PHYSICAL AND BIOLOGICAL STUDIES ON SOME ENZYMES

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PREFACE

The experimental work described in this thesis was carried out in the Agricultural Section of the Chemistry Department, University of Glasgow, under the supervision of Dr. W.R. Rees, from October 1964 to September 1967.

I gratefully acknowledge receipt of a Science Research Council Studentship during the period of this research project.

I thank Professor Robertson, F.R.S. for the use of facilities in the Chemistry Department, as well as in the Agricultural Section.

Also I would like to record my thanks to Dr. W.R. Rees for his supervision, constant encouragement and inspiration.

September 1967.

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Abbreviations used.(Many of the below abbreviations are more applicable to interpretation of tables etc. than to the general text.)

alk.	alkaline
AS	ammonium sulphate
BDH	British Drug Houses
CM-cellulose } CMC }	carboxy-methyl cellulose
cms.	centimetres
const.	constant
DCL	Distillers Company Limited
DEAE-cellulose	diethylaminoethyl-cellulose
ECTEOLA-cellulose	epichlorohydrintriethanolamine-cellulose
EDTA	ethylenediaminetetraacetic acid
Ex.,Exp.	experiment
g	gravity
g.,gm.	grams
GM	General Method
hrs,	hours
IR.	infra red
l.	litres
LKB	LKB-Produkter AB,Stockholm-Bromma-I
M	molar
max.	maximum

ml.	millilitre
min.	minimum
M.Wt.	molecular weight
N	normal
NaAc	sodium acetate
Nucleotides:	
U,UMP,&U3'P	uridylic acid or uridine 3'-phosphate
A,AMP,& A3'P	adenylic acid or adenosine 3'phosphate
G,GMP,& G3'P	guanylic acid or guanosine 3'phosphate
C,CMP, & C3'P	cytidylic acid or cytidine 3'phosphate
O.D.	optical density
prep.	preparation
ppt.	precipitate
pption.	precipitation
ref.	reference
RNA	ribonucleic acid
RNAse	ribonuclease
r.p.m.	revolutions per minute
w.r.t.	with respect to
TLC	thin layer chromatography
Tris	Tris(hydroxymethyl)amino methane
U.V.	ultra violet
vol.	volume

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INTRODUCTION

Protein catalysts or enzymes are involved in every chemical process of anabolism and catabolism in all living material. Probably the number of documented enzymes is only but a fraction of the total number of different types of enzymes involved in vital processes. Of these enzymes, the group known as nucleases have been the object of considerable research and in particular the ribonucleases and deoxyribonucleases. The reason that this group has attracted so much attention is twofold. Firstly, is that they are enzymes involved in the breakdown (and synthesis, since many of their reactions are reversible) of ribonucleic acid and deoxyribonucleic acid, both of which are involved in directive procedures like protein synthesis. Secondly, ribo- and deoxyribonucleases have been of interest because many of them are of a relatively small molecular weight and relatively easily characterized compared to many other types of enzymes. That is, their action is well defined and there seems to be little complication due to the necessity for cofactors and other activating compounds, as in the digestive system's enzymes for example.

Although the action of nucleases is well defined *in vitro* the exact metabolic role *in vivo* is still somewhat uncertain. In the case of the extracellular nucleases the function is more than probably one of breaking down unwanted polynucleotydic material - a scavenger function. A study was made of DNAse

(deoxyribonuclease) II in the developing mouse embryo (1) and it was found that generally DNAse activity was higher in organs and areas of the body concerned with metabolic breakdown, such as spleen and liver. Thus metabolic breakdown of DNA (deoxyribonuclease acid) in these areas would be one of the prime functions of DNAseII. Probably this could be said for extracellular ribonucleases as well.

The question of what function the intracellular nucleases play in metabolism is much harder to answer. These types of enzymes are abundant in variety, and although a degradative and repair role in cells could be visualised, it is an unsatisfactory theory since there seems to be little degradation of nucleic acids occurring in most functioning cells. With one major exception, the nucleic acids seem to be the stable end products of metabolism, and the exception is the small fraction, undergoing rapid turnover which is generally considered to be or to contain RNA.

There have been many suggestions as to the role both intra and extracellular nucleases might play <u>in vivo</u> but so far it has not been possible to reach a decision. However the role of nucleases <u>in vitro</u> is of great importance with respect to the investigation of nucleotide sequence in RNA and thus the key to understanding of protein synthesis (2, 3). The basic operation in this <u>in vitro</u> application of nucleases, is the enzymatic cleavage of RNA into oligonucleotides which are then

separated and characterized. As with a proteolytic enzyme approach to the study of protein structure (4, 5, 253) the chance of an unequivocal sequence determination is greater the more different ways there are to split RNA. This is a function of the degradative specificity of the nucleases available. Thus enzymes with new specificities would be useful. The alternative approach is to alter the RNA or DNA so that the nuclease enzyme breaks fewer bonds, thus yielding larger nucleotide fragments.

Ribonuclease enzymes with absolute specificity of action, and so of the greatest use in RNA sequence determination, are at present few in number. Most of the ribonucleases with their specificity established, so far show mainly a preferential specificity and not an absolute specificity. None the less ribonucleases having preferential specificity are of some use in RNA sequence determination.

The nuclease group of enzymes as a whole can be classified into two divisions according to the mode of action - exonucleases and endonucleases. The exonucleases hydrolyse from the end of a nucleotide chain in an orderly and stepwise fashion, whilst the endonucleases attack randomly at any point in the chain. The useful ribonucleases belong to the second group, however nucleases with exonuclease action are much used in RNA sequence determination, especially Venom phosphodiesterase, (6) and spleen phosphodiesterase (7) which have a complementary mode of action. The venom phosphodiesterase hydrolyses from the 3' hydroxyl end in

a stepwise fashion to release the 5'-phosphates, and spleen phosphodiesterase hydrolyses from the 5'-hydroxyl end to release the 3'-phosphates.

Summarized below are a few of the interesting and possibly useful endoribonucleases with their mode of action on RNA.

Bovine pancreatic RNAse (8, 9, also see section III of this thesis) The hydrolysis of RNA by bovine pancreatic RNAse is essentially a phosphorylation followed by an hydrolysis. It splits the phosphodiester bonds of 3'-cytidylic and 3'-uridylic acid initially to form the nucleoside cyclic 2', 3'-phosphates which are hydrolysed to the 3'-nucleotides of CMP and UMP. It is therefore a very specific enzyme and the products, besides the pyrimidine 3'-nucleotides, are the oligonucleotides of varying lengths always terminating in a pyrimidine nucleotide. An account of its practical use in conjunction with other ribonucleases and nucleases in interpreting the base sequence of ribosanal RNA is given by F. Sanger et al. (10)

Similar to Pancreatic RNAse in thermal stability and type of action, but not specificity, is Ribonuclease T_1 isolated from Takadiastase (Aspergillusoryzae.) (11, 12) RNAse T_1 is guanylic acid specific and in a similar way to pancreatic RNAse it splits the nucleotide chain between the 3'-guanylic acid groups and the 5'-hydroxyl group of the adjacent nucleotide forming an intermediary guanosine 2' 3'-cyclic phosphate. An enzyme

isolated from the mould Neurospora crassa has the same specificity (13)

Takadiastase also yields another ribonuclease, but this RNAse is non-specific and is designated RNAse T2. (12, 14) The T2 enzyme preferentially attacks phosphodiester bonds of adenylic acid, but not specifically, since all linkages are eventually attacked regardless of the nucleotide base. The mode of action is the same as pancreatic and T RNAses with the formation of intermediary 2'3'-cyclic phosphates.

Rushizky et al. (15) extracted seven new RNAses from various microorganisms in an attempt to find new specificities. The following were investigated:-

	strain No.
1. Bacillus pumilus	IFO 3028
2. Mucor genevensis	IFO 4585
3. Bacillus cereus	ATCC 10987
4. Lenzites tenuis	IFO 4946
5. Monascus pilosus	IFO 4486
6. Lipase B	
7. Lipase B	

1, 2 and 7 were found to have a specificity as in RNAse T_1, hydrolysing the 5' linkage to give 3'-GMP. Enzymes from micro-organisms – 3,4,5and 6 are non-specific giving the nucleoside 3' phosphates of all four bases.

At least three nucleases have been isolated from Phaseolus aureus - mung bean sprouts, (16) two of which are ribonucleases - ribonucleases M1 and M2. RNAse M_1 is an acid stable RNAse which acts to give a mixture of 2'3'-cyclic nucleotides. The cyclic purines are further hydrolysed to the 3'-phosphates, whilst the pyrimidines are not. However it is not definitely established that this enzyme, which produces the 2'3'-cyclic AMP and GMP, also produces the resulting 3'AMP and 3'GMP. A similar acting RNAse to M_1 is Tobacco leaf RNAse (17, 18) which is unspecific, cleaving all phosphodiester bonds to the 2'3'-cyclic esters and no further. Whilst these esters are inert to further enzyme action, the purine cyclic nucleotides are slowly hydrolysed to the nucleoside 3'-phosphate exclusively. This enzyme might be useful in the preparation of 2'3'-cyclic nucleotides.

Micrococcus pyogenes (20, 23) nuclease is a nuclease worth noting, (although not a ribonuclease) because it is often used in conjunction with ribonucleases in RNA sequence determination (19) The interesting features are an absolute requirement for calcium ions for activity, production of oligonucleotides bearing 3'- phosphate groups, and a possible combination of endonucleolytic and exonucleolytic activities (20, 21) It is unspecific (although preferential cleavage has been reported (22)) but it is specific in that when the 5'

phosphomonoester end group is present in the oligonucleotide, it is inhibited. It also does not cleave the 2',3'-cyclic phosphates. At all times of digestion mononucleotides form a significant proportion of the product even though substantial amounts of oligonucleotides remain undigested.

Rye grass RNAse (24), also an unspecific RNAse, hydrolyses oligonucleotides regardless of whether they are terminated in 3' or 5'-phosphates at roughly equivalent rates unlike the above Micrococcal nuclease. Similarly pancreatic RNAse does not differentiate between 3' or 5'-phosphate termination of oligonucleotide chains.

Another bacterial RNAse which has been found useful in sequence analysis of RNA is Bacillus subtilis RNAse (25) This enzyme is unspecific but preferentially attacks GpGp and GpAp linkages (Diagrammatic convention: p to the right of the nucleoside=3'-phosphate linkage, and similarly p to the left of the nucleoside=5'-phosphate, A,G,C and U=nucleosides) about 100 times faster than other phosphodiester bonds. Digestion is to the mononucleotides via a 2'3'-cyclic phosphate intermediate.

Mention of a RNAse isolated from hog spleen concludes this brief outline of ribonucleases with respect to their use as tools in RNA sequence determination. Acid RNAse activity is very widely distributed in different tissues and species; evidence is available that this enzymatic activity is due to more than one

enzyme (26) The purification of enzymes responsible for this activity has been attempted in only a very few cases, and very often the comparison of the partially purified preparations reported so far is difficult. The hog spleen acid RNAse reported by Bernardi and Bernardi (27) showed interesting features of specificity when natural and synthetic polyribonucleotides were digested with this enzyme. Polyuridylic acid was hydrolysed rapidly whereas polycytidylic and polyadenylic were highly resistant to this enzyme. Further elucidation of the specificity of this enzyme continues.

Early work leading to the present investigations

Because of the importance of ribonucleases with respect to their metabolic role with RNA and protein synthesis, interest was stimulated within this department by a report published in 1956 by Kaplan and Heppel (28) describing the purification and properties of a ribonuclease from calf spleen. They isolated a heat stable fraction, purified 700 fold with the reported same specificity as bovine pancreatic ribonuclease. The calf spleen were obtained fresh from the slaughterhouse, carefully packed in ice. Carrying out all operations at 3°C the spleen were homogenized in a Waring blender with pH 7 sodium acetate buffer; and then subjected to an ammonium sulphate fractionation at pH 3.5. The enzyme extract was subsequently heat treated at the

same pH of 3.5 and an enzymically active 60°C heat stable fraction obtained. Final purification, after another ammonium sulphate fractionation at pH7.0, dialysis, acetone precipitation and a final ammonium sulphate fractionation at an acid pH again, was by chromatography on Amberlite IRC-50 (XE64) resin, which brought the degree of purification up to a factor of 700. The pH optimum of this ribonuclease enzyme was between 6.0 and 6.5, and therefore according to pH optimum this is an acidic ribonuclease. The terminology is somewhat confused in the literature. The ribonuclease of Kaplan and Heppel (28, 29) above, although it has a pH optimum in the acid range, can be termed basic because of its behaviour on a cationic resin exchange column. Similarly there are acidic ribonucleases with respect to pH optima (29) which are also acidic with respect to their ion-exchange adsorption properties, accounted for by the constituent amino acid groups in the protein chain. Hereafter the term 'basic' ribonuclease will refer to its cationic properties.

The specificity of the basic calf spleen ribonuclease isolated by Kaplan and Heppel was reported to be the same as pancreatic RNAse (8, 28.) There was no evidence of any action upon DNA and this confirms it as being a ribonuclease and not merely a nuclease. Other properties that were reported as being in common with pancreatic RNAse were a stimulation of activity in the presence

of Mg^{++} ions ($MgCl_2$-0.01M concentration) and the response to the presence of various other metal ions.

The most interesting aspect of the paper was a note appended by W.R. Carroll on sedimentation measurements of the ribonuclease. These ultracentrifuge measurements indicated that the ribonuclease was of an unusually small size for an active protein. A molecular weight of 2,000 - 5, 000 was suggested. The implications and possibilities of isolating an enzyme with so low a molecular weight are far reaching. An enzyme of this size would be an attractive proposition for X-ray crystallographic analysis (viz: recent work on Pancreatic RNAse ref (30)), to further investigations such as amino-acid sequence determination, active-centre location, and, to eventually aid in the confirmation of information and hypotheses already available on the actual mode of enzyme attack and action.

It was with these possibilities in mind that work on ribonuclease from calf spleen was initiated in 1961 in this department (W.R. R. and J. E. - 37)

In the same year as Kaplan and Heppel's report (28) another paper was also published by Maver and Greco (31) which, among other nucleases, described a calf spleen ribonuclease. A procedure was enunciated for the purification of DNAse and RNAse activities of normal liver, spleen, two transplantable rat hepatomas, and a transplantable rat lymphosarcoma. (Maver and

Greco (32) had previously shown that there was RNAse activity present in catheptic preparations from calf spleen) Comparative studies on the pH optima, magnesium ion inhibition, and heat denaturation of the DNAse and RNAse activities from the above mentioned tissue sources were made. The studies revealed definite differences in the RNAse activities of liver and spleen, and indicated that each tissue may have characteristic RNAse activities. However resolution of the RNAses was incomplete and thus little could be concluded as to the identity and character of any RNAse at this stage - and no real comparison could be made with the low molecular weight RNAse mentioned by Kaplan and Heppel (28)

Maver and Greco 1959 (33) described the RNAse activities of fractions obtained when spleen nuclease preparations were subjected to chromatographic analysis on cellulose anion and cation exchangers. (34, 35). The RNAse activities were separated from the DNAse activity in the spleen nuclease preparations by means of the appropriate chromatographic techniques on columns of diethylaminoethyl cellulose (DEAE-cellulose). Several acid RNAse fractions with similar characteristics were eluted. Also a heat stable basic / alkaline ribonuclease was separated by chromatography or CM-cellulose anion exchanger at pH7.0 and therefore must be a fairly basic RNAse. This ribonuclease is probably the corresponding enzyme to the one isolated from calf spleen by

Kaplan and Heppel (28) although the information with respect to specificities was not in agreement.

In 1962 Maver and Greco (36) published an elaboration of the work appearing in 1959 (33). The chromatographic fractionation on DEAE-cellulose and CM-cellulose of the acid and basic RNAses of bovine liver and spleen nuclease preparations were described. These enzymes were separated from each other and from the following associated enzyme systems:- acid phosphatase, a non-specific phosphodiesterase and the deoxyribonuclease with which it is associated, and the enzyme which hydrolyses cyclic adenylic acid to 2'-adenylic acid. The effect of Mg^{++} ions and of heat on the acid and basic nucleases was demonstrated and also the specificity was investigated. Once again it is almost impossible to decide which basic ribonuclease corresponds to that reported by Kaplan and Heppel (28). The RNAses purified in Maver and Greco's 1962 paper (36) digested RNA to purine as well as pyrimidine nucleotides and guanylic and uridylic acids in both the cyclic and non cyclic form were found in the acid and basic RNAse digests. Although the ratios of adenylic to cytidylic acid differed; being higher in the acid digest, and lower in the basic RNAse digest, the specificity did not coincide with that reported by Kaplan and Heppel (28) for their basic ribonuclease which they reported to seem to have the same specificity as pancreatic RNAse. This point of confusion was further investi-

gated as part of the work of this thesis.

This was approximately the stage (1961)at which work in this department was commenced on purification of the heat stable basic ribonucleases of calf spleen (37). The work was mainly concerned with the perfection of purification and isolation of two basic RNAses from calf spleen using the techniques mentioned in previous papers (28, 29, 31, 32, 33, 34, 35, 36) as a broad basis, but with the accent on refinement of technique; and in some instances new techniques entirely were invoked, to obtain a reproduceable end product from the extraction procedure. The procedure was continually evolving over the period 1961-1964.

The investigation covered by this thesis was commenced in October 1964 at a stage where the extraction technique was fairly well defined (37), but low yields of the end products indicated the necessity of further extraction technique investigation.

The isolation of proteins from tissues, and their eventual purification represents one of the most important problems of biochemistry and is often a very complex problem with numerous interdependant factors to consider (40, 41) One of the principal considerations is the general lability of protein material to temperature, (42, 43) pH extremes (41), organic solvents (44), dilution factors (49), ultra violet light (45) and various other agents; leading to the eventual denaturation of the protein. Also denaturation (46) is a very general

term and it describes a change in protein structure with a loss of one or more of the protein properties. The steric structure of an enzyme fluctuates between a number of slightly different conformations within the same environment, but if this environment is changed the protein also suffers pronounced changes. Often these changes are irreversible with the breaking of non-covalent bonds and sometimes disulphide bridges, and new interactions within the molecule may occur which were not originally present in the native protein. Therefore all techniques in protein extraction and study have to be considered from a number of viewpoints and any changes in procedure are strictly monitored with respect to retention of enzyme activity and stability. The early idea of protein as an amorphous, ill defined substance has given way to the concept that proteins exist as specific and definable molecular substances (47). Using this concept it has been possible to treat the study of proteins as a true branch of chemistry, and it is on this basis that the problem of extraction and fractionation of ribonuclease from calf spleen was approached. From many lines of evidence it is generally agreed that each individual protein is a specific molecular entity with unique composition, sequence and 3-Dimensional configuration which endow the molecule with its particular biological usefulness and determine the special properties that are exploitable for identification and separation. The outstanding and most

utilized properties are: solubility, size, charge, lability, density and biological activity.

Unfortunately the classic criteria of purity (48) used by the organic chemist are of little use when working in the field of protein chemistry. Examples of such criteria of purity are crystallinity, melting point, and elementary analysis. Crystallinity is, in itself, no proof of homogeneity, as it has been shown that crystalline proteins contain substantial impurities (38, 39). Similarly with analysis, such as finding the number and type of amino-acids present in a protein is of little use as criteria of purity, since it is the arrangement of the amino-acids into primary and secondary structure with the resulting tertiary structure (which accounts for the folding of polypeptide chains into globular molecules) that characterizes a protein.

Scope of the present work

The work undertaken can be divided into three main divisions:-

Part I Extraction and purification investigations

Part II Aggregation properties of the enzyme

Part III Specificity investigations

Part I Extraction and purification

Besides the necessity of further investigations into the

extraction procedure, the lengthy operation was necessary to obtain material to work with in further studies (parts II and III). As mentioned previously, the extraction technique has been the subject of investigation and evolved over a period of three years and the initial extraction carried out approximates to the procedure resulting from this study. (J.E and W.R.R. 1961-1964, ref. 37). The two main objects in mind have been to increase the yield of enzyme extracted and to obtain as pure a product as possible. Yield increase attempts were approached from two directions. Firstly, attempts were made to extract more enzyme from the calf spleen starting material by use of acid extractions of various strengths, by the use of 2M urea present during the extraction process, by the use of detergents, organic solvents and by re-extraction of residues before their ultimate rejection. The second approach to yield increase was by ensuring that no active enzyme material was lost during any fractionation procedures. This care entailed the monitoring of every stage of fractionation, including both retained fractions and those normally discarded fractions. Changes were made in the procedure in each successive extraction programme in the attempt to increase the yield of the active enzyme.

In later stages of purification, use is made of both anionic DEAE-cellulose and cationic CM-cellulose ion exchange column chromatography and also 'Sephadex' cross-linked dextran

gel filtration. The difficulty of proof of homogeneity and the criteria that can be used with respect to protein material has been discussed, and in the light of the knowledge of protein purity-proof limitations, as many criteria of purity as possible have to be used. The criteria discussed and used are based on charge - CM-cellulose column chromatography, size - Sephadex gel filtration and ultracentrifuge studies, size and charge - disc gel electrophoresis, and finally specificity.

Part II Aggregation properties of the enzyme

The possibility of the enzyme being present as a dimer or aggregate was investigated. This particular line of investigation has largely been concerned with the behaviour of the enzyme in the presence of various concentrations of urea ranging from 2M to 8M urea. The action of urea, to the extent that it is understood is discussed in detail. Pancreatic ribonuclease enzyme maintains activity in urea concentrations of 8M (157, 158, 162, 104) but the elution pattern of the enzyme on CM-cellulose columns is very different at these concentrations as compared with its behaviour at 2M urea concentrations. A complete extraction of the ribonuclease from calf spleen in the presence of 2M urea at all stages up to and including column chromatography was carried out, in the hope that the hydrogen bond relaxing properties of urea would increase the yield of enzyme

recovered by possible disassociating it from other proteins.

The properties of the amino-acid leucine as an aid to studying aggregation was also investigated. It is believed (ref. 207 plus) that protein structure and protein organisation to some extent may be due to the folding of the amino acid chain in proteins so that areas of an essentially hydrocarbon or hydrophobic nature come together, that is, the parts of the amino acid chains which contain a high proportion of amino acids with hydrocarbon side chains such as alanine, valine, leucine, isoleucine, phenylalanine and tyrosine. Thus these types of amino-acids have a polar portion together with a non-polar end of an essentially hydrocarbon nature in the same way as have detergent molecules. These non-polar side groups will orientate themselves in the protein molecule in such a way as to come together to form hydrophobic centres or 'pools.' It was thought that by eluting CM-cellulose columns in the presence of leucine (or a detergent) that the solubilisation of the hydrocarbon 'pools' within the protein's secondary and tertiary structure would aid the separation of aggregates if indeed present. The reason for the choice of leucine and the results of its application to aggregation studies are discussed.

Part III Specificity investigations

This section of study occupied about a third of the time

spent on the complete enzyme investigation. Before the specificity work was initiated, time was spent on improving the yield of RNA obtained from Baker's yeast by trying a number of variations in procedure including the use of ethyl acetate in extraction, and chromatography on G-75 sephadex to obtain a purer fraction of high molecular weight material.

A number of digests of RNA with ribonuclease enzyme were performed and the products isolated and characterized. In some cases the digest products were separated using DEAE-cellulose column chromatography with a volatile ammonium carbonate elution system, but in another digest this same system was used but with subsequent application of the peaks to Dowex -I ~~cation~~ anion exchange columns for further separation of the nucleotide digest products. However since it was the mononucleotides that were mainly of interest to this investigation, other subsequent digests were analysed using Dowex -I ~~cation~~ anion exchange columns only, as the ammonium carbonate used with DEAE-cellulose made analysis and identification of the individual peaks collected difficult. The nucleotide products of digestion were analyzed and their identity as far as possible confirmed by the use of paper chromatography, paper electrophoresis, cellulose thin layer chromatography and ultra violet spectrophotometry.

Considerable experimentation on technique and some improvements were made on the thin layer chromatographic separation

of nucleotides on a cellulose medium.

The final results from specificity investigation have enabled some conclusions to be drawn as to the specificity of the basic ribonucleases from calf spleen.

PART I

PART I

EXTRACTION AND PURIFICATION

Introduction

Enzymes commonly occur *in vivo* together with complex mixtures of other enzymes, inert proteins, nucleic acids, polysaccharides and lipids. Even those that occur in true solution in the cytoplasm are often associated in molecular aggregates. It is important therefore in any extraction process that the required enzyme be separated from the other constituents present as gently and quickly as possible. Thus in the extraction of extremely labile active proteins it is necessary to observe one or two basic principles common to all such extractions of labile biological material from tissues:- One of the most difficult problems is to be sure of whether the enzyme remains in the same state as it occured *in vivo* when it has been isolated *in vitro*. The types of change that could occur on extraction are chemical reactions with breakage in some covalent bonds in the molecule, and a change in the association with other proteins or non-protein materials to which the protein molecule is bound. Any one, or a combination of all these factors could result in enzyme denaturation, which may terminate in the enzyme being irreversibly changed (46) in structure with change or complete loss of its characteristic properties. The following

elementary precautions are observed in the initial extraction stage.

a) Working at a low temperature

Proteins vary widely in their stability to temperature extremes and the ribonuclease being isolated is particularly stable to temperature variation. However at this point in the extraction procedure it is not so much the stability of the enzyme being extracted that is of concern (this is of course important) but the maintenance of a low temperature retains other enzymes such as proteases at minimal activity since these types of enzymes would digest part of the enzyme being extracted and so reduce the yield. Similarly a low temperature maintains micro-organism activity at a minimal level. Because of the possibility of proteolytic activity the initial stages of extraction are carried through as quickly as possible.

b) Surface denaturing effects

Attention should be rendered to the denaturing effect on proteins of surfaces, films and especially foams. Surface areas should be kept as small as is convenient and one should avoid foaming of extracts during pouring, homogenising and stirring of solutions. The denaturation caused by foaming can be quite considerable in dilute solutions and hence it is advisable to work with as concentrated solutions as possible since proteins seem more stable in concentrated solutions for other reasons (49, 50)

c) pH control

Adequate buffering of the extraction medium is important to maintain pH control, which is certain to vary otherwise, due to the release of varied cell constituents on maceration or homogenization or what ever method of tissue component release is being used.

d) Denaturing effect of some metal ions

Some metals, especially the 'heavy' metals such as lead, mercury and copper have a pronounced denaturing effect or inhibiting effect on enzyme activity (242, 244) A common source of such contaminants in extraction are the extraction vessels, metal stirrers, copper wire securing fixtures and often the large volumes of salts used in 'salting out' contain such impurities. It is therefore wise to include a suitable chelating agent in the extraction buffer. Ethylenediaminetetra acetic acid (E.D.T.A.) as the disodium salt is used in the below buffered extractant solution to 'take up' such metal ions. (243)

Perhaps an even more important function of E.D.T.A. is to 'chelate' metal ions that are needed for enzyme-substrate reactions and thus reduce these reactions. This is particularly important in the case of proteolytic enzymes since these are often metal-ion activated and by effectively removing the metal ion, their reaction is inhibited (51) The possible functions of metal-ions in enzyme-substrate systems are easily visualized:

(i) The metal may form a complex with donar atoms of either the enzyme or substrate and thereby enhance their tendency towards

reaction;

(ii) it may serve merely as a bridge through common co-ordination to bring the enzyme and substrate into proximity;

(iii) while serving function (ii) it may provide as well, a chemical activating influence; and

(iv) while co-ordinated to either the enzyme or the substrate it may appropriately orientate groups undergoing reaction.

It can be seen it is important that metal ions are as far as possible 'removed' by the use of E.D.T.A. and also that pure chemicals of 'Analar' grade and deionized water are used in making up solutions and buffers.

EXTRACTION I

Source of material

The spleen were removed from young calves immediately after slaughter. The calves were professionally slaughtered by initially stunning by the use of a 'bolt gun' and rendered unconscious, and then were hung up by their rear legs, and death brought about by exsanguination by cutting of the throat. A hollow needle was then inserted under the skin and air introduced subcutaneously to aid the removal of the skin. The spleen was removed along with other entrails and immediately placed in a stainless steel bucket and surrounded with small ice tips. The time gap between slaughter and processing was represented by the

journey from Girvan in Ayrshire to the Chemistry Department laboratories in Glasgow which was on average a lapse of three hours. During this intervening period the spleen were kept constantly surrounded with ice.

The average weight of a spleen was about seventy grams, but the actual weight varied considerably. The sought-after spleen were from young calves which had less accompanying fatty material attached to the spleen outer capsular layer. This capsular layer contains both fibrous tissue and unstriped muscle which has the power of contracting and squeezing the spleen at regular intervals. The main bulk of the spleen is made up of blood vessels and collections of lymphoid tissue surrounding small arteries, which are distributed fairly uniformly throughout the spleen (52). Kaplan and Heppel (28) used young or calf spleen, and for reproduceability this has been done here also.

As mentioned in the introduction ribonucleases are likely to be more active in tissues concerned with metabolic breakdown (1) and in a young calf these tissues are likely to be more active than in the adult where metabolic rate will be slower. During the second half of antenatal life it is reported (52) that the spleen is actively engaged in the formation of red blood corpuscles. In postnatal life spleen is also concerned with breakdown of haemoglobin and formation of bilirubin. It is also believed to destroy red blood corpuscles, lymphocytes and platelets

and is important in the defence reactions of the body and concerned with immune processes.

EXTRACTION I - STAGE I (See Experimental section - 1 and also Summary chart - page 120)

Preparation of spleen and homogenization in pH 7.2 buffer

All work on extraction and purification of spleen was carried out in a cold constant temperature room of 4° C. This temperature was monitored over the period of a week with a clock-work thermograph and the temperature variation was never more than two degrees, that is, the temperature never normally rose above 6° C.

All bench surfaces used in the preparation and chopping of the spleen were thoroughly washed using a commercial disinfectant solution ("Dettol") and hands were thoroughly washed, using soap, before handling the spleen. (It has been reported that human skin sweat contains ribonucleases, but sweating would be minimal at 4° C and amounts of contaminant so small, that this source of contamination was disregarded (53 and 54).

The outer capsular layer was removed from each spleen by stripping off manually. Likewise any fat material associated with this layer was removed with a sharp knife. The stripped spleen were then cut into thin sections and placed in a Waring blender with three times their own volume of pH 7.2, 10^{-3}M EDTA

disodium salt, 0.05M sodium acetate buffer and blended or "homogenized" for one minute at 12, 000 r.p.m. in a glass container. This treatment gave a degree of tissue breakdown that was not too fine to give centrifuging difficulties, but adequate without undue heating of the material, although the temperature naturally did rise somewhat. Frothing was minimal and care was taken when the homogenate was transferred from the blender to a 5litre beaker, to pour carefully down the inside of the beaker to avoid further frothing. The homogenate at this stage was a bright blood red.

This stage, and the following stage were carried through as quickly as efficiency permits, because of possible proteolytic action.

STAGE II A and B - ACID EXTRACTION and pH3.5 AMMONIUM SULPHATE FRACTIONATION

Introduction to the use of "salting out" in enzyme fractionation

(See Experimental section - Expt. 1) (58, 59, 60)

The process of salting out of a protein is a long established practice in the field of protein separation. However although the underlying assumption is, in most cases, that different proteins are precipitated at different salt concentrations providing pH and temperature are fixed; this is not always the correct assumption and the process is more complex than it initially appears.

The cause of salting out is not totally understood, but one approach is that the salt ions become hydrated removing part of the water, and therefore this is made unavailable as a solvent and the protein precipitates out of solution. If an electrolyte is progressively added to a protein solution there are two effects noticeable. Firstly there is an increase in the protein solubility, and secondly, as the concentration of the added electrolyte increases there is a reduction in solubility so the protein solubility passes through a maximum. The initial process of increased solubility is known as "salting in" and the important ions with respect to this process are the cations, where salts of multivalent cations are the most effective. But in the "salting out" region with decrease in solubility with increase in salt concentration, the critical ions are anions, and salts with multivalent anions such as sulphates, phosphates and citrates are desirable. The best type of salt for "salting out" should have a monovalent cation, and multivalent anion, should be very soluble in water, and be readily available in pure and large quantities. Ammonium sulphate is a salt that generally fulfills the requirements adequately.

There are a number of variables that can be adjusted to obtain the best results in salting out and these are:-

a) Nature of the ions

b) Ionic strength

c) Temperature

d) pH

e) rate at which salt concentration is raised.

The fractionation of protein may be separated into two principal types on the basis of these variables. Initially there is salting out at constant temperature and pH with the choice of ion and ionic strength variable, or there is salting out at constant ionic strength with variation of pH and temperature. The variability of pH and temperature during salting out are important and should be controlled carefully during salt fractionations.

The relative solubilities of two proteins may vary very rapidly with pH. A change in pH for example, may change the ratio of the solubilities of the two proteins at a given salt concentration by several thousand times.

Similarly solubilities are very sensitive to temperature, and a protein that may be soluble at 0° C may be virtually completely precipitated at room temperature. This can occasionally be a useful method of precipitation - the reverse temperature effect since most substances are more soluble at higher temperatures. (Use of this effect was tried in Extraction V)

It has been found that contrary to original theory, proteins do not appear in a fraction between characteristic fixed limits, but the limits of fractionation themselves vary with the concentration of a particular protein (all other variables being

constant) Theory (60) dictated that the width of a given peak in the distribution curve of separation was independent of protein concentration, but in a mixture of proteins the widths of the peaks will vary with the concentration of the mixture. Dilution of a mixture may separate or aggregate two peaks and which of the two that occurs depends on the proteins involved, because dilution shifts all peaks to higher concentrations. If dilution is too great this shift may move beyond the saturation limit of the salt and therefore never be precipitated, and anyway, precipitates at the saturation limits are difficult to work with and not desirable.

Although salting-out is more complex than it initially seems or has been interpreted, it is still relatively simple and a cheap method to operate. It is also a gentle method for delicate enzymic material.

Ammonium sulphate is about the best salt to use, but it also has certain disadvantages. The main difficulty is one of pH control due to loss of ammonia; also most commercial samples are acid. There is also the difficulty of determining the pH of solutions of ammonium sulphate above about 0.3 Molar concentrations because of salt error and junction potential at high salt concentrations.

