottles in the refrigerator. At the time of electrophoresis the small pore solution C was obtained in each case by mixing a volume of the freshly prepared ammonium persulphate solution D (page 329) with an equal volume of the C_p solutions of different pH to form the gel columns described on page These columns had to be prepared without delay as those gel solutions at pH 5.5, 6.5 and 7.5 polymerised quickly especially that at pH 7.5.

G.M.10. Procedure at disc electrophoresis [133].

a. Formation of the gel column

The glass tubes 0.4 cm. x 7 cm. were inserted into large plasticene moulds, sealed at the base to prevent leakage and very carefully set in a vertical position, Figure 54. The required amount of small pore solution C to fill the number of tubes set up was obtained by mixing, thoroughly and carefully to avoid aeration, equal volumes of solution Cp and freshly prepared solution D (page 329). Without delay the tubes were filled to 2.5 cm. from the top using a modified pipette tapered to a capillary at the outlet. The first few drops of solution C were ran down the tube wall slowly to ensure the

tube base was completely filled.

The acrylamide solution C was overlayed with about 0.1 ml. distilled water using the layering pipette, Figure 54, page 322. This manipulation was carried out with extreme care. The layering pipette was introduced into the tube until the wick knot just made contact with the acrylamide solution. The distilled water was added by very slow pipette action and a sharp interface formed between the two solutions. Samples were rejected if there was any deviation from a sharp refractile boundary. Usually within 30-40 minutes the acrylamide solution polymerised when the tubes were left undisturbed at R.T.

To avoid irregular polymerising times the acrylamide stock solutions were removed from the refrigerator and brought up to room temperature before use. After polymerisation, water and any unreacted monomer was removed by inverting and very gently shaking the tubes. The liquid was completely removed by draining on to a filter paper. Large pore solution was pipetted into the unfilled portion to rinse out, then drained free by absorption on filter paper after inverting the tube. A 1 cm. column of large pore solution was then added to each tube and overlaid with 0.1 ml. of distilled water in a similar manner to that applied to the small pore solution. Two 15 watt daylight fluorescent tubes were placed on either side of a row of gel tubes, Figure 56, page 324. The fluorescent tubes were at the same height as the large pore gel solution, parallel and equidistant to the line of gel tubes. The light tubes

were placed 15 cm. apart.

By 15 minutes the opalescent spacer gel had formed. The water layer was again removed, with great care taken not to disturb the 2.5% acrylamide spacer gel, by holding the tube on the horizontal and absorbing the solution with tissue paper. The space empty above the gel was again rinsed out as already described before adding the 1 cm. column of large pore spacer gel solution. The respective solutions made from an equal volume of large pore solution suitable for mixing with protein solutions (solution A), page 327, and protein solution were added to each tube after thorough but gentle mixing. The sample solution was polymerised in the manner just described for the spacer gel.

b. Electrophoresis

When gel formation was complete, the plasticene mould was cut away to prevent suction forces disturbing the gel base. The tubes, sample gel uppermost were inserted into the grommets of the upper buffer reservoir from underneath, then the reservoir filled with β alanine/acetic acid buffer after any air space in the top of each tube had first been filled with buffer. A hanging drop of buffer was then placed on the bottom of each of the eight gel tubes to prevent trapping of air bubbles. The upper reservoir was lowered to a level where all the tubes were immersed to 0.5 cm. depth in the lower reservoir buffer, see Figure 52, page 323. Electrophoresis was started within a half hour of the sample gel forming. The cathode terminal of the power supply was fitted to the lower electrode, the anode

terminal at the uppermost electrode then the power applied. The current was adjusted to between 6-8 m.amps per tube. Protein concentration and separation took place when the current was applied. The K ion boundary originally present at the buffer gel interface, moved into the gel toward the cathode sweeping up the protein components in the sample gel. In this region the protein sample was concentrated between the K ion boundary and β alanine boundary which took up the rear. The proteins present were separated into a series of discs stacked in order of electrophoretic mobility. The stack believed to be 10-25 microns thick could be observed in the spacer gel as a thin refractile band. In the small pore gel the proteins were separated from one another and several very thin refractile bands could be observed on the gel indicating the extent of electrophoresis. Electrophoresis was performed for 25 to 30 minutes or until the refractile bands had migrated 2-3 cm. into the small pore gel. The time of run varied with the conditions of the experiment, particularly the pore size of the gel. At the completion of electrophoresis the power was switched off and the buffers retained in separate containers.

c. Removal of the gel

The gel cylinders were dislodged from the tubes by rimming under water using a syringe needle through which a very slow jet of water passed[112]. The gel-glass interface was lubricated at both ends of the tube to a depth of 1.5 cm. by rimming while slowly rotating the tube. Much care had to be exercised to prevent mechanical damage to the gel.

When withdrawing the needle the tube had to be carefully rotated to prevent gel fracture on stretching. The rimming action was usually sufficient to free the gel which could then be gently driven out by applying a slightly increased water pressure through the needle at the sample gel end with rimming action.

d. Staining and destaining to locate protein bands

Each gel was fixed and stained overnight in a small test tube containing 2-3 ml. of fixative stain solution. A 1 hour staining time was not sufficient with the high acrylamide concentration gels as the protein discs appeared hollow after destaining. The excess fixative stain solution was decanted and retained then the gels were rinsed for a few minutes in a wash solution of 7% acetic acid to remove excess dye. An electrophoretic destain of uncombined dye in the gel was performed in an exactly similar apparatus to that depicted in Figures 52 and 53, page 323, where eight gels were destained simultaneously.

Each stained gel was placed in a destaining tube and retained by the polyacrylamide gel plug, Figure 55. The viscous polyacrylamide destaining solution was pipetted into the vacant space in each destaining tube. This solution reduced convection and backflow of the free dye to the upper vessel. Contact was made with the cathode terminal to the upper reservoir electrode. Each buffer reservoir contained 7% aqueous acetic acid. Again care was taken to ensure air bubbles were excluded from the system. Uncombined anionic dye migrated down the gel and destaining solution into the lower anode

reservoir. Destaining was usually completed in 1 hour at 15 m.amps per tube. After destaining the gels were washed free of destaining solution with 7% aqueous acetic acid then stored in sample phials containing 7% acetic acid as a preservative.

G.M.11. The determination of phosphorus according to King's method[181]

Reagents A 60% Perchloric acid A.R.

B 5% Aqueous Ammonium Molybdate A.R.

C "Amidol"

"Amidol" reagent contains 0.5 g 1 amino 2 naphthol sulphonic acid, 30 g. $NaHSO_3$ 6g Na_2SO_3 dissolved in 250 ml. distilled water. The solution was filtered and kept in a well stoppered brown-glass bottle. The reagent is not stable thus renewed stocks were prepared within fortnightly intervals.

Inorganic phosphate as phosphorus

The calibration curve, range 0-80 μg.P, was prepared by taking aliquots from a standard solution containing 20 μg Phosphorus/ml. (0.0439 g KH_2PO_4 AR in 500 ml. distilled water). The samples containing phosphorus were pipetted into 15 ml. graduated pyrex tubes and the reagents added in the order:

1.2 ml. A

1.0 ml. B

0.5 ml. C

Distilled water to the 15 ml. graduation mark.

The sample was mixed by inverting and swirling and the colour intensity read after 5 minutes in the EEL colorimeter filter 608 (680mμ)

using matching 1 cm. cells and distilled water as reference.

Total phosphate as phosphorus

1 ml. of phosphate sample to which 1.2 ml. reagent A had been added was digested for about 2 minutes over a bunsen flame with care and constant manual agitation of the pyrex tube to prevent spurting. A reddish brown colour often appeared as charring took place. This colour disappeared indicating that the organic phosphorus had been released to inorganic phosphorus. The tube was cooled and approximately 5 ml. distilled water added to wash down the inside of the tube. Reagents B and C were then added in the same proportions and order as for inorganic phosphate. The solution was made up to 15 ml. graduation with distilled water and the colour intensity measured as before.

The time of 5 minutes alotted to colour development made the procedure limited with respect to the number of estimations possible. It was found that the colour intensity developed little in 20 minutes over that for 5 minutes[182], thus it became standard practice to use a 20 minute time interval before reading. The phosphorus content of samples was read from the calibration graph. A reagent blank was carried out by omitting the phosphorus sample. Inorganic phosphorus was determined on samples when total phosphorus estimations were made, to obtain a value for organic phosphorus.

G.M.12. Estimation of residual ammonium sulphate concentration in protein solutions

The method involved the use of the modified Nesslers reagent as

described by Paul[175] after the precipitation of the protein with 6% T.C.A.

Reagents A Modified Nesslers Reagent

B 2N NaOH

Nesslers reagent (modified) was prepared by adding a solution of 3.5 g of gum acacia in 750 ml. of deionised water to a solution of 4 g. potassium iodide and 4 g mercuric iodide in 25 ml. deionised water and adjusting the volume to 1 litre.

The calibration curve range 0-20μg N was prepared by taking aliquots from a standard nitrogen solution containing 10μg N/ml. of a stock solution of NH_4Cl A.R. at 100 g/ml (0.38214g NH_4Cl per litre) and diluting with deionised water to 250 ml. in a graduated flask.

Preparation of the test solution

3 ml. of 6% T.C.A. was added to 3 ml. of protein solution mixed thoroughly, cooled in ice, then centrifuged to remove the protein precipitate. 2 ml. of the supernatant was diluted to 500 ml. with deionised water in a graduated flask. If the protein solution was 10% saturated with ammonium sulphate, a 2 ml. sample from the 500 ml. solution should contain nitrogen in the range 0-20 μg N per ml. (i.e. $\simeq$ 11 μg N per ml. for 10% saturation). It was customary to carry out the determination of nitrogen on the range 0.5, 1, 1.5 and 2 ml. with deionised water added where appropriate to make the test solution 2 ml.

Estimation of nitrogen

2 ml. of reagent A was added to 2 ml. of the test solution and

mixed by swirling action. 3 ml. of solution B was added and the solution mixed again. After 15 minutes the yellow colour was measured at 470 mμ on the EEL colorimeter filter 602 in matched 1 cm. cells using deionised water as a reference. A reagent blank was carried out using 2 ml. deionised water as test solution. A comparative reagent blank was carried out on a sample prepared in the same way as the protein test solution using 3 ml. of deionised water in place of the protein solution.