STAGE II A. Acid extraction at pH 3.5 and 30% ammonium sulphate saturation

The pH of three litre aliquots of the freshly homogenized material from Stage I was slowly brought to pH3.5 by the addition of INHCℓ dropwise from a burette whilst the solution was mechanically stirred. The slow addition was necessary so that local areas of ~~high~~ low pH did not occur in the solution which might lead to denaturation of the enzyme. Samples were taken at intervals during the addition of the acid and the pH read using a pH meter. Due to the rather heterogeneous mixture of tissue material this could only be an approximate reading. By the time the pH of the medium had reached 3.5 it had turned from a bright pink-red to a light chocolate brown colour, and this change in colour also gave some indication of the acidity of the solution and the end point (pH3.5) Stirring was continued for a few minutes to ensure that the extract was homogeneous with respect to pH. Care was taken to check that the stirring speed was not over vigorous so as to bring about excessive frothing.

The "Analar" grade ammonium sulphate was added slowly by hand until the concentration was equivalent to 30% (56) saturation. The salt was added in solid form to prevent any further dilution and slowly added with stirring since rapid addition would result in transient precipitation and redissolution of the protein and this favours enzyme inactivation and also salting out of an amorphous precipitate which is a slow process. For reproduce-

ability the salt was also added at a constant rate.

The amount of ammonium sulphate needed to bring a solution to a specific level of saturation is given in published tables (55, 56, 57). The amounts of ammonium sulphate quoted in the below precipitation stages were figures for work carried out at room temperature, and therefore the actual percentage saturation value will be a little higher than stated since the temperature worked at was 4°C. But because the working temperature was always constant, and this method of interpretation was constant, the results were reproduceable.

A 30% saturated solution starting from an ammonium sulphate free system is quoted to require 176 grams per litre. But due to incomplete homogenization and the large volume of solid material present, such as connective tissue etc, to have added 176 grams of ammonium sulphate would have raised the effective salt concentration up to a level exceeding 30% saturation. (This normally discarded fraction on centrifuging is found still to contain activity and therefore to discard any more than is necessary is undesirable - see later results) It was estimated that 10% of the homogenate was solid material and the amount of ammonium sulphate for this initial precipitation was reduced by 10% to 160 grams per litre (see Experiment I in the Experimental section for salt calculations.) That the salt concentration should be exactly 30% saturated was not so

important at this stage as this was a crude initial fractionation step in which many proteins, muscle fibres, connective tissue, blood vessels, and nerves etc. were removed.

The homogenate, now at pH3.5 and approximately at 30% saturation, was left to stand overnight to allow full precipitation . For the sake of reproduceability this interval was maintained approximately the same in subsequent preparations.

The precipitate obtained on standing overnight contained much fatty material which tended to come to the top as a foam. This was removed by hand and the remainder centrifuged at *9,000 r.p.m. at 2^{o}- 3^{o} C in polypropylene bottles. The solid material (see details of later extraction where the solid was retained and reextracted, page 62) was discarded and the supernatant retained for stage II B.

*The relationship between the centrifuge speeds quoted in r.p.m. and 'g' value is given in the Experimental section - EXP I.

Stage II B - 80% Ammonium sulphate fractionation

The supernatant from stage II A was then made 80% saturated with respect to ammonium sulphate by the addition (56) of 356 grams per litre, which was also added slowly whilst the solution was mechanically stirred. The rate of addition was not more than 150 grams per minute. The solution was allowed to stand overnight and then centrifuged at 10,000 r.p.m. at 2-3^{o} C and the

precipitate retained this time. Often there was a separation within the standing solution prior to centrifuging, with heavier material sinking to the bottom and a lighter (probably more fatty) floating layer, and so it was possible to save centrifuging time by syphoning off as far as possible the intervening clear solution which was to be discarded.

The bulked precipitates were dissolved in about one litre of 0.05M sodium acetate, 10^{-4}M EDTA, pH7.2 buffer with continuous stirring to produce a dark red solution which was then subjected to heat treatment in stage three.

STAGE III - HEAT TREATMENT

General introduction (61, 62)

This is an extremely valuable and efficient method of fractionation of proteins providing the protein one wishes to retain is itself relatively heat stable. In the case of the ribonuclease being investigated, it has been shown to retain its activity after being held at a temperature of 60°C for ten minutes (28)

The destruction temperature of proteins is usually quite sharply defined and different for each protein, and therefore by heating a solution of protein to a temperature just below that which would destroy the wanted protein, all other proteins with

lower thermal denaturation values would precipitate out of solution. The reason for this precipitation is that a decrease in solubility usually accompanies the denaturation of a protein. The unwanted protein material can be centrifuged off to leave the wanted protein still present in solution. The presence of the substrate in the case of enzymes often enables one to heat to a higher temperature than would otherwise be possible without loss of enzyme; and thereby obtaining a more efficient fractionation (251)

Denaturation is a process of finite rate and therefore both time and temperature must be accounted for. In order to establish the optimal conditions for thermal denaturation, both these factors must be varied systematically in addition to the optimum conditions of pH and ionic strength. (In later work, variation of time and temperature of heat treatment were investigated in the search for optimal conditions.)

The state of purity of a protein at the time of heat treatment is also a critical factor. It generally seems that less pure mixtures are more resistant to heat denaturation, and also greater purification is achieved by the first heat treatment than succeeding ones, and the less stable proteins evidently are just those which are removed first.

Kaplan and Heppel (28) used heat treatment as a method of

fractionation and found approximately 70% of the ribonuclease activity of the original homogenate was destroyed in this heating step in their procedure. Most of this activity lost will be that of the acid ribonucleases reported by Maver and Greco (31,33) Thus in Kaplan and Heppel's purification of a basic ribonuclease fraction from spleen this was a useful purification step and was likewise carried out as follows:-

Procedure

The pH of the dark red solution obtained from stage II B was adjusted to pH3.5 again by the careful dropwise addition of IN Hydrochloric acid with continuous mechanical stirring. Sometimes at this stage on reducing the pH a small white membranous type of precipitate was obtained. The pH of the solution was returned to neutral and the solution centrifuged. However on return to neutrality most of the precipitate redissolved. When the pH was once again returned to pH3.5 the same precipitate reappeared. It was decided to ignore this since much other material would be brought down in the heat treatment stage in any case.

In carrying out the heating, three water baths were set up, one at the desired temperature of 60°C, one at a temperature of 80°C and one filled with ice surrounding a glass beaker. The respective temperatures were maintained using bunsen burners under the water baths, and with a little adjustment of the flame the

temperature was held at the desired level with little trouble. The solution to be treated was divided into 500 ml. aliquots in one litre capacity beakers supported by an exactly fitting retort ring and therby lowered into the 80°C water bath to such a point that the level of the protein solution in the beaker was below the level of the water in the bath, but not to the extent of resting on the bottom of the bath which might have resulted in local overheating. The solution was stirred constantly by hand as the temperature rose. When the solution had reached 60°C the beaker was quickly transferred to the bath being maintained at 60°C, and by means of the supporting retort ring it was held in this bath with constant stirring for exactly ten minutes. At the termination of the heating at 60°C the protein solution was poured gently into the beaker that was cooling in the ice bath, in order to lower the temperature to 5°C or so as quickly as possible.

For reproduceability of results this exact procedure was observed with all subsequent heat treatments, and although some experimentation with temperature was undertaken (see later results) apparatus, size of beakers etc. were maintained constant.

The pH of the suspension of precipitated denatured protein material resulting from this treatment was adjusted to pH7.2 and then the solution was high speed centrifuged at 14, 000 - 15,000 r.p.m. at 3-4°C. The compacted solid material obtained was resuspended in pH7.2 sodium acetate buffer and recentrifuged at

15, 000 r.p.m. to ensure all activity had been collected. The two supernatants were combined and carried forward to stage IV.

STAGE IV SECOND ACID AMMONIUM SULPHATE PRECIPITATION AT pH2

The solution was adjusted carefully to pH2 with IN HCI (a small buff coloured precipitate was obtained which was probably due to proteins that were partially denatured by the heat treatment, but were not rendered insoluble until a lower pH was experienced.)

There was still residual ammonium sulphate present in the solution at this stage, and in order to estimate the concentration of ammonium sulphate to add in order to raise the level, this residual amount must be estimated. Kaplan and Heppel (28)quote the use of a Barnstead conductivity meter, but as this instrument was not available the ammonium sulphate was previously estimated (J.E., 37) by the Nessler method (65). This method produced a value of about 10% saturation with respect to ammonium sulphate. However this estimation requires much dilution and is a lengthy procedure and so in all future determinations the residual salt level was accepted as being approximately 10% ammonium sulphate. This estimate is not critical, since ammonium sulphate levels, as previously discussed, are not as critical and specific as originally thought and the purpose is to obtain a broad spectrum separation of products and not a narrow defined specific separation.

The ammonium sulphate concentration was brought up to

between 40% amd 50% saturation and a slight precipitate was centrifuged down at 10,000 r.p.m. at 3-4°C for thirty minutes. The supernatant resulting was retained and then made 85% saturated with ammonium sulphate observing the same precautions of addition as previously described. The solution was allowed to stand overnight and a light buff coloured precipitate resulted. This was centrifuged at 10,000 r.p.m. at 3-4°C and the supernatant discarded. The precipitate obtained was dissolved in 0.005 M TRIS/HCI, 10^{-4} EDTA, pH5.5 buffer by adding slowly to a stirred solution until all of the precipitate had dissolved. The precipitate was dissolved in the minimum volume of buffer in order to retain a small volume because larger volumes will increase still further on dialysis in the next stage, and difficulty of handling and denaturation factors will become important considerations.

STAGE V DIALYSIS

General introduction to dialysis (66, 67)

The use of dialysis in biochemistry dates from the time Thomas Graham separated inorganic salts (and sugars) from proteins by the use of a sac made of parchment paper. But by far the most common material used now is extruded cellophane or Visking tubing. Briefly, there are two theories of the mechanism by which dialysis operates. In one the membrane is considered to

have the properties of a sieve with more or less rigid pores of fixed size; whereas in the other theory the membrane is thought to act as a solvent permitting solute to dissolve at the interface on the side of the highest concentration, to diffuse through the membrane and to emerge at the interface of lowest solute concentration. However it has been demonstrated that cellophane membranes do not function in this latter way.

When cellophane tubing is wetted in water, electron microscope studies have shown (75) it to be like a very thin sponge with tortuous anastomosing pores or microcavities of various shapes and sizes. It has not a lot of resilience to stretch and therefore care must be observed in the handling of this tubing because stretching may result in the pores being enlarged and thereby resulting in loss of standardization of sieving capabilities.

Procedure

The dissolved precipitate from stage four was dialysed in one inch diameter visking tubing against 0.005 M Tris/HCI, 10^{-4} M EDTA, pH5.5 buffer in clean six litre capacity polyethylene buckets for a total of twenty-four hours with three changes of dialysing buffer, and occasional agitation of the buffer around the tubing. The tubing was rinsed internally with deionized water prior to use and also checked for perforations. During the dialysing procedure there was only slight precipitation of material and this

was removed by centrifugation at 10,000 r.p.m. at 3-4°C for ten minutes.

The dialysis will prepare the protein solution for the next stage of purification, column chromatography on CM-cellulose, by removing the salt and any small protein or peptide fragments that can get through the membrane, but retaining the large active protein molecules. Dialysis can lead to a loss of enzyme activity and results of previous experiments with ribonuclease by W.R.R. and J.E. (37) had given rise to doubts with regard to the expediency of dialysis at this stage. The dialysis problem will be dealt with in further detail in later work. (page 89)

STAGE VI CARBOXY-METHYL CELLULOSE COLUMN CHROMATOGRAPHY

Introduction to the use of CM-cellulose.

Ribonucleases can in some circumstances be separated quite successfully by the use of the cationic ion-exchange resin Amberlite XE-64 (68, 69, 70, 71.) The latter resin has also been applied to the chromatography of relatively basic proteins (72) and was employed by Kaplan and Heppel (28) in the isolation of the basic calf spleen ribonuclease. These workers reported a 40% loss of activity and it is therefore a somewhat less than ideal method of purification and separation.

Ion exchangers prepared from cellulose by the attachment of a limited number of ionizable groups (73) provide a wide range

of affinities for essentially all types of proteins. The open structure of cellulose ion exchangers permits ready penetration by large molecules, resulting in a high capacity for the adsorption of proteins. The stability of the covalent bonds through which ionizable groups are attached allows the use of an almost unlimited variety of buffer species over a wide range of pH.

The chromatography of proteins on cellulose ion-exchangers involves primarily the establishment of multiple electrostatic bonds between charged sites on the surface of the absorbent and sites bearing the opposite charge on the surface of the protein molecule. The number of such bonds that can be established will determine the concentration of the competing ions required for the release of the bound molecule. Thus a separation of proteins may be achieved by the virtue of the fact that proteins differing significally in charge density, or in number of charges by virtue of size, may be expected to differ in their requirements for elution. Charge distribution can also be regarded as a factor, and the total effect of these factors will determine the affinity of the protein for the adsorbent. Differential elution is accomplished by reducing the number of charges on the protein molecule through appropriate changes in pH, or by decreasing the effectiveness of existing bonds by increasing the salt concentration.

Because a basic ribonuclease was being isolated, Rees and

Edmond (37) decided to use a cation exchanger, but not the Amberlite XE-64 cation exchanger which caused considerable loss of enzyme activity when used by Kaplan and Heppel (28) The material of choice was the substituted cellulose cation exchanger - carboxymethyl cellulose (or CM-cellulose) Maver and Greco (29) similarly used CM-cellulose for the separation of their basic RNAse fraction which was not held by the anionic D.E.A.E. - cellulose ion exchanger.

The commercial grade of CM cellulose used was Whatman CM-70 carboxymethyl cellulose.

Preparation of the commercial adsorbent (74) - See General Method 3 (a)

The dry powder was suspended in a solution of 0.5 N sodium hydroxide made 0.5 N with respect to sodium chloride, and mechanically stirred for twelve minutes. The presence of salt reduces the possibility of disruption of the particles. It was then centrifuged at 1500 r.p.m. in 600 ml. cups for about five minutes, the supernatant poured off and the residue of CM-cellulose washed repeatedly with deionized water until it was free of base as indicated by litmus paper. The CM-cellulose was then suspended in the buffer at the pH which the column was initially to be. "Fines" were also decanted off after allowing fifteen minutes for the suspension to settle. More buffer was added, and the CM-cellulose suspension was placed in the cold room

with mechanical stirring overnight to equilibriate with the buffer and to attain the correct temperature.

CM-cellulose column filling technique

The technique of filling described below was standard procedure for filling all columns. (See General Method - G.M.5) A clean glass column made to a specific size with a tap key, was fitted, after the column had been filled with a little initial buffer, with a glass wool plug and clamped firmly into a vertical position. It was found in later work that for the attainment of uniform and symmetrical peaks, absolute verticality of the chromatographic column was essential, and this could be checked by means of a spirit level placed against the side of the column or less accurately by means of a simple plumb-line. Then a large filter funnel was fitted to the top of the column with a mechanical stirrer positioned over the funnel to stir the contents (fig. 1)

The whole apparatus was filled about two inches from the lip of the funnel and topped up with a suspension of the CM-cellulose. The stirrer was set in motion and a slow flow of buffer through the column was allowed by opening the bottom tap to a small extent.

Thus in this way the column is filled slowly and evenly. It is important the column is filled without interruption as this produces 'banding' or interruption of homogeneity. As the column will operate in the cold room, all column filling was carried

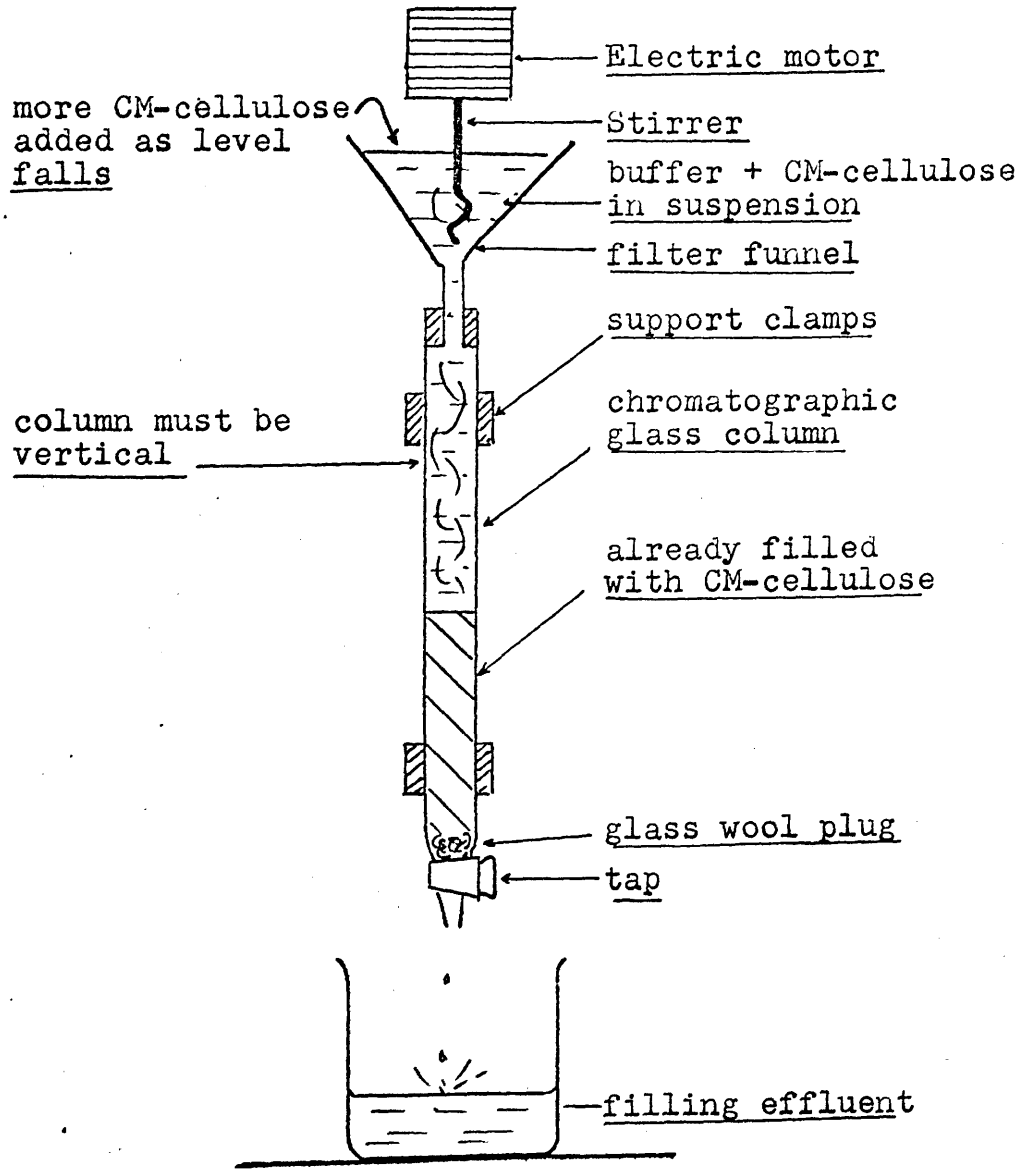


Fig.I. Column filling technique, CM-cellulose.

out at this temperature to avoid contraction difficulties within the column which would occur if it had to be transferred from a room temperature of 23°C to the 4 °C of the cold room.

Application of protein to column and gradient elution (66, 67)

Introduction to gradient elution

The term 'gradient elution' refers to a procedure for the mechanical mixing of the eluting agents to provide smooth and reproduceable increase in eluting power. The technique is especially suitable for the resolution of complex mixtures of unknown or widely varying affinity for the exchanger. In addition the eluted compounds are usually obtained in smaller volumes with less tailing when gradient elution is used (66)

The system used below results in a convex gradient curve since it was found (37) that this type of gradient with its initial sharp increase in eluting power gave the best separation of active ribonuclease from other inactive proteins.

As shown in fig. (2) the apparatus consists of two distinct chambers; the first of which is the reservoir which contains the eluting agent solution which is used for development of the column; and the second, the constant volume mixing chamber which contains the solvent in which the column is packed. The mixing chamber is equipped with a stirrer which mixes the liquid already present and the liquid drawn from the reservoir vessel.

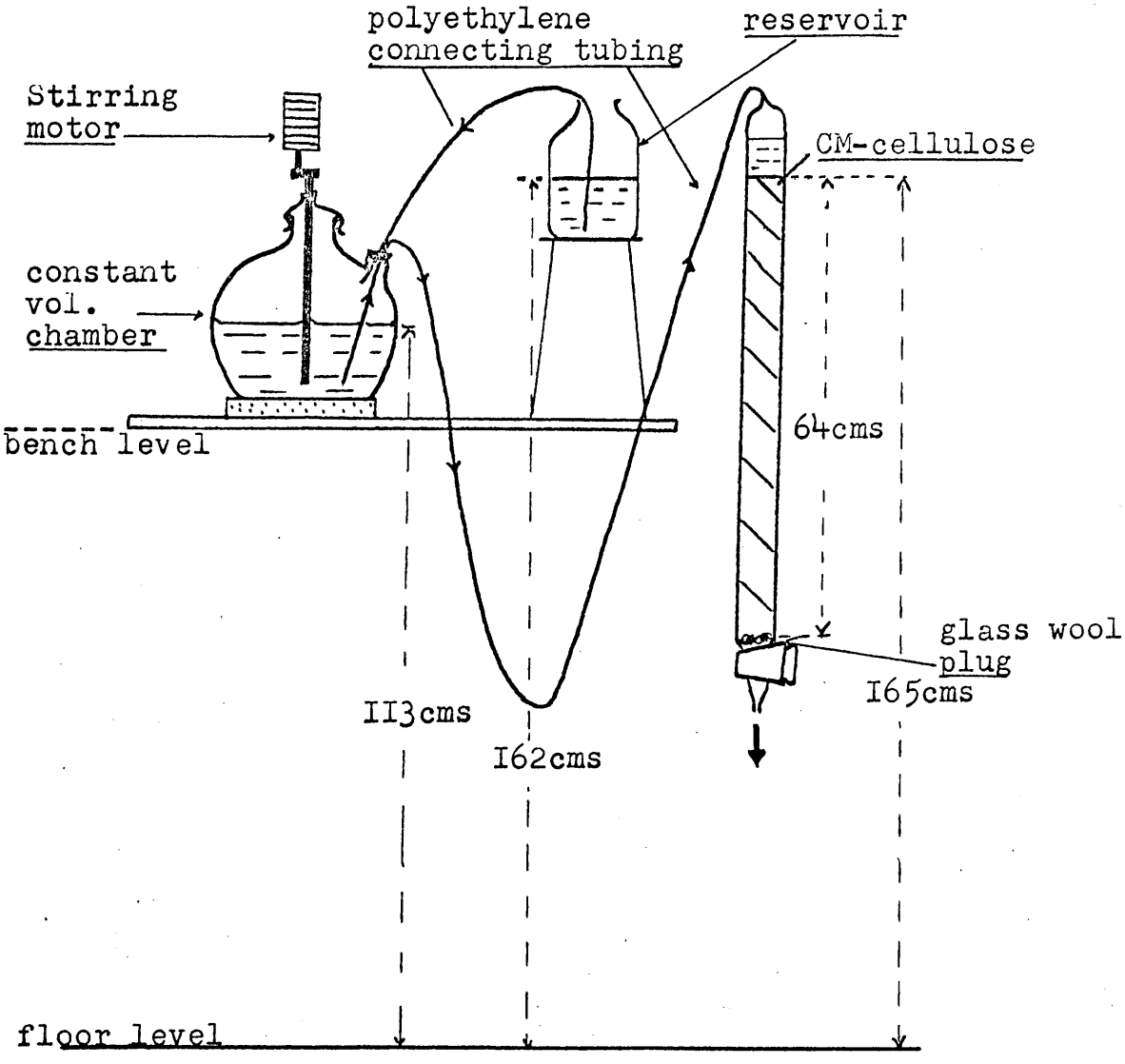


Fig.2. Apparatus for column gradient elution(CM-cellulose)

The operational procedure is simple. The column is set up and the substances to be examined are applied to the top of the column in the initial solvent which itself has little or no eluting action on the substances applied and wanted. After a short period of washing with the initial solvent, the column is connected to the mixing chamber, which in turn is connected to the reservoir which has an hydrostatic superiority over the mixing chamber and column base outlet. Since the mixing chamber is now a sealed airtight constant volume system, there is a pressure on the contents and as the first solvent starts to move down the column, the second solvent is drawn into the mixing chamber. The gradient of the second solvent applied in this way is expressed by:- $2.303 \log_{10} \frac{X}{X-x} = \frac{v}{V}$ where x = concentration of the second solvent in the mixing chamber when a volume v has flowed into this chamber, and X is the concentration of the second solvent in the reservoir.

V = volume of the mixing chamber solution.

A plot can be made of $\frac{x}{X}$ in the mixing chamber against v. The shape of the plot is independent of X but is dependent on V. The steepness of the curve can be altered by change in the volume V. v can be replaced by $V_1 - V_2$, where v_1 = volume of effluent

v_2 = dead volume of the column,

providing the eluting agent travels without retention volume.

Procedure

The protein solution was applied slowly to the column top by means of a slow dripping separating funnel. Care was taken not to disturb the level CM-cellulose surface or allow the column to 'run dry' at the top. Once all the protein solution had been applied, the liquid level was allowed to fall almost to the cellulose surface, and then a small amount of starting buffer was very carefully pipetted on to the column of CM-cellulose. The column was then washed with 1500 mls. of initial buffer at pH5.5. The mixing chamber and reservoir were then connected up as described under the "Introduction to gradient elution" section above and as shown in fig. (2) to set the gradient elution in motion.

The buffer solutions used in the gradient were:-

In the reservoir 5 litres initially and then another 5 litres of 0.005M TRIS/HCI pH8.2, 10^{-4}M EDTA; 0.32M NaCl.

In the mixing chamber

(constant volume chamber) 3.75 litres of 0.005M TRIS/HCI, pH5.5, 10^{-4}M EDTA.

In this extraction I column, fifty ml. aliquots were collected by means of a 50 ml. siphon on a "Towers" rotating fraction collector. The rate of flow was adjusted to 175 mls. per hour. The 1500

mls. of initial buffer column wash at pH5.5, eluted off that protein not held by the column. A total of 150 tubes were collected so giving a total eluent of seven and a half litres.

Method of location of protein and enzyme activity

There are several well known methods for estimation of protein, but few are very accurate for the estimation of low concentration of protein, and all methods have certain distinct disadvantages. The four common methods discussed are the Turbidimetric, Folin Ciocalteau, biuret and the use of u.v. absorption.

The turbidimetric techniques (92) are rapid and convenient but yield different values with different proteins, and thus in the case of separating a mixture of proteins, before and after comparisons are of little use.

The Folin Ciocalteau reagent is used in a method described by Lowry (79) and the final colour is the result of a biuret reaction of protein with copper ion in the alkali, and a reduction of the phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophan in the treated protein. The major disadvantage is:— that the amount of colour varies with different proteins. In this respect it is less constant than the biuret reaction, but more constant than the U.V. absorption method at 280 mu. Furthermore the colour is not strictly proportional to concentration. It is however very sensitive to low

concentrations, and is 10-20 times more sensitive than U.V. absorption at 280 mu and a 100 times more sensitive than the biuret reaction.

That substances containing two or more peptide bonds form a purple complex with copper salts in alkaline solution, is the basis of the biuret method of protein estimation (80) This method is virtually specific to protein present in biological materials and does not suffer from colour interference from other substances as do the previous two methods and also the U.V. method of protein determination. However more material is required for assay by the biuret method and it cannot be used in the presence of ammonium salts.

Most proteins exhibit a distinctive U.V. light absorption maximum at 280 mu due primarily to the presence of tyrosine and tryptophan. Since the tyrosine and tryptophan content of various enzymes normally only varies within reasonably narrow limits, the absorption at 280 mu has been used as a rapid and fairly sensitive method of protein estimation (81). Nucleic acids absorb more strongly at 260 mu than 280 mu, the reverse situation of proteins and so Warburg and Christian (81) used this fact to eliminate the interference of nucleic acid in protein estimation by this method. Although the technique may give considerable error due to different proteins and nucleic acids not having the same absorption, it is very quick, is non-destructive and can be carried out in the

presence of ammonium sulphate and other salts. On the basis of these latter points this method was generally used for protein location and estimation throughout this investigation. The protein concentration is given by the formula (81):-

Protein concentration in mgs/ml $= F \times \frac{1}{d} \times$ optical density (O.D.) at 280 mu where d= cell width in centimetres and from the value of $\frac{\text{O. D 280 mu}}{\text{O. D 260 mu}}$ the conversion factor F, which accounts for nucleic acid u.v. absorption, can be obtained from published tables (81)

The protein fractions obtained by gradient elution of a column are monitored by reading a sample from each tube (or each alternate tube etc.) in a 1 cm. quartz cuvette in a Unicam SP500 spectrophotometer.

The method of location of enzyme activity in the fractions was by assay of a sample from every tube with yeast RNA. The method is founded on the fact that during digestion of RNA by RNAse, 40% of the total nucleic acid phosphorous is converted into a form soluble in acid uranyl acetate (82)

Method of routine assay for RNAse (See General Method 4)

0.25ml aliquots of a solution of high molecular weight yeast RNA (preparation page 262) were placed in ten ml. capacity conical centrifuge tubes and this was followed by 0.25 ml aliquots of the sample being assayed. The substrate and sample were

thoroughly mixed and immersed in a constant temperature water bath at 37°c for thirty minutes to digest. The RNA solution used was made up in a 0.1 M Na succinate buffer, pH6.5 and 0.05M with respect to magnesium ions since Kaplan and Heppel (28) had reported that the presence of Mg^{++} ions stimulated activity by 25-35%, in a similar way to pancreatic RNAse. The concentration of RNA substrate was 10mgs/ml.

The digest was terminated after thirty minutes by the addition of 0.5 ml of MacFadyen's reagent (83) which was 0.25% uranyl acetate in a 2.5% trichloracetic acid solution. The tube, now containing 1 ml of material, was well shaken and immersed in a bath of iced water to ensure all reaction had ceased. Particular care was taken with drops of solution held by surface tension on the walls of the tube and it was ensured that they were well mixed. After a cooling period of about thirty minutes, each tube was centrifuged for five minutes at 1500 r.p.m. All protein and oligonucleotide material, other than perhaps a small quantity of dinucleotides, should be precipitated by the uranyl acetate-trichloracetic acid to leave only the mononucleotides in solution as the uranyl salts. (84)

0.1 ml of supernatant was withdrawn from each tube and pipetted into a boiling tube and diluted to four mls. (i.e. x 40) and then read at 260 mu in the U.V. spectrophotometer.

The optical density reading at 260 mu, and similarly the

protein optical density at 280 mu, were each plotted against the fraction number, and thereby the fractions with enzyme activity corresponding to protein presence could be pinpointed in readiness for bulking of similar fractions for further study.

Results of CM-cellulose column chromatography of material from Extraction I

After the collection of 150 fractions of 50 ml dimensions each the column was washed with 2.5 litres of pH8.2 buffer which was made molar with respect to sodium chloride, to ensure all protein was removed from the column. The strong salt solution also has the function of regenerating the CM-cellulose column in the Na^+ form. Finally the column was washed copiously with the initial buffer at ph5.5 ready for reapplication of fresh material.

The result of the fractionation on CM-cellulose is shown in fig (3) with three initial protein peaks which had no RNAse activity, and then two subsequent peaks which corresponded to two RNAse activity peaks.

This elution pattern was the reproduceable and predicted result of the extraction and purification procedure developed (37) up to the stage from which further investigations were continued by the writer.

It can be seen from these results that there are two regions of activity and from the position of the activity peaks in the

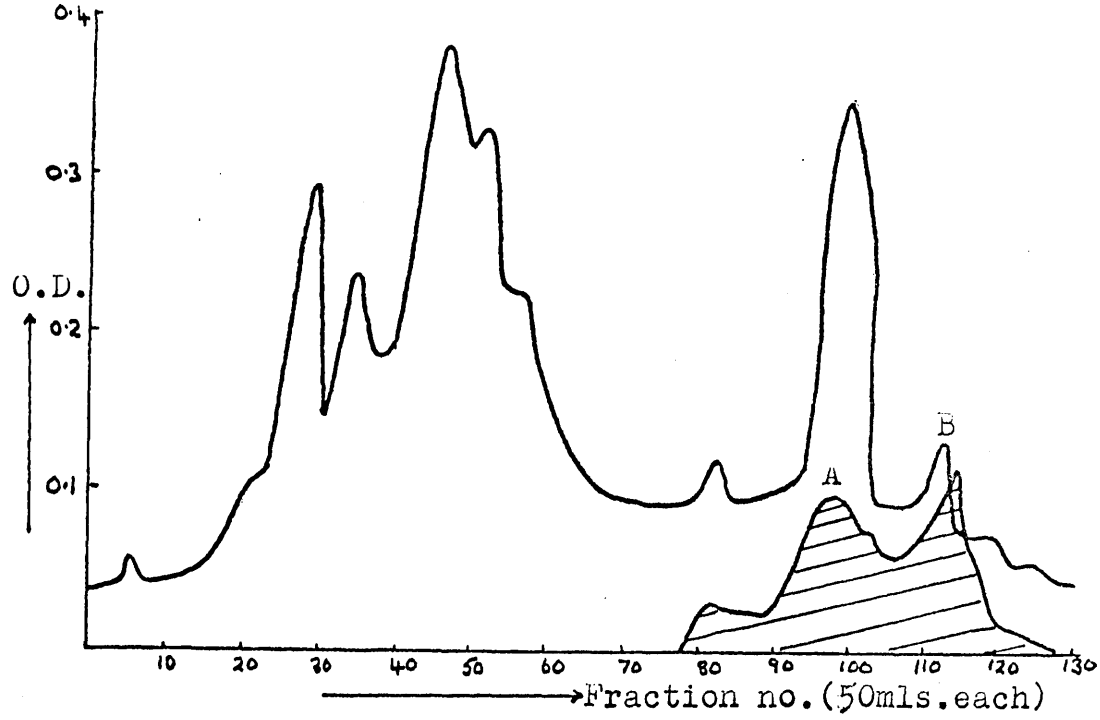


Fig.3. CM-cellulose column chromatography of Extraction I.