From the calibration curve and subsequent calculation the amount of ammonium sulphate in the protein solution could be determinated, then by using the formula[73]

$$X = \frac{533\ (S_2 - S_1)}{1 - 0.3\ S_2}$$

Where X = g solid sulphate of ammonia added to 1 litre of solution of saturation S_1 to bring it to saturation S_2

S_1 = initial fraction of saturation

S_2 = final or desired fraction of saturation at 23°C

S_2 the present saturation could be calculated as X was now known and S_1 = zero. Blanks were very essential and care had to be taken that the atmosphere was free of ammonia fumes.

G.M.13. Detection of sucrose by the phenol-sulphuric acid method[183,184]

Reagents: Analar grade

A 5% w/v Phenol in water

B Concentrated H_2SO_4

Method

1 ml. of A was mixed with 1 ml. of the aqueous solution containing the sucrose sample (10-90 μg range) in a boiling tube. 5 ml. of B was added from a wide bore pipette so that the stream hit the surface directly, to produce good mixing and even heat distribution. Each tube was agitated during the acid addition and exactly the same procedure practised throughout a particular range of samples. After 10 minutes each tube was cooled by immersion in a 25° water bath for 20 minutes. The absorbency of the yellow-orange colour was measured at 490 mμ filter 603 on EEL colorimeter after transfering the sample to a matched colorimeter tube against a blank as reference. The blank consisted of the aqueous solution without sucrose developed by the method above.

G.M. 14. The assay procedure for spleen ribonuclease

(a) The assay

The first attempt at assay involved the application of the method used by McDonald[51] to estimate pancreatic ribonuclease. The method was insensitive with the spleen enzyme and no significant amount of activity was observed. A simplification of the assay method used by Kaplan and Heppel[8] was adopted and spleen ribonuclease activity estimates were based on the ability of the enzyme to produce "Uranyl and acid soluble nucleotides". The minute scale of the digest operated by these workers was considered not practical enough to initiate the work and the many pipettings (five in all) to make up the digest could be a considerable source of error.

"Uranyl and acid soluble nucleotides" were estimated by recording the O.D. on the DU spectrophotometer. Each digest contained one volume of substrate solution and one volume of enzyme solution. For most estimations 0.25 ml. substrate and 0.25 ml.enzyme solution were used. However, in the initial stages of the work phosphorus estimations by G.M.11 were carried out to determine the "Uranyl and acid soluble organic phosphorus"[51] release at assay. At this stage the volume of the digests was considerably larger at 2 ml. to allow for suitable sample volumes for the determination of inorganic and total phosphorus. These digests were comparable proportionally with later digests of lower volume as the volume increase was four fold and O.D. estimates of "Uranyl and acid soluble nucleotide"[51] release were recorded. After the incubation period the reaction was stopped by the addition of an equal volume of R.N.A. - protein precipitant to the digest.

(b) Solutions

(i) Substrate solution

The concentration of the substrate solution was calculated to compare with the digest conditions reported by Kaplan and Heppel[8]. The substrate solution was prepared fresh on the day of assay by dissolving the appropriate amount of R.N.A. in the stock buffer solution. The stock buffer solution was 0.1M sodium succinate/succinic acid pH 6.5 containing 0.05M $MgCl_2 6H_2O$. This solution was made up by dissolving 11.81 g of succinic acid in 300 ml. of deionised water by titrating to pH 6.5 with N NaOH. After 10.17 g. $MgCl_2 6H_2O$ had been

included the volume was made up to 1 litre, the solution boiled briefly, cooled and the pH checked.

On the day of assay freeze dried R.N.A. at the rate of 15 mg/ml was dissolved in a volume of stock buffer. The volume of stock buffer required was determined by the number of digests envisaged. Before use the substrate solution was filtered through Whatman No.1 and the pH checked.

(ii) Enzyme solution

The enzyme solution was prepared before the assay at a suitable dilution in order that only enzyme solution need be added to the digest. The amount of enzyme varied with the ribonuclease activity content of the sample and was adjusted according to the definition of activity required to produce optimal conditions, page 345 . By trial and error assay the enzyme solutions were diluted to the required activity content, the pH adjusted to 6.5 and the appropriate sample added at assay.

(iii) R.N.A. - Protein precipitant

MacFadyens reagent[114] contained 0.25% Uranyl acetate in 2.5% T.C.A. The solution was made up by dissolving 5 g T.C.A. and 0.5g Uranyl acetate in deionised water to 200 ml. This reagent was stored in the refrigerator and was cold when used.

(c) The method of assay with 0.5 ml. digests

The digests were carried out in 10 ml. conical centrifuge tubes made of pyrex glass and numbered to prevent confusion. 0.25 ml.

substrate solution was pipetted into each tube and the tubes placed in a water bath (Grants' constant temperature bath) at 37.5° for 6 minutes. 0.25 ml. of enzyme solution was added and the solution thoroughly mixed by swirling and rotating the tube carefully. When very particular assays to determine specific activity and total activity were carried out, a sample of the protein solution was held at incubation temperature, before pipetting, for 5 minutes. Where a series of 50-60 digests were required to locate an active region from, e.g. a column effluent and very many samples had to be scanned 0.25 ml. samples were pipetted direct into the centrifuge tube.

The digests were incubated at 37.5° for 30 minutes. Several digests could be carried out concurrently over a period of 29 minutes. Particular care was taken not to contaminate pipettes and enzyme solutions with R.N.A. on return to the stock samples. After the 30 minutes digest, the reaction was halted by the addition of 0.5 ml. cold R.N.A. protein-precipitant. The contents were carefully mixed by swirling and rotating the tube to ensure the reaction had ceased, especially on the tube walls. The tube was then placed in ice for 30 minutes at 0° then centrifuged for 10 minutes, six at a time, to pellet the insoluble R.N.A. and protein.

0.1 ml. of the supernatant was pipetted into a test tube and diluted x 40 by the addition of 3.9 ml. of deionised water. The O.D. of this solution was measured at 260mμ in a Beckman DU spectrophotometer SP500 using a 1 cm. light path and deionised water as reference. An ordinary blank, corresponding to incubation without enzyme, was

substracted. 0.25 ml. deionised water was added instead of enzyme. Enzyme blanks were carried out especially when the enzyme source was crude. A blank value corresponding to incubation without substrate was also taken into consideration. 0.25 ml. deionised water made up the volume. This was carried out similar to the ordinary blank and estimated endogenous reaction without substrate supplied. A check was also made for the presence of compounds which would give a U.V. absorbency in the protein samples. In this case the equivalent of a zero time blank was carried out by adding 0.5 ml. of cold R.N.A. - protein precipitant to 0.25 ml. enzyme solution in the 10 ml. conical centrifuge tubes with thorough mixing. To this volume 0.25 ml. substrate solution was added, mixed, then placed in the ice bath and dealt with similarly to the enzyme digests.

(d) Estimation of ribonuclease activity by the O.D. method

It was made a condition of the assay that for optimal activity the amount of enzyme was restricted so that the net O.D. of the final diluted sample did not exceed 0.1 (0.05-0.1 was the optimal range)[8].

The unit of enzyme activity was taken as the amount which caused a density increment of 2 in the final diluted sample[8].

Specific activity was defined as units per mg protein[8].

The ordinary blank had an O.D. of 0.06 usually.

example

Ordinary blank = 0.06

Digest = 0.14 :- average of three or more digests

Net O.D. = 0.08 in the final diluted sample

$$\text{Units per digest of 0.25 ml. enzyme sample} = \frac{0.08 \times 10}{2}$$

$$= 0.4 \text{ units}$$

Appropriate adjustements were made to the calculations for total activity if the enzyme sample was diluted prior to assay.

"Uranyl and acid soluble organic phosphorus" estimates were carried out in the early assay work to compare the activity values with those from O.D. measurements from the D.U. spectrophotometer. 1 ml. samples were taken for total phosphorus and 0.5 ml. samples were used to estimate inorganic phosphorus by G.M.11. The increase in "Uranyl and acid soluble organic phosphorus" was expressed as μg phosphorus released per 1 ml. protein for total activity or as μg phosphorus released per mg protein for specific activity. The estimation was abandoned when it proved insensitive.

G.M.15. Measurement of protein

(a) The Biuret Test[185]

The reagent was prepared by stirring a solution of 10% sodium hydroxide (150 ml.) into a solution containing copper sulphate pentahydrate (0.75 g) and Rochelle salt (3g). The volume was made up to 500 ml. with water and the reagent stored in polythene bottles.

Protein concentration

1 ml. of sample containing 1 to 10 mg protein was mixed with 4 ml. of reagent and allowed to stand at R.T. for 30 minutes. The intensity of the colour produced was measured in the EEL colorimeter (Filter 605, 550 mμ) and compared with a standard graph. The system was calibrated using solutions of bovine serum albumin of known concentration.

(b) Folin - Ciocalteu method[185]

The following reagents were required.

Reagent A — 2% Na_2CO_3 in 0.1N NaOH.

Reagent B — 0.5% $CuSO_4$ $5H_2O$ in 1% sodium tartrate.

Reagent C — 50 ml. of reagent A are mixed with 1 ml. of reagent B. This solution is retained for no longer than 1 day.

Reagent E — Sodium tungstate (100 g), sodium molybdate (25 g), 85% phosphoric acid (50 ml.), conc. hydrochloric acid (100 ml.), and water (700 ml.) were refluxed for 10 hours. The solution was filtered and diluted to 1 litre. The concentration of acid was determined and adjusted by dilution to 1N.

Protein concentration

The estimation was carried out by mixing 1 ml. protein solution with 5 ml. reagent C and allowing the reagent to stand for 30 minutes at R.T. 0.5 ml. of reagent E was added with shaking, and after 10 minutes the blue colour was read in the EEL colorimeter

(filter 608, 660 mμ). Bovine serum albumin (Armour laboratories) was used to prepare the standard curve, range 0-100μg protein per ml.

(c) Protein estimation by ultraviolet absorption[185]

I. To find the protein concentration of an unknown sample the formula: protein concentration (mg/ml) = $F \times \frac{1}{d} \times D_{280}$ [185] can be used. This formula compensates for any interference from contamination by nucleic acid.

The optical density of an appropriately dilute protein solution was obtained at both 280 mμ and 260 mμ in 1 cm matched quartz cells against the solvent as reference. From the ratio R of the optical density at 280 mμ to that at 260 mμ the factor F was obtained, Page 453 of Methods in Enzymology III edited by S. P. Colowich and N. O. Kaplan, Academic Press 1957. d was the cuvette width in centimeters and D_{280} was the optical density at 280 mμ

II. Total U.V. absorbing units

Total U.V. absorbing units were used to estimate the proportion of protein retained or discarded by the ion exchange columns (Section III B). Total units were obtained by taking the value for the optical density at 280 mμ of an appropriately dilute protein solution, then multiplying this value by the total volume of the sample after making adjustment for any dilution effect.

III. Column effluent

Column effluent elution patterns were constructed by recording the optical density of each effluent fraction in the U.V. spectro-

photometer at 280 mμ in 1 cm matched quartz cells against the eluting solvent as reference.

G.M.16. Preparation of R.N.A.

(a) R.N.A. for general use, e.g. detection of ribonuclease activity in column effluents

Commercial yeast R.N.A. (L. Light and Co., Ltd., Colnbrook, England), was further purified by extensive dialysis in 32/32" visking tubing against a x 10 volume of deionised water at 0-4° for 36-48 hours with a charge of deionised water every 6 hours. Before dialysis the commercial sample was dissolved in water, aided by a trace of alkali to pH 7. 3/4 the length of the dialysis bag was left free to accommodate volume increase by osmotic flow. A spectrophotometric examination of the diffusate solution on the final dialysis period at 260 mμ in a 1 cm cell gave the same optical density reading as the deionised water reference. The R.N.A. believed to be a minimum in molecular weight of 10,000[78] was freeze dried, G.M.17 then stored as a fluffy white solid in sealed 4 oz. bottles at -12° in the deep freeze.

(b) R.N.A. for accurate assay purposes, e.g. digests where ribonuclease activity units and specific activity were determined

R.N.A. was prepared from fresh bakers yeast by the detergent method of Crestfield et al[186] as set out in Methods in Enzymology III[187].

Preparation of the detergent sodium dodecylsulphate

500 g. Empicol L.Z. batch 5255 (supplied by Marchon Products Ltd.)

was added to 3 litres boiling ethanol to reflux on an electric isomantle. After 10 minutes the solution was filtered hot through a Buchner funnel using a celite filter aid. The filtrate was allowed to cool at R.T. and crystalline sodium dodecylsulphate was obtained, (200 g.)

Extraction of R.N.A.

500 ml. of an aqueous solution containing 2% sodium dodecyl-sulphate 4.5% Ethanol 0.0125M primary and 0.0125M secondary sodium phosphate was heated in a 4 litre beaker on a sand bath. The beaker was covered with a plate containing slots for a thermometer and mechanical stirrer. On boiling 150 g yeast cut into fine pieces was added in one batch to the boiling aqueous solution with stirring and the beaker cover replaced. On the addition of yeast the temperature of the mixture fell to 82-84°. Within 4 minutes the temperature was raised to 92-94° and after eight minutes had elapsed the mixture was cooled to 4° by pouring it into a 2 litre stainless steel beaker which was immersed in a solid CO_2/acetone cooling mixture. The mixture was stirred manually to prevent local freezing.

After the mixture was centrifuged at 3,000 r.p.m. on the M.S.E. magnum centrifuge at 0° for 30 minutes to remove cellular debris, etc., the supernatant was poured into 2 volumes of cold ethanol and the suspension formed was collected by centrifugation as already described. The suspension was washed twice with 150 ml. portions of 67% aqueous ethanol. Dispersion of the solid after centrifugation in 67% ethanol was carried out by the gradual addition of the liquid

with thorough mixing first of all to form a paste which was increased in volume until 150 ml. was added. Before centrifugation at 3,000 r.p.m. as above 6 drops of 2N NaCl was added to the suspension to aid flocculation. The washed precipitate was left overnight in the refrigerator suspended in 80% ethanol.

The precipitate was recovered from the 80% ethanol by centrifugation at 3,000 r.p.m. and dissolved in 150 ml. water. The pH was adjusted to 7 with 1N acetic acid and the solution clarified by high speed centrifugation at 30,000 x g at 0° for 20 minutes. The decanted supernatant was made 1M by adding 8.7 g NaCl and after the mixture was left overnight at 0° in the fridge, RNA was obtained by centrifugation at 3,000 r.p.m. on the M.S.E. Magnum centrifuge for 1 hour at 0°. The gel was washed with three successive 150 ml. portions of 67% aqueous ethanol then centrifuged after 1 ml. of 2M NaCl was added to aid flocculation. The washed gel was dissolved by the gradual addition of water at pH 7 and extensively dialysed in 32/32" visking tubing sacs as described for the commercial yeast R.N.A. preparations, G.M.16a. Before freeze drying to a white solid the dialysed solution was passed through a bacterial filter (H. A. Jones). The lyophilised sample (500 mg) was stored in sealed screw-cap 4 oz. bottles at -12° in the deep freeze.

G.M.17. Procedure at freeze drying

Spleen ribonuclease preparations and R.N.A. solutions were lyophilised on a Model 10P freeze drier supplied by Edwards High Vacuum Limited. Before freeze drying R.N.A. samples were extensively dialysed

against deionised water, while ribonuclease preparations were dialysed against the buffer 0.005M tris HCl 10^{-4} MEDTA or 10^{-4} MEDTA, all at pH 7. Solutions were frozen completely as a thin uniform film on the inside wall of round bottom flasks by rotating the flask in a cold bath of solid CO_2/acetone mixture. The sample volume occupied not more than 15% of the volume of the container for satisfactory results, e.g. samples of 150 ml./1 litre flask x 5 were freeze dried overnight or within 12 hours. Freeze dried samples were carefully collected and stored in air tight screw cap bottles in the deep freeze at -12°.

EXPERIMENTAL SECTION

Experiment 1. Extraction and salting out of calf spleen ribonuclease at pH 3.5

All operations were carried out in the cold room at 0-3°C. The calf spleen carefully packed in ice crystal tips were processed through the main extraction to that point where the pH was taken to 3.5, within 2-3 hours. The outer capsular layer was stripped off and any non splenic material removed. The tissue was sliced into small pieces then homogenised in a Waring Blendor with 3 volumes of extracting buffer (G.M.2) for 1 minute. This was carried out quickly and accurately by placing two sliced spleen in a measuring cylinder, adding 200 ml. buffer, noting the increase in volume over 200 ml. due to the volume of spleen and multiplying this spleen volume by three. The amount of extractant yet to be added to the spleen in the measuring cylinder was calculated by deducting 200 ml. from this product.

The homogenised spleen had the colour and consistency of thin tomato ketchup. Each homogenisation involved between 150-200 ml. spleen and 450-600 ml. extracting solution. It proved time saving to have a three litre graduation on the five litre beakers into which the homogenised material was transferred. The homogenate was strained through muslin previously washed to remove starch and other contaminants. This step removed blood vessel fragments which could be bulky and interfere with centrifugation procedures at a later stage. The straining was best carried out directly after homogenisation at the

point where the newly homogenised spleen was decanted into a five litre beaker. The operations were arranged such that the muslin draped and supported over the beaker permitted natural straining while the next spleen pair were prepared. The final straining was carried out manually by squeezing the muslin until all the liquid was expressed.

Three litre aliquots of homogenate were adjusted with continuous mechanical stirring to pH 3.5 by the dropwise addition of $\leq$ 200 ml. 1N HCl. On nearing pH 3.5 100 ml. portions of homogenate were removed from the bulk and the pH tested. Before taking the samples for test the acid addition was discontinued and the mechanical stirring extended for 2-3 minutes to ensure thorough mixing. The pH meter for stability reasons was situated at room temperature. Prior to reading the pH the meter was set by the use of control buffers pH 4.01 and pH 6.99 at 25° and during the proceedings frequent checks were made for deviations. The pH of the homogenate samples were quickly taken after the temperature controller of the pH meter was set at 5°. The tested portions were returned to the bulk homogenate and more acid was added until the sample taken had reached pH 3.5. The five litre beakers were placed in 12 litre polythene buckets and insulated by packing ice crystal tips in the intervening space. This measure permitted the safe transport of the homogenate from the cold room to the pH unit and acid titration assembly close by.

To each litre of acidified homogenate 160 g ammonium sulphate was added carefully by sprinkling the solid into the stirred mixture.

This quantity brought the saturation to 30%. After 30 minutes of continuous mechanical stirring to ensure all the solid had dissolved, the mixture was allowed to stand for 1 hour. After this time the impression was obtained that settling out would take place if the mixture had not a thick consistency. The mixture was now a light chocolate brown colour. Each 3 litre portion required $\simeq$200 ml. 1N HCl to acidify, which brought the total volume to 3.2 litres. There was consistently little variation with this manipulation thus it proved time saving to have available 512 g amounts of ammonium sulphate previously weighed out. By setting up two stirring devices, one at the pH assembly for acid addition and one to stir at salting out it was possible to arrange a smooth programme of events through the continuous series of homogenisation, pH adjustment and salt addition with several homogenate portions, only if the considerable apparatus assembly and reagents were extensively prepared.

The mixture after salting out and standing for 1 hour was centrifuged in 6 x 250 ml. cups in the M.S.E. high speed centrifuge at 15,000 x g for 10 minutes at 0°. After centrifugation the supernatant was bulked by decantation into a cold clean 1 litre measuring cylinder, the volume noted then transferred into a 5 litre beaker already insulated in a polythene ice bucket as earlier described. The precipitate was retained for re-extraction.

Re-extraction was achieved by adding an equal volume of re-extracting solution to the precipitate and stirring the slurry for about 2 hours (sometimes overnight). This mixture was centrifuged

as above. The precipitate was rejected and the supernatant decanted into a measuring cylinder and the volume noted. The supernatants from the extraction and re-extraction were combined. On occasions the supernatants had to be centrifuged at 30,000 x g in 6 x 80 ml. polypropylene cups for 7 minutes at 0° to completely clarify the solution.

To each litre of supernatant 356 g solid ammonium sulphate was slowly added with care to bring the solution to 80.5% saturation. As the last few grams proved difficult to dissolve it was concluded that the solution was probably near saturated at $0-3^{\circ}$. The solution was stirred mechanically at the salt addition and thereafter until the ammonium sulphate dissolved, usually in $\frac{1}{2}$ hour. The protein precipitate produced had a pale pink colour and settles out overnight. On occasions the colour varied from almost white to brown at the other extreme. Some of the precipitate floated as a pad on the surface while the remainder sedimented to form a fine layer at the beaker bottom. Considerable economy in time was possible by siphoning off the clear supernatant from the beaker using a polythene tube and gravity flow.