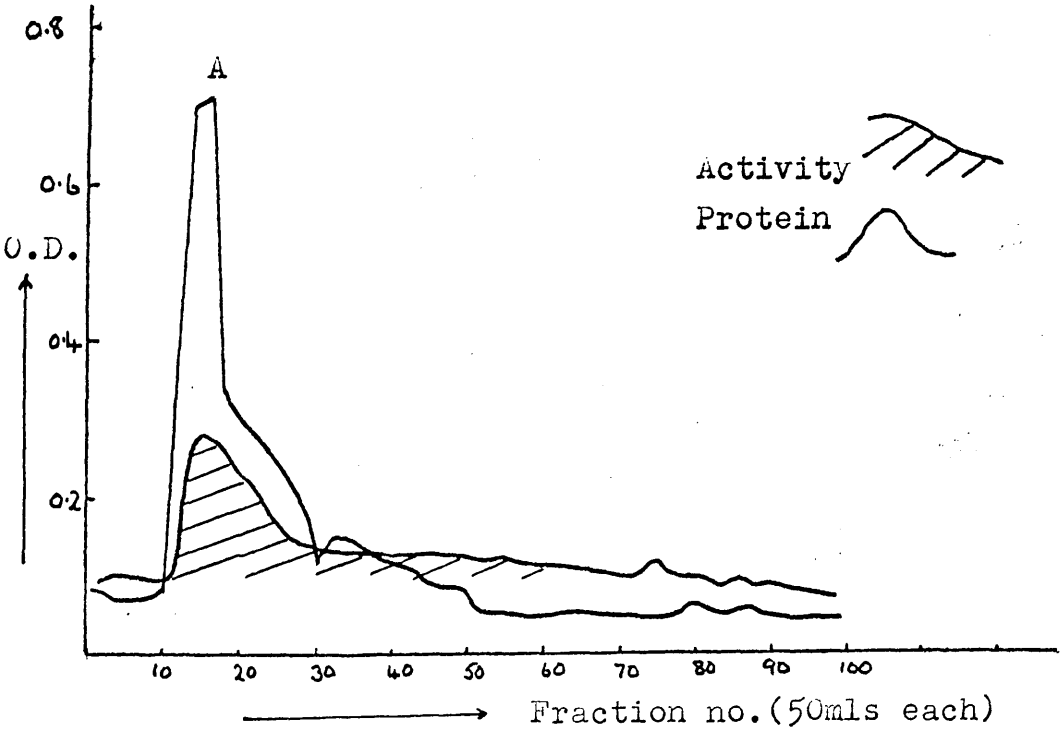


Fig.4. Rechromatography of peak A from CM-cellulose chromatography of Extraction I (fig.3).

elution sequence, they must be attributed to a fairly basic RNAse. The active peaks were labelled RNAse A and B in order of elution, but the naming has no intentional relationship with any other RNAse A or B reported elsewhere other than by Rees and Edmond (37)

The active protein peaks were bulked as follows:-

Tubes 90-105 inclusive as peak A.

Tubes 107-115 inclusive as peak B.

- and separately dialysed against 0.005 M TRIS/HCI pH8.2, 10^{-4}EDTA over a period of twenty-four hours with three changes of buffer of six litres each change. The A and B fractions were then concentrated by the use of small CM-cellulose concentrating columns.

Protein Concentration - general

In protein isolation one is often presented with the problem of having to concentrate large volumes of dilute protein solutions. There are a number of methods of doing this, but each has its difficulties because of the special nature and properties of proteins which are generally extremely sensitive to physical and chemical manipulations and changes in environment. Proteins are also more likely to suffer changes that may lead to denaturation if maintained in dilute solution, and therefore besides ease of handling, concentration is important for other reasons.

There are seven commonly used techniques of protein concentration (85) and these are: adsorption, evaporation and sublimation, precipitation, osmotic removal of solute, water

adsorption by cross-linked gels, ultrafiltration and finally centrifugation.

Of these seven methods the two that have been made use of are the first two, namely adsorption, and evaporation and sublimation.

Because proteins are sensitive to heat, few can withstand normal evaporation techniques and thus a low temperature evaporation or lyophilization is used. In this technique the protein is frozen and then subjected to a high vacuum which removes the water from the protein by sublimation so reducing the volume of the protein solution. The difficulties are that if any salts are present, as the volume is reduced, the salt concentration increases and similarly with changes in pH. The process of freezing itself can actually harm some proteins also.

The possibility of using the adsorption properties of ion exchangers in concentration of proteins was suggested by Peterson and Sober, 1956 (86). The basis of this method is the adsorption of the protein solution of low ionic strength onto a suitable short ion exchange column followed by displacement in a sharp band by a strong eluting agent. The disadvantage of this approach to concentration is that the required eluting solvent may have an undesirable composition for the next procedure, but this can be rectified by adding an extra step of dialysis.

Protein concentration - procedure

The column was prepared by taking an 18cm x 1 cm length of glass tubing, which was drawn to a small opening at one end, and plugging this end with a bung of glass wool. The column was filled up to the 3.5 cm level with CM-cellulose in the correct form and equilibriated with 0.005 M Tris/HCI pH8.2, 10^{-4}M EDTA buffer. The column was pretreated by washing with 250 mls. of the above buffer (initial buffer) before protein application. The dialysed bulked protein solution of peak B (fraction 107-115 inclusive - 450 mls.) was then applied to this small column. The protein was visibly seen to adhere to the top of the column as a brown band. On completion of protein application, the column was washed with 100 mls. of pH8.2 buffer and then the protein was eluted off by the passage of buffer made 1 Molar with sodium chloride.

As elution proceeded the protein band was seen to move down the small column due to the displacing action of the Na^+ ions. Small fractions were collected just before the band reached the glass wool plug and the band was completely eluted in a total volume of 2mls. A further six mls. of eluate were collected and retained for enzyme assay purposes to ensure all activity had been collected. The concentrated sample of RNAse B was sealed and stored in the deep freeze for further use.

Note on the U.V. absorption of RNAse B at 280 mu

Although the activities of RNAse A and B peaks on CM-

cellulose are approximately similar, it was found in this and subsequent preparations of RNAse B that the degree of U.V. absorbence at 280 mu was unusually low compared with activity. The reason may perhaps be suggested that this particular protein fraction is particularly short of the chromophores that are responsible for U.V. adsorption in this range, namely the aromatic amino acids - tyrosine, tryptophan and phenylalanine (252). Absorption at 280 mu may vary by a factor of five or more for equal concentrations of protein due to variations in the aromatic animo acid content.

Further chromatography of RNAse peak A (fraction 90-105, CMC-I)

Artifacts are possible in any procedure and a component isolated by one technique must be examined by other criteria before its validity is accepted, and to avoid being deceived by spurious fractions, it is advisable to subject the isolated fraction to a repetition of the original separation experiment. Thus rechromatography can establish that the peak in question migrates as a chromatographic entity in the absence of other fractions as well as their presence; or it may reveal that the peak actually comprises portions of other components eluted together because of factors such as overloading, channeling, anomalous pH effects, and improper elution etc. The formation of dissociable complexes must also be considered and indeed it was thought that RNAse B might be a subunit of the dissociable complex A, but there was no

proof of purity or molecular weight estimation at this stage that could give any more credence to this beyond mere conjecture. However previous work (37) had found when activity region A was reapplied to the recycled CM-cellulose column at pH8.2 and eluted with the same salt gradient as previously (CMC-I), a similar corresponding activity region A and B were obtained. When peak A from this rechromatography of the initial peak A was itselfagain rechromatographed under the same conditions, only the one peak corresponding in position of elution to peak A was obtained.

Two possible explanations for this phenomenon came to mind and the initially favoured explanation was that A was a dissociable complex of B. Also it was thought that due to overloading of the column and high protein concentration present, resulting in peak A occluding portions of peak B, portions of peak B would be eluted with peak A. Rechromatography of A in the presence of lower total protein concentration, should therefore allow separation of B from A; and indeed this did occur (37) and was supported by the evidence of re-rechromatography of peak A to give one product.

Thus peak A from the present separation (CMC-I) was reapplied to the same recharged column at pH8.2. The column was washed with 1.5 litres of the initial pH8.2 buffer and then a 0.32 M sodium chloride gradient was applied to the column as previously, with the ommission of any pH gradient.

The buffer solutions used in the gradient were:-

In the reservoir — 1 litre of 0.005 M Tris/HCl, pH8.2, 10^{-4}M EDTA, 0.32M Na Cl

In the constant volume chamber — 0.4 litre of 0.005M Tris/HCl, pH8.2 10^{-4}M EDTA.

Ten ml. fractions were collected. 74 fractions were collected in the first instance and then 1 litre of buffer made molar with respect to sodium chloride was passed through the column and a further 76 fractions collected.

The protein and enzyme location was carried out as before and the results plotted in fig. (4) ———— CMC-II

Therefore the reapplication of peak A only gives one peak on chromatography on CM-cellulose and confirms the eventual results of previous workers (37) discussed in the above text and therefore the theory of peak A being a multiple aggregate of peak B, in the light of these results, cannot be confirmed.

Tubes 10-30 from CMC-II were bulked and the material dialysed against initial buffer at pH8.2 with three changes of four litres each. This material was then concentrated on a small CM-cellulose column as previously described (page 58) and the protein solution stored in the deep freeze at -22°C.

Further extraction procedures to obtain larger supplies of the purified enzyme (both A and B) were carried out. Attempts to improve yield and extraction techniques were the main considerations in these subsequent extraction procedures. The

procedure already described forms the basis of the further four spleen extractions which were undertaken between October 1964 and January 1967. Experimental details of each extraction are recorded largely in the text due to the importance of changes in experimental procedure which are reflected in the results, but details are also to be found in the Experimental section (EXPS. 1-7). Also summary charts of extraction procedures are to be found in figs. 17-21. The changes in procedure are discussed in the following section, together with the reasons and theory involved in these changes and also the results of changes are discussed when possible. In a long extraction and purification process such as described, there are so many variable factors that it is not always possible to correlate changes of procedure with the corresponding change in results. Each topic of change is considered below in the order that it would have occurred in the initial extraction - I.

I A Consideration of the re-extraction of normally discarded residues

A Re-extraction with initial buffer.

Edmond (37) reported the yield of purified protein to as far as the CM-cellulose stage VI to be about 1 mg. of protein per spleen extracted. In Extraction I this was approximately confirmed, since 15 mgs. of RNAse B were obtained from 25 spleens. The quantity of RNAse A obtained was not measured, but judging

solely from activity considerations the quantity is unlikely to be any more than obtained for RNAse B. Also, if the U.V. absorption method of protein estimation is taken into account, a yield of approximately 1 mg of enzyme per spleen is a justified conclusion for extraction I. However, it was thought possible that an active organ such as the spleen, especially in a young growing animal, would be a larger potential source of this ribonuclease than in fact it appears at present.

The most obvious point of the procedure at which to initiate increased efficiency of extraction investigations was the initial extraction step - stage I. It will be recalled that the normal process of extraction was an aqueous extraction with 0.05M sodium acetate, 10^{-4}M EDTA pH7.2 buffer, and then this same buffer at pH3.5 with the ratio of homogenised spleen to buffer of 1:3. Note that phosphate buffers are not used as these are reported to give poor activity with basic RNAses (90). The extract was then made 30% saturated with solid ammonium sulphate and precipitated material and cell debris centrifuged and discarded.

It was not wanted to leave the homogenate to extract for any longer length of time before the first ammonium sulphate precipitation because of proteolytic attack and other harmful protein - protein interactions. Besides, it was doubtful whether an increase in the length of extraction period alone could increase extraction of the enzyme in question, and so a more

positive approach to efficient enzyme extraction was sought.

The solid material obtained after 30% ammonium sulphate fractionation and centrifugation was discarded in Extraction I, but in the extractions 2-5, the solid material was bulked in glass beakers and two volumes of initial buffer were added and the mixture stirred until all conglomerations of material had given way to a thin brown suspension. The mixture was then brought up to 30% saturation with respect to ammonium sulphate and centrifuged. The supernatant from this and the initial extraction and precipitation were combined and taken on to the next stage in the procedure.

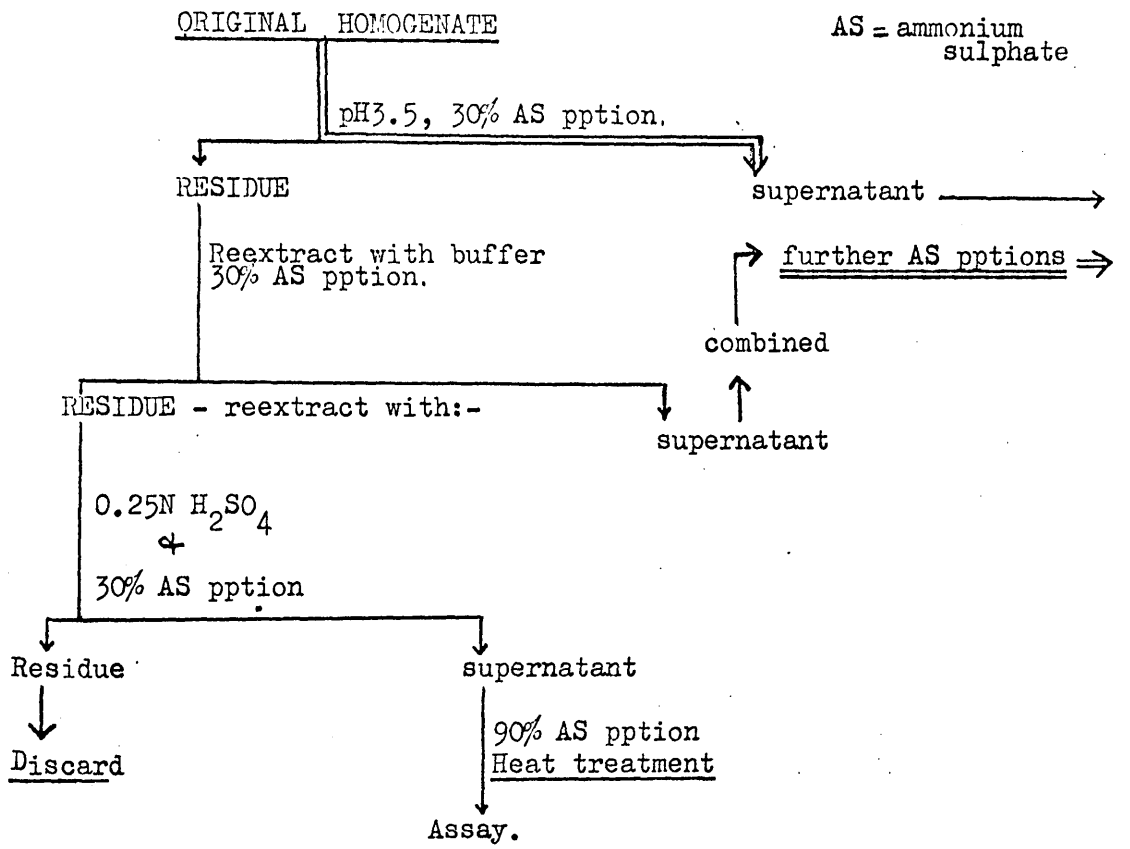
B Reextraction with 0.25N sulphuric acid

Extraction 5 also showed that further reextractions of the above reextracted residue with 0.25N sulphuric acid yielded further active protein. The extraction with a sulphuric acid seems a little drastic with respect to enzyme denaturation even for an acid stable RNAse as was being isolated, but its use was reported by Kunitz (89) where, in his classic isolation and crystallisation of bovine pancreatic RNAse, the minced pancreas was allowed to extract in cold 0.25N $H_2 SO_4$ for eighteen to twenty-four hours. However no reasons for its use were given. Roth (90) found that treatment with the same strength of acid increased the alkaline ribonuclease activity from liver fractions by ten times. This increase in

activity was thought to be due to the destruction of a natural inhibitor of the ribonuclease present in the homogenate. Much research into RNAse inhibitors has been done since Kunitz's isolation of pancreatic RNAse. In 1965 Roth (91) suggested that the inhibitor present in the supernatant of rat liver may be a protein capable of binding considerable quantities of alkaline ribonuclease possibly through -SH groups on the inhibitor. However this inhibitor is heat labile, is destroyed by heating at 65°C for five minutes and is not dialysable. Beard and Razzell (87) also used 0.25N sulphuric acid in their extraction process of an alkaline ribonculease from pig liver.

It was with these reports in mind that extraction of the reextracted material was carried out with 0.25N sulphuric acid. One volume of the acid was made 30% saturated with respect to ammonium sulphate and slowly stirred into the reextracted residue (at cold room temperature) The now acid mixture (pH approximately 1.2) was stirred mechanically for four days, and then was centrifuged at 10,000 r.p.m. The residue was discarded and the supernatant retained, but not neutralised at this stage in the manner of Kunitz (89)

Summary of initial reextraction of Extraction V



Further precipitation was accomplished by raising the ammonium sulphate saturation to 90%, and the precipitate material was collected by centrifugation. The sample was then heat treated at 60°C for ten minutes and centrifuged and its further development arrested at this stage - with the retention of the supernatant for assay and activity determinations.

Measurement of enzyme activity

Enzyme activity was assayed as described previously (page 52) with an incubation time of thirty minutes at 37°C. A unit of enzyme activity following this procedure is defined as that amount of enzyme which causes an optical density increment of 2.0 at 260 mu in the final diluted sample. The unit was defined by Kaplan and Heppel in their original paper (28); the assay system also being essentially the same in order to permit more reliable comparisons. For best results it was desirable to restrict the net optical density (O. D.) to 0.10 in the final diluted sample. This ideal was approximately achieved by experimentation in dilution of the enzyme solution being assayed.

The net result of the 0. 25 N sulphuric acid treatment at the post heat treatment stage was that approximately 36% of activity obtained by the acid treatment was retained. This method of extraction was pursued no further because of time limitations, but a method that extracts 3.1% activity from already re-extracted material and then retains 36% of the activity after heat treatment warrants further investigation with respect to heat stable basic spleen RNAse extraction. (see fig. 37 and accompanying data table.)

C Comparison of m-butanol, urea, triton X-100 and Cetab . as extraction aids.

i) N-butanol - general

Enzymes are often located in tissues bound to lipid material. The presence of fatty material was noticeable in the extraction process especially during the initial ammonium sulphate fractionation stages (page 34). In order to separate protein from any possible lipoprotein complexes a method must be found that will separate the protein from any associated lipid constituent without denaturation of the protein. The use of butanol was introduced by Morton (94) and has subsequently been modified to a number of separation uses. Through experiment, n-butanol in particular has been found to be the least damaging to enzymes. Other organic solvents such as: carbontetrachloride, chloroform and toluene have proved of little use because of their denaturing effects. The unique behavoiur of butanol may be attributed to its very marked lipophilic character and this results in a rapid penetration of the complex. Similarly it has a concomitant hydrophilic property since it is soluble in water to the extent of 10.5% at $0^{o}C$. Thus butanol has a detergent like action being both water and fat soluble, and unlike most other organic solvents, lipids can be extracted satisfactorily from aqueous solution.

Procedure

A 100 gms. of frozen material that had been retained from the initial 30% ammonium sulphate precipitation and stored in the deep freeze, were allowed to thaw at room temperature in polypropylene

centrifuge bottles. Then a 100 mls. of 0.05M sodium acetate pH7.2 buffer was made 30% saturated with respect to ammonium sulphate to maintain the same conditions of protein precipitation as in the extraction procedure. The solution was stirred and placed in the cold room and was designated as the control extraction.

To a similar 100gms. of material, n-butanol was added to give a 6% butanol solution. In the same manner a 20% butanol extraction was set up. Thus at 6% and 20% butanol mixtures, a one and two phase procedure respectively was being operated. In the one phase procedure, there should be a dissociation of lipoproteins, whilst in the two phase procedure there is a similar separation, but the emulsion of fatty material is taken up into the butanol layer whereas the proteins remain in the clear aqueous layer. However the ribonuclease may not be associated with lipid material in this residue of 30% ammonium sulphate precipitation.

Both solutions were stirred intermittently for thirty minutes and then centrifuged at 10,000 r.p.m. for forty minutes, filtered, and the supernatants were assayed with RNA.

ii) Detergents - general

Detergents act as solubilising agents with respect to their action as used in protein extraction. Solubilization is the spontaneous passage of solute molecules of a substance insoluble in water into an aqueous solution of soap or detergent in which a

thermodynamically stable solution is formed (98)

In the case of the protein being investigated, the RNAse is not insoluble in water in normal circumstances, but it is possible that it may not be "available" to normal aqueous extraction processes due to attachment or interaction with other molecules such as lipids, to form lipoprotein complexes.

Synthetic detergents all have basically the same structure which consists of a long hydrocarbon chain moiety or "tail" which is hydrophobic in nature, and a hydrophilic "head" containing either an ionic group which can render the detergent cationic or anionic or else the detergent can be non-ionic, the hydrophilic group in this case being a neutral entity. Thus detergent molecules are often represented as :- "tail" ———o "head" molecules diagrammatically.

Interaction between proteins and detergents may conceivably involve electrostatic attraction between oppositely charged ionic groups as well as mutual association of non-polar residues. The action of a detergent on a lipid is that the non-polar hydrocarbon "tails" orientate themselves so as to be closest to the lipid surface or to "dissolve" in the surface, whilst the polar end of the molecule remains free and the resultant effect is that the lipid presents, in effect, a polar surface to the exterior due to the detergent polar groups and therefore allows the lipid to be dispersed in aqueous solution.

The action of detergents on proteins depends on the conditions of study, and often may result in denaturation, precipitation or complex formation. The action of detergents on biological systems is therefore much more complicated. Protein denaturation by detergents often takes place at a much lower concentration of detergent than it would occur with neutral denaturants such as urea or guanidine hydrochloride (0.008M versus 8M.) The amount of detergent to do this also depends on the concentration of the protein.

Detergents also inhibit the coagulation of denatured proteins, whilst the same detergent can precipitate the native protein. Excess detergent leads to a dispersal of the precipitate.

Though initially the use of detergents seems an attractive proposition with ubiquitous uses in protein extraction, it is discovered in practice that their value is somewhat smaller. Frequently their use is in an 'all or none' category and the reason appears to be that their mode of action involves strong bonding between the lipophilic portion of the protein and of the detergent molecule. This complex formation is often very difficult to reverse, and therefore in the preparation of a pure enzyme sample, detergents are of little use. Invariably also, denaturation of the protein follows. The large amounts of detergent bound to protein components, present similar surfaces to the solution, thus largely hiding the inherent differences in the protein components and causing them to behave similarly in any fractionation

procedure.

Tergitol NPX is a non-ionic detergent and was used to aid the release of an acid RNAse (99) Sucrose homogenates were being prepared from brain and cervical spinal cords of teleostian fish and an amphibian, and the homogenates were separated into a nuclear fraction, a microsomal fraction and a non-sedimentable supernatant by preparative centrifugation. The original homogenate and each of the separated fractions were assayed for acid phosphatase and an acid RNAse. The addition of Tergitol NPX to the homogenate by Jones and Janoff (99) caused the release of nearly 100% of the granule associated RNAse into the non-sedimentable phase of the homogenate.

If a similar release of associated ribonuclease from any precipitated material in the extraction of basic RNAse from calf spleen could be obtained by the addition of a non-ionic detergent, this would be a valuable aid and improvement on the present extraction without the use of detergents.

Triton X-100 is also a non-ionic detergent, and this particular non-ionic detergent was chosen more or less empirically as a solubilizing agent for extraction since it was readily available. It was hoped that a neutral non-ionic form of detergent would be somewhat milder in action than a cationic detergent. Use was made of this detergent in the solubilization of particulate cytochrome oxidase (95). Similarly Triton X-100 has been recently reported

(96) to have been used in the release of RNAse activity in rat liver particulate study. It was stated in this paper that the release of enzyme activities with increasing concentrations of detergent could be attributed solely to the effect of this detergent on the intracellular structure perhaps of the membranes. The increase occurred up to a maximum concentration of 0.1% Triton X-100.

Because of availability of cationic detergents, cetyl-trimethyl ammonium bromide (Cetab.) was chosen as the cationic detergent to test the effect of solubilization on enzyme extraction. A cationic detergent was thought worthy of investigation because of the relatively basic nature of the enzyme being isolated and as with Triton X-100 it was hoped that it would aid the extraction of the enzyme from the homogenate.

The exact mode of the action of the two detergents chosen for experimentation is not fully understood and the choice, and concentration decision is largely empirical. The possible effects of detergent use has been reviewed comprehensively by Putnam (97)

Procedure

A 100 mls. of buffer at pH7.2 was made 1% (v/v) with Triton X-100 and added to 100 gms of the homogenate and 30% ammonium sulphate precipitated material. Similarly a 1%

solution of Cetab. in buffer was added to a 100gms of the precipitate. Both solutions were stirred for ten minutes and centrifuged at 10, 000 r.p.m. for ten minutes or fifteen minutes in the case of 'Cetab' extraction since this proved difficult to centrifuge. Aliquots of the supernatant were taken and assayed for activity.

(iii) Use of urea

The use of urea and its effect with proteins is discussed in greater detail in the next section (page 185), Part II of this thesis.

The extraordinary solvent action of urea solution with proteins has long been known, but the action of urea still remains unexplained. However there is no shortage of theories about its possible mode of action with proteins at various concentrations of urea. One of the principal theories is that urea is a competitive breaker of hydrogen bonds (100) Studies on model compounds of urea in solution do not support the view that urea facilitates the breaking of hydrogen bonded structures in protein.

Whatever the theory behind urea action, the employment of the concept of urea as a competitive breaker of hydrogen bonds has been very useful. For example it has been shown that urea can dissociate the complex enzyme flavocytochrome b_2 into a haemoprotein, a flavin, and a polynucleotide (101) Urea has also been found an effective extracting agent for separating pituitary

tissue proteins (102) and is presumed to act in reducing protein interaction. It is precisely along these lines that the use of urea was considered in the case of the spleen RNAse extraction.

It was thought that after homogenization, probably the majority of the RNAse activity would be extracted with the initial buffer at pH3.5 and not precipitated with 30% ammonium sulphate. However, it was also thought very probable that there would be considerable quantities of active enzyme, 'trapped' in the normally discarded material. The manner in which this 'trapped' protein might be held was open to speculation. Lipoprotein complexes have already been considered, but more likely is some type of protein-protein association of the enzyme either with a non specific protein by hydrogen bonding, or a more specific protein-protein association of a coenzyme type compound or enzyme-inhibitor complex. Thus some aid to diminish hydrogen bonding and so release more enzyme molecules was sought and the urea hydrogen bond breaking theory seemed applicable to the problem.

The theory had been invoked by Tsaryuk (103) whilst extracting a proteinase from brain homogenates. Following brain homogenate incubation in urea, there was a facilitated release of alkaline RNAse up to a maximum of 3M urea concentration. Higher concentrations were reported to denature the enzyme. It was also reported that similarly to pancreatic RNAse (104), an alkaline RNAse (from liver) is stable in urea and therefore urea

in moderate concentrations seems to exert a stabilising effect on RNAse (105)

Procedure

Two portions of a 100 mls. of initial pH7.2 buffer were made respectively 6M and 3M with 'Analar' grade urea and stirred for ten minutes and then left to stand overnight. The two samples were centrifuged and aliquots of the resulting supernatant were taken for assay with RNA.

Results of control (page 69), butanol, detergent and urea extractions are tabulated below:-

	Zero blank	Assay O.D. 260	Difference
Control	0.112	0.505	0.373
n-butanol 6%	0.103	0.323	0.220
20%	0.124	0.300	0.176
Urea 6M	0.124	0.400	0.276
3M	0.124	0.447	0.334
Triton X-100 1%	0.135	0.495	0.360
CETAB 1%	0.133	0.515	0.382

A 'zero blank' digest was carried out and 0.25 ml of RNA solution were pipetted into the digest tube and then 0.5 ml of

uranylacetate /TCA solution. The test solution was then added and the tube thoroughly shaken and placed in an ice bath. Thus this was a zero time blank. The digests were carried out by the methods previously described except that the incubation period at 37°C was forty-five minutes instead of the usual thirty minutes. The results were merely expressed as the optical density readings at 260 mu from the U.V. spectrophotometer, but as all the protein samples were of the same source and all other factors were constant (except the extracting agents) the results are comparable.

With the exception of n-butanol as an extractant there seems to be little significant difference between the other methods. n-Butanol is therefore of little use in extracting active enzyme material under these conditions. Similarly 6M urea seems a somewhat low result compared with 3M urea, though probably the result is of no significant difference.

II Changes made in the ammonium sulphate fractionation procedure

As previously discussed precipitation of enzymes by 'salting -out' with ammonium sulphate is far from an exact and predictable method of protein isolation. Besides such controllable factors such as temperature, salt concentration, and pH, the precipitation is also dependent on the concentration of protein present and in the fractionation procedure described this is not controllable.

Therefore ammonium sulphate as a tool of initial purification, is used not as a refined narrow spectrum precipitation technique but as a wide spectrum precipitant. The object, therefore, is to precipitate the enzyme wanted over a wide range so as to ensure that absolutely all activity is collected.

There is also another factor to be taken into consideration which is that enzymes are often in the form of multiple aggregations of a monomeric form. Chesbro (1966) (108) has shown, working with a staphylococcal nuclease with active units possessing molecular weights from 3,000 or less to about 30,000, that the position of fractionation with ammonium sulphate depended on molecular weight, and indeed there was a fraction that was still soluble and not precipitated by saturated ammonium sulphate. This soluble fraction in ammonium sulphate had molecular weights ranging from about 6,500 and under. Thus if the conditions leading to dissociation are not understood a wide fractionation range should be taken with ammonium sulphate precipitation. Undoubtedly much unwanted protein will also be precipitated at the same time, but further and more predictable techniques can be used to remove these impurities. The use of ammonium sulphate as a fractionation procedure can be likened to the use of a large fork to dig the garden, and then more refined cultivation techniques can be applied.

In extraction I in accordance with the method of Kaplan and

Heppel (28), a second acid fractionation at pH2.0 was performed, but this step was omitted completely from extraction 2 because it was considered that little could be achieved by a repeat precipitation under similar conditions of pH as in stage IIB of extraction I. The purification in this latter case proceeded directly to dialysis after heat treatment.

The results after chromotography on CM-cellulose (CMC-2, fig. 5) were little different from those of extraction I which had a stage IV pH2.0 ammonium sulphate fractionation - giving two activity peaks - A and B (fig. 5).

The large protein peak labelled 'x' with a very small associated activity peak may have been the result of the elimination of the second acid ammonium sulphate fractionation at stage IV since this was not present in CMC - I (fig. 3) column extraction I.

Therefore, by the exclusion of this step, there was a fourth largely inactive protein peak on CM-cellulose chromatography and, as a result, the second ammonium sulphate fractionation at stage IV was retained in all future extractionations but at pH7.0, and not pH2.0 since there seemed little justification for this low pH.

In extractions I and II at the ammonium sulphate precipitation at stage IIB before heat treatment, the concentration of the salt was always increased to 85% saturation. Nevertheless it was always suspected, and in extractions 4 and 5 confirmed, that a large proportion of the active enzyme was not precipitated even

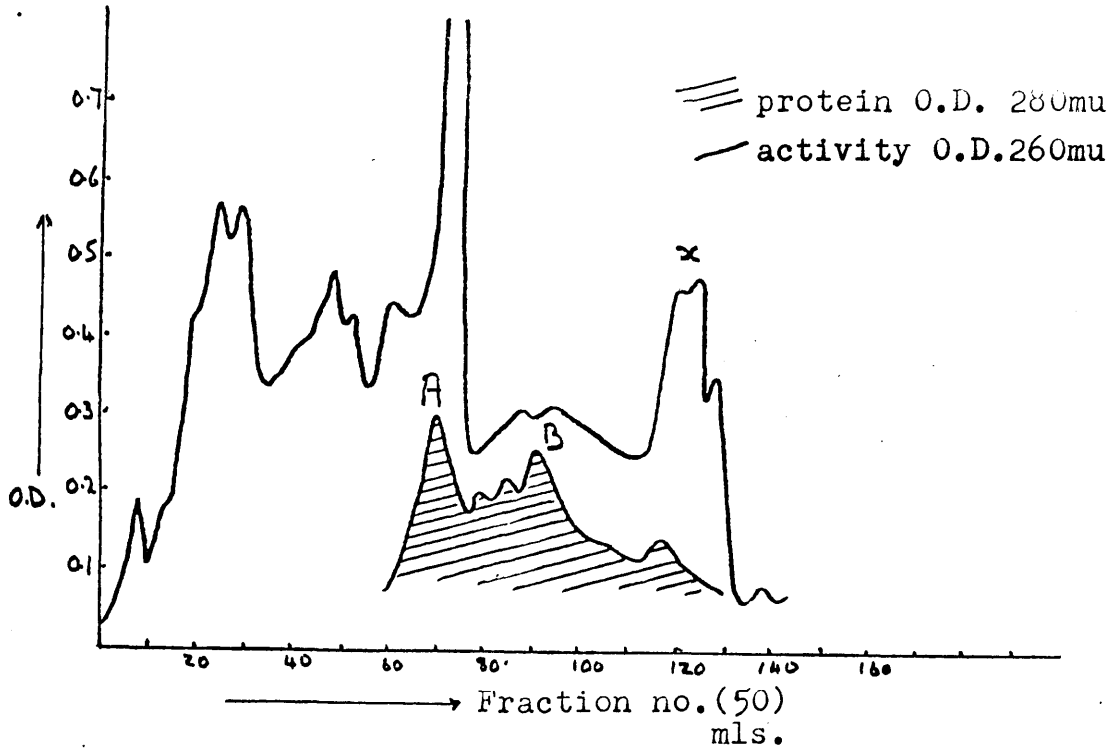


Fig.5. CMC-2. Extraction2, application after dialysis and heat treatment. No pH 2 second AS precipitation.

Extraction 4.-Diagram of path of activity during extraction.

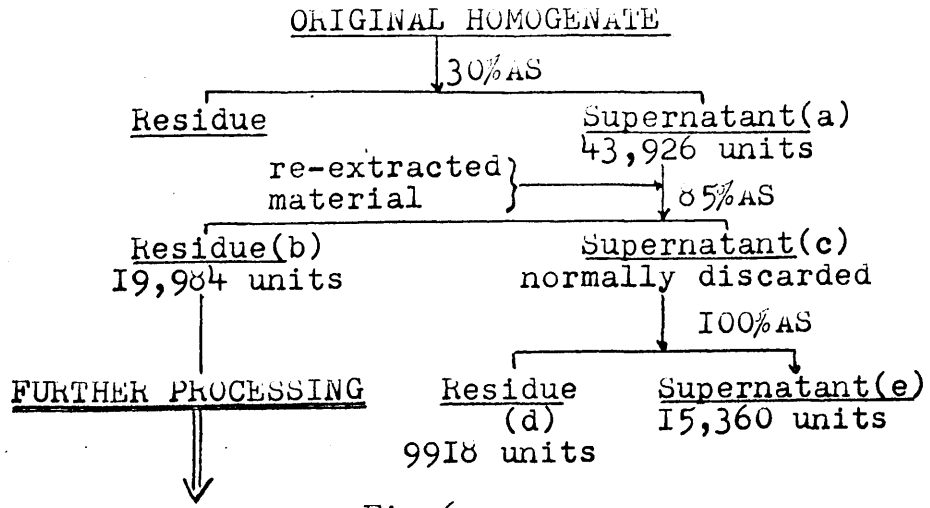


Fig.6.