The precipitate was collected by centrifugation at 30,000 x g in 6 x 80 ml. cups for 7 minutes at 0°. The solid was collected after discarding the supernatant by decantation and draining the cups by inverting them on to filter paper. The solid was dissolved in an appropriate amount of dissolving buffer (G.M.2). One litre was required for every 20 spleen to give a 20-fold reduction in volume

from the homogenate volume. Solution was effected by carefully adding a small portion of buffer to the solid and stirring first to a paste, then as solution occurred, larger quantities of buffer were added until the total volume was included. The solution was stirred slowly using a magnetic stirrer until all the protein was in solution.

The operations undertaken to this stage from the time the calf spleen arrived at the cold room, were complete usually within 48 hours. Mechanical and manual stirring were carried out at such a rate the minimum of frothing occurred. The transfer of liquid from one container to another at various stages in the procedure was carried out carefully by tilting the receiving container. This measure ensured the solution ran down the wall of the container with the minimum of disturbance and froth production.

Experiment 2. The preliminary experiment to extract and salt out calf spleen ribonuclease at pH 3.5

The techniques already described in Experiment 1 to extract and salt out the ribonuclease activity from the spleen homogenate were developed from the many trials which were undertaken using the same ammonium sulphate saturation limits described by Kaplan and Heppel[8] for step II, page 26 . These trials followed a similar procedure to that described in Experiment 1 and exactly the same precautions and experiences applied as the process became familiar. The difference to note was the amount of ammonium sulphate necessary to effect fractionation in the preliminary method listed under Experiment 2 and

was as follows. To each litre of acidified homogenate obtained and processed, as described in Experiment 1, 266 g ammonium sulphate was added per litre to bring the saturation to 40%. The precipitate was discarded after centrifugation. The supernatant was brought to "80%" saturation by the addition of 258 g ammonium sulphate and the precipitate collected by centrifugation after the mixture was left overnight to settle out and much of the clear supernatant was siphoned off. The solid was dissolved in 0.05 M sodium acetate 10^{-4} MEDTA ready for the heat treatment.

Experiment 3. The heat treatment at pH 3.5

The precipitate obtained from the centrifugation of the mixture after salting out by raising the ammonium sulphate saturation from 30% or 40% to 80.5% was taken into solution with 0.05M sodium acetate 10^{-4} MEDTA pH 7.2. and diluted until the protein concentration was 12-16 mg/ml. The dark red solution was reacidified to pH 3.5 by the addition, at a dropwise rate down the inside wall of the beaker, of 50 ml. 1N HCl per 500 ml. of solution with continuous mechanical stirring in a manner similar to that outlined in Experiment 1 for the acidification of the homogenate.

Two baths, one containing water heated at a constant temperature of 80°, the other at a constant temperature of 60° were made available. Retort stands fitted with retort rings which could hold a 1 litre beaker by the rim were placed over each bath at a sufficient height to allow the beakers to immerse to 3/4 their height without touching the bottom of the water bath. 500 ml. portions of the spleen enzyme

preparation were heated to 60° in the 1 litre beakers by immersion in the water bath controlled at 80° with stirring sufficient to prevent local overheating and ensure an even distribution of temperature effect. To raise the temperature of a 500 ml. portion of spleen solution required 7-10 minutes, but varied from 2-7 minutes when small volumes were treated. On reaching 60° the mixture in the 1 litre beaker was transferred to the second water bath controlled at 60° and kept constant at this temperature for 10 minutes with stirring as before.

The mixture was cooled rapidly to 0-3° by transferring it to a four litre beaker previously immersed in an ice bath, with occasional stirring. The pH of the solution was now readjusted to 7 by the addition of 1N NaOH dropwise down the wall of the beaker as already outlined for adjusting the pH to 3.5 before the heat treatment. The precipitate was removed by centrifugation at 30,000 x g for 7 minutes at 0°. The precipitate was re-extracted by resuspending it in two volumes of 0.05M sodium acetate 10^{-4} MEDTA pH 7 and the mixture recentrifuged. The initial supernatant containing the heat stable ribonuclease enzyme and wash were combined.

Experiment 4. Salting out with ammonium sulphate at pH 7.

Fractionation by salting out with ammonium sulphate at pH 7 was carried out on the supernatant from the mixture after heat treatment and centrifugation. Before centrifugation to remove denatured protein the pH was adjusted to exactly 7. The residual ammonium sulphate concentration of the solution was determined by the modified

Nessler's method of ammonium ion estimation, G.M.12, and the ammonium sulphate saturation calculated from the formula on page The ammonium sulphate concentration was usually in the region 10% saturation. Precautions similar to those taken to ensure reproducibility of procedure at salting out in Experiment 1 were included at this fractionation. The amount of salt required to produce the first fraction was calculated from the formula on page 340 where the initial saturation S was calculated from the Nessler's determination of ammonium ion concentration. Assuming a residual saturation of 10% then the heat treated extract containing 5-8 mg protein per ml. was taken to 50% saturation by the addition of 233 g ammonium sulphate per litre with continuous mechanical stirring, prolonged for 10 minutes after the addition of the solid salt, to equilibrate the mixture. The mixture was allowed to stand for 15 minutes then the precipitate was collected by centrifugation at 25,000 x g for 7 minutes at 0°. The precipitate was retained for examination by assay.

The supernatant was taken to 85% saturation by the slow addition of 230 g ammonium sulphate per litre in a manner similar to that used to bring the saturation to 50%. After the mixture was set aside for 15 minutes the precipitate was collected by centrifugation at 25,000 x g for 10 minutes at 0°. The supernatant was discarded and the precipitate dissolved in 0.05M sodium acetate 10^{-4} MEDTA pH 7.2 to a protein content of $\simeq$ 20 mg protein per ml. Small quantities of this buffer were added stepwise to the solid until solution was effected.

Experiment 5. Concentration by salting out at pH 7

The fractionation described in the previous experiment was modified and used as a protein concentrating step. The same procedure already described for salting out proteins was used to obtain a concentrated sample of spleen ribonuclease activity which precipitated when the salt concentration was increased from 40 to 85% saturation. If a 10% residual ammonium sulphate saturation was calculated then the clear supernatant from the heat treatment stage at pH 7 was taken to 40% saturation by the slow addition of 190 g ammonium sulphate per litre and the precipitate produced, if any, discarded after centrifugation. On occasions no precipitate was formed at this stage though on others a quantity of brown coloured protein was removed at centrifugation. The saturation of the supernatant was increased from 40% to 85% by the addition of 330g ammonium sulphate per litre and the precipitate retained after centrifugation. The supernatant was discarded. The precipitate was dissolved in suitable buffer solutions containing 10^{-4} MEDTA then desalted on Sephadex.

Experiment 6. The first acetone fractionation attempt

By the Biuret method, G.M.15, the ribonuclease enzyme solution from Experiment 4 after dialysis, G.M.3, was found to contain 2.7 mg protein per ml. The solution was concentrated by freeze drying then redissolved in 50 ml. deionised water and the protein concentration was 5 mg protein per ml. 6.25 ml. 1M sodium acetate was added to 25 ml. of the concentrated protein solution (1:4 ratio). To 25 ml. of this solution, 20 ml. of acetone at -10° was added and the first

fraction precipitated and centrifuged off at -15°, i.e. 44.4% acetone by volume. The precipitate was dissolved in 20 ml. cold 0.05M sodium acetate 10^{-4} MEDTA pH 7 and stored at -12° until assayed, (Fraction A).

To the supernatant at -15°, 30 ml. acetone at -10° was added to precipitate the second fraction B which was collected and dissolved in cold 0.05M sodium acetate 10^{-4} MEDTA pH 7 (22 ml.) then stored similar to fraction A. To investigate if any activity remained in solution a third fraction was collected by adding 25 ml. acetone at -10° to the supernatant from fraction B and the temperature was allowed to fall from -10 to -20° by the end of the addition at 75% acetone by volume. The precipitate was collected by centrifugation and dissolved in 13 ml. cold 0.05M sodium acetate 10^{-4} MEDTA then treated exactly similar to fraction A. All fractions were assayed and the protein concentrations estimated by the biuret method.

Experiment 7. The second acetone fractionation attempt

A 23 ml. portion of the concentrated enzyme solution from the original 50 ml. volume in Experiment 6 was diluted by the addition of 5.75 ml. 1M sodium acetate. To this volume (28.75 ml.), 25.5 ml. acetone at -10° was added to obtain the first precipitation at -15°, i.e. to 45% acetone by volume, and fraction A was obtained. 34.8 ml. acetone at -10° was added to the supernatant from fraction A at a constant temperature of -15° and the precipitate, fraction B was collected by centrifugation. A third fraction C was produced by

adding 20 ml. acetone (-10°) to the supernatant from the second fractionation until the content was 73% v/v acetone at -20°. The precipitates, fractions A, B and C were drained after centrifugation as detailed in the G.M.4, then dissolved in 37, 25 and 10 ml. of cold 0.05M sodium acetate 10^{-4} MEDTA respectively then stored at -12° in the deep freeze until assayed.

Experiment 8. The third acetone fractionation attempt

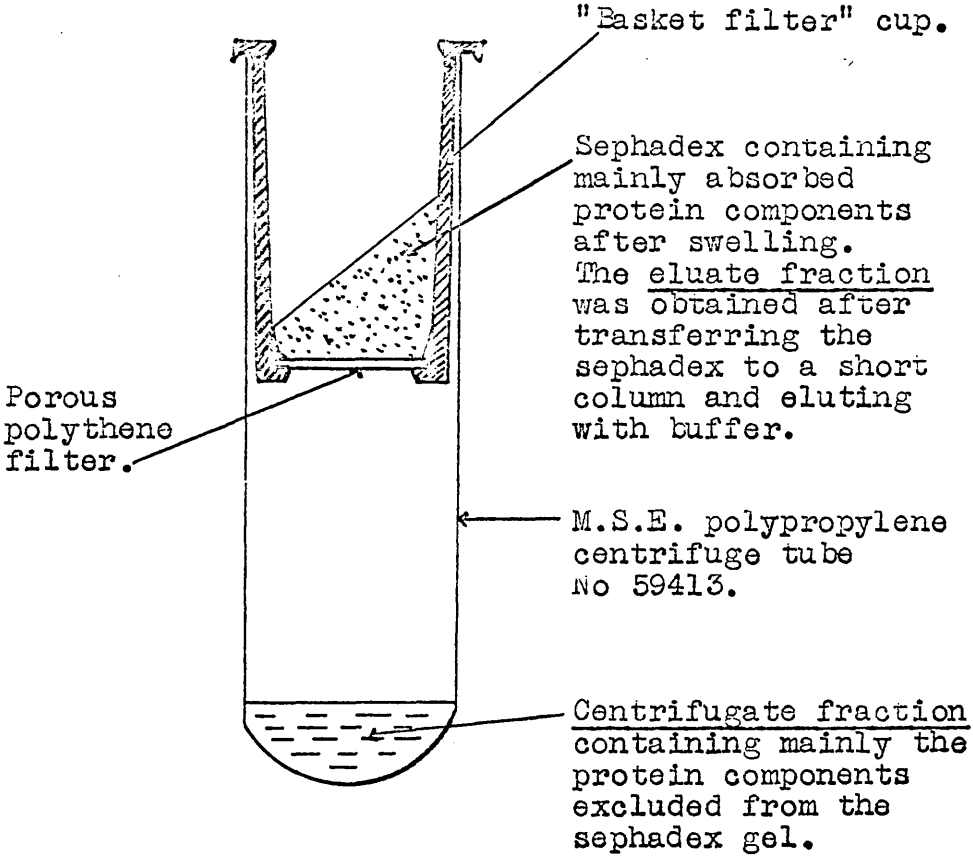
The enzyme sample fractionated was obtained by a similar purification procedure but from a different trial and purified to the same degree as the sample tested in Experiments 6 and 7. 300 ml. of enzyme solution which was dialysed as described for Experiments 6 and 7 contained 1.6 mg protein per ml. as estimated by the biuret method. The sample was freeze dried to concentrate then taken up in exactly 72 ml. of deionised water ready for the acetone fractionation. To this 72 ml., 18 ml. of 1M sodium acetate was added, then 72 ml. of cold acetone (-10°) to bring the acetone content to 44.4% by volume. After 5 minutes the mixture was centrifuged at 13,000 x g for 3 minutes and the precipitate dealt with in a similar manner to that in Experiments 6 and 7. To the supernatant at -15°, 108 ml. acetone at -10° was added (66.6% acetone by volume) and the precipitate retained by centrifugation, allowed to drain at -10° then dissolved and stored as described for the first fraction. The supernatant was discarded without a third fraction being precipitated and both fractions obtained, fractions A and B were assayed and estimates made of the protein concentration.

Experiment 9. The batch treatment of a spleen ribonuclease sample with dry Sephadex G-50.

The spleen ribonuclease sample from Experiment 2 (page 357), obtained by dissolving the precipitate formed by raising the ammonium sulphate concentration 40 to 80% saturation in 0.05M sodium acetate pH 7 10^{-4} MEDTA was taken for the treatment. The solution was not heat treated at 60° for 10 minutes at pH 3.5 according to the details in Experiment 3, page 358. 6.5 g dry Sephadex G-50 medium grade powder was added with stirring to 35 ml. of the spleen preparation in a beaker previously immersed in an ice bath. The Sephadex formed a gel like consistency as water and low molecular weight solutes were imbibed. The mixture became solid, crumbly then dry in appearance on stirring and a further 35 ml. of 0.05M sodium acetate 10^{-4} MEDTA pH 7 was added carefully with stirring to produce a mixture of very thick consistency. The mixture was left for 1 hour with occasional stirring, then centrifuged in a polypropylene basket-filter arrangement, Figure 14, at 200-300 r.p.m. for 5 minutes at 0° on the medium head of the M.S.E. Magnum centrifuge. The centrifugate 21.5 ml. was retained, Figure 14. The gel matrix was transferred to a short column and eluted with 0.05M sodium acetate 10^{-4} MEDTA pH 7.2 to elute the remainder of the preparation. Bulked samples of the eluate were collected. The various fractions were examined for protein concentration, total ribonuclease activity and specific activity by G.M.15a and G.M.14 respectively.

Fig. 14.

The "basket filter" arrangement of the sephadex batch treated protein sample after centrifugation



Experiment 10. The batch treatment of the eluate from Experiment 9 with Sephadex G-25

The eluate from the previous Experiment 9 was freeze-dried on a model 10P freeze dryer made by Edwards High Vacuum Ltd., G.M.17. The solid was taken up in water to a volume of 10 ml. To 5.8 ml. of this solution 1.5 g Sephadex G-25 was added with stirring in a beaker immersed in ice. Approximately 2 ml. 0.05M sodium acetate 10^{-4} MEDTA pH 7 was added to give the mixture an even and thick consistency. After setting aside for one hour at 0° with occasional stirring, the mixture was centrifuged as described in Experiment 9, Figure 14. The centrifugate 3 ml. was retained and the Sephadex matrix transferred to a short column where it was eluted with 0.05M sodium acetate 10^{-4} MEDTA pH 7.2 to remove the absorbed protein. The centrifugate and eluate were examined for ribonuclease activity and protein concentration by G.M.14 and G.M. 15a respectively.

Experiment 11. The batch treatment of a heat treated spleen ribonuclease sample

A similar protein sample to that used in Experiment 9 was heat treated to remove heat labile ribonuclease activity and protein, Experiment 3, page 358. 6.0 g dry Sephadex G-50 medium grade was stirred into 70 ml. of the ribonuclease solution. The mixture was left to stir for one hour before it was basket filter centrifuged then the Sephadex matrix eluted in a small column. The centrifugate and eluate were examined for protein content and ribonuclease

activity by the biuret test G.M.15a and G.M.14 respectively.

Experiment 12. Gel filtration on Sephadex G-25

A column containing Sephadex G-25 equilibrated with 0.05M sodium acetate 10^{-4} MEDTA pH 7.2 and 2.5 x 17.5 cm. gel dimension was prepared from 16 g. dry G-25 Sephadex and made operational in the cold room at 4°. A medium sized glass-wool plug was fitted into the lower end of the column to support the gel while a Whatman No.54 filter paper disc cut to 2.5 cm diameter was placed over the gel top to protect the surface from disturbances on sample application. Full details of general preparations are outlined under early Sephadex column procedures G.M.5 (iiia, iva). The average rate of flow of the column was 10 ml. per hour. The fractions collected were assayed for ribonuclease active content and protein content estimated by the Folin method, G.M.15b. This column was not a technical success.

a) The Locarte automatic drop counting fraction collector proved inconsistent in the cold room owing to an electronic failing of the drop recording mechanism and the sample size of fractions varied considerably.

b) The sample volume applied to the column was too large at 3 ml. (6.5 mg protein per ml.) to expect a refined resolution of separable components.

c) A steady decrease in the flow rate was attributed to the initial wash procedure when too high a hydrostatic pressure was applied.

The nature of the protein and ribonuclease activity pattern at elution was reported on page 129. The Locarte automatic drop

counting fraction collector functions adequately at R.T. thus the other columns in this series were made operational and eluted at this temperature-20-22°.

Experiment 13. Gel filtration on Sephadex G-25

A second Sephadex G-25 column was established by procedures similar to that for the column in Experiment 12, and described above. 13 g dry Sephadex G-25 was conditioned for gel filtration and by using a 1 x 55 cm 50 ml. burette a gel column of effective length 1 x 50 cm was prepared and equilibrated with 0.05M sodium acetate 10^{-4} MEDTA pH 7 at R.T. A 1 ml. spleen ribonuclease sample (15-20 mg per ml.) was applied to the column and eluted with 0.05M sodium acetate 10^{-4} MEDTA pH 7. 0.6 ml. samples were collected at a flow rate of 30 ml. per hour. Protein and ribonuclease activity content were estimated by the Folin reagent and ribonuclease assay method (G.M.15b, G.M.14) respectively. The elution pattern appears on page 131.

Experiments 14 - 18. Gel filtration on Sephadex G-50

A Sephadex G-50 column was made up by the procedures described in G.M.5 (iiia, ivb). 50 g dry Sephadex G-50 medium grade was conditioned for use and by using a glass column 2 x 70 cm a gel column of effective dimensions 2 x 66 cm was prepared, equilibrated with 0.05M sodium acetate 10^{-4} MEDTA pH 7.2 solvent until the effluent was found to have the same UV absorbency and pH as the eluting solvent. Between each experiment the column was eluted in this way to wash.

TABLE 21

Experiment	Sample applied	Fraction size	Rate of flow ml/hr	Assays used for total activities
14	57 mg/ml	1 ml	10	Uranyl acetate trichloroacetic acid soluble organic P by Kings Method G.M.11
15	22 mg/ml	1 ml	5	Increase in U.V. absorbency from "Uranyl and acid soluble nucleotide" release. G.M.15.
16	7.8 mg/ml	1 ml	8	
17	5 mg/0.5 ml	1.5 ml	8	
18	3.5 mg/0.5 ml	1.5 ml	8	

A fresh Whatman No.54 filter paper disc was fitted over the gel between each run. The practical aspects for the operation of these columns are described under early gel filtration procedures (G.M.5). Protein in each tube was estimated by the Folin method, G.M.15b. The nature of the samples applied is detailed in the discussion, page 130. Table 21 gives the essential details for each experiment.

Experiment 19. The prototype column. Ion exchange chromatography of crystalline pancreatic ribonuclease on carboxymethyl cellulose according to the method of Taborsky[90].

The column 0.9 x 31 cm containing suitably charged C.M. cellulose was prepared as described for column procedure and ion exchange chromatography (G.M.7). The ion exchanger was equilibrated with 0.005M tris HCl pH 8 prior to packing into the column. It was then eluted copiously with this buffer in the column until the effluent had an optical density at 280 mμ in a 1 cm light path of $\simeq$0.01 against the equilibrating buffer as reference. 100 mg of crystalline pancreatic ribonuclease previously prepared in this laboratory by the method of Kunitz[50] as modified by McDonald[51] and crystallised x 3 from ammonium sulphate then x 2 from 95% ethanol was dissolved in 6 ml. 0.005 M tris HCl pH 8 and the pH checked at 8 before the solution was applied to the column. The solution was carefully added to the column and washed on with a small quantity of equilibrating buffer.