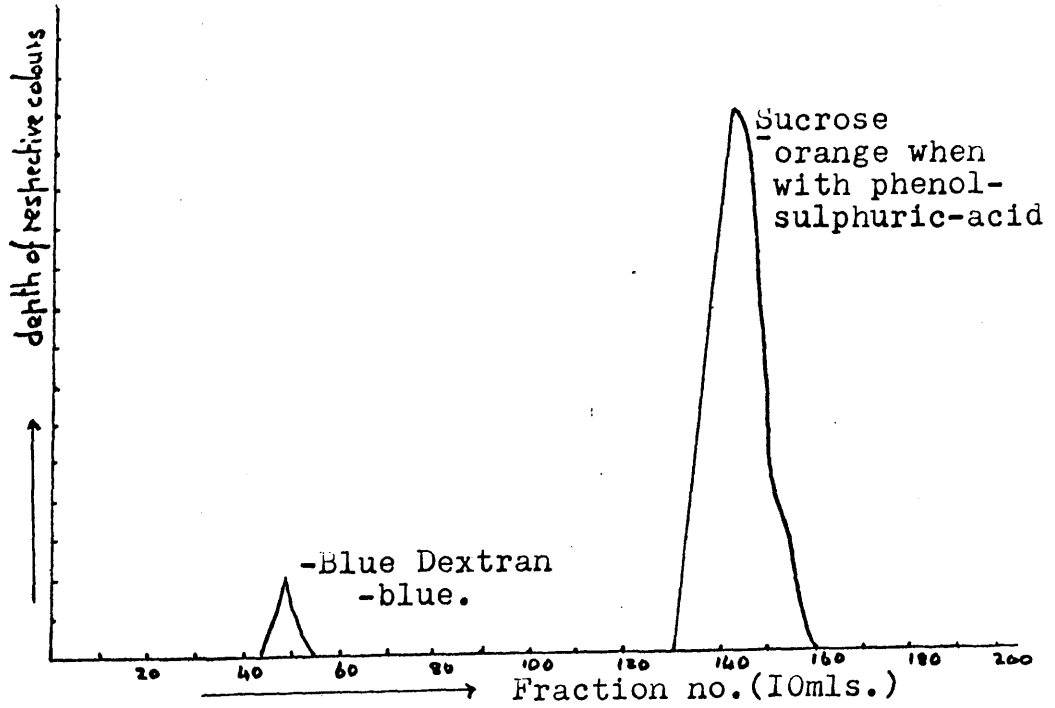
at this high salt level. In extraction 4 the number of activity units discarded was more than those retained (see fig. 6). A sample of this normally discarded supernatant from the final 85% saturation level was retained and increased to saturation level and allowed to stand overnight. There was, as a result of this treatment, a further precipitation of active protein which was removed by centrifugation and dissolved in buffer for assay purposes along with all other samples taken during extraction 4 and the results obtained as shown - fig. no. 6

From this diagram (fig. no. 6) it can be seen that even at saturation with ammonium sulphate there is yet unprecipitated activity in the supernatant solution - Stage 'e' fig. 6.

Extraction 4 was accomplished completely in the presence of 2M urea from the initial homogenizing step, but extraction 5 was executed without urea and the same results were obtained where large proportions of the entire activity were soluble in solutions of 90% ammonium sulphate saturation and above. (See fig. 7 for comparison of Extraction 4 and 5 results).

a,b,c,d,e,- with ref.to fig.6 letters.	Extraction 4.	Extraction 5
Step a	43,926 units activity	237,770 units activity
Step b	I9,984	I49,250
Step c	30,720	24,846
Step d	9,9I8	9,058
Step e	I5,360	26,296

Fig.7. A comparison of activities for extraction 4 & 5 at stage IIB as above.



Sephadex G-75 column. Void volume & column Calibration

Fig.8.

It is noted that the loss due to unprecipitated activity was appreciably higher in the presence of urea. This may be due to the effect urea has on dissociation of protein molecular *aggregates *(for full discussion see "Aggregation" Section II) thus giving a larger proportion of dissociated enzyme molecules perhaps in their monomer form, and therefore not precipitated by saturated ammonium sulphate in a similar and parallel fashion to the staphylococcal nuclease preparation reported by Chesbro (108)

Possibly this saturation-point soluble RNAse activity does not belong to a basic RNAse at all, so a portion of this fraction of supernatant from extraction 5 was subjected to heat treatment in the usual manner of 60°C for ten minutes, in order to estimate the proportion that was heat stable and therefore likely to be of interest.

Heat treatment

Procedure for 90% ammonium sulphate soluble activity from extraction 5 (see fig. 37)

The 90% saturated material was removed from the cold room at 5°C and allowed to rise to room temperature at 16°C. This was done because often solubilities are very sensitive to temperature and as previously stated a protein that may be soluble at 0°C in a certain ammonium sulphate solution may be completely precipitated out at room temperature. Consequently this reverse temperature effect was tried. The results were disappointing

and there was no evidence of an increase in precipitation over and above that achieved at 5°C, and so the material was replaced in the cold room to return to 5°C. After centrifugation at 10,000 r.p.m. for fifteen minutes a soft pale precipitate was obtained and was dissolved in initial buffer at pH7.0. A sample was retained for assay and the rest was adjusted to pH3.5 and subjected to 60°C heat treatment for ten minutes. On cooling the pH was readjusted to pH7.0 and the heat treated material centrifuged at 10,000 r.p.m. Assays were carried out on the resultant supernatant.

The result of the assays was illuminating since it showed that only 9% of the activity that was soluble in near saturation point salt, was active after heat treatment. Therefore at stage IIB the technique enables most of the activity to be recovered when the ammonium sulphate concentration is taken almost to saturation in this precipitation preceding heat treatment.

Similarly as with stage IIB, in the final ammonium sulphate fractionation after heat treatment at stage IV (see fig. 21 and 37) there is considerable loss of activity which is still soluble at 85-90% salt saturation. Since all RNAse activity by this stage is heat stable, this fraction must be considered as wanted enzyme and also this solubility in high concentrations of ammonium sulphate has re-occurred since stage IIB, because all the material heat treated was insoluble in ammonium sulphate at 85-90% saturation prior to the heat treatment. There seems to be some kind of

equilibrium established between enzyme which is still soluble and enzyme which is insoluble and precipitated at 85-90% salt saturation.

The explanation which best fits the facts is that founded on the recent work of Chesbro et al. (108); that the enzyme (like many other proteins) exists in a number of states of aggregation. Those with the smaller molecular weights are soluble in ammonium sulphate concentrations which near saturation point. Therefore when the larger aggregates were removed at stage IIB by virtue of their insolubility at near saturation point, and subjected to heat treatment, there was probably a dissociation of aggregates to give the smaller multiples of the monomer unit. The stimulus to dissociate may have been due to thermal effects, or protein dilution effects, or a combination of these and other changes in environment.

The total activity destroyed by heat treatment in extraction 5 was 89%. The sudden fall in protein concentration in solution after heat treatment could also be used to explain the soluble and unrecoverable activity by virtue of altering the ammonium sulphate fractionation spectrum to put some activity beyond the saturation limit. But this does little to explain why all the protein should not be soluble, and not as in the present situation where some protein was soluble and some was insoluble. The first expounded theory, summoning deviation in molecular sizes is more

attractive.

Summary of the efficiency of ammonium sulphate as a fractionation procedure

By increasing the ammonium sulphate to at least 90% saturation at stage IIB, the majority of the active heat stable enzyme can be precipitated from solution, and that which is not is only a small proportion. Disc electrophoresis (detail page I40) indicated that the remaining 10% heat stable enzyme which was soluble at near saturation level contains the characteristic bands of the wanted protein as well as other proteins. Evidence strongly suggests that this latter 10% heat stable RNAse was of low molecular weight approaching that mentioned by Kaplan and Heppel (28)

The problem of loss of enzyme during the final ammonium sulphate fractionation (stage IV) still remains and no method was devised to recover the unprecipitated activity.

The presence of urea appears to have no effect on fractionation procedures.

III Temperature variation experiments

Kaplan and Heppel (28) studied the lability of their basic splenic ribonuclease and found it quite stable when held at 60°C for ten minutes at pH2.0, but lost 20% of its activity after treatment at 80°C for ten minutes at pH3.3. But Maver and Greco

(29) reported at least four acid ribonucleases which were heat labile and these were destroyed during the 60°C heat treatment and probably account for Kaplan and Heppel's loss of 70% activity on heat treatment (90% loss in extraction 5)

Roth (106), and Beard and Razzell (105) have isolated a protein inhibitor of alkaline ribonuclease from liver fractions. The inhibitor has been partially purified by Roth and was reported to be heat labile. In 1965, Roth (91) also described an inhibitor from the supernatant fraction of rat liver which may be a protein capable of binding considerable quantities of alkaline ribonuclease. The inhibitor was also heat labile and was destroyed by heating at 65°C for five minutes.

Whether the inhibitor would be destroyed by heat treatment used in the preceding extraction I is debatable and its destruction could not be stated as certain. Thus there are grounds for use of a higher temperature for heat treatment of the RNAse. Previous data (28) shows that even a temperature of 80°C for ten minutes, only results in a 20% loss of active RNAse, so a slightly higher temperature could be accommodated without any loss of basic RNAse activity.

In extraction 2 (which was performed prior to knowledge of Roth's report on inhibitor lability limits) the heat treatment of the protein solution was carried out at 67°C for ten minutes, using an 85°C bath to bring samples up to the treatment temperature.

This change was made with the aim of using this step as an even greater separative procedure with the removing of the three or so initial inactive peaks obtained on CM-cellulose chromatography (see CMC-I fig. 3) of the post heat treated stage. The higher the temperature that can be used without loss of wanted activity, the more efficient the treatment becomes.

Results

From the results obtained from CM-cellulose chromatography which immediately followed heat treatment in extraction 2, (CMC-2 fig. 5) no difference in elution pattern was experienced that could be attributed to the use of a higher temperature.

Extraction 3 saw the introduction of a D.E.A.E.-cellulose column step after dialysis or desalting, and the temperature of heat treatment was returned to 60°C since there was no apparent advantage of using 67°C and to continue without good evidence of its superiority was an unnecessary risk with respect to activity loss of the basic RNAse. An added incentive to return to the 60°C treatment, together with the use of D.E.A.E.-cellulose chromatography was that it had been reported (107) that a protein inhibitor of alkaline RNAses from rat liver had a strong affinity for the basic ion exchanger D.E.A.E.-cellulose. Thus the use of an anion exchanger could possibly remove any inhibitors not heat denatured and precipitated.

IV Dialysis, desalting and the use of Sephadex G-75

The stage V dialysis step was used for the removal of ammonium sulphate and other salts before application to CM-cellulose column chromatography. However it had been reported when working with certain RNAse enzymes that there was a loss of activity during dialysis. Beard and Razzell (105) mentioned being repeatedly frustrated by the tendency of their alkaline RNAse from pig liver homogenates to diffuse through common Visking dialysis membranes. This loss of enzyme activity had also been experienced by Edmond (37) and confirmed.

It was in the light of these two reports that dialysis in stage V of extraction was replaced by another form of desalting using the molecular sieving action of Sephadex.

Sephadex gel filtration - general

Sephadex is a modified cross-linked dextran with a three dimensional network of polysaccharide chains. Sephadex is strongly hydrophilic because of its high content of hydroxyl groups and therefore the Sephadex beads swell considerably in water and electrolyte solution. This degree of swelling is an important characteristic of the gel formed, and gels in which the matrix is a minor component are used for fractionation of high molecular weight substances, whereas compact gels are used for low molecular weight compounds.

The principle of gel filtration is such that molecules larger than the largest pores of the swollen Sephadex, that is above the exclusion limit, cannot penetrate the gel particles and therefore they pass through the bed in the liquid phase outside the particles, and are eluted first.

But small molecules penetrate the gel to a varying extent depending on their size and shape, and are impeded to varying extents with the result that these molecules are therefore eluted from a Sephadex bed in order of decreasing molecular size. Thus the separation is a purely physical one, no ion exchange properties are involved and therfore no gradient salt elution is necessary. When all the molecules have passed through the Sephadex bed the column is ready for re-use. This regeneration is one of the advantages of gel filtration.

The Sephadex types of the G-series differ in degree of cross linking and thus in swelling properties. The property of swelling ability or water regain value is used to characterise the types of Sephadex G-series available. The water regain value represents the amount of water imbibed by the gel grains on swelling and does not include the water between the grains. Sephadex G-200 for example will have a water regain value of 20 mls water per gram of dry Sephadex.

Desalting using Sephadex

The use of dextran gels for desalting was reported initially

by Porath and Flodin (109) in which they separted a solution of serum albumin containing 40% ammonium sulphate. There was a complete separation of the serum and ammonium sulphate, and so the result of gel filtration is often the same as dialysis. One disadvantage of gel filtration is the unavoidable dilution but this can be contained within reasonable limits by the suitable choice of experimental conditions. The method is very rapid and effects the complete separation of two or more substances if the differences in molecular size are sufficient, and the scale of the procedure may be increased upwards without any difficulty.

The absorption of proteins on Sephadex

It was noted (110) that during studies on gel filtration on G-50 columns with proteins, that in the absence of salts some proteins including RNAse, were strongly absorbed to the gel. Although this binding capacity, due to the hydroxyl groups, is very small compared with that of ion-exchange celluloses, the property may acquire considerable significance when small quantities of protein are being used. However all proteins are quickly removed from the column by use of a dilute salt such as 0.IM sodium chloride, but often if the eluent has a maximum strength of 0.02M it is quite sufficient to discourage absorption. Not all proteins tested behave in this manner, but it is a phenomenon that must be heeded when desalting proteins in this way.

Choice of sephadex type and the setting up of the column

G-75 was the grade chosen to use for the setting-up of a large preparative and desalting G-75 column, and it has a fractionation range of approximately 3,000 to 70,000. Therefore besides acting as a desalting step, it will also act as a purification procedure by possible separation of some unwanted proteins by virtue of differences in molecular size. The small molecular size of ammonium sulphate will result in its later elution than the larger protein molecules, so effecting a desalting of the applied solution.

General Procedure of G-75 column preparation

The dry form of G-75 was suspended in water with gentle stirring for twenty-four hours at room temperature to de-aerate. "Fines" were removed by three successive decantations. The gel was then stirred with 0.005M Tris/HCI, pH7.5, 10^{-4}M E.D.T.A (and 2M urea in the case of extractions 3 and 4) in the cold room to equilibriate. The column of G-75 was supported by a scintered glass disc, but the material was not packed directly on to this as the gel grain would probably clog the disc and cause a flow stoppage, and so about half a centimetre of exhaustively acid washed sand was allowed to settle down, through the previously buffer filled column under gravity. Care was taken to ensure that the column was absolutely vertical by means of a spirit level because a skew column will produce a non-horizontal zone on elution,

which in turn will result in asymmetric peaks.

The glass column, measuring 80 cms x 4.8 internal cms, was then filled at 4-5°C, by the method of column filling previously described (page 44 also (111) and General Method 5) to a height of 78 cms. The filling was carried out slowly over a period of twelve hours, with the buffer being allowed to flow slowly through the column, after an initial 3-4 cms of gel had settled under gravity action on the sand, at the rate of 4-5 mls per minute to aid packing. More buffer was added at the same rate as the effluent drains. A slowly rising horizontal surface of gel indicates uniform packing.

When the column was full, copious amounts of buffer were allowed to flow through the column to the extent of ten litres and more.

If the G-75 column has not been in use for a period of weeks, bacterial and algal growths might occur. These contaminating influences were effectively removed by eluting the column with buffer and 0.02% sodium azide and then eluting with buffer alone to clear the column of azide. The procedure was carried out periodically as a matter of routine between column runs (II2).

Testing and calibration of G-75 columns

Before the freshly compiled G-75 column was used for any specific purpose, the homogeneity of the bed was checked and also the void volume was measured - Vo.

The total volume (V_t) of a Sephadex column is the sum of the volume outside the gel grains (Vo) plus the volume inside the gel grains (Vi) and the volume of the gel matrix (Vg) or substance itself. $\therefore \quad Vt = Vo + Vi + Vg.$

The outer or void volume (Vo) is the volume of liquid required to elute a substance through a column if the molecules are completely excluded from the gel particles. The elution volume (Ve) of a substance depends on the volume external to the gel particles (Vo), and on the distribution coefficient (Kd)

$$\therefore \quad Ve = Vo + Kd \cdot Vi$$

Where Kd, the distribution coefficient indicates the fraction of the inside volume accessible to the molecules of a particular substance.

The substance used to find the void volume and to test homogeneity of packing was a blue high molecular weight dextran polymer marketed by "Pharmacia" called Blue Dextran 2000. It is made from dextran 2000 and has an average molecular weight of 2,000,000 and was readily soluble in the buffer solution used. Because of its high molecular weight it is completely excluded from all Sephadex types.

This Blue Dextran (B.D.) was used for checking the column packing as well as the void volume determination by application of a band of 10mls of buffer solution which was 0.25% with respect to BD. The progress of the band down the column was seen visually

but additionally fractions were collected by a volume fraction collector and located by reading in a colorimeter (E.E.L. type) with a 608 filter or since B.D. has an absorption peak at 260 mu a U.V. spectrophotometer could be used.

In some G-75 desalting columns, sucrose was also added along with the B.D. to give some idea of elution volume of low molecular weight molecular species. Elution was followed by developing an orange colour with a phenol sulphuric acid reagent (113) and reading at 490 mu in the spectrophotometer (Unicam SP 500)

The presence of sucrose with B.D. made application to the column top easier because of the increase in specific gravity or density. Application from a small pipette, was made by careful layering of the more dense solution on the surface of the gel beneath the normal buffer surface. This layer gradually passes into the gel and then the column supernatant buffer can be topped up before the column is connected up to the elution reservoir of pH7.5 (or 7.0) buffer. Flow rate was 70 mls per hour and ten ml. fractions were collected.

From the results shown in fig. 8 it can be seen the peaks were virtually symmetrical, indicating horizontal zone elution and a homogeneous gel bed. The void volume was 50 x 10 = 500 mls, which gives a separation distance of about 900 mls before small molecules like sucrose are eluted.

Procedure and results obtained from G-75 desalting

Procedure - Extraction 3 (see fig. 19 - summary chart)

The use of G-75 for desalting was first applied to extraction 3 which was carried through entirely in the presence of 2M urea. Thus the G-75 column was thoroughly equilibriated with 0.005M Tris/HCI, pH7.5, 10^{-4}M E.D.T.A made 2M with respect to urea.

Aliquots of 5-6 mls of the protein material obtained from stage IV and dissolved in pH7.5 buffer were applied by the layering technique already described for Blue Dextran 2000 application. The column was eluted with the pH7.5, 2M urea buffer at a flow rate of 84 mls per hour. Fractions of 10 mls were collected by a fraction collector and the tubes from the initial column were assayed for RNAse with RNA and the optical density at 280 mu read to locate the protein.

As a matter of routine, after ten tubes had been collected on the fraction collector (100 mls), ten mls. of a solution which was 4 molar with respect to NaCl was layered carefully on to the top of the column and elution continued as before. The purpose of this application was to ensure all protein was removed from the column because it was found that protein can sometimes be adsorbed (110). Since most proteins are removed by elution with 0.1M sodium chloride, all protein will certainly be removed by the elution with 4M sodium chloride.

A total of nine applications and elutions were carried out

for material from Stage IV of extraction 3. It was not necessary to read the optical density at 280 mu every time or even to assay every time. The first column was assayed but as the protein distribution was the same in desalting G-75 columns III, IV, VI, VII and VIII of extraction 3, it was assumed the activity distribution would be the same, all other factors being equal. Column VIII was checked with respect to activity to ensure the distribution had not changed since column I, and this was found to be consistent with the previous results.

Results

Fig. 9 shows the protein distribution during elution of columns I and IV, and also the activity distribution of column I. Thus in G-75 columns I-IX, fractions 71-113 inclusive of all columns were bulked as active and retained for subsequent purification steps. The column void volume was estimated with Blue dextran 2000 and found to be 500 mls (fig. 8)

It was observed that in all of the nine desalting columns of extraction 3, there was a precipitation of protein that occurred in the form of a turbidity starting from fraction 117-125 and continuing for approximately thirty tubes to fraction number 148-150. This turbid region was assayed but no activity was evident. The turbid precipitation was presumed to be protein because of the high ultra violet absorbance at 280 mu that lead up

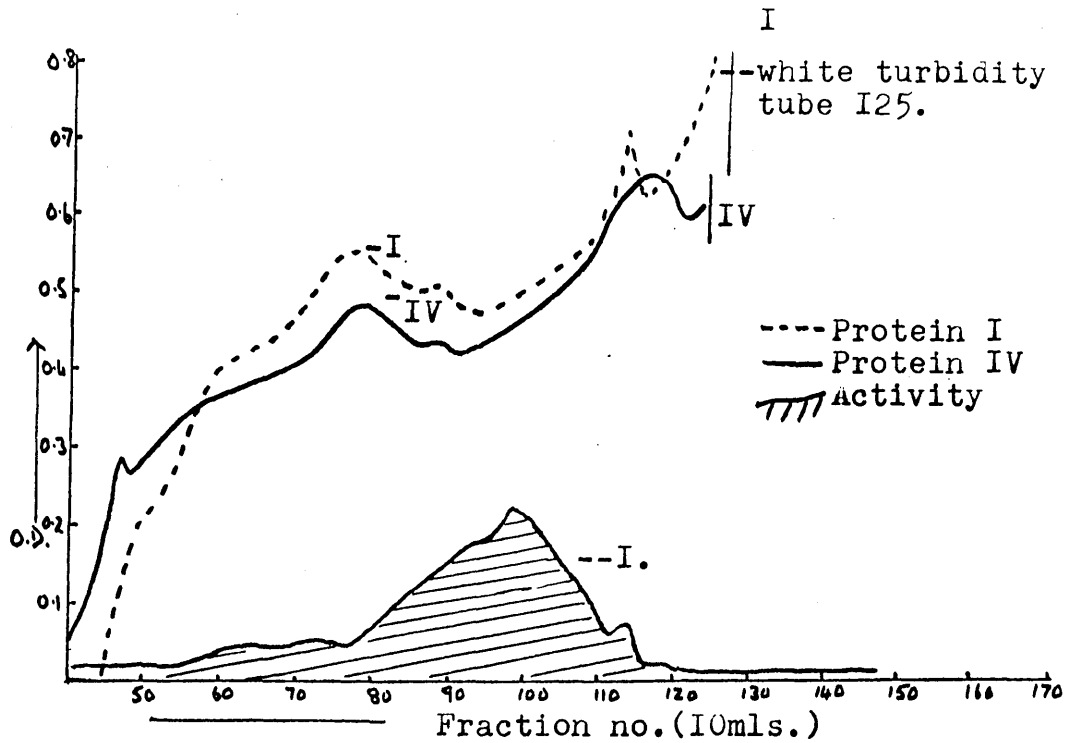


Fig.no.9- G-75,Extraction 3, columns I&IV in 2M urea.

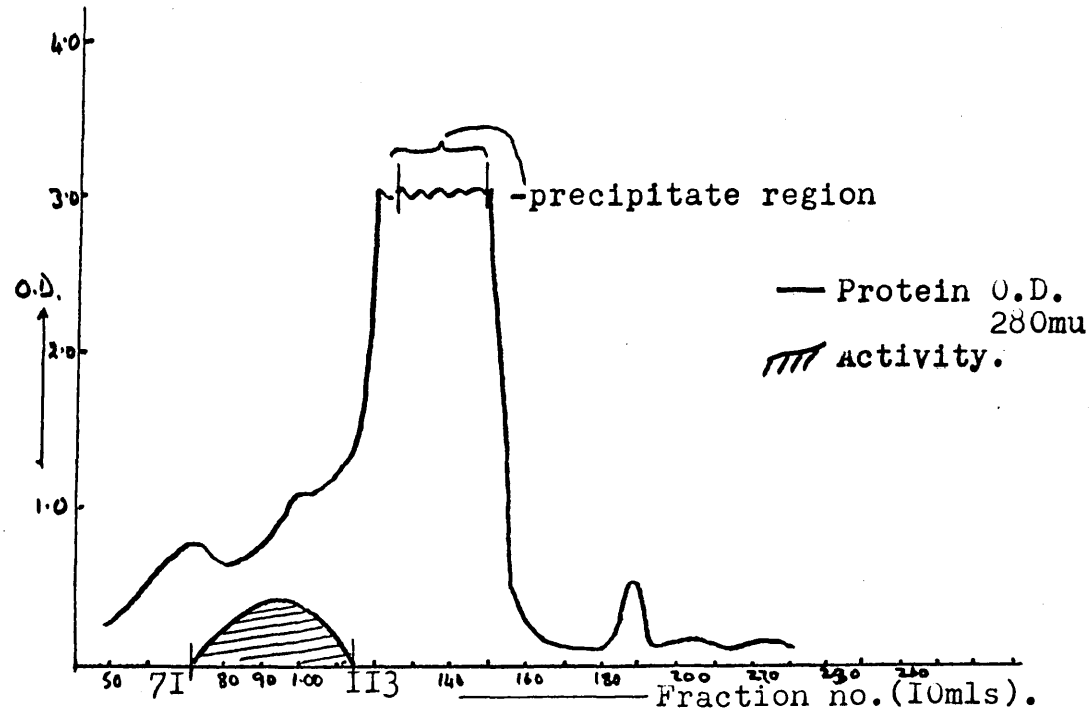


Fig.no.I0.-G-75, Extraction 3, column III

Total range of protein spread in 2M urea.

to the appearance of the turbidity in the eluted material, which of course had an optical density at 280 mu that was so high as to be unrecordable on the spectrophotometer without substantial dilution.

Procedure - Extraction 4 (see fig. 20 - summary chart)

The application and elution procedure used was the same as for desalting in extraction 3, except that this time the path and separation of the ammonium sulphate was monitored using a modified Nessler determination (General Methods 1) to ensure there was a separation that was an efficient desalting process.

Seven columns were run, collecting ten ml. fractions and this was achieved in this extraction by means of an LKB "Radirac" fraction collector. The location of active regions for bulking was initially done by assay, and relation of this activity region to the region in which ammonium sulphate was eluted was established by the Nessler method (65) of ammonium nitrogen determination. In subsequent columns the position of salt elution alone was verified, and the active region was bulked on the basis of this, since columns were consistent with respect to position of eluted constituents.

Results

Fig.I2 is a summary of the fractions bulked from extraction 4 G-75 desalting columns, together with an indication of the method used for confirmation of activity regions.

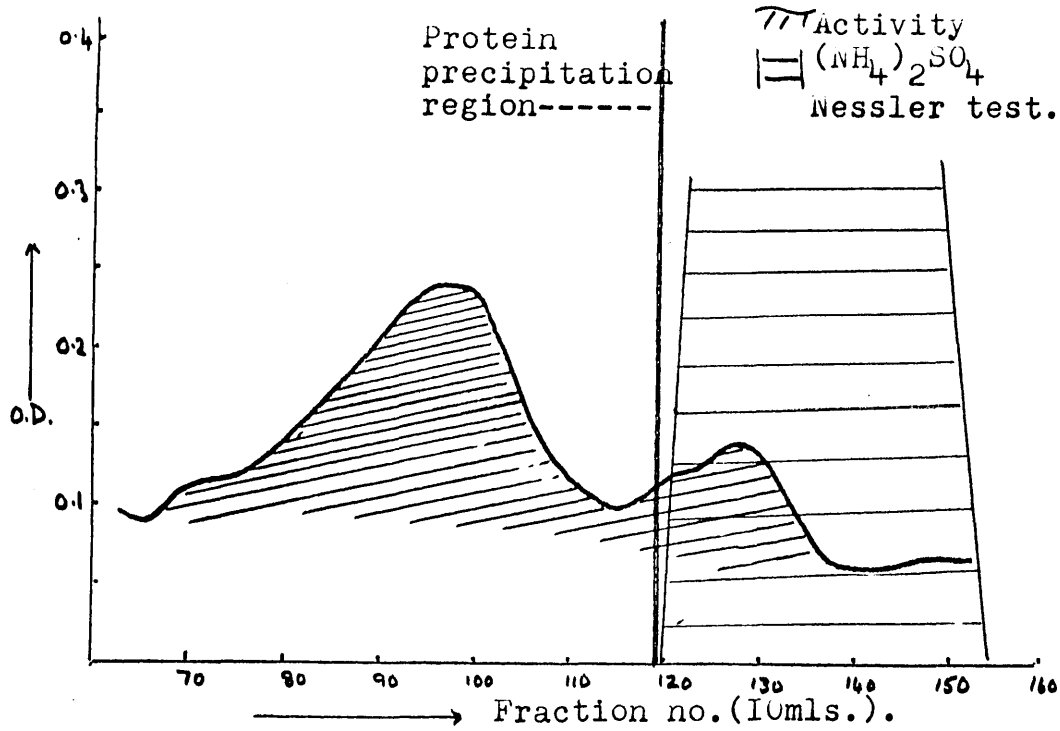


Fig.II.G-75, Extraction 4, column I.

Extraction 4	Fractions bulked	Location
Column no. I	78-118	Assay
II	40-82	Nessler & assay
III	75-117	Nessler & assay
IV	60-114	Nessler.
V	" "	Nessler & assay
VI	" "	Nessler.
VII	" "	Nessler.

Fig.I2.G-75, Columns no.I-VII, bulking and methods of location, Extraction 4.

Column II was unusual in that too much protein solution was applied and 'channeling' of the front occurred with the result that horizontal elution of a band of material was not achieved. The protein solution being applied was fairly concentrated and viscous and the application of sixteen mls. of this was too massive for the column head to retain without 'channeling' occurring.

As in the previous extraction 3, extraction 4 had also been performed entirely from homogenization in the presence of 2M urea, and again turbidity was obtained in : - fractions - 117 onwards. If fig. (11) is studied, it can be seen that the protein precipitation exactly coincides with the region at which the ammonium sulphate is eluted.

Procedure - Extraction 5 (see fig. 21 - summary chart)

Similarly the procedure carried out was the same as for extractions 3 and 4 but with one important exception, and that was that this extraction had not been performed in the presence of 2M urea throughout all stages.

For extraction 5, two columns of G- 75 were prepared afresh, and were thoroughly equilibriated with 0.005M Tris/HCI, pH7.0, 10^{-4}M E.D. T.A. buffer. The columns had the following dimensions:

Column A : 5 x 78 cms of G-75

Column B : 5 x 73 cms of G-75

The void volumes of column A and B were measured using Blue

dextran 2000 and found to be 21 tubes (500 mls) and 19 tubes (450 mls) respectively. The fraction size collected was 23.8 mls or approximately 24 mls. In a similar manner to that previously described, to each column, five mls. of buffer 4M with respect to sodium chloride was applied as a band. The actual point at which this was applied was not important so long as it was not applied at the same time as the protein solution, since it would have no chance of catching up with these faster eluting protein molecules in normal circumstances. Therefore in the table (fig 13) giving the details of all the protein applications, the stage at which the sodium chloride band was added can be seen to vary over a wide range. Care was taken to see the sodium chloride was well eluted from the column before further protein application was made. The elution position of the NaCl was checked with columns I, II and IV using a flame photometer and Na^+ specific filter.

A total of twelve applications of protein from stage IV (see fig. 21) were made and more or less consistently similar results were obtained from each column. Because of the reproduceability of results, it was not necessary to assay for activity in every column, once the constancy of the initial G-75 columns of A and B series had been verified by assay. Enzymically active regions of further column elutions could be collected and bulked on the evidence of previous columns. The methods of

Column	No.	4M NaCl appld.	Bulking	Blue Dextran	prot. applied	O.D. 280mu	assay	Na^+	Nessler
Column A. 5x78 cms. Void vol. =500mls (21 tubes)	I	17	15-70		I	✓	✓	✓	
	II	20	15-70		I	✓	✓	✓	
	III	62	31-86	✓	14	✓ 0.05M NaCl	✓		
	IV	55	15-70		I	✓		✓	
	V	19	"		I				
	VI	49	"		I				
	VII	20	15-60		I	✓	✓		✓
	VIII	20	"		I				
Column B. 5 x71 cms. Void vol= 450mls. (19 tubes)	I	140	35-90	✓	21	2M urea ✓	✓		
	II	72	15-56		I				✓
	III	24	"		I	✓	✓		
	IV	25	"						

(The numbers=to fraction numbers.)

Table of G-75 desalting columns in extraction V.

Fig.13.

monitoring are summarized in the table in fig 13 .

One of the twelve columns runs, column B-I, was equilibriated with 2M urea and the sample of protein applied to this was made 2M with urea before it was layered on to the top of the column. Elution of this column was with the pH7.0 buffer used for all desalting G-75 columns in extraction 5, but was made 2M with respect to urea.

It is to be noted that the pH of the elution buffer in extraction 5 was 7.0 and not the 7.5 of extraction 3 and 4. This change was merely one of convenience and standardization since the extraction step was carried out at pH7.0 and the alteration should have little effect on the behaviour of protein material on desalting.

A small test was also carried out to check the concentration of Na^+ ions in 2M urea solutions relative to deionized water and standard buffer at pH7.0, since the presence of urea may exert an ionic effect during elution due to Na^+ ions as an impurity in the commercial urea. A flame photometer with a sodium specific filter was used to estimate the Na^+ ions (if any) but the estimation was only relative and not an absolute reading since no standard graph was drawn.

Results:

	Scale reading
Water - deionized. Used for zero setting on the flame photometer - control	0.0
Buffer (0.005M Tris/HCI, pH7.0,10-^{4}M EDTA)	10.5
Buffer - as above but 2M w.r.t. UREA	6. 5

Estimation of relative Na^{+}content of G-75 eluting buffer

It can be deduced from this test that the ionic effect due to the presence of urea was negligible with respect to Na^{+} ions and Na^{+} content in the buffer with, and without urea at 2M concentration, was shown to be approximately the same.

After the one desalting in the presence of 2M urea had been carried out, the column was eluted free of urea by the passage of copious quantities of buffer. Similarly, as a matter of routine, after every column run, the columns were washed with buffer at the maximum flow rate for a minimum period of twenty-four hours, which approximately represents 1.5 litres of wash. All column heights with respect to eluting reservoirs etc. are described in the Experimental section.

Results

Fig (14) shows an elution pattern of column A-II which is typical of any A or B desalting column. Blue dextran peaks on A and B series of columns show the packing to be even and homogeneous.

One of the most important aspects of this series of desalting columns for extraction 5 (which was NOT carried out in the presence of 2M urea, see summary chart - fig 21) was that the activity was eluted in two peaks (fig 14) The second peak of the two was eluted more or less where one would expect the protein to be eluted in accordance with size, but the elution of the initial peak approximately coincides with the void volume. If fig 14 of G-75 column A-II is examined, the first activity peak has a maximum at about tube 21, which exactly corresponds with the void volume defined by the use of Blue Dextran 2000. Also this corresponds to a turbidity or precipitation in these initial tubes of high protein content. For example in column A-II fraction 21-26 showed turbidity, and similarly in column B-I (fig 15) the initial large protein peaks corresponding to elution in the void volume shows turbidity over a range of six or so tubes. This turbidity in the protein eluted in the area corresponding to the void volume was common to all the Extraction 5 desalting columns, including B-I which was 2M with respect to urea.

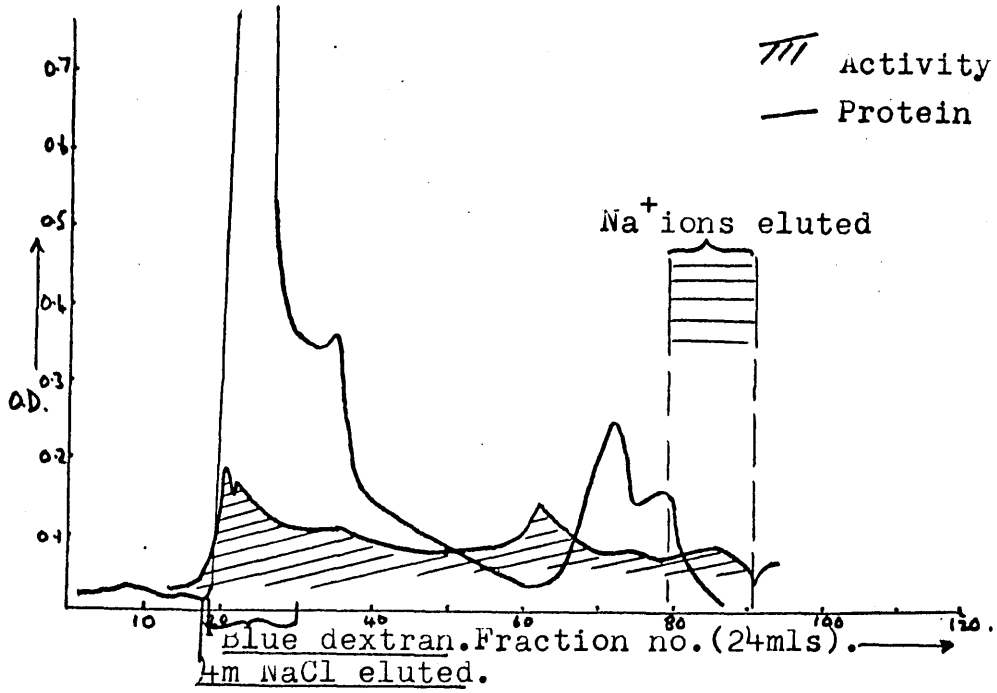


Fig.I4. G-75, extraction V,Column A-II.

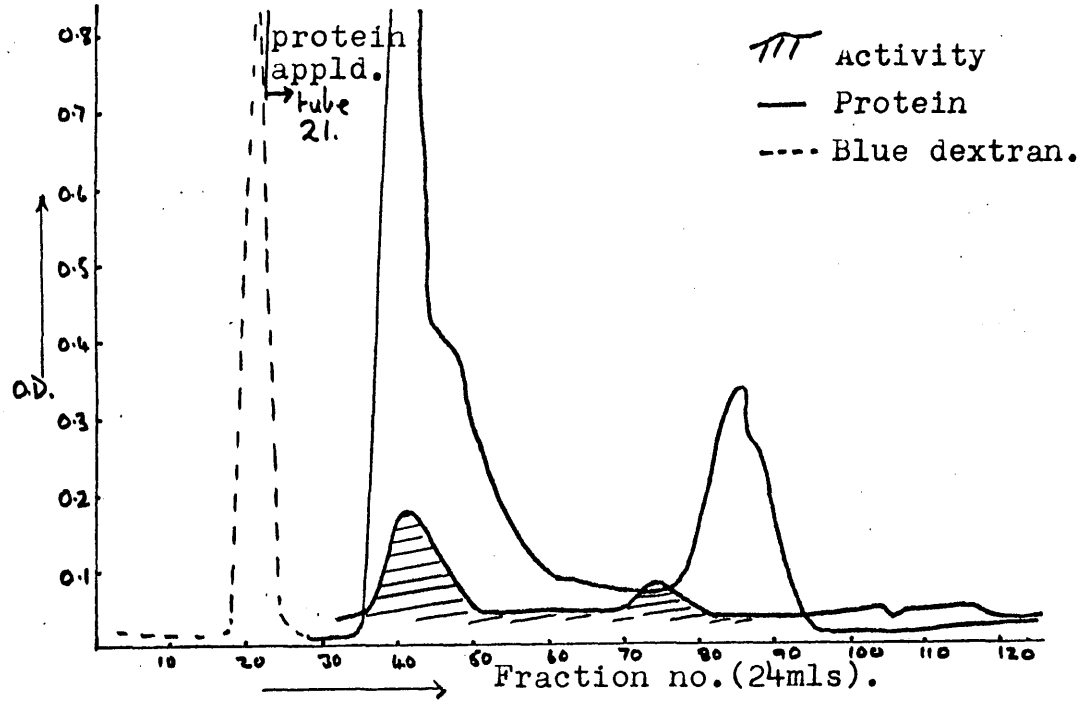


Fig.I5. G-75,extraction V,Column B-I.

Possible explanation of results of G-75 desalting column anomalies from extractions 3, 4 and 5.

Extractions 3 and 4 were conducted throughout from the raw spleen stage, through initial ammonium sulphate precipitations and heat treating stage in the presence of 2M urea unlike extraction 5, and up until the desalting stage this factor of urea presence or absence seems the outstanding cardinal difference between the extractions. (See extraction summary tables, figs 19, 20 and 21 for comparison)

One might expect the G-75 desalting elution patterns to be much the same for all extractions, but they are very dissimilar in two respects.

Firstly, activity in extractions 3 and 4 was eluted in the region where one might expect to find it with respect to molecular size; whereas in extraction 5 the larger part of the activity was eluted in the void volume and further activity in a more anticipated elution position.

The second difference is at which point protein precipitation occurs, since precipitation was common to all desalting procedures conducted. In G-75 column runs of extractions 3 and 4, protein appears in a precipitated form in later fractions after about 1.2 litres of eluted material, which is in a column region that would indicate a very small molecular weight, but this is obviously not the case.

A tentative explanation can be given for these facts. It was reported in extraction I (see page 40) that there was some precipitation of protein inside the Visking sac on dialysis (stage V, extraction 1) This is a common occurrence on removal of salt from proteins and is the reverse effect of 'salting in' since certain proteins are often precipitated or even denatured in the absence of any salt or a lower salt concentration. Edmond (37) also found that precipitation occured during dialysis and perhaps part of the loss of enzyme activity during this stage can be attributed to RNAse precipitation, and this precipitation was centrifuged down and discarded. However the possible precipitation of RNAse may not be as simple as that, and a more complex precipitation in the form of a co-precipitation with another protein could be envisaged with the RNAse physically occluded and/or chemically bound to other protein. Therefore besides loss of enzyme through the membrane, loss in dialysis could possibly result from this internal precipitation on reduction of salt concentration.

Thus the protein precipitation in dextran gel desalting could be explained correspondingly; that is, as the protein solution moves down the column there is a separation of the smaller salt particles from the protein due to the sieving effect, thus causing the protein to precipitate.

The question of the difference in position of precipitation exhibited is a little more complex to explain. The reason for

this difference seems more than likely to be associated with the outstanding difference in preparation of the enzyme up to this stage, namely an effect produced because of the presence of urea.

In extraction 5, in which no urea was used, it is suggested that as the protein solution travels down the column, desalting occurs and the salt concentration falls sufficiently for some protein to come out of solution, and in so doing aggregation to larger units occurs resulting in rapid elution with the void volume due to size increase. The precipitation seems to cause elution of a portion of the active protein also, since this was also in part eluted with the void volume. The elution of the active RNAse may, as suggested in the antepenultimate paragraph, be via some sort of protein-protein interaction or physical occlusion due to large amounts of the precipitated protein.

In extraction 3 and 4, in which urea was used throughout the extraction process, the protein precipitate was not eluted until a very late stage and appeared to have been retained by the column since one would expect elution of precipitated protein to occur in the void volume fraction as above. The explanation suggested is that the concentration of protein in solution from an extraction in the presence of 2M urea is higher due to the solubilizing effects of urea. Thus the protein solution obtained from stage IV for stage V dialysis may contain more protein other than RNAse, and when applied to Sephadex G-75 (also 2M with

respect to urea), there is separation of protein from salt, and the resulting precipitation is large enough physically to retard elution. Protein retention by the gel may also be a factor. The active RNAse, however, does not seem to precipitate from solution and is eluted in a predictable manner, whilst the precipitated material makes slow progress to be eventually eluted at a point which is normally associated with small salt molecules. Because of this elution position it was thought that the final elution of the precipitated material was due to the salt front finally catching up with the protein and causing its elution. Fig 11 of extraction 4 column I shows the position of elution of the precipitated protein.

One may ask why, if the salt and precipitated protein are eventually eluted together, and if it is assumed protein precipitates because of salt separation, does the protein not go back into solution again in the presence of salt? Edmond (37) found that the protein precipitated during dialysis was not soluble again when the salt concentration of the solution was raised to its pre-dialysis level, indicating an irreversible protein precipitation probably attributable to changes in structure on salt withdrawal resulting in denaturation. Probably, therefore, for the same reasons, that even when the protein was eluted in the presence of salt again, it remains in a precipitated state.

This leaves unexplained the fact why extraction 5 column B-I

(fig 15) which was made 2M with respect to urea just for the one protein application, does not show the same behaviour pattern as G-75 columns for extraction 3 and 4. From the table in fig (13) the column B-I shows behaviour in agreement with all other extraction 5 desalting columns. The protein material applied to this column was the same as all the other eleven columns of extraction 5 and was not the result of extraction and processing in the presence of urea, but only made 2M with urea prior to application to column B-I. Since results show no differences in elution pattern from other extraction 5 G-75 columns, it must be assumed that it is not merely the presence of 2M urea whilst desalting on G-75 that causes the results obtained in extractions 3 and 4 to be so different from extraction 5, but the differences in previous stages of extraction. The presence of urea throughout extraction is suggested as the main difference from which result divergences of G-75 desalting behaviour stem.

It will be noted from the table in fig (13) that column A–III extraction 5 was equilibriated with pH7.0 buffer made 0.05M with respect to NaCl and also this buffer was used in the elution of this particular column. It was thought that the presence of this concentration of sodium chloride salt would enable the protein to remain in solution. In fact the presence of this concentration of salt made no appreciable or discernible difference to either activity elution patterns or precipitated protein elution.

The preceding explanation of the experimental results obtained during desalting operations from extractions 3, 4 and 5 remain only speculative suggestions on the basis of the facts available.

V

Introduction to the use of DEAE-cellulose in RNAse purification procedure

General

Maver and Greco (33) in their 1959 publication described the purification and characterization of RNAses from calf spleen using D.E.A.E.-cellulose chromatography in the separation of enzyme at pH8.0 and 0.005M sodium acetate buffer. At this pH an RNAse active fraction which they referred to as A, was not retained by the DEAE-cellulose and passed straight through. The other fraction, B, was held and by gradient elution separated out to four activities which were not heat stable to treatment at 80°C for ten minutes. But the material not held by the D.E.A.E.-cellulose at pH8.0 was the alkaline or basic RNAse and this fraction was heat stable to the described treatment. Thus the prospect of an effective chromatographic separation of the heat stable basic ribonuclease from calf spleen on the anionic cellulosic ion exchanger D.E.A.E. becomes apparent.

Occasionally when a protein of interest is either more acid

or more basic than the contaminating proteins, a rapid separation from the latter can be achieved by using an ion exchanger bearing charges of the same sign as those predominating on the enzyme to be isolated. When the enzyme is applied to a column of the appropriate ion exchanger it can emerge in a highly purified form although it was never absorbed.

This method was used as a technique of separation of acid RNAse by Maver and Greco in a 1962 report (36) on the chromatographic separation of acid and alkaline RNAses of bovine spleen and liver. The technique was similarly used by Edmond (37). A series of D.E.A.E. cellulose columns were also used by Rushizky and Sober (114) in the purification of RNAse T_1 from Taka-diastase powder. Subsequent reports of DEAE-cellulose use in protein purification procedures include the report by Smellie et al. (115) where D.E.A.E. was used as a purification step in the preparation of a rat liver nuclease in which there was protein with RNAse activity adsorbed by the ion exchange medium at pH8.0.

There are recent reports (107, 91) that the introduction of a D.E.A.E.-cellulose step may be particularly important in connection with the removal of ribonuclease inhibitors, which other than being protein contaminants, actually reduce or inhibit RNAse activity. An alkaline ribonuclease inhibitor from liver was reported (107) to have a relatively strong affinity for the basic ion exchanger D.E.A.E.-cellulose.

There was strong evidence that this inhibitor was a protein but with a possible carbohydrate nature also. The role and distribution of ribonuclease inhibitors is still currently the subject of much research. Thus if the basic RNAse from calf spleen is associated with any kind of inhibiting protein of the above described nature, the use of a D.E.A.E.-cellulose purifying step is further warranted.

Procedure and Results (See General Methods 3b)

The D.E.A.E.-cellulose powder (Whatman DE50 batch 727-735) was subjected to the washing and preparative procedures prior to filling a column, described in refs. 34 and 74 by Peterson and Sober, who did much of the early work on the use of cellulosic ion exchangers.

The protein peaks from G-75 desalting were applied and all the effluent collected (together with a small volume of washings) since the active and wanted basic ribonucleases should not be adsorbed and held by this anion exchange material.

Fig. 16 summarizes the use of D.E.A.E.-cellulose with respect to extractions 3, 4 and five. The protein concentrations were calculated by the U.V. spectrophotometric method previously described. From an estimation of the protein concentration applied to the column, and also of the concentration of protein in the effluent, the percentage of protein retained and the percentage passing straight through without absorption were calculated. A

Extraction number.	Column size.	Total protein applied.	(%protein) %not held & thus unwanted.	held & thus wanted.	Conditions & buffer.
3. a).	I.8xI3 cms	322mgs.	59%	4I%	pH 7.0 2M urea
b).		737mgs.	6I%	40%	
4.	2x8.5 cms	I795mgs.	29%	7I%	pH 7.0 2M urea
5.BatchI	2.2xI8 cms	768mgs.	64%	36%	pH 7.0
Batch 5. A.	2.4xI9 cms	I854mgs.	77%	23%	pH 7.0
B .					
5. 2M urea	2.2xI2 cms	223mgs.	5I%	49%	pH 7.0 2M urea

Fig.I6. Table of protein distribution on DEAE-cellulose chromatography.

sample of the protein retained by each column after application of protein from G-75 desalting, was assayed for RNAse activity and found to be inactive. This protein for assay was removed by elution of the D.E.A.E.-cellulose with buffer made 2M with respect to sodium chloride.

With the exception of extraction 4, the amount of protein removed was in all cases over 50% of that applied, and since this portion was inactive, it can be said that D.E.A.E.-cellulose produces a purification of at least 50% and seems to be an exceedingly useful and efficient purification step.

As mentioned, extraction 4 results seem to be anomalous in that they indicate that less than a third of the protein was removed by the purification step. This abnormal result may be due to the fact that the method of estimation of protein retention was different to all others. Instead of retention being estimated from a knowledge of protein applied and protein in the effluent, it was estimated from protein applied and the actual protein retained and measured after its elution from DEAE-cellulose by means of 2M sodium chloride buffer. The general procedure of extraction 4 does not differ in any way that would result in this difference in results than from extraction 3. The material retained by the anion exchanger and removed with sodium chloride elution may not entirely equal protein applied minus protein in effluent of unabsorbed material, but estimation in extraction 5 has

shown there to be approximate correlation of results and so the anomaly of extraction 4 can not be explained by tight column retention of protein against sodium chloride eluting power. A pertinent factor however is the inherent error in U.V. spectrophotometric estimation of protein concentration. Different proteins vary in their degree of absorbance of U.V. light, and this difference will be accentuated on such a separation of proteins that has been accomplished by the use of D.E.A.E.-cellulose.

There seems to be little statistically relevant difference in the application of this purification step in the presence of 2M urea from when no urea was used. Extractions 3 and 4 were carried out completely in the presence of urea and if extraction 3 results are compared with extraction 5, much the same separation was achieved.

The extraction 5 material that was desalted on a Sephadex G-75 column made 2M with urea, was also subjected to DEAE-cellulose treatment in the presence of 2M urea. As can be seen from fig 16 an approximate 50% purification was achieved which is a little lower than the other results, but whether this is significant is doubtful.

Summary

The use of D.E.A.E-cellulose was an effective purification step since the protein removed was inactive. However disc-gel electrophoretic (see later) analysis of the protein retained by the anion exchanger, did show some evidence of bands of protein showing

RNAse protein characteristics. These could possibly belong to acid RNAse that survived heat treatment since it is unlikely that the basic RNAse being isolated would be held by an anionic column at pH7.0. The position of elution on CM-cellulose cationic exchanger indicates considerable basicity and therefore unlikelihood of absorption to an anionic cellulose.

Summary of extraction techniques considered so far.

Thus concludes a consideration of the changes made in extraction procedures (sections I to V) begun after the detailed description of the initial fractionation procedure I on page 62 .

Part I of the thesis will be terminated by a consideration of the overall effects of the changes and improvements made in each extraction, as reflected in CM-cellulose chromatographic results since by this stage the enzyme was in a relatively pure form.

Figs 17, 18, 19, 20, 21 summarize in outline the changes discussed in detail in sections I-V above for each of the five extractions made between October 1964 and March 1967.

A comparison of extraction procedures 1-5 as reflected in the results of CM-cellulose chromatography

General considerations

The theory of column chromatographic separation of proteins on cellulosic ion exchangers has already been discussed to some extent

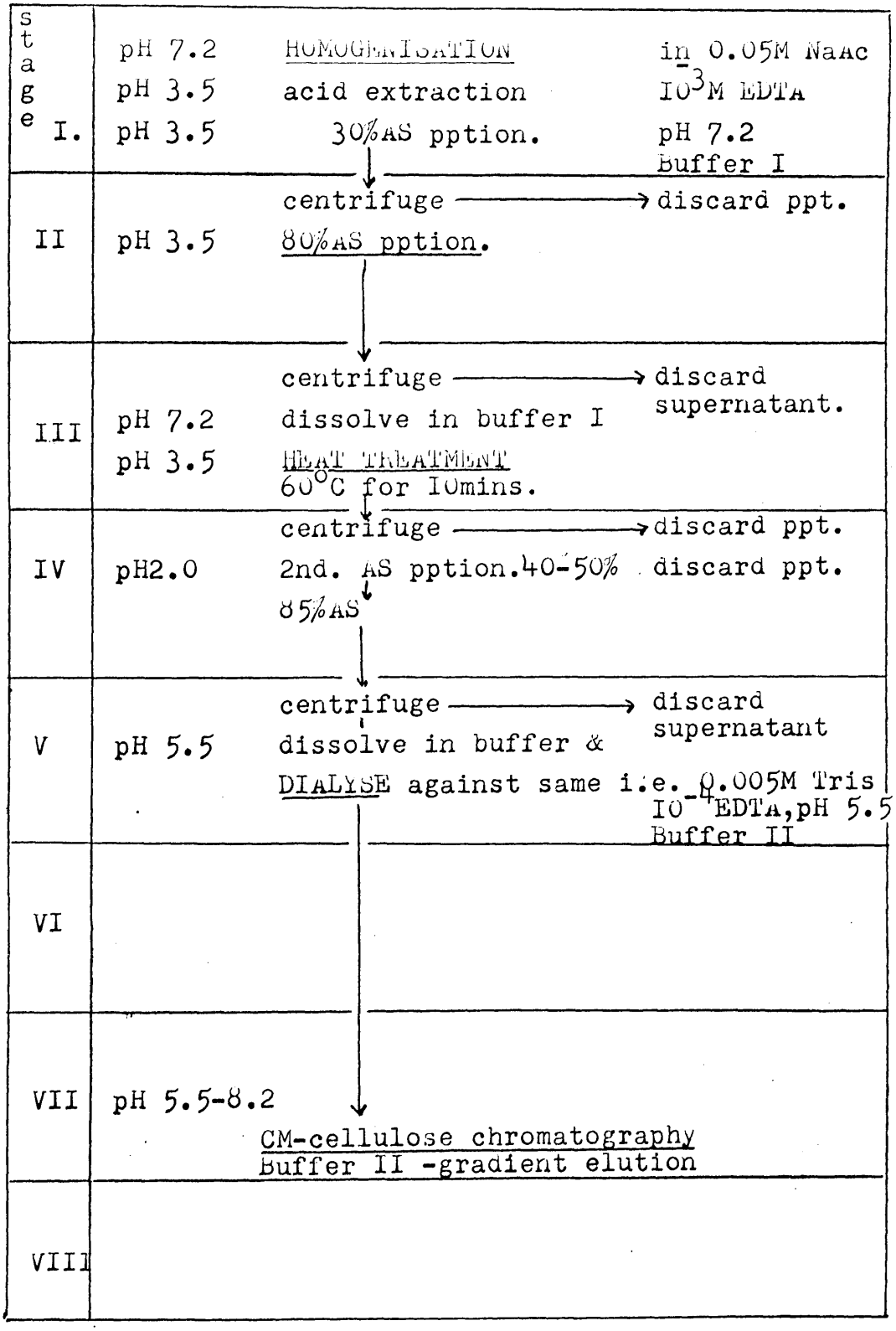


Fig.I7. Summary chart of Extraction procedure I.

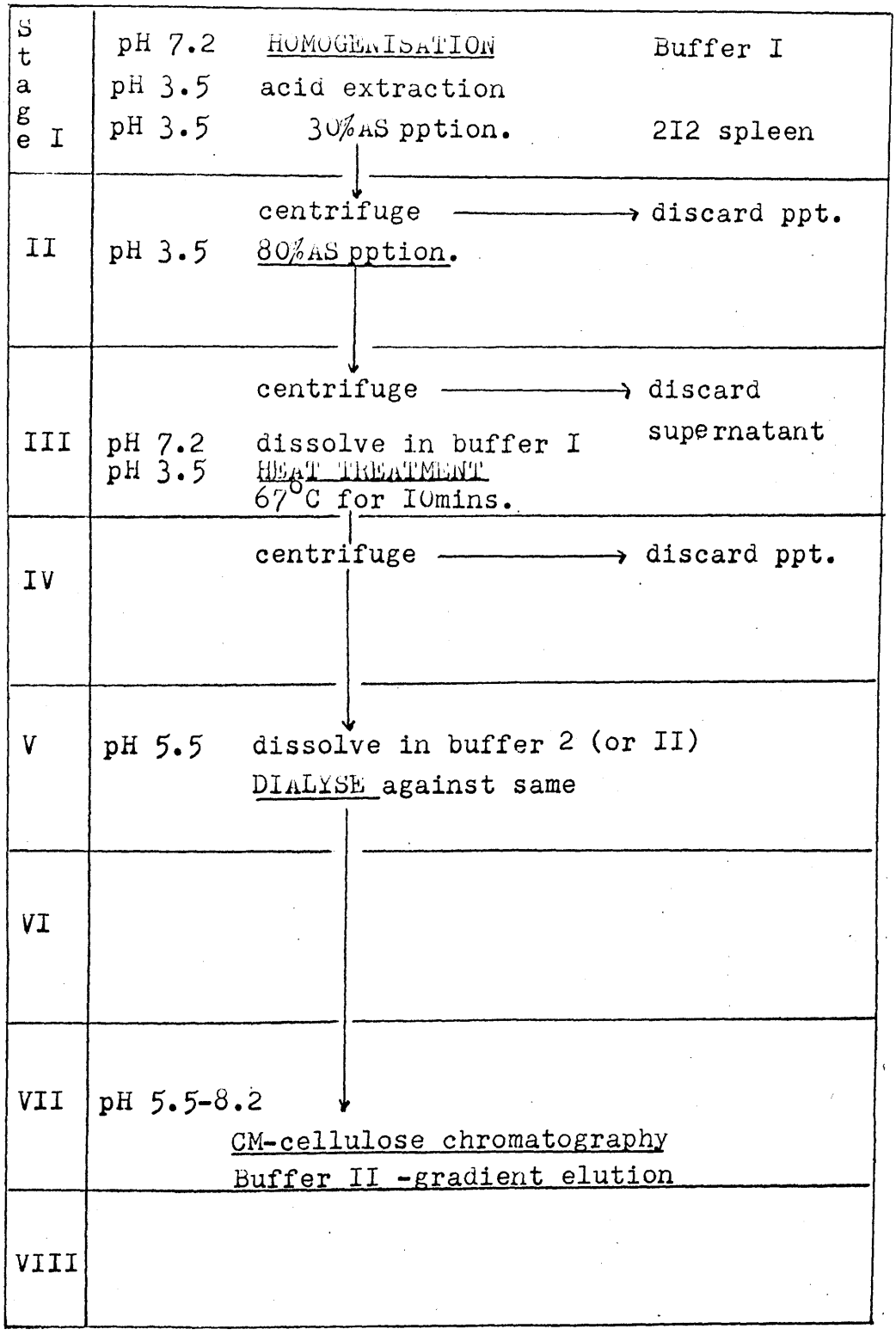


Fig.18. Summary chart of Extraction procedure II.(or 2)

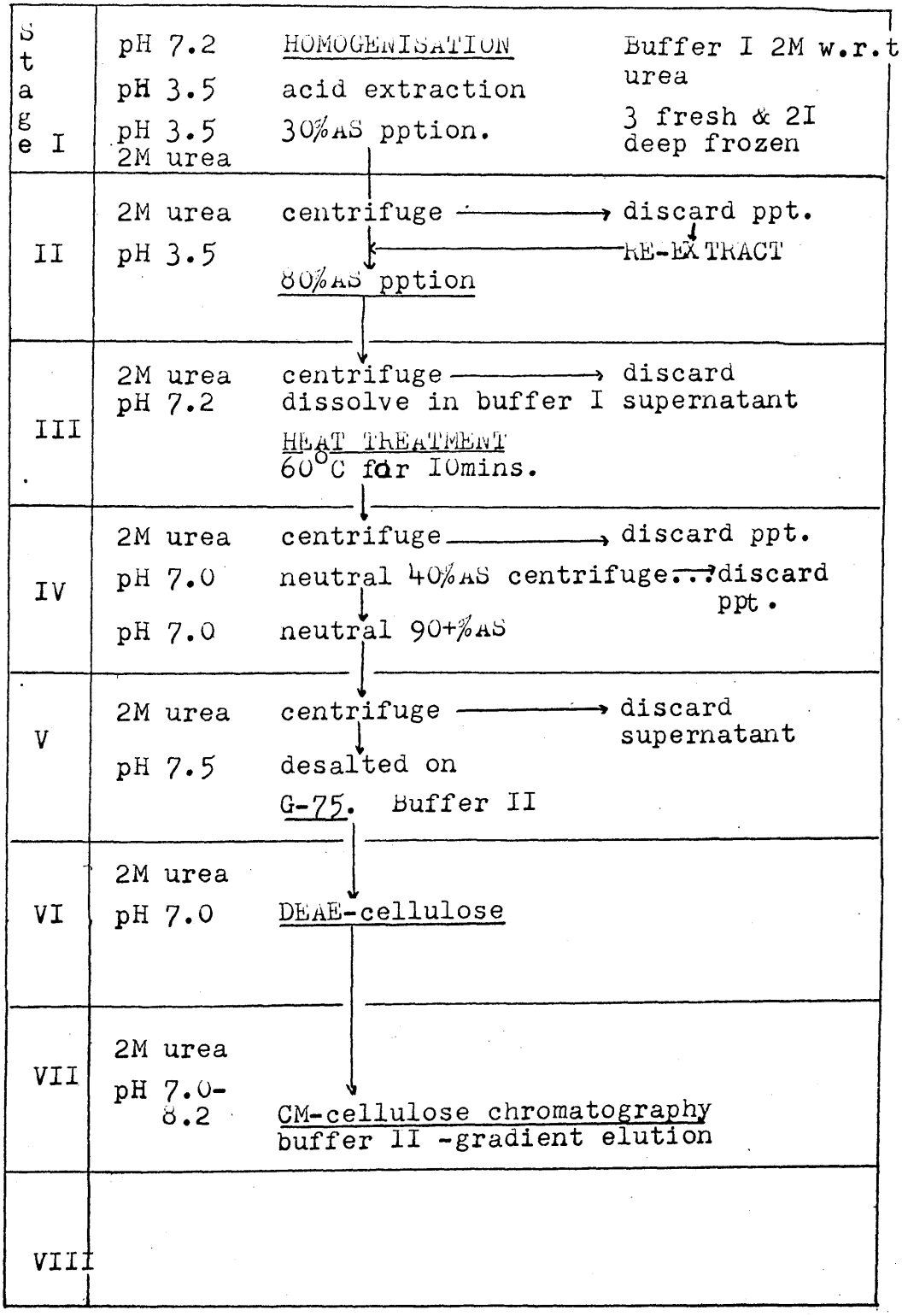


Fig.I9. Summary of Extraction procedure 3.

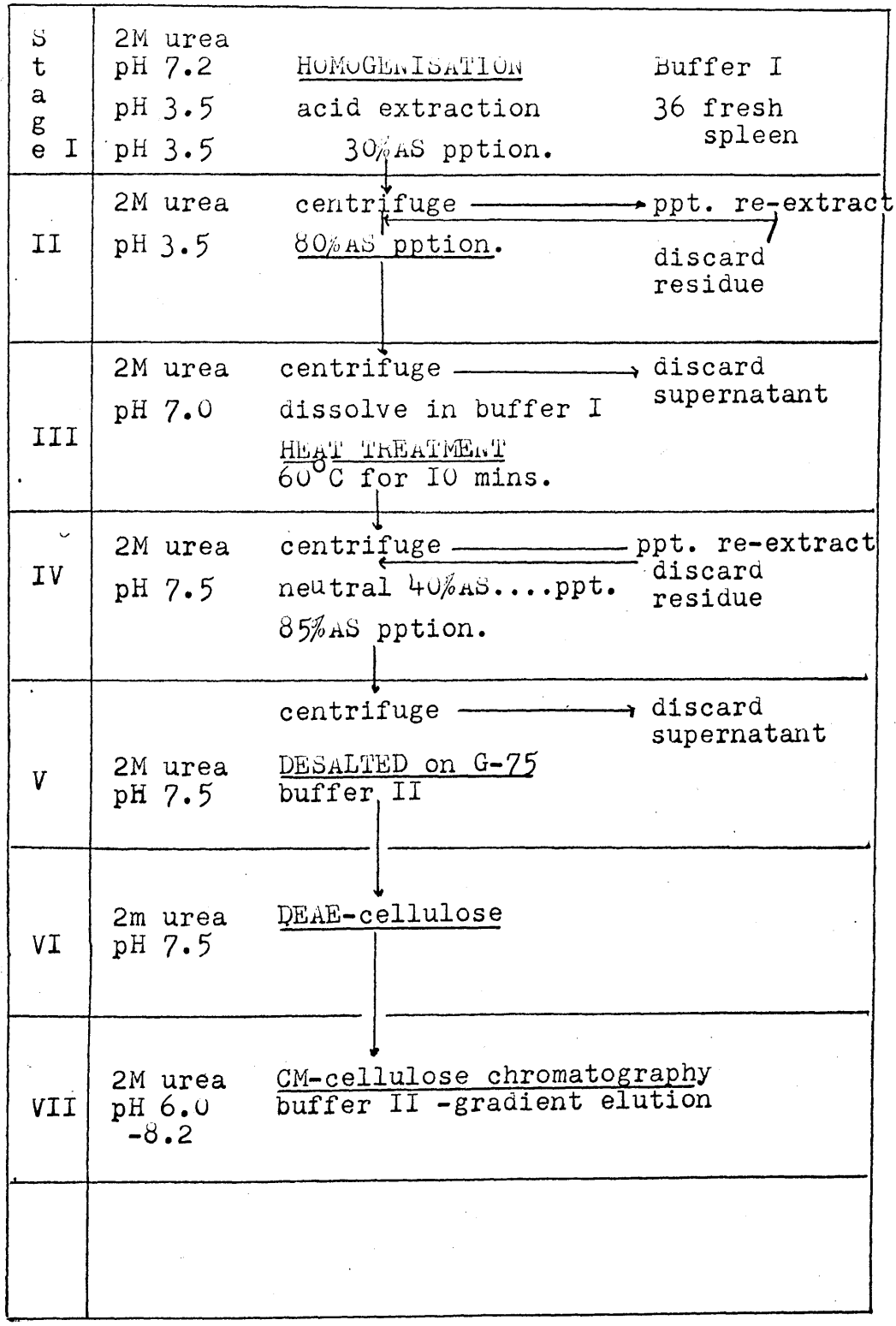


Fig.20. Summary chart of Extraction procedure 4.