A linear gradient elution was applied by the gradient elution

system depicted in Figure 35, page 312 . The salt concentration rose from zero NaCl to 0.1M NaCl. The mixing chamber contained 500 ml. 0.005M tris HCl pH 8 while the stock reservoir chamber of similar dimensions and at the same solvent level contained 500 ml. 0.005M tris HCl pH 8,0.1M NaCl. The gradient was applied immediately and after 870 ml. had passed from the mixing chamber this solution was replaced by an eluting solution of 0.005M tris HCl pH 8 1M NaCl to remove any protein residue from the column. 3 ml. fractions were collected at a flow rate of 10 ml. per hour. A measure of the protein concentration in each fraction was made by reading the optical density at 280 mμ in a 1 cm quartz cell against distilled water as reference. The elution pattern is presented on page 162 Figure 18 and the protein peaks A to E were assayed for pancreatic ribonuclease enzyme by the method of McDonald[51].

Experiment 20. An attempt to fractionate the crude spleen enzyme sample by cation exchange chromatography at pH 7.5 with a strong salt gradient

A column 0.9 cm x 35.5 cm was packed with C.M. cellulose, prepared thenequilibrated with the starting buffer 0.02M tris HCl pH 7.5 and set up according to the general procedure already described, G.M.7. After the column had been eluted copiously with starting buffer 0.02M tris HCl pH 7.5, 420 mg of the crude spleen preparation, page 118, was dissolved in 10 ml. of this buffer and the pH checked. The solution was applied to the column and washed on with a few ml. of starting buffer.

A linear gradient elution system where the salt concentration rose from zero NaCl to 4M NaCl over a volume of 1 litre was immediately applied. The mixing chamber contained 500 ml. 0.02M tris HCl pH 7.5 and the second chamber contained 500 ml. 0.02M tris HCl pH 7.5 4M NaCl. 2.5 ml. fractions were collected on the Towers Automatic siphon balance fraction collector at a flow rate of 15-18 ml. per hour. After 105 fractions were collected a solution of 0.2M tris HCl pH 7.5 4M NaCl was washed directly through the column to remove residual protein and thirty six fractions were collected. The protein concentration of each fraction was measured by reading the optical density of the solution at 280 mμ in a 0.5 cm light path against the starting buffer as reference on the U.V. spectrophotometer. Each tube was assayed for ribonuclease activity as described in G.M.14. The elution pattern illustrating the distribution of protein and ribonuclease activity eluted is presented on page 162, Figure 19.

Experiment 21. An attempt to fractionate the crude spleen enzyme sample by cation exchange chromatography at pH 6.8 with a strong salt gradient

A column 0.9 cm i.d. x 36 cm containing C.M. cellulose was set up in a similar manner to that described in Experiment 20. The equilibrating and starting buffer was 0.02M tris HCl pH 6.8. 500 mg of the crude spleen sample dissolved in 10 ml. of the starting buffer was very carefully applied to the column after the pH was adjusted to 6.8. This spleen preparation like that used in the previous

experiment was taken from the same bulk sample prepared by salting out with ammonium sulphate at pH 3.5, heat treated at pH 3.5 according to Experiment 3, page 358, then dialysed before freeze drying and stored at -12° in the deep freeze, G.M.17. After the 10 ml. sample was applied the column was eluted to wash with 60 ml. of the starting buffer before the gradient was applied. The mixing chamber of the gradient system contained 500 ml. 0.02M tris HCl pH 6.8 while the reservoir chamber at the same solvent level contained 500 ml. 0.02M tris HCl pH 6.8, 3M NaCl. The gradient was linear over 1 litre from zero NaCl to 3.0M NaCl. 5 ml. samples were collected on the Towers Automatic siphon balance fraction collector at a flow rate of 15 ml. per hour. The protein concentration of each fraction was measured by the Folin method, G.M.15b, and by measuring the optical density of the contents on the U.V. spectrophotometer at 280 mμ in a 0.5 cm quartz cell against the eluting buffer. Each fraction was examined for ribonuclease activity by the assay method described in G.M.14.

Experiment 22. An attempt to fractionate the crude spleen enzyme sample by cation exchange chromatography at pH 6.8 with a convex gradient of low salt strength

A C.M. cellulose column prepared and set up by the general procedures described in G.M.7 was 1 cm. i.d. x 58 cm. effective length. 500 mg of the crude spleen preparation from the bulk supply sample was dissolved in 7 ml. of 0.005M tris HCl pH 6.8 and the pH checked. This volume was applied with care to the column top then

the column eluted with 50 ml. of starting buffer. The convex gradient elution device, Figure 36, page 312, produced the convex gradient effect from zero NaCl to 0.25M NaCl over 1 litre. The fixed volume was 400 ml. 0.005M tris HCl pH 6.8 in the mixing chamber and the stock supply reservoir contained 0.005M tris HCl pH 6.8 0.25M NaCl. 2.5 ml. fractions were collected on the Towers automatic siphon fraction collector at a flow rate of 20 ml. per hour. The fractions were examined for protein at 280 mμ in a 0.5 cm light path on the U.V. spectrophotometer against the starting buffer as reference.

After 1080 ml. from the system had eluted the column an eluting solution containing 1M NaCl 0.005M tris HCl pH 6.8 was passed through the column to remove residual protein. All the fractions were assayed for ribonuclease activity, G.M.14. Figure 22, page 173, illustrates the elution pattern.

Experiment 23. A pretreatment of the crude spleen enzyme preparation on a D.E.A.E. cellulose column before cation exchange chromatography

D.E.A.E. cellulose and a D.E.A.E. cellulose column 1.8 cm i.d. x 38 cm effective length were prepared by the procedures described in G.M.7. The equilibrating and eluting buffer was 0.005M tris HCl 10^{-4} MEDTA pH 7. 1.5 g by weight of the freeze dried, spleen enzyme preparation was dissolved in 12.5 ml. of equilibrating buffer, taken to pH 7 and briefly dialysed before application to the column. The column was eluted with 0.005M tris HCl 10^{-4} MEDTA pH 7 and 5 ml.

samples were collected at a flow rate of 15 ml. per hour. After 46 fractions were collected the column was eluted with the same buffer which contained 0.5M NaCl until 30 fractions were eluted. After this the same buffer was made 1M with NaCl and a further 20 fractions collected. Figure 24, page 178, illustrates the elution pattern.

Experiment 24. An attempt to fractionate the ribonuclease active effluent from the DEAE cellulose pretreatment of Experiment 23 by cation exchange chromatography on C.M. cellulose with a convex gradient of low salt strength.

The column preparation techniques described were used to prepare a C.M. cellulose column 1 cm i.d. x 61 cm effective length, G.M.7. The equilibrating and initial eluting solvent was 0.005M tris HCl 10^{-4} MEDTA pH 6. Prior to the application of the enzyme sample 1.5 litres of equilibrating buffer was passed through the column. The ribonuclease active preparation not retained by the DEAE cellulose pretreatment of Experiment 23 was taken to pH 6 then dialysed briefly against the equilibrating buffer before it was applied to the C.M. cellulose column under gravity flow. The last of the solution was eluted through with 100 ml. of the equilibrating buffer. The convex gradient system, Figure 36, page 312, was then set in motion. The fixed volume reservoir contained 400 ml. 0.005M tris HCl 10^{-4} MEDTA pH 6 while the stock buffer reservoir contained 0.25M NaCl in 0.005M tris HCl 10^{-4} MEDTA pH 7. The gradient was continued over 1 litre then replaced by 1.5M NaCl in 0.005M tris HCl 10^{-4} MEDTA pH 7 which eluted any protein

residue retained after the convex gradient was discontinued.

5 ml. fractions were collected at a flow rate of 20 ml. per hour on the Towers automatic siphon balance fraction collector. Figure 25, page 178, illustrates the elution pattern and distribution of ribonuclease activity.

Experiment 25. A preliminary attempt to fractionate the spleen ribonuclease enzyme preparation on C.M. Sephadex C 50

100 g C.M. Sephadex, medium grade, lot No. To 160M with exchange capacity 4.7M.eq/g dry powder was cyclised then equilibrated with solvent according to G.M.7b. The equilibrating solvent was 0.005M tris HCl 10^{-4} MEDTA 0.05M NaCl pH 6. The column conditions and technique were essentially the same as that used with C.M. cellulose in Experiment 24.

A C.M. Sephadex column 1.5 cm i.d. x 38 cm effective length was prepared by the column packing procedure G.M.7c and 1.2 litres of the equilibrating solvent 0.005M tris HCl 10^{-4} MEDTA 0.05M NaCl pH 6 was passed through the column to wash and ensure thorough equilibration and stabilisation of the gel. 600 mg of the crude spleen enzyme preparation, by weight, was applied to the column after the lyophilised sample was dissolved in the equilibrating buffer then equilibrated against the same buffer by dialysis with three changes of diffusate solution over 12 hours. The enzyme solution (18 ml.) was applied by gravity flow, then the column eluted with 200 ml. of equilibrating buffer to wash. The fixed volume in the convex gradient elution system applied was 400 ml. of 0.005M tris HCl 10^{-4} MEDTA

0.05M NaCl pH 6 and the stock solution reservoir contains 0.05M tris HCl 10^{-4} MEDTA 0.3M NaCl pH 7. The column was eluted by this gradient system until 725 ml. passed through the column. At this point the solution in the stock reservoir was replaced by 0.005M tris HCl 10^{-4} MEDTA 0.6M NaCl pH8 and the column was eluted until a further 580 ml. passed through the column. Finally the column was eluted with 1.2M NaCl in 0.005M tris HCl 10^{-4} MEDTA pH 8 to desorb any protein residue adsorbed to the column. 5 ml. fractions were collected on the Towers automatic siphon balance fraction collector at a flow rate of 25 ml. per hour. Figure 26, page 191, illustrates the elution pattern.

Experiment 26. Column chromatography of the crude spleen ribonuclease preparation on C.M. cellulose where the procedure was scaled up by a tenfold increase in capacity

A large stock of C.M. cellulose was recyclised then equilibrated as described in the general procedure for the preparation of this exchanger G.M.7a. A column of effective dimensions 3 cm i.d. x 60 cm long was packed by the prescribed method, G.M.7c, with equilibrating solution containing 1M NaCl present. A circular pad of Whatman 3 mm filter paper was placed at the column top then the column was eluted copiously with 4 litres of initial solvent 0.005M tris HCl 10^{-4} MEDTA pH 5.5.