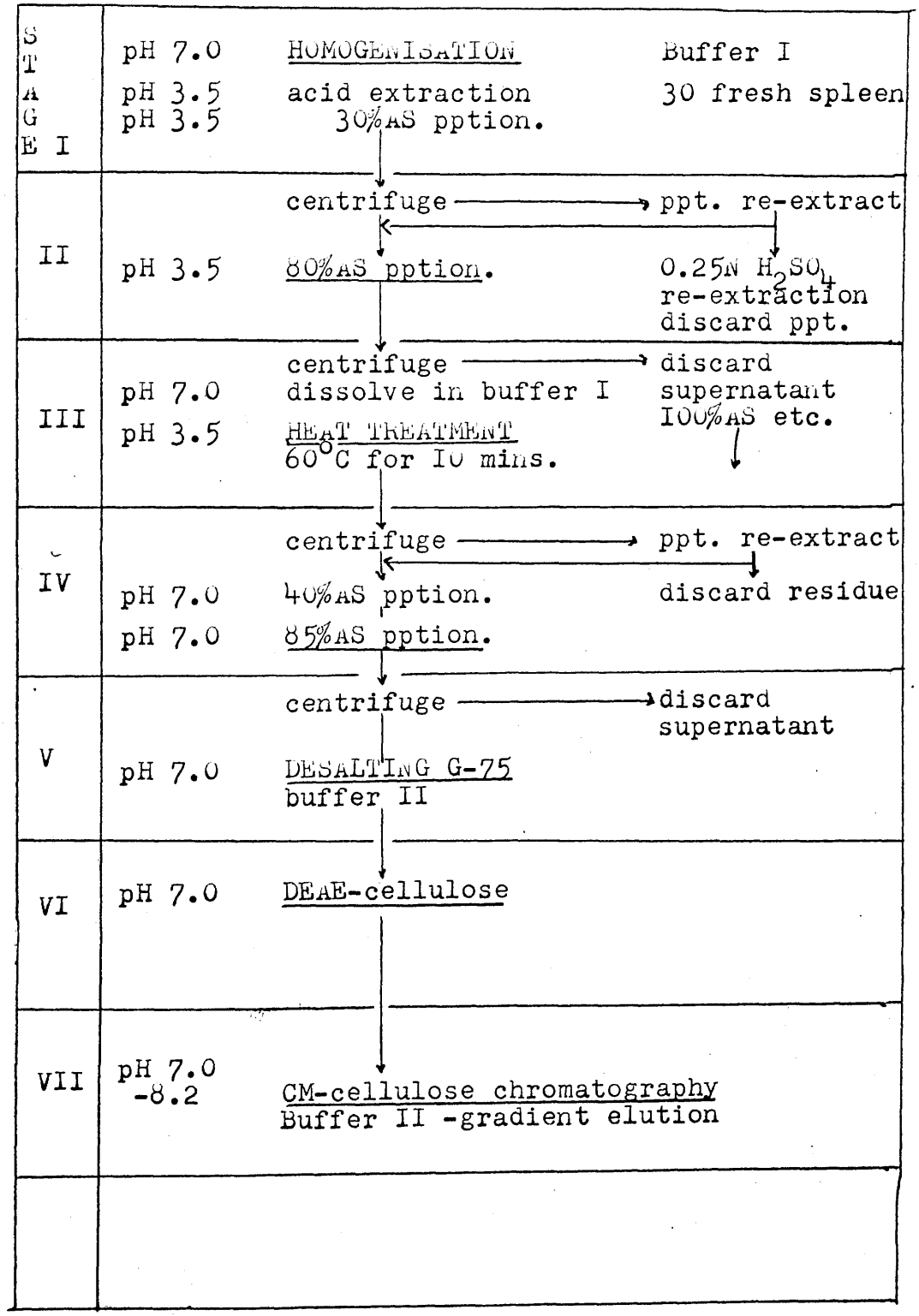


Fig.2I. Summary chart of extraction procedure 5.

(page 41). The degree of binding of the protein to the absorbent and by what type of bonds, and to what extent the elution sequence is predictable, is not easily answerable because of the very complex nature of proteins themselves.

The binding of protein to the ion exchanger involves the formation of electrostatic bonds between protein and ion exchanger, and since the protein and ion exchanger are polyelectrolytes, there is the likelihood of interaction at several points. The number of such bonds that are established will determine the concentration of the competing ions required for the release of the bound molecules. Thus the theory is that a separation may be achieved by virtue of the fact that proteins differing in charge density, or number of charges by virtue of size may be expected to differ in their requirements for elution.

It can therefore be seen that two quite different proteins may be eluted together because they have the same net charge. For example one protein may be small with a high charge density, whereas the other may be large with a small charge density. Similarly if a protein is subject to dissociation into sub-units, and if the sub-units are assumed to have the same charge density, the aggregates of larger molecular weight and therefore higher net charge may be eluted last in a gradient elution since these are likely to form more bonds with the ion exchanger and so there will be a separation on the basis of size in this case.

Other factors such as the spatial arrangement of ionizable groups on the molecule can be expected to contribute. This is especially important in conditions that are likely to alter the molecular configuration. Any change in configuration such as unfolding of the peptide chains due to the severence of S-S linkages, or the severence of intramolecular hydrogen bonding perhaps aided by urea, will result in a change in the arrangement of ionizable groups and the exposure of new ionizable groups, or the reverse situation of screening already available groups. Any of these changes will affect the degree of adsorption to the cellulosic ion exchanger and therefore to some extent, dictate the position of elution from the column on the application of a salt and/or pH gradient. Elution by raising the pH alters the number or sign of charges on the protein (or adsorbent), and, elution by increasing the salt concentration decreases the effectiveness of existing electrostatic bonds between the protein and adsorbent.

More recently the non-ionic binding forces of proteins have been considered and it is thought that they may contribute to binding even on ion-exchange surfaces. But to what extent, is, as yet, not fully explored. In part II of this thesis the lyophilic nature of the RNAse was investigated to some extent by CM-cellulose chromatography in the presence of agents that possess lyophilic as well as hydrophilic properties.

The protein profile obtained on elution and read by U. V.

spectrophotometric methods alone is not enough, since the absorption at 280 mu is a summation of the individual contributions of what may be a large number of protein species, displaced from one another significantly but often overlapping in sufficient degree to prevent discrimination on the basis of protein alone. Thus for full realization of the resolution attained, enzymic assay was carried out. This was of particular importance with respect to the RNAse being examined since a portion had very little U. V. absorbance at 280 mu. (page 58)

Protein molecules are complex and abstraction of any conclusive decisions from cellulosic ion exchange separation behaviour will be equally complex; and every consideration has to be given to conditions of protein configuration change and its bearing on adsorptive properties.

Procedure and comparison of results

The application of samples to CM-cellulose cation exchange columns was described in Extraction I (page 46) All buffers were made 0.005M with respect to Tris/HCI and 10^{-4}M with respect to E.D.T.A. and the pH of each particular eluent can be found in the table (fig 22)

a) The last three extractions (Nos. 3, 4 and 5) differ from the first two in two major respects and that is the introduction of desalting using Sephadex G-75 and the use of DEAE-cellulose as a further purification step. Sephadex also acts as a major

Extraction No.	I	2	3	4	5	5	5	5
Column No.	CMC I	CMC 2	CMC 3	CMC 4	CMC 5-II	CMC 5-I	CMC 5-III	CMC 5-IV
Gradient: starting pH	5.5	5.5	7.0	6.0	7.0	6.0	6.0	8.2
Gradient final pH	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2
Final salt concentration	0.32 M	0.32 M	0.32 M	0.25 M	0.25 M	0.32 M	0.32 M	0.32 M
Urea concentration	-	-	-	2M urea	-	-	2M urea	-
Column size	64x I.7	5Ix I.7		68x I.7	49x 2.5	46x 2.5	20x 2.2	I8x 2.2
Fraction size	50 mls	50 mls	5.0 mls	I0 mls	I0 mls	Io mls	I0 mls	I0 mls
Bulking A	90-I05	60-80	70-I00	44-54	66-I03	60-95	40-90	20-56
Bulking B	I07-II5	80-II0	?	68-82	I04-I56	II0-I40	?	-

Comments:

CMC 3. NO urea with CM-cellulose column, but present in rest of preparation.

CMC 4. New elution volumes for subsequent CMC 4&5 Columns

CMC 5-III Gradient steeper.

CMC 5-IV Re-application of CMC 5-I, peak 60-95 at pH 8.2

Size of reservoir	5+51.	5+51.	I1.	I.51	I.51	I.51.	I.51	I.51
Size of const vol. chamber	3.751	3.751	0.51	0.81.	0.81.	0.81.	0.81	0.81.

Fig.22. Table of elution data of the principal CM-cellulose chromatographic separations

purification step because of the non-active protein that was separated from RNAse activity during the desalting process. Thus as a result of the introduction of these two additional purification stages, the total protein applied to CM-cellulose in stage VII will be considerably reduced. In a likewise manner the elution volumes were reduced to the more manageable proportions as shown in fig 23 and unless otherwise stated these volumes are adhered to in all CM-cellulose elutions in extractions 4 and 5. This change in elution volume was of course reflected in a change of elution position of active peaks (after the difference in size of the fractions collected is accounted for - see fig 22). CMC columns of extractions 3, 4 and 5 did not have the characteristic initial non-active protein peaks of CMC-I and CMC-2 and of those similarly reported by Edmond (37). The reason for their absence is more than likely their removal either on G-75 or else by adsorption on the anionic DEAE-cellulose.

b) If the CMCellulose separations of extractions 1 and 2 are compared it will be seen that the overall elution profile with respect to activity is virtually the same (figs 24, 25). Columns CMC-1 and-2 show the same initial inactive protein peaks eluted in the early stages and two main peaks of activity (designated A and B) eluted in corresponding places in extractions 1 and 2. However the protein profile of extraction 2 (CMC-2) differs from extraction 1 (CMC-I) by the possession of a large protein peak 'x' appearing after

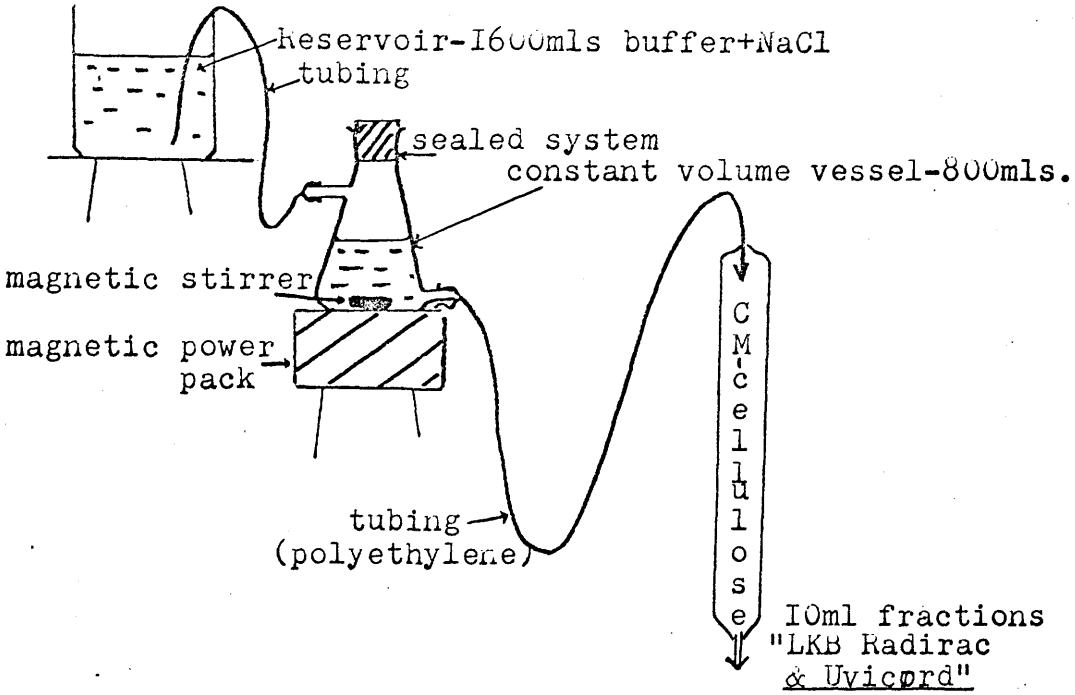


Fig.23. Apparatus & elution volumes used in columns CMC 4&5 series.

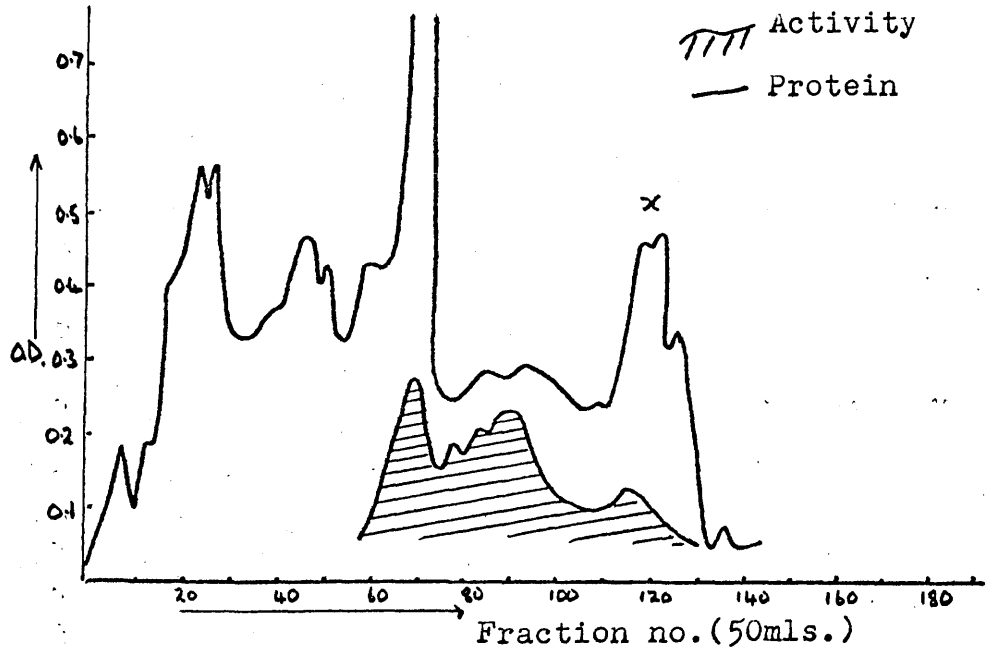


Fig.25. CMC2.

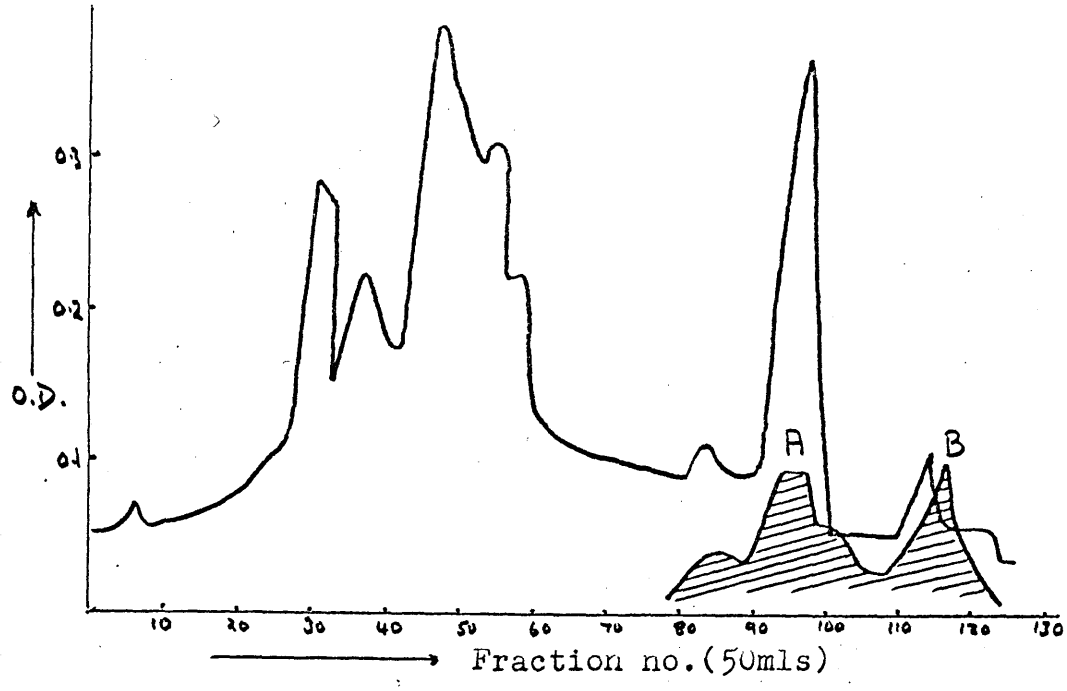


Fig.24. CMC-I

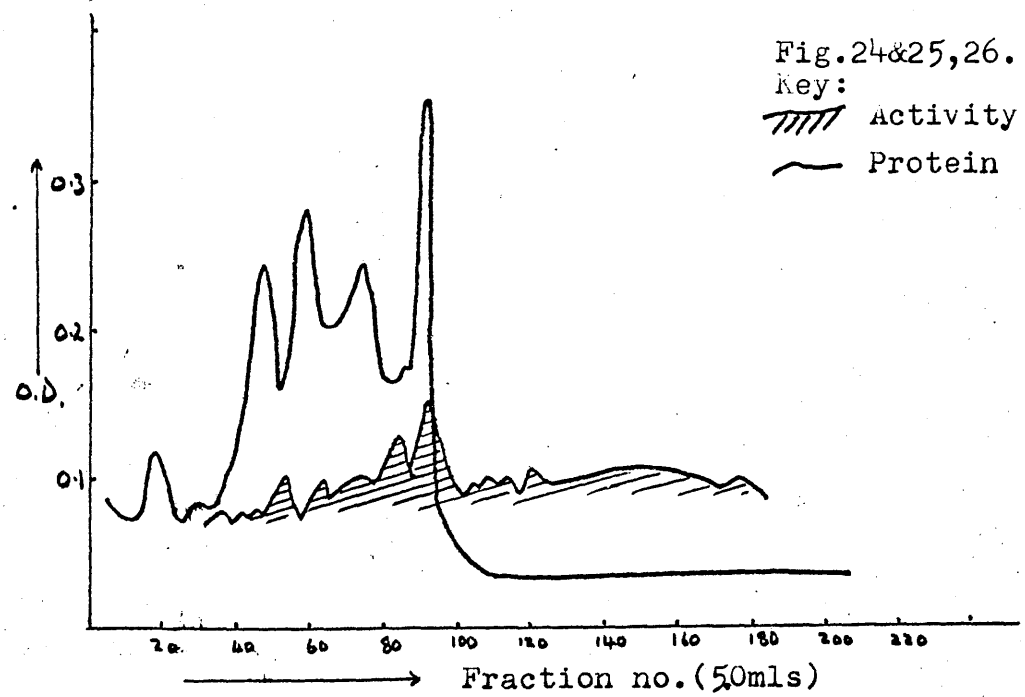


Fig.26. CMC-3.

most of the activity has been eluted. There was a very small peak of activity associated with peak 'x.' Extraction 1 and 2 differed in the main due to the fact that extraction 2 did not have any second ammonium sulphate (Stage IV) precipitation and possibly the reason for the non-appearance of this peak in CMC-I was that this particular protein fraction was precipitated during the final ammonium sulphate precipitation at stage 4.

c) Although extraction 3 was carried out entirely in the presence of 2M urea, the CM-cellulose chromatographic separation was not. Elution took place at room temperature at 17-18°C due to an absence of cold room facilities for a period. The usual expected profile of two activity peaks has given place to a rather indistinct pattern of peaks (fig 26) with perhaps what might be described as a major peak corresponding to peak A of CMC-2, though this peak of CMC-3 was a bifurcated peak.

Little information can be deduced from this column and the indistinct nature of the protein and activity profile may perhaps be attributed to operation at room temperature. The higher operational temperature may result in some dissociation or association of molecular groupings thus causing elution to extend over a larger range because of the range of molecular sizes and therefore molecules with a range of different net charges.

d) CMC-4 (fig 27) was the result of CM-cellulose chromatographic separation of protein material that had been extracted and conducted

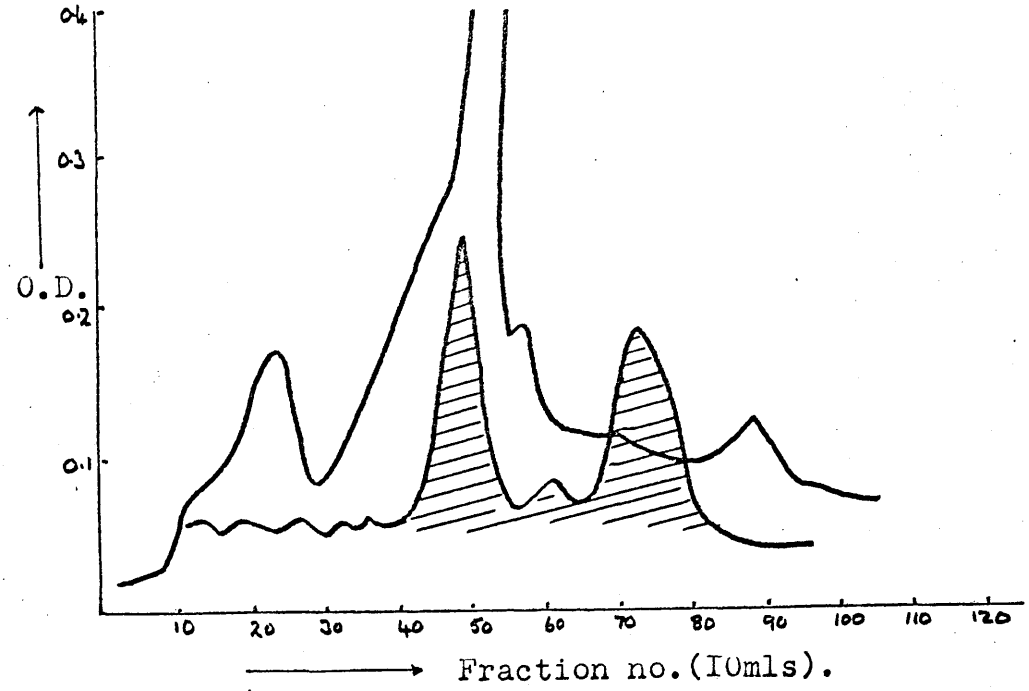


Fig.27. CMC-4

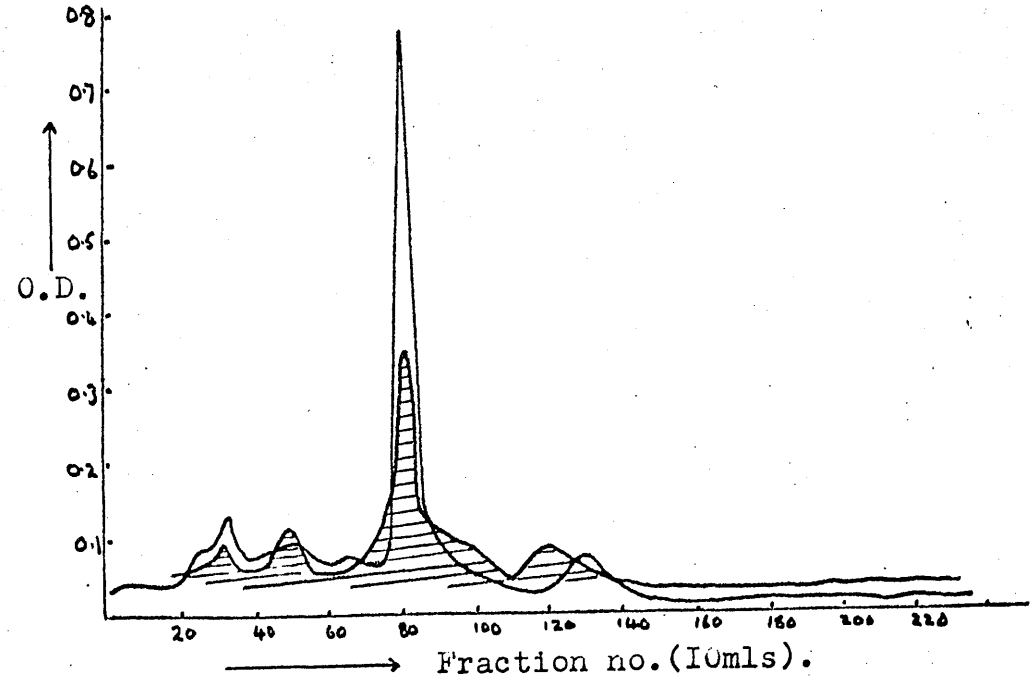


Fig.28. CMC-5-I.

through all purification stages in the presence of 2M urea. This column can most profitably be compared with CMC-5-I (fig 28) since the protein has had much the same extraction history except that urea has been used during the extraction and purification process. Similarly comparable with respect to two peak activity elution profile was CMC-1 (fig 24), however which cannot strictly be compared because of the differences in the extraction and purification stages. The distinctive pattern of two major activity peaks occurs in CMC-4 (as in CMC-1) but the activity peaks in CMC-5-1 do not correspond in elution position even when column lengths are taken into consideration. However disc-gel electrophoresis results (see page I5I) show that fractions 60-95 and 110-140 of CMC-5-I have the peak A and B characteristics in accordance with Edmond (37) If the two peaks of CMC-4 correspond to those of CMC-5-I or A and B, the presence of 2M urea, (being the outstanding difference in preparation) appears to have had the effect of causing earlier elution of the active protein from the ion—exchanger. Providing the concept of urea as a hydrogen bond dissociating agent is assumed (and this theory has not been proven) then it may be suggested that owing to the presence of urea on the column, adsorption to the column medium has been reduced, thereby reducing the salt concentration, necessary for elution of the protein. However it could be reasoned that the omnipresence of 2M urea in processing may have caused the dissociation of:-

i) — The protein into smaller aggregates or units

ii) — another non-enzymic protein that may have been attached to the RNAse protein by hydrogen bonding, so that on elution in the absence of 2M urea on CM-cellulose, this association of protein would present a stronger bonding action to the ion exchanger resulting in later elution.

iii) — or merely reduced any hydrogen bonding, that may occur as part of the ion exchange adsorption process, to a minimum.

All three suggestions might result in early elution. The explanation on the basis of the results can only be in the form of theory.

e) Evidence against the suggestion that the elution pattern of CMC-4 was a 2M urea column effect was demonstrated by column CMC-5-III (fig 29) The protein solution applied to this column was processed in the absence of 2M urea up until the G-75 desalting stage and then was in the presence of 2M urea up to and including the CMC-5-III column. (fig 29) The elution pattern was totally different to CMC-4 in that only one activity peak is obtained. If the fact that the column size (see table fig 22) was appreciably smaller and the salt gradient was steeper is considered, the elution of this peak was fairly retarded and approximates to peak A of CMC-5-I. Disc gel electrophoresis also indicates that this CMC-5-III peak of activity has similarities with activity B of CMC-5-I (gels 3

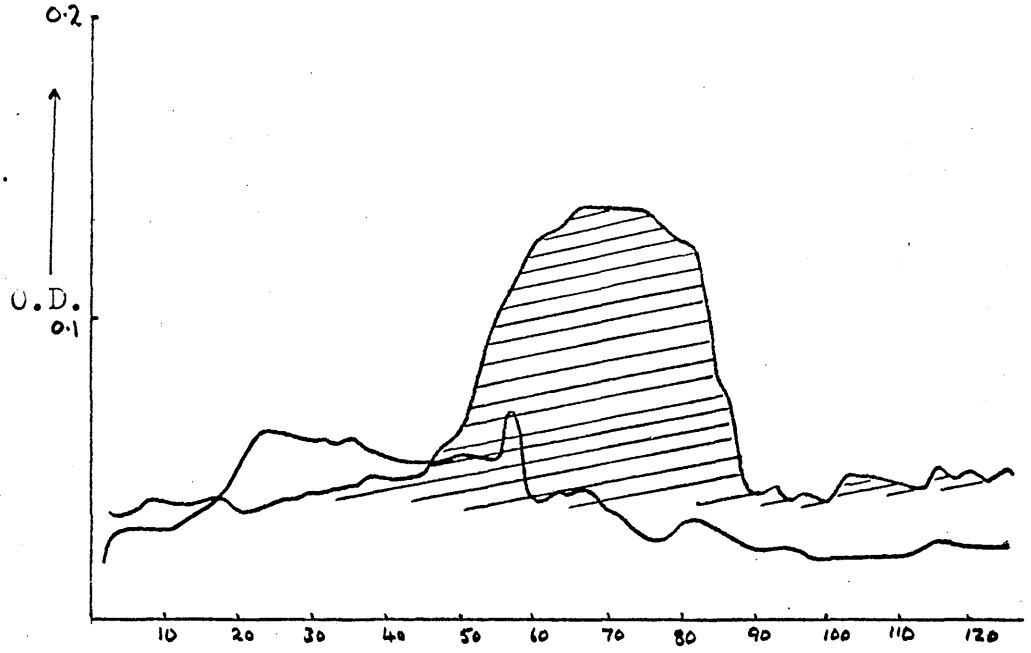


Fig.29. CMC-5-III

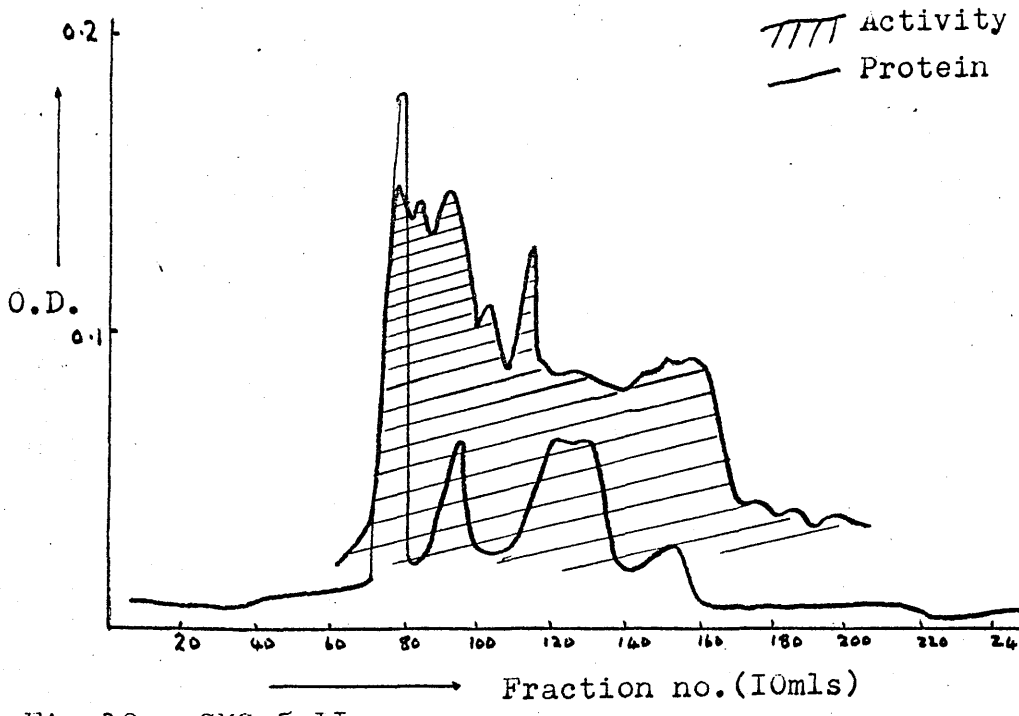


Fig.30. CMC-5-II

and 5 respectively, on page I51), and also the low U.V. absorbance at 280 mu corroborates the possibility of identification as 'B' peak. The whereabouts of the usual large protein and activity peak corresponding to A, can not be explained. The presence of urea in CMC-4 does not affect the elution of two major activity peaks which have come to be expected and characteristic. The material was stored in the cold room at 4-5°C for a period of about one month before application to CMC-5-III and possibly could have suffered some molecular change or denaturation of the protein normally eluted as activity peak A (or B?) The conceivability that activity peaks A and B (see CMC-1, page I3I) could be related forms of different aggregate size, also arises, and that in CMC-5-III there has been elution as one aggregate size. But evidence so far (page 60) and evidence demonstrated later by disc gel electrophoretic studies of peaks A and B, show this latter theory to be less likely and the more credible theory is that A and B are two separate protein species of RNAse. None the less, often more than two activities are found on elution (vis. CMC-2, CMC-3, CMC-4, CMC-5-I) and these may be sub units or multiples of the RNAse two main activity species.

f) There was no intentional difference in the preparation applied to CMC-5-I (fig 28) and II (fig 30). The protein applied to both these columns only differed in that there was a difference in batch as applied to D.E.A.E., but exactly the same procedure of desalting and CM-cellulose elution was carried out on all batches from DEAE

cellulose chromatography of extraction 5. But the protein and activity profile of CMC-5-II has little in common with CMC-5-I. If the bulked peaks of CMC-5-I (60-95 and 124-146) are compared with the two bulked peaks of CMC-5-II by disc gel electrophoresis (gels 4 and 5, and 2 and 1 respectively, pages I53, I52) they are shown to be very similar indeed. The evidence points to aggregation differences which are reflected in elution.

g) The initial activity bulked from fractions CMC-5-I, 60-95, and probably corresponding to the 'A' fraction of CMC-I, were dialysed against a pH8.2 buffer and reapplied to the same regenerated CM-cellulose column at pH8.2 and subjected to a standard gradient elution of salt. This experiment (CMC-5-IV fig 31) was conducted as a parallel experiment to the reapplication and rechromatography of RNAse A from the original extraction CMC-I, page 59). The same results were achieved, namely a single activity corresponding to a protein peak. In CMC-5-I, fraction 60-95 or 'A' contained one protein peak and this separated further on rechromatography into two protein peaks, of which only one had the majority of activity associated with it (fig 31 CMC-5-IV). The separation of proteins was not reflected in the disc gel electrophoresis of CMC-5-IV fraction 18-50 (gel 12, page I55) when compared with CMC-5-I 60-95 (gel 4, page I53) Thus as far as CMC-5-I fraction 60-95 is concerned, it shows no tendency to form any further activity peaks on rechromatography which may be eluted within the RNAse B region, and like

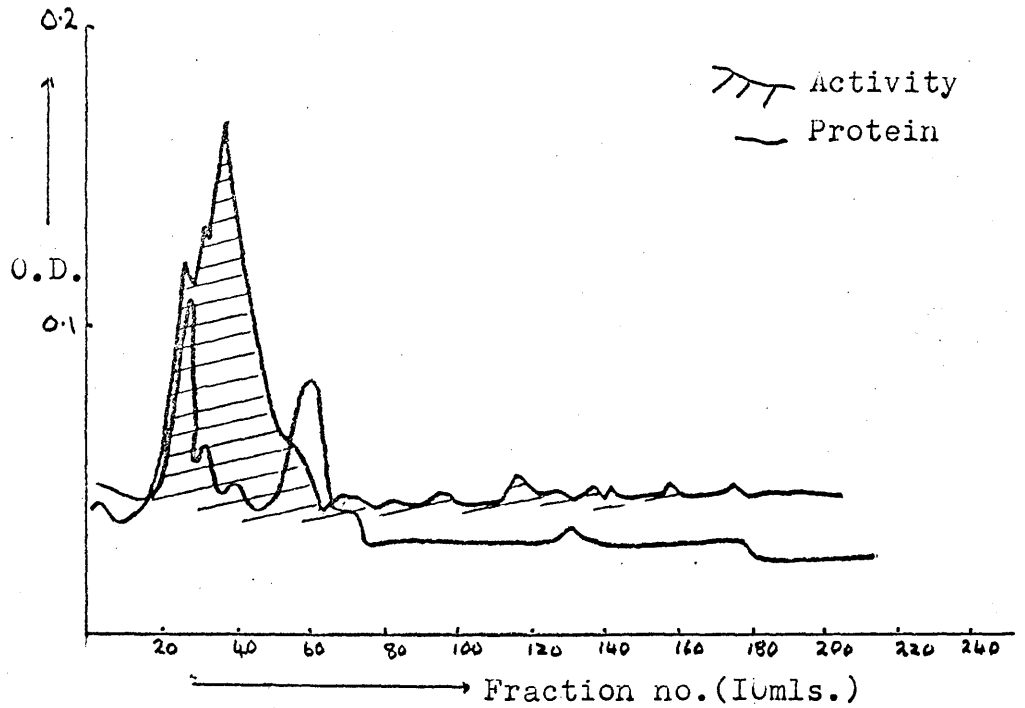


Fig.3I. CMC-5-IV

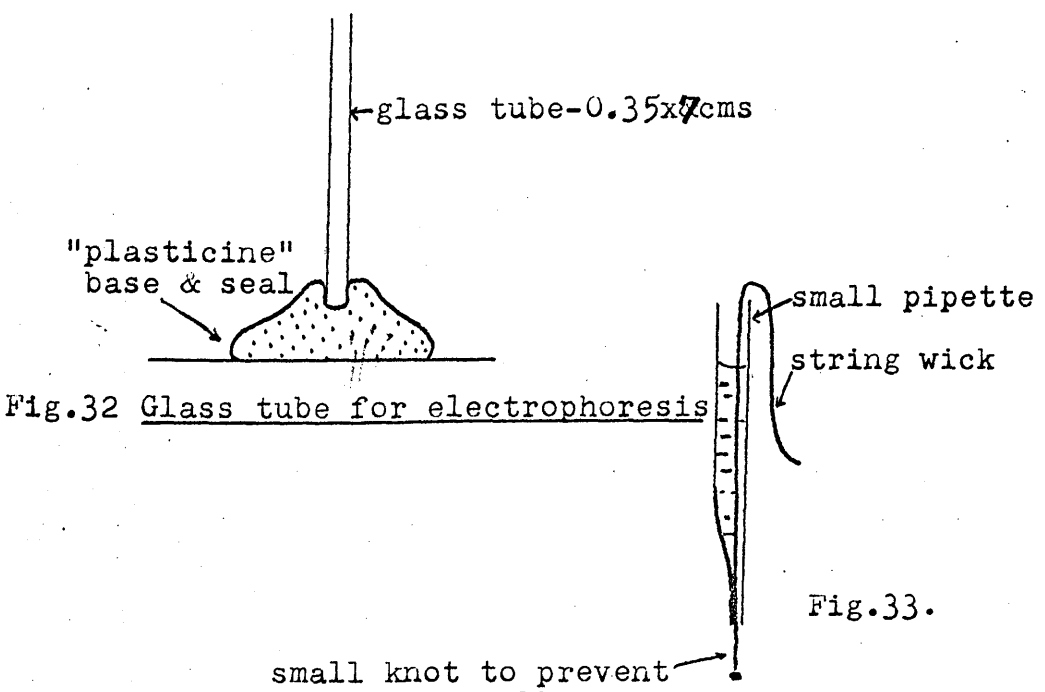


Fig.32 Glass tube for electrophoresis

Fig.33.

extraction I reapplication of peak A, the theory of RNAse being a separate entity from RNAse B is further supported by this result (page 59)

Summary of chromatography on CM-cellulose for extractions 1-5

i) The results were not always predictable with respect to activity and protein profiles, although as shown later (page 151) analysis by disc gel electrophoresis demonstrated the same pattern of proteins, even though protein profiles often gave a contrary impression from examples that had virtually identical histories. This indicates the possibility of the enzymes existing in more than one form and being eluted accordingly.

ii) There was a predominance of two activity peaks, although other activities were present to a less extent.

iii) The effectiveness of G-75 and D.E.A.E. cellulose as purification steps was demonstrated.

iv) The presence of urea did not appear to hinder purification to any great extent or aid the process. The exact role of urea at 2M concentrations is obscure as yet.

"Disc" electrophoresis and its use as a criterion of purity and protein homogeneity

Procedures for electrophoresis on polyacrylamide gels were worked out independently by Ornstein and Davis, (116) and Raymond and Weintroub (117) Polyacrylamide gels in contrast to starch

gels are transparent, thermo-stable, non-ionic, and the pore size can be varied over a wide range by changing the concentration of the acrylamide or the cross-linking agent. The absence of charged groups reduces endosmotic flow to negligible proportions. Polyacrylamide gels are also superior to starch gels for high resolution and sensitivity in performance, but unfortunately due to their low electrical resistance, there is a limit to the scale of operation and best results are therefore attained on small columns.

The method that was used in the disc gel electrophoresis of various stages of extraction 5 was the modified procedure of Reisfeld et al. (118) for acrylamide gel electrophoresis of basic proteins. The method permits excellent resolution of protein mixtures as small as 50 μ-gm. within as little as twenty minutes. The method achieves this sensitivity and resolution by concentrating the components of dilute samples into very thin starting zones and by utilizing the frictional properties of the gel to aid separation by molecular sieving.

Procedure

Details of buffers and stock solutions not given in the text below are given with all other relevant details in - General Methods 8.

The electrophoresis of protein samples was carried out in small glass columns measuring 0.35 x 7 cms. These columns were

made up with three types of gel:-

(i) A large pore anti-convection gel containing the protein sample.

(ii) A large pore 'spacer' gel in which electrophoretic concentration takes place.

(iii) A small pore gel in which electrophoretic separation and molecular sieving takes place plus the crosslinking agent of ammonium persulphate which provides the radicals to catalyse the crosslinking process.

The proportions of each type of gel in each tube were 1:1:4 by volume respectively for the above listed gel layer types, but a small space of 2-3 mm. was usually left at the top of each tube for tray buffer during the electrophoresis.

Eight such tubes were used in each electrophoretic assembly. Each tube was erected into a vertical position by embedding in plasticine, which also acted as an effective seal to the tubing end and retained the solution before polymerisation had taken place (fig 32)

The tubes were initially filled to about $\frac{2}{3}$ full with <u>small pore gel solution</u> which had been mixed in a 1:1 ration with the cross-linking agent solution of ammonium persulphate (see General Method 8 and fig 34 (a) also). Care was taken to exclude any air bubbles that might be occluded at the foot of the tube, by tapping the plasticine base gently up and down on the bench. The small pore

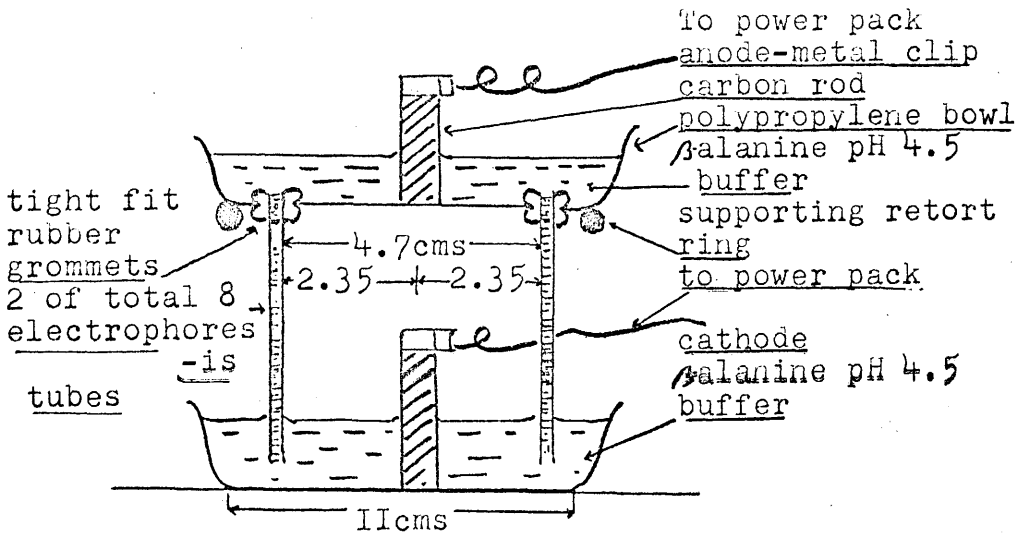


Fig.35. Apparatus for electrophoresis

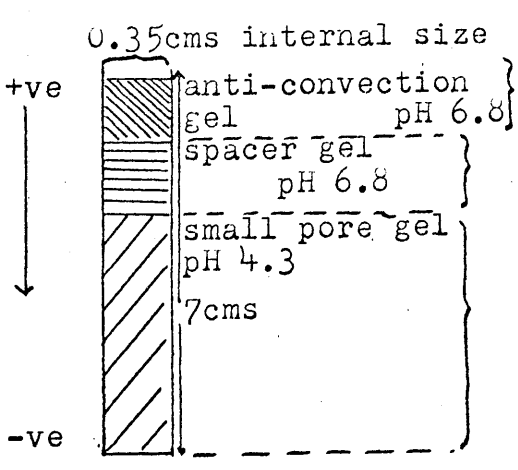


Fig.34(a). Types of gel in electrophoresis tubes.

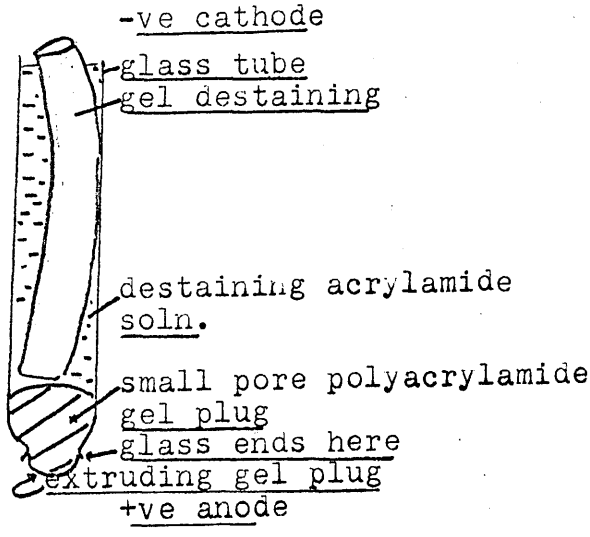


Fig.34(b). Destaining tube

gel solution was then gently overlaid with about 2mm of water (deionized) by the use of a wick and small pipette (fig 33). This was important since oxygen in the air inhibits the chemical polymerization process. The tubes were then left for about half an hour to polymerise, and sometimes when polymerisation was slow, a little heat via a closely placed lamp was used to speed the process a little. The small pore solution was constituted such as to give a 15% acrylamide solution.

The water used to overlay the small pore gel was removed after polymerisation, by a wrist-flicking action as is used for reducing the mercury reading on a clinical thermometer. The remainder of the tube above the small pore gel, was rinsed out with a little large pore solution, and about half of the remaining volume was filled with large pore gel solution and similarly overlayed with 2 mm. of deionized water. This large pore solution contained $2\frac{1}{2}\%$ acrylamide.

The tubes were then subjected to photopolymerization by placing between two 15 watt fluorescent tube lamps which were six inches apart and at the same level as the tubes for maximum illumination because this was a light activated reaction. The point at which polymerisation occured was easily observed because of the opaque nature of the polymerised large pore gels. The time taken for the polymerisation to occur was normally in the order of twenty minutes.

On polymerisation, the overlayed water was removed and sub-

-sequent to further rinsing with large pore gel solution, the protein sample was applied as a 1:1 mixture with large pore gel solution. The dilution of the large pore solution due to the addition of the sample solution, was compensated for by adjusting the dilution of stock solutions in compounding the large pore gel solution. The sample containing solution was overlayed with deionized water and photopolymerised as before.

The time taken for polymerization to occur in this final step varied enormously depending on the constitution of the sample solution applied. High salt concentration seem to inhibit polymerisation completely and thus samples of the early stages of fractionation containing high ammonium sulphate levels, had to be dialysed to some extent before succesful analysis by this method could be achieved. But the use of dialysis was avoided as much as possible because loss of protein as a precipitate (page 109) or through the Visking tubing (page 40) was feared, thus leading to a false conception of the sample at a particular stage with regard to protein content.

After removal of the overlayed water the tubes were ready for assembly into the electrophoresis apparatus (fig 35). The remaining space at the top of the electrophoresis tubes was filled with β-alanine pH4.5 tray buffer, and the tubes were removed from their "plasticine" bases and then fitted, sample gel uppermost, into the polypropylene bowls of the anode compartment by means of small

rubber grommets. A hanging drop of buffer was attached to the bottom of each tube to ensure exclusion of all air bubbles and good electrical contact on immersion into the cathode buffer tray. The anode compartment, supported by a retort ring, was placed so the tubes were immersed about $\frac{1}{4}$inch into the buffer contained in the cathode department as shown in the diagram (fig 35). A D.C. power pack was used to provide a current of 64 milliamps at initially 50 volts, but owing to heating effects, the voltage continued to rise over the electrophoresis period eventually to reach nearer a hundred volts.

The tray buffer contained β-alanine at pH4.5, and the other important ions were those of potassium which constituted part of the small and large pore gel solutions (see General Method 8) . Thus when a current was applied, the β-alanine -potassium ion boundary, originally present at the buffer-gel interface, moved into the gel towards the cathode sweeping up protein components. The proteins were concentrated in the spacer gel between the potassium and β-alanine in a series of discs in order of electrophoretic mobility. When the stack of discs passed into the small pore gel, the β-alanine potassium ion boundary was accelerated in this region of lower pH and the protein discs were left behind. The discs were now subject to a linear voltage gradient, each in a very thin starting zone and separation was henceforth with respect to charge and also molecular size.

The actual time taken for electrophoresis varied depending on the proteins present, but as far as possible the time was standardized to fifty minutes for the sake of comparison of results.

After the electrophoresis was finished the polymer gels were removed from their tubes using a 22-gauge syringe needle through which a gentle stream of water was allowed to pass. This needle was used to rim the gels, with the water acting as lubrication, and after careful manipulation of this kind the gels were released from the tubes.

The gels were then placed in small test tubes (taking care to remember the order and number of each gel) together with a 1% amidoschwarz (Naphthol blue-black) protein stain and allowed to absorb the stain overnight.

Destaining was accomplished electrophoretically using open ended tubes which had been plugged with a small portion of small pore gel to allow electrical conductance but at the same time contain the gel to be destained (see fig 34 b). The gel lengths were placed in the tubes described which were then filled with a viscous but mobile polyacrylamide solution that had only been partially polymerised by the action of light in the presence of riboflavin and acrylamide. The purpose of using the more viscous medium for destaining other than 7% acetic acid as used by Reisfeld et al. (118) was to reduce the loss by convection currents of dye into the upper cathode tray. The buffer reservoir was filled

filled with 7% acetic acid and the apparatus was assembled in the same manner as was used for initial electrophoresis except that now the cathode was the upper tray because of the negative charge of the dye molecules and electrophoresis of the stain was from the cathode to the anode. All excess dye, not absorbed by the protein bands appeared in the bottom buffer tray.

Destaining took place at the maximum current and voltage that the power pack was able to deliver, which was in the region of 220 volts. The process of destaining can take up to two hours and after which the gels are stored in labelled tubes in the presence of 7% acetic acid. Although time has the effect of reducing the stain intensity, the gels can be stored without any further deterioriation for long periods. Because of this loss of colour intensity, it is advisable to make more permanent records of the gels by photography and/or densitometer scanning procedures. The colour of the bands was also quite diagnostic since RNAse bands tended to be a brighter blue than any other bands present and had a pink tinge if held in front of a bright I.R. lamp.

Assessment of the value of disc gel electrophoresis

The method was extremely sensitive and the heterogeneity revealed by the application of this technique to samples obtained in earlier stages of the extraction of RNAse enzyme, posed the problem of how to identify the individual bands for comparison with other samples. Although care was taken to obtain reproduceable results

it proved extremely difficult to standardize the procedure sufficiently to permit identification on the basis of migration distance in an absolute sense. Internal standards close to the band in question were used in order to establish relative migration. Only after examination of a purified sample of calf spleen RNAse and noting its characteristics and colour could the earlier purification stages be followed by this method, and then results were often confusing. As separation was a function of net charge density as established by the buffer, and of the relationship of molecular size to gel porosity, it was very difficult to make any conclusions as to the nature of a protein with respect to distance travelled in the gel.

Also, although there was considerable latitude in the protein concentration, volume, salt concentration, and pH of the protein sample applied, where a high degree of reproduceability is required these parameters would need to be more closely controlled than they were.

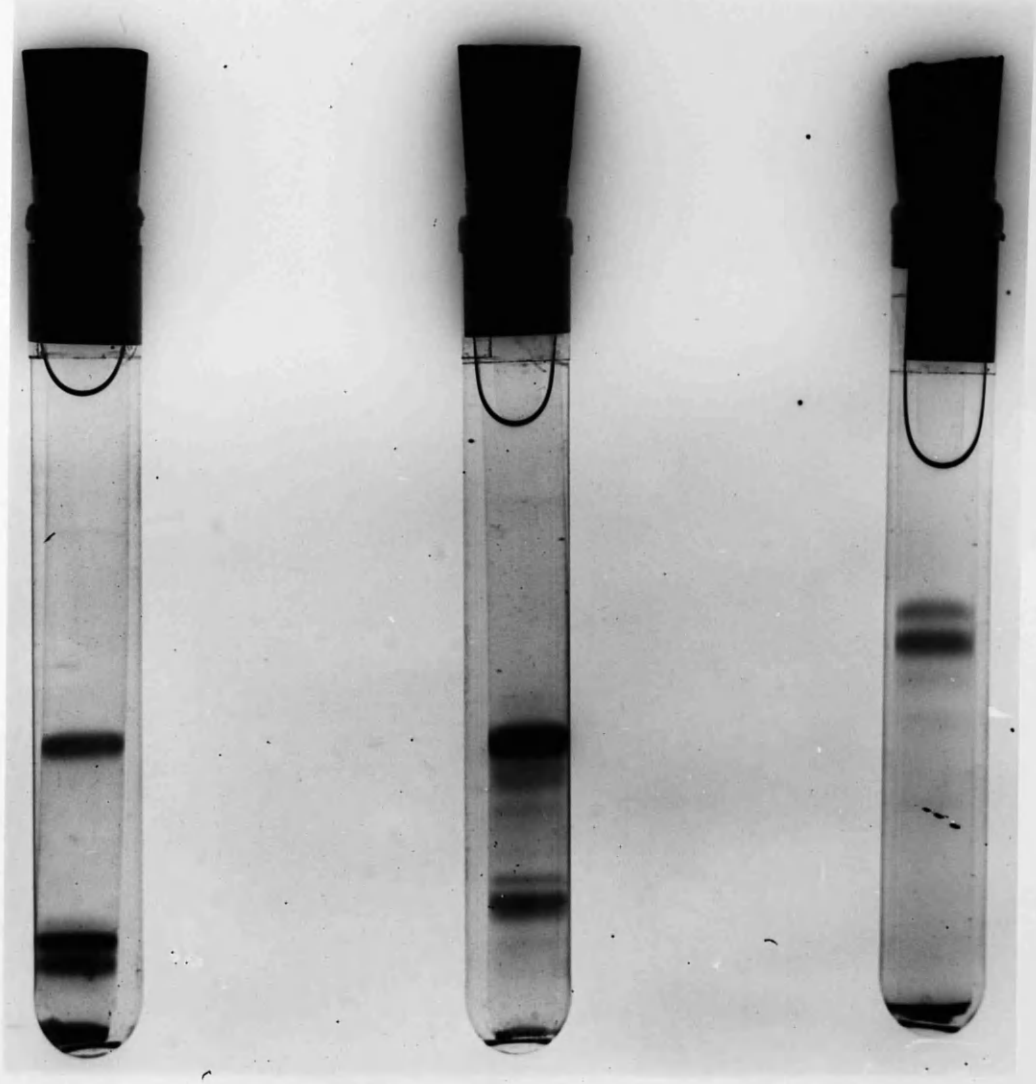
None the less as a criterion of purity, the technique was extremely useful and although the existence of one band does not necessarily mean that purification to a single protein entity had been achieved, it was an indication that a high level of purification had been attained.

Index to photographs of gels

GEL NO.	COLUMN NO. or Sample		Fraction No.
1	CMC-5-II	fig 30	104-156
2	CMC-5-II	fig 30	66-103
3	CMC-5-III	fig 29	45-90
4	CMC-5-I	fig 28	60-95
5	CMC-5-I	fig 28	110-140
6	Example of gel during earlier extraction		
7	Tubes 56 (or 60)-70 Non-bulked G-75 fraction		
8	Not held by CMC-5-I at pH6.0		
9	Held by D.E.A.E. at stage VI		
10	CMC-5-II reapplication fraction -		8-20
11	CMC-5-II " " "		56-109
12	CMC-5-IV reapplication 60-95		18-50

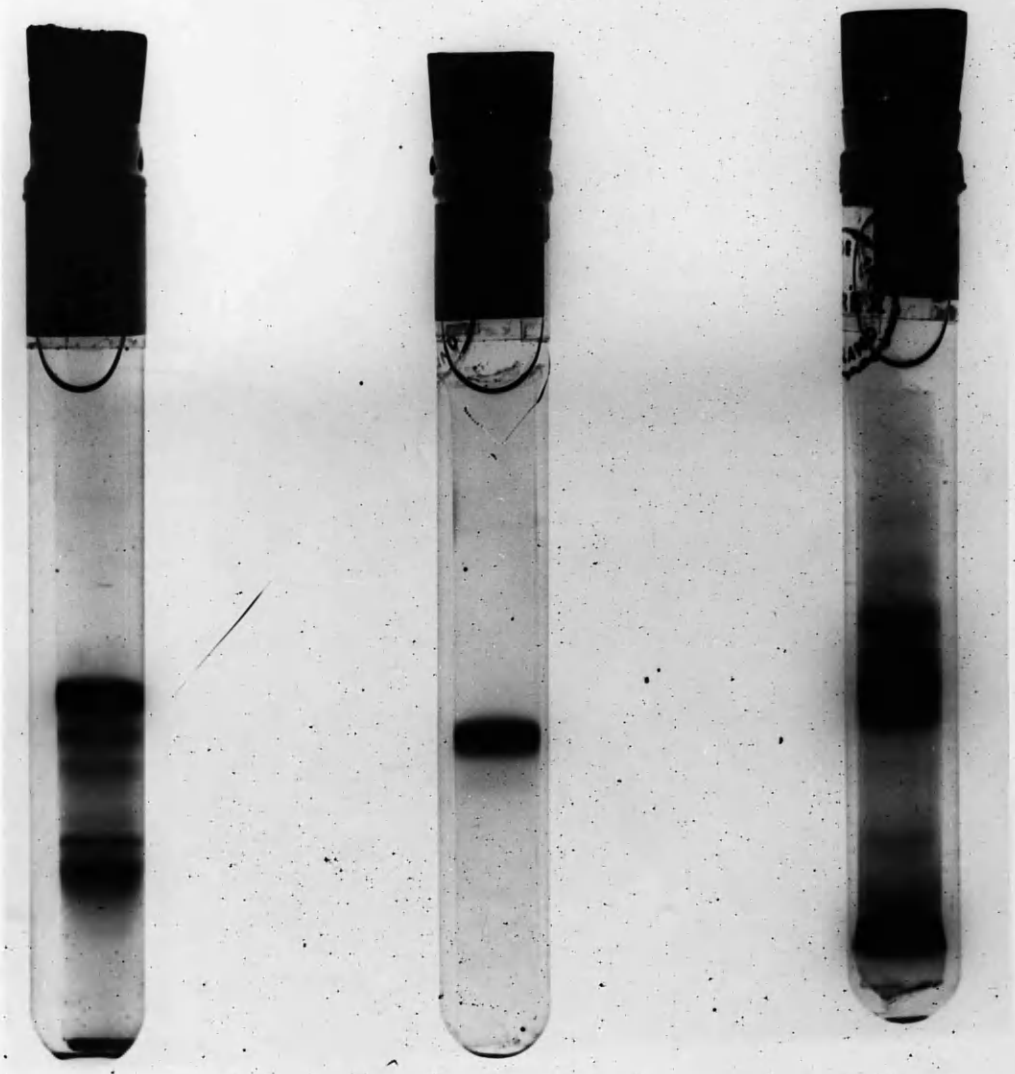
The below discussion of some of the gels obtained from disc-gel electrophoresis will aid interpretation of those gels that have not been made fully self-explanatory by allusion to them in the text.

1. Gels 2, 4, 1 and 5 The two columns, CMC-5-I and II should



Gel no.I. 2. 3.

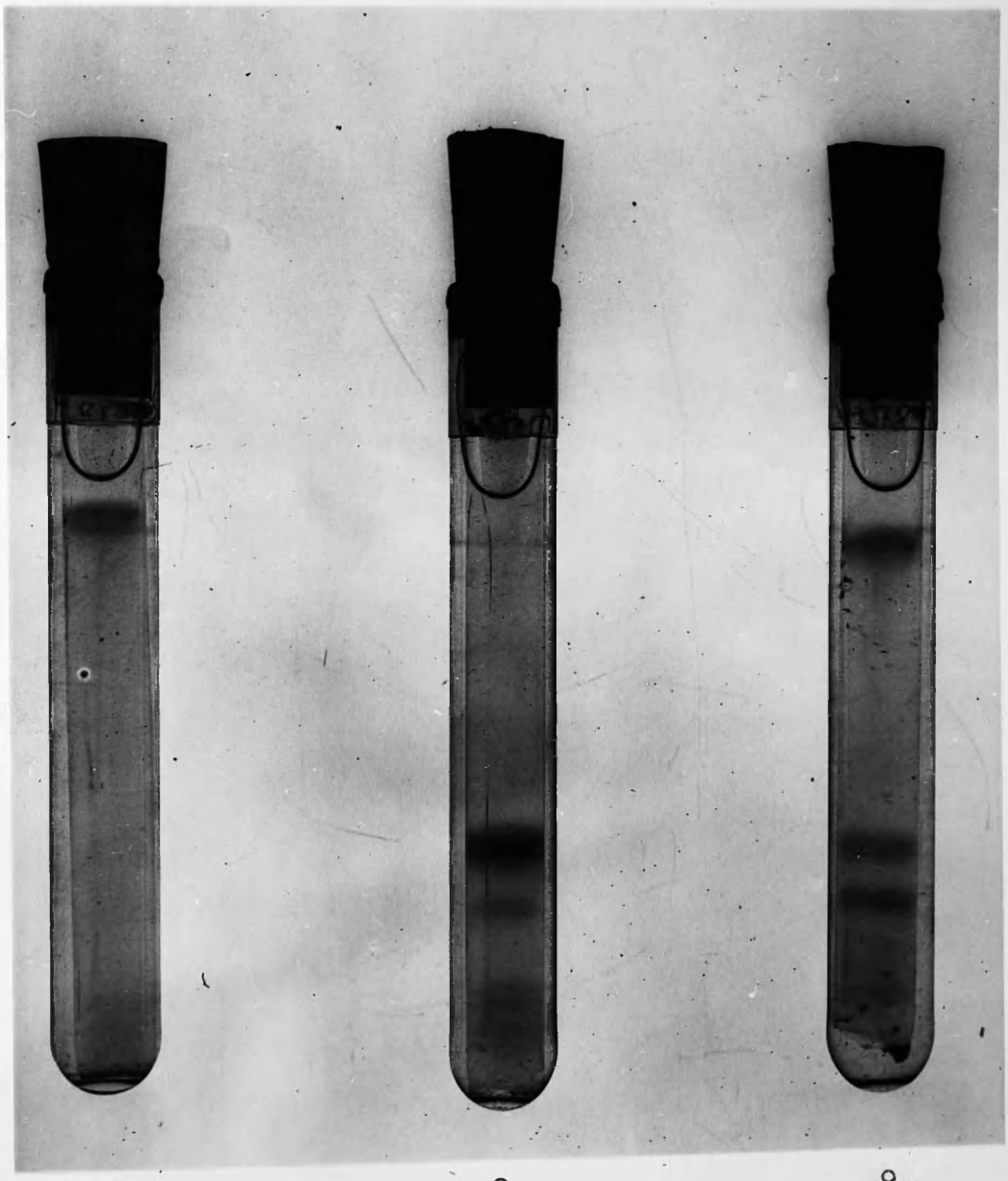
I52.



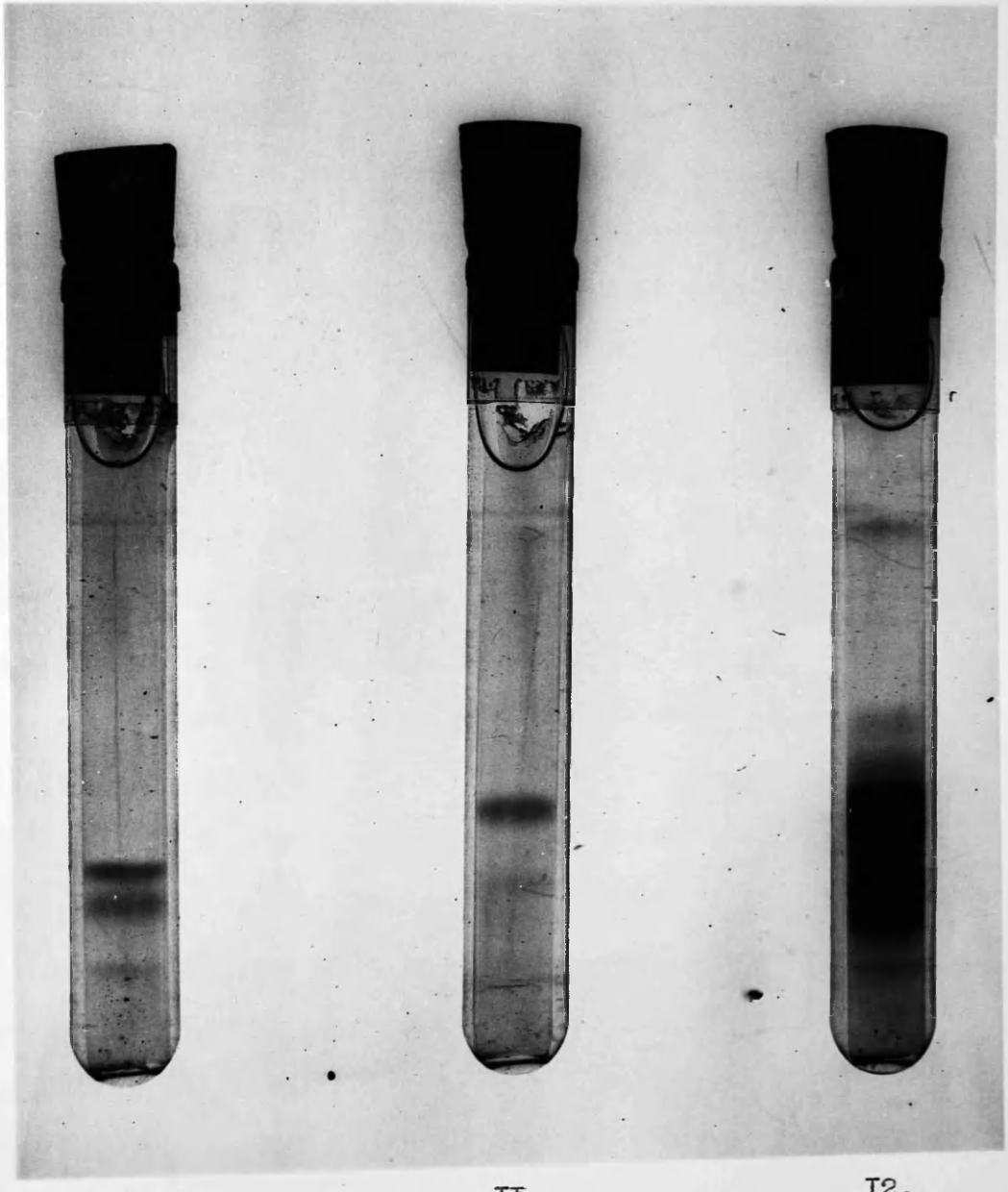
Gel no.4.

5.

6.



Gel no.7. 8. 9.



Gel no.I0. II. I2.

I55.

correspond closely with regard to results because of their having the same source of enzyme (Extraction 5) and very similar elution conditions, and a comparison of gels 2 and 4, and gels 1 and 5 show this to be the case. Gel 5 shows that according to disc electrophoresis, a high degree of purification had been attained, which was not quite matched by gel 1 where there were three main protein components whereas there was only one main component in gel 5. Gels 1 and 5 correspond to that fraction designated RNAse B with respect to CM-cellulose chromatographic separation and was obtainable in a relatively pure form.

The fraction designated RNAse A with respect to CM-cellulose chromatography was represented by gels 2 and 4. The dark uppermost band had the characteristic lighter blue colour that was also common to RNAse B (gel 1 and 5) and similar if not the same light blue bands seem to be present in all active samples and thus the colour may be characteristic of the RNAse enzyme under study.

2) Gel 12 The RNAse A fractions obtained by Edmond (37) gave similar results to those obtained in gels 2 and 4. The RNAse A fraction was composed of at least six proteins of which five were very prominent. Rechromatography of A at pH8.2 on CM-cellulose (page I38, fig3I, CMC-5-IV) and disc gel electrophoresis of the resulting fraction 18-50 (gel 12) gave much the same results as gel 4 from CMC-5-I fraction 60-95.

3) Gels 10 and 11, 8

When protein material was applied to CMC-5-I and II for chromatography it was noticed that material was not held by the column at pH7.0 (gel 8) and passed straight through the column. This unadsorbed protein was found to be active when assayed with RNA as substrate and thus the material from CMC-5-II was adjusted to pH6.0 and applied to another CM-cellulose column upon which the RNAse activity was seemingly held. The following gradient elution was applied.

Reservoir 1600 mls 0.005M Tris, 10^{-4}M EDTA, pH6.0

Constant volume chamber 800 mls 0.005M Tris, 10^{-4}M EDTA, pH8.2 0.32M NaCl.