4.5 g by weight of the crude enzyme preparation was taken up in the minimum possible of initial buffer solution then dialysed against the initial buffer with x 3 charges of diffusate solution of 1 litre

over eight hours. The sample was applied to the column by the technique outlined in the column procedure, section G.M.7. In all 63 ml. of the equilibrated enzyme solution was applied slowly with the usual precautions to facilitate even adsorption. The column was eluted with 2.2 litres to remove material not retained by the exchanger. A convex gradient system, Figure 36, page 312, where the fixed volume reservoir A' contained 4 litres of initial equilibrating solution 0.005M tris HCl 10^{-4} MEDTA pH 5.5 and the stock solution in the reservoir B' was 0.005M tris HCl 10^{-4} MEDTA 0.3M NaCl pH 8.2, was applied to the column. After 7 litres of the effluent had been collected the convex gradient was discontinued and the column eluted directly with 2 litres of 1.3M NaCl in 0.005M tris HCl 10^{-4} MEDTA pH 8.2.

50 ml. fractions were collected on the Towers automatic siphon balance fraction collector at a flow rate of 84 ml. per hour. The elution pattern presented on Figure 28, page 187, illustrates the distribution of protein and ribonuclease activity.

Experiment 27. Ion exchange chromatography of the spleen ribonuclease active peak "B" which was obtained in the effluent of Experiment 26 (Figure 28, page 187), on a C.M. Sephadex C 50 column at pH 8.

The column 1.8 cm i.d. x 26 cm effective length containing C.M. Sephadex was prepared and equilibrated as described in G.M.7b. The sample labelled ribonuclease active peak "B" on Figure 28 (tubes 144-161 inclusive containing 800 ml.) was dialysed in 18/32" visking

tubing against the column equilibrating buffer 0.005M tris HCl 10^{-4} MEDTA pH 8, after adjusting the pH of the bulked samples to 8. The equal volume of diffusate solution was charged x 3 over 6 hours. The protein solution was applied to the C.M. Sephadex from a sample reservoir and 50 ml. fractions were collected, assayed for ribonuclease by G.M.14, then rejected when the tests proved negative. The flow rate at application was 50 ml. per hour.

After sample application the column was eluted with 1 litre of initial eluting buffer 0.005M tris HCl 10^{-4} MEDTA pH 8 to wash the column. The convex gradient system, Figure 36, page 312, was applied. The fixed volume chamber A' contained 400 ml. 0.005M tris HCl 10^{-4} MEDTA pH 8 and the stock solution chamber B' contained the final eluting solution 0.005M tris HCl 10^{-4} MEDTA 0.3M NaCl pH 8. Over the gradient span 10 ml. fractions were collected at a flow rate of 50 ml. per hour. After the gradient was discontinued the column was eluted with 1.5M NaCl in 0.005M tris HCl 10^{-4} MEDTA pH 8. Figure 29, page 190 , depicts the elution pattern for the chromatogram.

Experiment 28. Ion exchange chromatography of the ribonuclease active peak "A" obtained in the effluent from Experiment 26 (Figure 28, page 187) on the same C.M. Sephadex column used at Experiment 27 at constant pH = 8

Experiment 27 was repeated exactly as described above. The same column conditions relating to eluting buffers and gradient system was retained. 350 ml. of spleen ribonuclease active peak "A" (tubes 119 - 125) was dialysed after adjusting the pH to 8 as described for

the active "B" sample in the previous experiment. Before the application, the column was washed free of salt by eluting with 1 litre of the equilibrating buffer then repacked and requilibrated. The distribution of protein and ribonuclease activity after the column was developed is presented on Figure 30, page 190.

Experiment 29. Rechromatography of the ribonuclease active peak "A" which was obtained in Experiment 28 (Figure 30, page190) on the same C.M. Sephadex column at pH 8.

The experimental detailed previously in Experiments 28 and 29 was repeated exactly as already described. The peak of ribonuclease activity labelled "A_1" (160 ml.) and now characterised by its close association with the protein peak "x", Figures 28 and 30, was bulked, dialysed briefly at pH 8 then applied to the requilibrated C.M. Sephadex column. The column was eluted by the convex gradient system exactly similar to that already described for Experiments 27 and 28. 10 ml. samples were collected at a flow rate adjusted to 50 ml. per hour. The elution pattern is presented in Figure 31, page 191.

Experiment 30. The determination of void volume and potential range of the elution pattern of a preparative scale gel filtration column of Sephadex G-75.

The large scale column of Sephadex G-75 was set up as described in G.M.6. A 5 ml. sample of equilibrating buffer containing "blue dextran 2000" (2%w/v) supplied by Pharmacia, and sucrose at 5 mg/ml. was layered on to the gel top, G.M.5 iv.b, page 298 , and the column

eluted with the equilibrating buffer. 0.05M tris HCl 10^{-4} MEDTA pH 7.5. 10 ml. fraction samples were collected by the Towers automatic siphon balance fraction collector at a flow rate of 90 ml. per hour.

The location of the "blue dextran" component was determined visually and the contents of the fractions estimated colorimetrically at 680 mμ (Filter 608) on the EEL colorimeter. The elution pattern for sucrose was established by the phenol sulphuric acid method, G.M.13. The results are expressed on the elution pattern, Figure 32, page 149.

Experiment 31. The gel filtration of bovine plasma albumin on the preparative scale Sephadex G-75 column as a test protein sample.

100 mg of crystalline bovine plasma albumin from lot No.43 supplied by Armour laboratories was dissolved in 10 ml. 0.05M tris HCl 10^{-4} MEDTA pH 7.5 and after centrifugation to remove a little insoluble material. 7 ml. of the sample was applied to the 5 cm x 75 cm column by exactly the same method as used in Experiment 30. 10 ml. fractions were collected on eluting with buffer. Each fraction was examined for protein by measuring the optical density of the contents at 280 mμ in a Beckman spectrophotometer with a 1 cm light path, against the equilibrating buffer as reference. The elution pattern is illustrated in Figure 33, page 149.

Experiment 32. Gel filtration of the heat stable spleen ribonuclease sample prepared by the preliminary purification procedure cutlined on page 118 on the preparative scale Sephadex G-75 column.

The ribonuclease active fraction precipitated with ammonium sulphate at pH 7 within the 40-85% saturation limits, Experiment 5, was dissolved in 0.05M tris HCl 10^{-4} MEDTA pH 7.5 then centrifuged to remove any undissolved protein. 7 ml. of the ribonuclease active spleen sample was layered on the column by exactly the same method as described for Experiments 30 and 31 and the column eluted with the buffer to develop the molecular sieve process. 10 ml. samples were collected and the protein concentration estimated by measuring the optical density of the solutions at 280 mμ on the U.V. spectrophotometer with a 1 cm light path against the buffer as reference. The distribution of ribonuclease activity was established by assaying each fraction for ribonuclease activity G.M.14. The protein and ribonuclease activity patterns are illustrated on page 149, Figure 34.

Experiment 33. Experimental conditions at disc electrophoresis to obtain the gel electrophoretograms presented in the photographs (Figures 39-49, pages 228 - 251).

All the experiments undertaken were based on the procedures set out as G.M.9 and 10, pages 320 - 336. . It is convenient to describe the conditions for the disc electrophoresis runs with reference to each figure and to the labelled gels of each set of electrophoretograms rather than by a series of experiment numbers.

The amount of protein applied at each electrophoresis cannot be included as in most cases it was not determined. From time to time protein samples were diluted with spacer gel solution if over concentrated, or greater volumes of enzyme solution were applied if the solution gave a poor electrophoretogram, until a suitable electrophoretogram was obtained.

Figure 39.

The gel column A 3 contained 15% acrylamide polymerised at pH 4.3 and was from a set of eight tubes exposed to a 13 minute electrophoresis at 16 m.amps and 25 volts per tube. A prototype power pack was in use for this experiment and the current and voltage applied were excessive. After staining overnight the gels were destained electrophoretically at 100 m.amps and 200 volts.

The gel columns A_1, A_2 and A_4 contained 22.5% acrylamide set at pH 4.3 and were from a batch of eight tubes submitted to electrophoresis at a controlled 6 m.amps and 17 volts per tube. The run extended over 25 minutes then the gels were stained overnight. They were destained at 100 m.amps and 200 volts with six gels only in combination.

Figure 41.

The gel columns B_1, B_2 contained 15% acrylamide set at pH 4.3. They were submitted to electrophoresis over 25 minutes with four other tubes in a balanced arrangement about the electrodes at 10 m.amps and 21 volts per tube. The gel B_1 was stained overnight then destained at

80 m.amps and 200 volts with three other tubes containing gels.

The gel columns B_3, B_4 contained 22.5% acrylamide polymerised at pH 4.3 and were from a batch of eight tubes submitted to electrophoresis for 25 minutes at 6 m.amps and 25 volts per tube. Gel B_3 was stained overnight then destained.

Gels B_2 and B_4 were treated separately. They were washed in distilled water then placed in tubes 1 cm x 10 cm which contained 0.25M sodium succinate buffer made 0.05M W.R.T. Mg^{++} at pH 6.5 and 6 Mg RNA/Ml. The whole gels and bathing substrate solution were incubated at 37.5° for six hours. The gels were removed, copiously washed with distilled water to remove excess R.N.A. solution then placed in a solution of MacFadyen's reagent[114], page 343 . The gels became opalescent in those regions containing R.N.A.

Figure 40.

The gel columns C_1 - C_3 contained 7.5% acrylamide set at pH 4.3 and were from a batch of eight tubes submitted to electrophoresis for 28 minutes at 7.5 m.amps and 17.5 volts per tube. The gels were stained for two hours then destained at 100 m.amps and 160 volts over 1 hour.

Figure 42.

The gels D_1 - D_4 contained 7.5% acrylamide set at pH 4.3 and were submitted to electrophoresis for 20 minutes at 7 m.amps and 17 volts per tube with an eight tube electrophoresis set up. The tubes were stained for several hours then electrophoretically

destained at 100 m.amps and 160 volts.

Figure 43.

The gels E_1 - E_4 contained 7.5% acrylamide polymerised at pH 4.3.

Figure 44.

The gels F_1 - F_4 contained 15% acrylamide polymerised at pH 6.5.

Figure 45.

The gels G_1 - G_4 contained 22.5% acrylamide and G_5 7.5% acrylamide polymerised at pH 4.3.

The samples on Figures 43, 44 and 45 were submitted to electrophoresis for about 30 minutes at 7 m.amps and 16 volts per tube when the electrophoresis assembly was fully loaded with eight tubes on each occasion. After staining overnight the gels were destained at 90 m.amps and 220 volts.

Figure 46.

The gels H_1 - H_4 contained 30% acrylamide polymerised at pH 6.5 and were submitted to electrophoresis with two similar gels, i.e. six in all placed equidistant from a central electrode for 1 hour 45 minutes at 2 m.amps and 9 volts per tube. The gels were stained overnight then destained electrophoretically.

The times for each run in Figures 43, 44 and 45 were not accurately recorded. In these samples the refractile band was allowed to travel half way down the gel before the current was switched off. With the samples on Figure 46 the same effect was observed over 1 hour 45 minutes.

Figure 48.

The gel columns I_1, I_2 contained 15% acrylamide polymerised at pH 4.5 and gels I_3 - I_5 contained 15% acrylamide polymerised at pH 5.5. These gels were submitted to electrophoresis at 7 m.amps and 17 volts per tube for about 30 minutes when the refractile band had travelled half way down the gels. The gels were stained then electrophoretically destained.

Figure 49.

All the gel columns contained 15% acrylamide. J_1 - J_3 were polymerised at pH 4.5, J_4 & J_5 at pH 5.5, J_6 & J_7 at pH 6.5 and J_8 & J_9 at pH 7.5. The electrophoresis lasted 30 minutes at 7 m.amps and 13 volts per tube. The gels were stained overnight then destained electrophoretically at 120 m.amps and 150 volts.

Experiment 34. An estimation of the molecular weights of the spleen ribonuclease active fractions from the C.M. cation exchange columns by molecular sieve chromatography on a Sephadex G-75 column calibrated with proteins of known molecular weight.

25 g Sephadex G-75 lot No. To 5079 particle size 40-120μ was allowed to swell in equilibrating buffer for 3 days as described in the paragraph on gel preparation (G.M.5i). The suspension was deaerated under reduced pressure before packing into a column of the B type depicted in Figure 15, page 296. The column 2.3 cm i.d. x 55 cm effective length was prepared, equilibrated, stabilised and run

as described in detail in the paragraphs on Sephadex column procedure in G.M.5, iii, iv, v. Extensive care was taken to ensure the column was packed evenly at the vertical. The equilibrating and eluting buffer was 0.05M tris HCl pH 7.5 0.25M NaCl and the column was further equilibrated and stabilised by eluting with buffer over 2-3 days. Over this period the flow rate was adjusted to 18-22 ml. per hour by raising or lowering the stock reservoir to an appropriate level after which the column maintained a constant height of 55 cm. The column was allowed to elute continuously throughout the series of experiments to maintain gel bed stability and a constant stock buffer level per run was obtained by replenishing the reservoir two hours before each run every day to a predetermined level in the reservoir with respect to the position of the column ($\simeq$ 480 ml./day during the course of the columns use). The gel filtrations were carried out in the chromatography room at $20^{\circ} \pm 2^{\circ}$.

1 ml. sample volumes were applied to the column top by the layering procedure depicted on Figure 16, page 299 , as a well defined thin disc on the gel top under 3 cm of buffer head. The sample was layered accurately without mixing with gel particles or buffer as a zone 2mm thick.

Five protein standards were used to calibrate the column and details of the concentrations used are given in Table 29, page 389.

1. Glucagon was a crystalline sample obtained as a gift from Eli Lilly & Co., Ltd.
2. Pancreatic ribonuclease was available as it had been prepared

previously in this laboratory from ox pancrease and extensively crystallised (page 370).

3. Crystalline trypsin inhibitor from soya bean meal and
4. Crystalline ovalbumin were commercial samples supplied by Koch Light Laboratories Ltd.
5. Bovine serum albumin Fraction V was a commercial sample supplied by Armour Pharmaceuticals Ltd.

Blue dextran 2,000 supplied by Pharmacia, Upsalla was introduced to determine the void volume of the column, to act as a periodic check on column packing and serve as a control in suitable chromatograms at a solution strength of 0.2% w/v.

Sucrose (B.D.H. reagent grade) was included at the rate of 2.6-10 mg/ml. to any protein sample of low viscosity. This was sufficient to produce a layering effect at sample application.

The ribonuclease activity "A" used for the molecular weight estimation was that activity eluted from the scaled up chromatography on C.M. cellulose first then rechromatographed at pH 8 until a refractionation did not occur, i.e. the activity "A_1" depicted in Figure 31, page 191 , of Section III.

The spleen ribonuclease activity "B" was that activity eluted last from the scaled up column of C.M. cellulose then rechromatographed at pH 8 to yield a single ribonuclease activity peak "B", see Figure 29, page 190 , Section III.

Protein standards, enzyme samples, Blue dextran and sucrose were made available in the eluting buffer at the desired concentrations as listed in Table 29.

TABLE 29.

Solute	Concentration	Column effluent examined
Glucagon	1 mg/ml	at 230 m 1 cm cell U.V. Spectrophotometer.
Pancreatic ribonuclease	1 mg/ml	by assay (page 390)
Trypsin Inhibitor	0.75 mg/ml	at 230 m 1 cm cell U.V. Spectrophotometer.
Ovalbumin	1 mg/ml	" " " " "
Bovine Serum Albumin	1 mg/ml	" " " " "
Blue dextran	0.2%	at 260 m " " "
Sucrose	2.5-5 mg/ml	by colorinmeter at 490 m filter 603
Spleen ribonuclease activity A	60 units/ml	by assay G.M.14
Spleen ribonuclease activity B	100 units/ml	by assay G.M.14

The column effluent was collected as 3 ml. fractions on the Gallenkamp Towers automatic siphon balance fraction collector. Each gel filtration commenced with an empty siphon immediately the 2mm protein layer entered the gel top. The column was turned off at the stopper while the samples were applied and care was exercised to retain procedure sequence. After application of the sample and the empty siphon was in position the column tap was turned full on before the supply tube, fully charged with buffer from the reservoir was placed in position. In this way the same starting conditions were in force throughout. Favourable column techniques described in the section on Sephadex column procedure were rigidly observed.

Examination of the column effluent.

The effluent samples were examined for U.V. absorbency in the U.V.

spectrophotometer at suitable wavelengths, using 1 cm matched quartz cells, against the equilibrating buffer as reference, Table 29. The sucrose peak, used to determine the end of the elution span, was detected by the phenol sulphuric acid method G.M.13. Spleen ribonuclease activity was located by the assay technique described in G.M.14, page 341, and bovine pancreatic ribonuclease was estimated using this technique modified to a 10 minute digestion time at 20° with very dilute enzyme samples to record the activity peak.

In all 20 gel filtration trials were successfully completed and the elution patterns of the protein standards and ribonuclease samples examined. The effluent volume Ve, Table 30, page 271, for each sample applied was obtained from the elution pattern by extrapolating both sides of the solute peak to an apex. This point represented the region of maximum concentration of each solute. The number of chromatographic runs carried out and the samples applied on each occasion are presented in Table 30 along with the Ve values obtained. The results are presented on page 272 and Figure 50.

Experiment 35. Anomalous molecular sieve chromatography

Several spleen ribonuclease active samples were molecular sieved on the 2.3 cm i.d. x 55 cm Sephadex G-75 column at the time the 20 suitable gel filtration trials were carried out for Experiment 34. These spleen samples exhibited irregular elution profiles which were not used at the molecular weight estimation experiment. The conditions for these irregular elution results are reported briefly here as Experiment 35.

The elution profiles of the spleen ribonuclease active samples concerned are presented in Figures, 57, 58, 59 and 60, page 262. In each gel filtration 1 ml. of enzyme solution was layered on the column as a finite band between the gel and buffer head without intermixing. Irregular elution could not be attributed to imperfect application of sample. Figures 59, 61, 62 and 63, pages 262, 263, depict the elution patterns, for spleen ribonuclease active fraction B, sucrose, pancreatic ribonuclease and trypsin inhibitor and were taken from the results of Experiment 34. Since the same column was used for both sets of figures, all seven figures can be directly compared.

The molecular sieve column technique has already been described in Experiment 34 and G.M.5, page 292.

Figure 57.

This figure depicts the elution profile after the gel filtration of 1 ml. of a sample of spleen ribonuclease active fraction "A_1" from Figure 31, page 191 , and from Experiment 29, page 380. The enzyme sample had been freeze dried then stored as a white solid for several months in the deepfreeze at -12°. Before freeze drying the sample had been dialysed against 0.005M tris HCl 10^{-4} MEDTA pH 7. 7 mg by weight of the freeze dried powder, which must contain salts from 0.005M tris HCl 10^{-4} MEDTA, was dissolved in 1 ml. of 0.05M tris HCl, pH 7.5, 0.25M NaCl and applied to the column. The column was eluted at 16 ml. per hour and 3 ml. fractions were collected. Sucrose was not present in the sample when applied.

Figure 58.

The ribonuclease active sample "A" applied to the column was not freeze dried at any time. The solution had been concentrated on a small C.M. cellulose column G.M.8d after the fractionation on C.M. cellulose as Experiment 28, page 187. The solution had been heat treated at 70° for 10 minutes at pH 3.5 as described in Section III, page 358, before a sample was molecular sieved in the molecular weight estimation experiment.

1 ml. of the enzyme solution containing ≃ 60 units of ribonuclease activity was applied to the 2.3 cm i.d. x 55 cm Sephadex G-75 column and eluted with 0.05M tris HCl pH 7.5 0.25M NaCl as was the sample in Figure 57. Sucrose at 5 mg/ml was included in the 1 ml. sample applied to aid the layering effect. Rate of flow was 18 ml/hr and 3 ml. samples were collected.

Figure 60.

The elution pattern was obtained by the gel filtration of spleen ribonuclease active sample "B" from Experiment 27, page 378, as depicted in the elution pattern of Figure 29, page 190. The active fractions from this elution pattern had been bulked, dialysed against 0.005M tris HCl 10^{-4} MEDTA pH 7 then freeze dried to a white fluffy powder and stored in the deep freeze at -12°. 4 mg by weight of this powder was dissolved in 1 ml. 0.005M tris/HCl pH 7.5 0.25M NaCl then applied to the Sephadex G-75 column and molecular sieved. 3 ml. fractions were collected at a flow rate of 18 ml./hr.

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