The activity regions obtained werebulked as follows:-

Fractions 8-20

Fractions 56-109 fig 36

The bulked fractions were subjected to disc gel electrophoresis (gels 10 and 11). The results were interesting in that they had little resemblance to those of CMC-5-II with respect to the position of elution of activity or to gel electrophoretic results. The elution pattern should have been comparable to CMC-5-II since the same column was used after regeneration of the cellulose and except for the difference in salt concentration (0. 32M instead of 0.25M NaCl.) the gradients were identical. The difference in salt concentration only made a difference in the elution position of

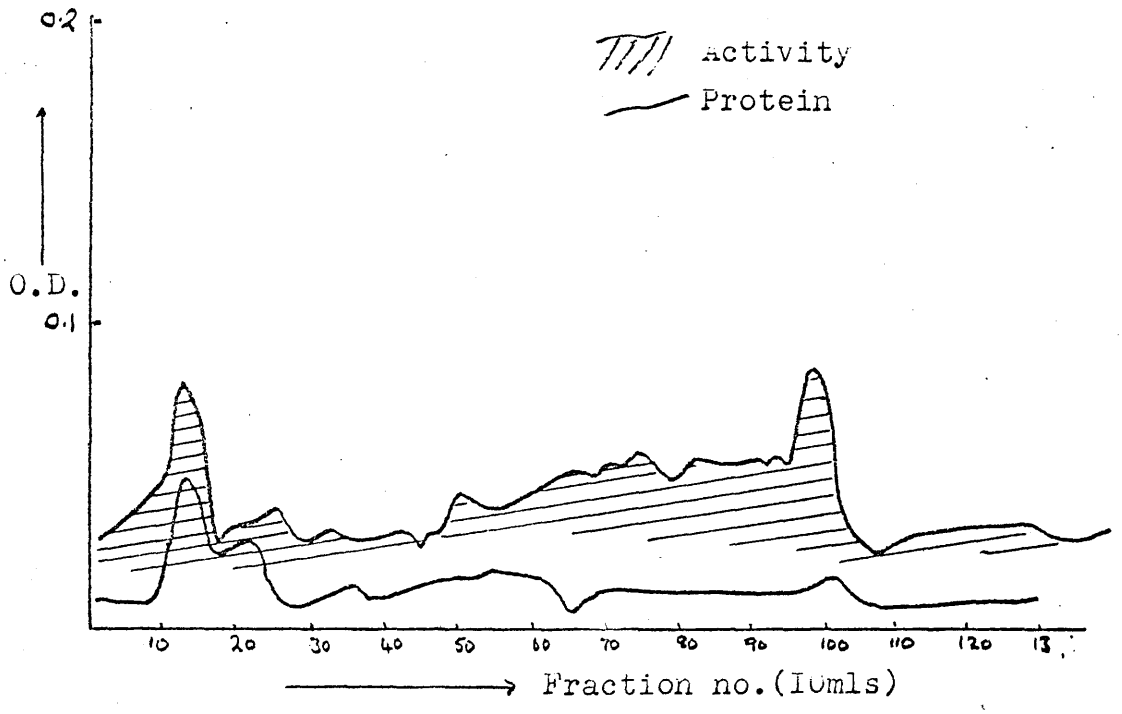


Fig.36. Re-application to regenerated CMC-5-II of material that was not held by CMC-5-II previously.

RNAse A, in CMC-5-I and II, of six fractions.

The nature of the enzymically active material, eluted between fraction 8-20 from this reapplication column, had little in common to any other peaks from CM-cellulose columns because of its very early elution position. Gel 10 indicated that this fraction was not of the typical RNAse A type (gels 2, 4). Perhaps this could be explained by the theory that this fraction owes its early elution to the protein being of a very small size and therfore with a smaller net charge than a larger unit. Alternatively this particular RNAse fraction had little basic nature and as a result was barely retained on the cationic exchanger at pH6.0

The second fraction bulked from this column, fraction 56-109, had a gel behaviour similar to the RNAse B types (gels 1 and 5), but the elution position was more compatible with the previous elution of RNAse A (CMC-5-I and II) Thus again either a small protein, maybe a subunit of A or B, or a less basic protein was possibly indicated. Other protein changes which could possibly lead to changes in behaviour of elution, such as certain protein three dimensional or structural changes seem unlikely because conditions were standard to all the columns (unless otherwise stated)

An explanation of the behaviour of the RNAse active material not initially held by the CM-cellulose (and barely held the second time of application) cannot be given with any degree of certainty

and the explanations put forward remain only as suggestions.

Other gels not discussed above are mentioned in their relevant contexts.

The course of enzyme purification during extraction 5 (see fig.37).

Extraction 5 was the last extraction of calf spleen to be made and represented the culmination of twelve weeks work within itself. It also represented the culmination of all the techniques investigated during extractions 1-4 and as shown by disc gel electrophoresis the isolation of a reasonably homogeneous RNAse active entity was achieved by this technique, namely RNAse activity peak 'B' (gel 5). However as will be seen from the following analysis, much RNAse activity was lost at various stages especially those stages which involved ammonium sulphate fractionation.

At each stage of purification of extraction 5 a sample was retained for assay and was stored in a stoppered glass flask in the deep freeze. Before assay each sample was thawed out slowly at room temperature. The total volume from which the sample was taken was noted in order to calculate the total number of units of activity present in the original material.

The enzyme activity was measured in units as defined by Kaplan and Heppel (28), viz. the amount of enzyme which causes an optical density increment of 2.0 at 260 mu in the final diluted

sample. For best results it was desirable to restrict the net optical density to as near 0.10 as possible in the final diluted sample. This adjustment of optical density level was achieved by a system of trial and error experimentation in dilution of the enzyme-containing sample which was to be assayed. It will be seen that it was important that this dilution factor was noted and incorporated into the calculation of the total number of activity units.

Assay procedure and calculations

The assay procedure was carried out as previously described (page 52) but as this assay was not merely for the location of RNAse activity in column fractions, but was a quantitative estimation of the enzyme present in a particular fraction, care was taken to observe correct timing and reproducibility of results by accurate procedure standardization.

The source of RNA used for these determinations and indeed all column chromatography RNAse location in extraction 5, was commercial yeast RNA (L. Light and Co. Colnbrook, England) The commercial yeast RNA was treated by dissolving 25 grams in 1.25 litres of deionized water and stirring with a magnetic stirrer. The RNA was not very soluble at this stage so the pH was brought to neutrality by the dropwise addition of normal sodium hydroxide. It was then exclusively dialysed against deionized water for three days with several changes of water. This took place in the cold

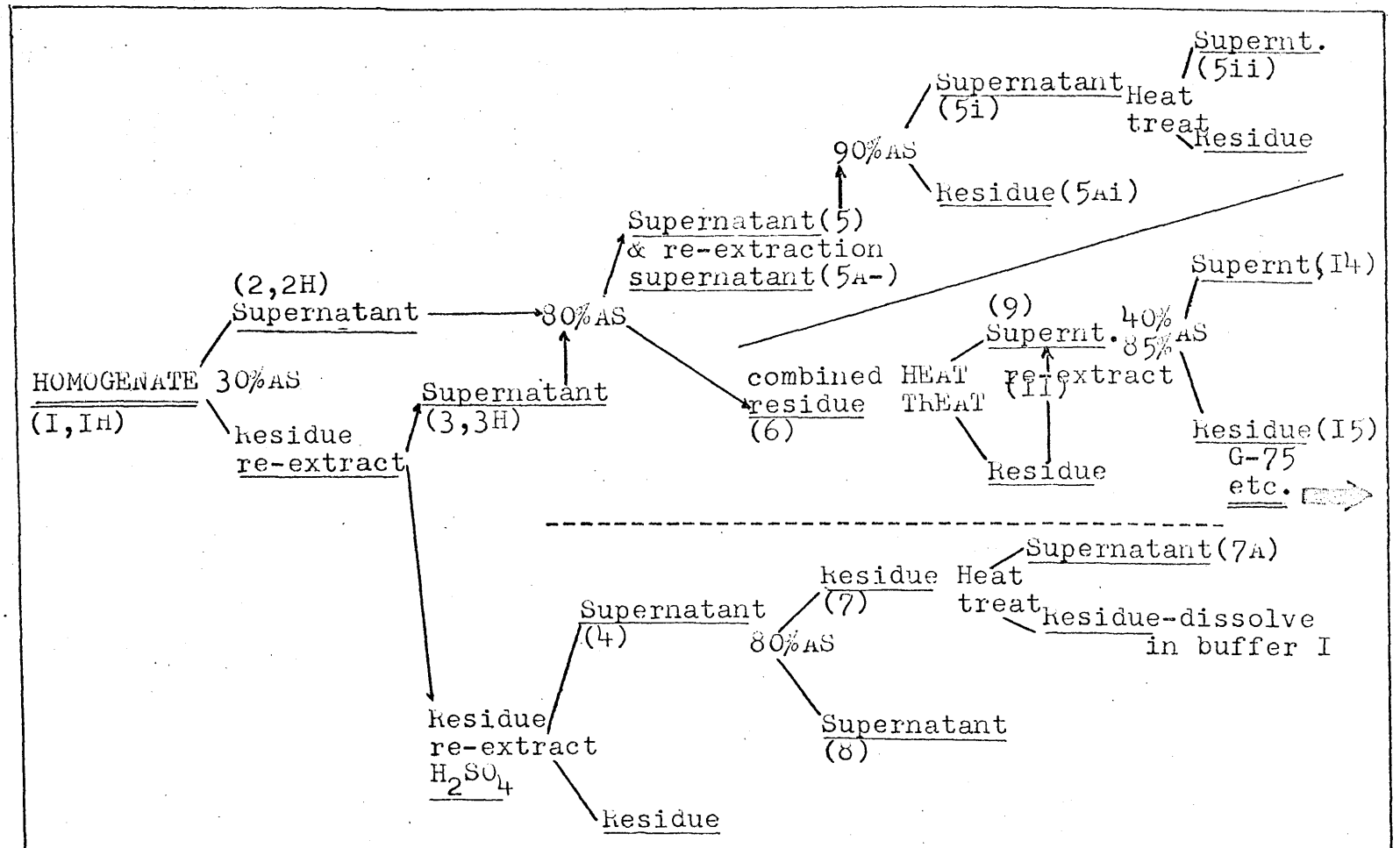


Fig.37. Flow sheet -Extraction 5 with reference numbers for units of activity at each stage on page

Sample number	Treatment	No. of UNITS ACTIVITY
1		97,428
1H	Heat treatment, centrifuge, assay ($60^{\circ}C$ for 10 minutes)	38,447
2		237,770
2H	Heat treat, centrifuge, assay	146,972
3		38,766
3H	Heat treat, centrifuge, assay	17,189
4		7,333
5		16,530
5 A		8,316
5 Ai		9,058
5 i		26,296
5 ii		2,427
6		149,250
7		3,618
8		9,370
7 A		2,611
9		15,888
11		298
14		5,919
15		3,792

List of Units of activity at each stage as in fig 37 for Extraction 5

room at 4-5°C using 12 litre stainless steel buckets to avoid any contamination. The RNA was then lyophilized and the dry product stored in sealed glass bottles in the deep freeze between use (at -22°C).

A zero-time blank estimation was conducted for each sample by initial precipitation of RNA solution with a 0.25% uranylacetate and 2.5% trichloracetic acid solution (UA/TCA), and then by inactivation of the sample protein solution when it was added to the RNA-U A/TCA mixture. The digest tube was then immersed in iced water for one hour and finally centrifuged at 1500 r.p.m. at room temperature. The cooling in iced water ensured curtailment of any residual enzymic activity, which was unlikely in the presence of U A/TCA but the low temperature was an added precaution. After the correct sampling and dilution procedure had been carried out (page 52) the zeroblank optical density was read at 260 mu.

The assay procedure for each sample was carried out as below:-

1. Sample + RNA — digested for 30 mins. } average of 1 and 2= a
2. " + " " " " " }
3. RNA+ U.A/TCA+ sample — cool } average of 3 and 4= b
4. " " " "

∴ Net optical density= a-b = c

∴ Total number of enzyme activity units = $c \times \frac{10}{2} \times 4 \times V \times d$.

Where V= total volume from which sample was taken
d= dilution factor of sample

The division by two was necessary to conform to the Kaplan and Heppel (28) definition of a unit of activity, and the multiplication factor of 10 x 4 was inserted to account for the final dilution of the sample in the assay procedure, which, if the same procedure as detailed on page 52 is followed, will remain constant.

The flow sheet on page 162 , fig 37 should be interpreted along with the summary chart of extraction 5 on page 124 fig 21.

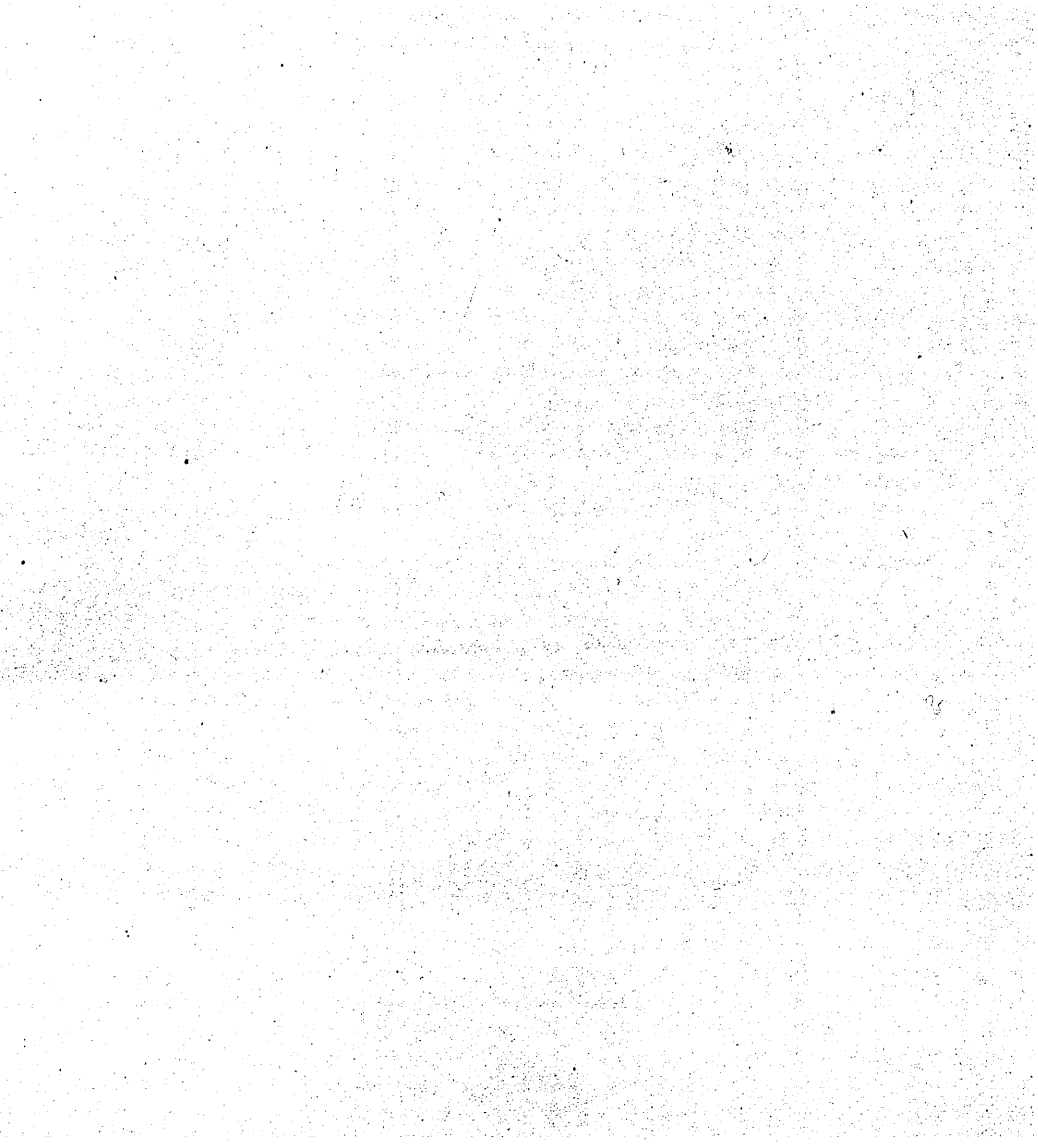
Concluding comments on extraction 5 (see summary charts in figs 21, 37)

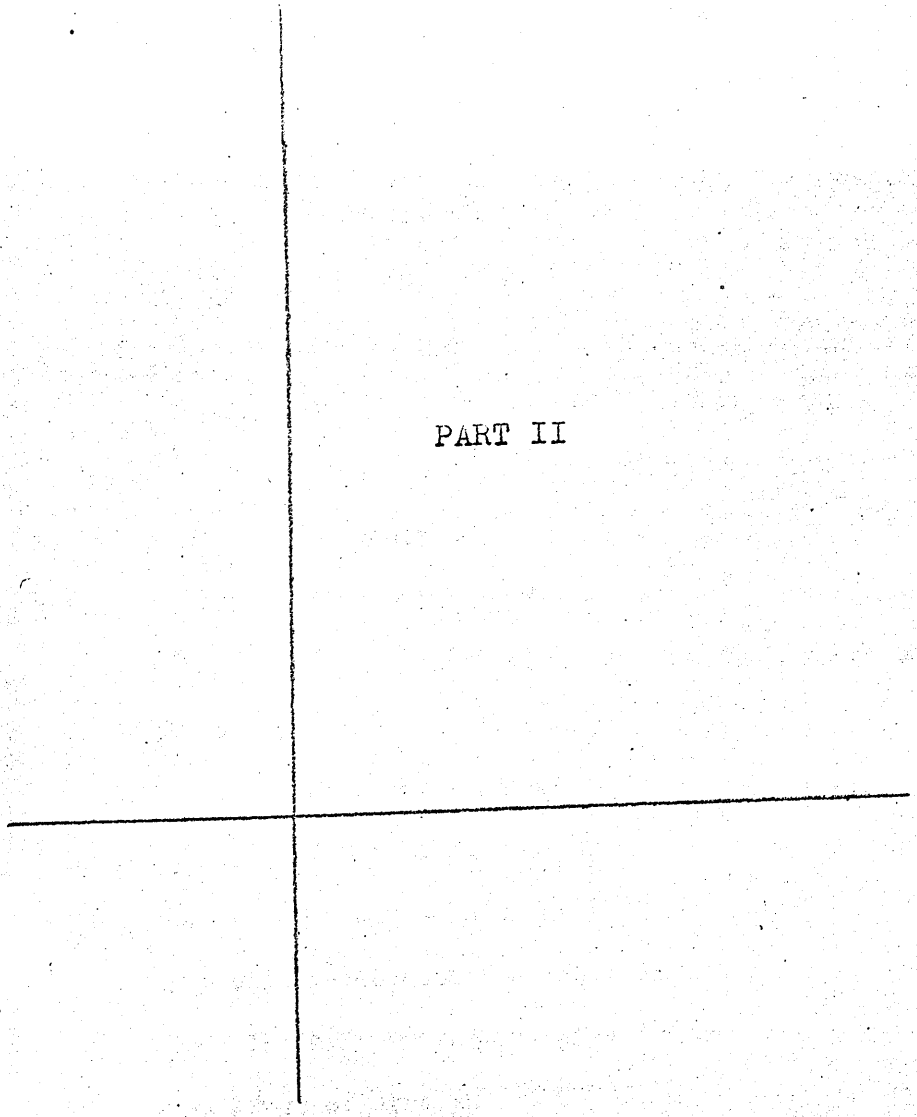
The loss of activity in normally discarded residues, and also from enzyme material that was soluble in ammonium sulphate at even saturation point, still remains a problem. (page 86) Careful reextraction in extraction 5 of normally discarded residues, recovered appreciable amounts of active enzyme (fig 37) and the result of investigations into the use of 2N H_2SO_4 certainly merits further examination (page 64) The loss of activity by virtue of the solubility of material with RNAse activity in concentrated ammonium sulphate solutions both at pre-and post-heat treatment stages, also accounts for irretrievable enzyme during this extraction. In the light of similar reports by Chesbro (108) of low molecular weight nucleases soluble in saturated ammonium sulphate, the possibility of finding and isolating very low molecular weight forms of a heat stable ribonuclease must be considered.

Extraction 5 still exhibits the characteristic two main activity peaks A and B (page 54) on CM-cellulose chromatographic separation and all evidence seems to indicate these are quite separate entities (pages 59, I37, I38, parts II&III). A relatively homogeneous sample of RNAse B was obtained from this extraction with respect to disc gel electrophoretic analysis (gel number 5)

Extraction 5 probably represents the most efficient extraction of ribonuclease from calf spleen that can be obtained using

the type of purification procedure described.





PART II

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A UREA

PART II

Aggregation properties of the enzyme

Introduction - A UREA

In recent years conclusive evidence has been presented (125, 126, 127) establishing that the amino-acid sequences of proteins are determined by the nucleotide sequence of genetic material. Specific groups of nucleotides in genetic material are translated into the amino-acids of proteins, and there appears to be a linear correspondence between the sequence of nucleotides in genetic material, and the sequence of amino-acids in the polypeptide chain. To establish unequivocally this mechanism directly it would be necessary to have nucleotide and amino-acid sequence information for a gene and messenger RNA and the corresponding protein. Although it is possible to obtain amino-acid sequence information, no-one has succeeded yet in isolating a single gene or messenger RNA molecule corresponding to a single polypeptide chain. However evidence is sufficient to assume that the genetic map is colinear with protein structure.

The primary structure of protein is the sequential arrangement of amino-acids in the polypeptide chains. The folding of the polypeptide chains to produce a three dimensional structure constitutes what is known as the secondary structure of proteins where near neighbours of a chain frequently bear a fixed spatial arrangement (or orientation) relative to one another. This

level of organisation can be accounted for in terms of bond distances, bond angles, and the stereochemical considerations of restricted rotation and non-covalent bonds. A tertiary order of structure accounts for the folding of the polypeptide chains into compact globular molecules, and this order deals with the spatial relationships among remote segments of the same polypeptide chain or even different chains and can be considered as depicting interchain structure.

According to present views the characteristic secondary and tertiary structures of protein molecules are a direct consequence of the sequential arrangement of the amino-acids in the polypeptide chains (176) In support of this view, disorganized polypeptide chains produced by the denaturation of proteins were transformed readily into biologically active molecules having the properties and architecture characteristic of the native protein (245, 246)

However before this "renaturation" process of protein can be discussed the process and the definition of denaturation must be established. Denaturation is a very general term and describes a change of protein structure with a loss of one or more protein properties. The steric structure of an enzyme is considered to fluctuate between a number of steric conformations within the same environment, however, the structure suffers pronounced changes if this environment is changed.

Thus if the three dimensional structure of a native protein

can be destroyed and reconstituted, it is difficult to avoid an important conclusion; namely that amino-acid sequence alone is enough to guide organisation into three dimensions. One of the most spectacular examples of the renaturation process was the reversible denaturation of tobacco mosaic virus. In 1959 three groups of investigators (119, 120, 121) reported that the virus protein could be dissociated or denatured reversibly by alkali, 67% acetic acid or 8M urea. In each case a minimal sub-unit of 17,400 was obtained but the sub-units were randomised in three directions as well as dissociated from each other. These remarkable successes in re-establishing an original array from the random array are all the more remarkable in view of the claim by Aach 1960 (128) that there exist two kinds of similar sub-units in one virus particle or that the identical sub-units are arranged in pairs as mirror images.

Another example is that of ribonuclease (pancreatic) in which it seems that the activity of the enzyme depends on the secondary structure being stabilized by four disulphide linkages. Initially it was shown that the RNAse disulphide groups could be fully reduced with thioglycollate in 8M urea, and the resulting inactive protein was partially reactivated by oxidation of the sulphydryl groups by bubbling air into the preparation. (123, 124) The reductive conditions abolished secondary and tertiary structure to a point where there was little specific structure or activity remaining. It should be noted that on reoxidation of the

sulphydryl groups to give the reconstituted tertiary structure, 105 isomers are possible, all giving the necessary four disulphide bonds possessed by the native protein. But as the same position of disulphide bonds is found in the oxidized protein as in the normal native protein, this is strong evidence for the suggestion that the amino-acid sequence in RNAse possesses all the information that is required for the determination of the specific secondary and tertiary structure of the protein.

Thus, accepting that the secondary and tertiary structures of protein molecules are a direct consequence of the sequential arrangements of amino-acids in the polypeptide chain, consideration must be given to the forces that are responsible for maintaining this three dimensional structure.

The forces responsible for maintaining native configuration

An understanding of how and why protein molecules change their configuration, requires and understanding of the various kinds of internal bonds that might be expected to form in the native protein and the means by which the denaturing agents can break these bonds. The possible forces responsible for maintaining configuration in the native state are:-

1. Hydrogen bonds between peptide bonds.
2. Hydrophobic bonds.
3. Salt linkages
4. Hydrogen bonds other than between peptide links

5. Stabilization by electron delocalization.

6. Dispersion forces.

7. Effects of disulphide groups and other cross linkages on the stability of the folded form of the peptide chain.

1. Hydrogen bonds between the oxygen atoms of the carbonyl groups and the hydrogen atoms of the amide groups of peptide linkages are assumed to play a basic role in determining the pattern of folding of polypeptide chains.

It has been possible by the study of the diffraction of X-rays by peptides and protein crystals to show that polypeptide chains tend to twist or coil upon themselves. (129) A polypeptide chain can assume an infinite number of configurations, but in fact the most stable configurations are those in which all NH-groups of of peptide bonds are hydrogen bonded to C=O groups, and thus a polypeptide chain will tend to take a conformation in which the hydrogen bonding is at a maximum. This leads to two extreme types of bonding that can exist between polypeptide chains. Firstly sheets, with the hydrogen bonding between polypeptide chains (interchain hydrogen bonding), and secondly, coils or helices, in which H-bonding exists between the peptide bonds of the same polypeptide chain (intrachain hydrogen bonding.)

The shape of the helices in the latter case depends on the

number of amino acid residues in one turn of the helix.

2. Hydrophobic bonds

Most proteins will contain a high proportion of amino acids with non-polar side chains, such as the isopropyl group of valine and the secondary and isobutyl groups of leucine. The proportion of the non-polar side chain amino-acids in protein is somewhere in the region of 30% on average. Then there are also the semi-polar types like tyrosine. Thus if proline, alanine and tryptophan are included among the non-polar side chains, the percentage becomes 35-45% Since the non-polar side chains have a low affinity with water, those polypeptide chain configurations which have large numbers of these groups in contact with each other (e.g. in an alpha - helix configuration), and hence tend to remove them from the aqueous phase, will be more stable than other configurations, other things being equal. One can consider that these non-polar type side chains will form intramolecular micelles analogous to the micelles which are known to occur in solution of soaps and detergents. The tendency of non-polar groups to adhere together in aqueous environments is known as hydrophobic bonding.

After several decades of absolute reliance on the peptide hydrogen bond as the main force in contributing to the stability of folded polypeptide chains in globular proteins, the hydrophobic bond (a term introduced by W. Kauzmann - 130) has taken its place as the principal non-covalent attractive force in the stabilizing

of the folded chains (131) of native protein.

By study of the behaviour of small hydrocarbon molecules (132) tentative conclusions may be drawn as to the thermo-dynamic properties of the hydrophobic bond. Firstly these bonds are largely stabilized by entropy effects. For each non-polar aliphatic side chain that leaves the aqueous environment and enters a non-polar region of the protein, there is a gain in entropy of the order of twenty entropy units. Secondly, the transfer of an aliphatic side chain from water to a non-polar region in the protein is endothermic to the extent of 1000-2000 calories per mole of groups, and hydrophobic bonds involving aliphatic side chains are more stable at room temperature than they are at 0°C because of the endothermicity of the transfer of non-polar groups from water to a non-polar environment.

Thus if hydrophobic bonds are weakened at 0°C and if urea brings about denaturation of some proteins by breaking interpeptide hydrogen bonds, and if both hydrophobic and hydrogen bonds are necessary to maintain the native structure, then any weakening of the hydrophobic bonds (such as lowering of temperature) will make it easier for urea to bring about denaturation.

An alternative view has been formulated by Klotz (170) who stressed the effect of the interactions between non-polar side chains and the water in determining the conformations of polypeptide chains in proteins. But this view, although based on the same

thermodynamic studies of hydrocarbons in water, is markedly different from that proposed by Kauzmann since an alternative model system was employed in its development.

According to Klotz, non-polar side chains form crystalline hydrates with water (as do many hydrocarbons) and the coalescence of the hydration "icebergs" produces stable ice lattices in which the non-polar residues, rather than being on the interior of the protein molecules, are at the exterior in contact with the solvent.

But despite the divergence of views, the disagreement is over the nature of the hydrophobic bond, and not over the importance of non-polar side chains in stabilizing tertiary structures.

In agreement with the original proposal of Waugh 1954 (133) that a protein molecule had an essentially polar outer volume and non-polar inner volume, and contrary to the above interpretation of Klotz of hydrophobic bonding, experiments by Fisher (134) indicated that the phenyl-alanine residues in the enzyme glutamic dehydrogenase were totally absent from the molecular surface. X-ray diffraction studies of the myoglobin molecule (135) show that most non-polar residues are buried, whereas most polar residues are exposed to the outside with the result that most of the interior of a protein molecule is anhydrous.

The insolubility of denatured proteins in water is undoubtedly due in part to intermolecular hydrophobic, hydrogen and disulphide bond formation. The fact that denatured proteins

have a greater tendency to dissolve in stronger urea solutions indicates that urea possibly weakens hydrophobic bonds as well as hydrogen bonds.

3. Salt linkages

It has been suggested frequently that salt linkages are an important factor in maintaining the structure of native proteins by the attraction between positively charged amino and guanidino groups, and negatively charged carboxyl groups. However Jacobsen and Linderstrøm (136) have given various reasons for believing that in typical proteins only a small fraction of the charged groups can be involved in bonding of this kind.

It is evident that both salt linkages (137) and hydrophobic bonds are stabilized predominantly by entropy effects rather than by energy effects. On the addition of electrolyte to an aqueous medium and the addition of a non-polar substance there is a difference of behaviour between the two types of bonds. Salt linkages would be strengthened by lowering the dielectric constant of the medium (which will follow from the addition of a non-polar substance such as dioxane to the aqueous environment of the protein), but hydrophobic bonds are weakened by adding a non-polar substance to the aqueous medium.

4,5, and 6.

The other forces of hydrogen bond linkage (other than that

of peptide linkage (138) and delocalisation (139) and dispersion (140) forces are of less importance in maintenance of configuration of proteins.

7. Disulphide bonds

Most of the large multichain proteins do not contain disulphide bonds, and therefore the maintenance of their interchain tertiary structures can be attributed to non-covalent bonds. Even for those proteins that do contain disulphide bonds, the bonds are generally intrachain, and rarely interchain. In alkaline phosphatase for example most of the disulphide bonds are intrachain and only a few are involved in crosslinking various chains. All disulphide bonds in proteins react with sulphite ($Na_2 SO_3$) in the presence of mercuric chloride and thus this reaction can be used to estimate the total number of disulphide bonds. But sulphite ions alone react with interchain S-S bonds, whereas the majority of intrachain bonds are not broken by the presence of sulphite. This approach to the estimation of disulphide bonds was used by Cecil and Wake in the case of the insulin molecule (141)

Many single chain proteins, both small and large contain an appreciable number of disulphide bonds and both pancreatic RNAse (30) and lysozyme (142) are noteworthy examples of small proteins with disulphide bonds. These enzymes exhibit unusual resistance to denaturing agents and their stability as well as their striking

capacity for reversibility of denaturation can be attributed to the disulphide crosslinks. (four each with respect to RNAse and lysozyme enzymes.)

Association and dissociation of protein structures

Studies of molecular weights of proteins have revealed a structural conformation above that of tertiary structure, which has been designated quaternary structure. Many proteins seem to exist as aggregates in solution and may be composed of several units to give one aggregate structure. Thus the quaternary structure is composed of aggregates of sub-units, where the term sub-unit is taken to consist of tertiary structured units.

An excellent example of the association of protein units to form an aggregate quaternary structure is that of crystalline yeast hexokinase. This enzyme was reported (143) to have a molecular weight of 95,000 and to be inactivated in solutions of high or low pH with a concomitant dissociation into quarter molecules or sub-units with a molecular weight of 24,000. The enzyme, which exists in solution as essentially globular, compact and highly ordered particles, loses a large part of its helical conformation when it dissociates in alkali or acid, but very little of its tertiary structure.

On neutralisation with acid or alkali, inactivated enzyme rapidly regains lost activity to the extent of 80% of its original activity, and the chemical, physical and biological properties are

identical with those of the native molecule.

The yeast hexokinase molecule in its native state exists as a reversible mixture of whole and half molecules and the active half molecule is favoured at pH values above 5.5 in the presence of salt.

Besides aggregation of sub-units within the structure of one enzyme (homo-association), there is a similar aggregation of different enzymes (which in turn may have quaternary structure) to form multi enzyme aggregations or complexes (heteroassociation) which can be dissociated much in the same way as can single enzyme aggregates. A particularly spectacular example (144) of a multi-enzyme complex is found among the enzyme systems that catalyse the lipoic acid mediated oxidative decarboxylation of pyruvate both from pigeon breast muscle and from E.coli, and have molecular weights of 3.4 and 4.8 million respectively. Similar complexes to this pyruvate dehydrogenation complex have been found which catalyse α-ketoglutarate decomposition.

The E.coli pyruvate dehydrogenation complex was separated with the aid of urea, and is composed of at least three enzymic components:-

Pyruvate dehydrogenation complex (P.D.C.)

1. Pyruvic carboxylase
2. Lipoic reductase - transacetylase (L.R.T.)
3. Dihydrolipoic dehydrogenase - a flavoprotein (D.H.D.)

It is not certain whether LRT comprises two enzymes, a

reductive acetylase and transacetylase, or whether both activities reside on the same enzyme. The pyruvate dehydrogenation complex (P.D.C.) of E.coli was separated into the flavoprotein component (DHD) and a complex of LRT, and pyruvic carboxylase on calcium phosphate gel in the presence of 4M urea.

The three isolated components could be combined to reconstitute the original P.D.C. with composition and enzymic activities resembling the original. L.R.T. is itself an aggregate of 64 subunits of molecular weight 26,000 per subunit and this can be dissociated and reconstituted also, and when reconstituted LRT are combined with DHD and pyruvic carboxylase in the correct amounts a PDC is similarly produced with molecular weight etc. corresponding to native PDC.

Similar separation (145) and reconstitution of the E.coli coenzyme A and diphosphopyridine nucleotide linked α-keto-glutarate oxidation soluble complex of enzymes was reported. The complex was similarly dissociated in the presence of urea, and then the three components reconstituted to a large unit of molecular weight 2.4 million resembling the native α-keto-glutarate dehydrogenase complex in all respects.

One can imagine that the next step is aggregation of aggregates until eventually the order becomes so high that aggregates of mitochondrial dimensions could be visualized. The aggregation is not a random process it seems, and aggregates are thought to have specific sites of attachment because of the fact that some aggregates

can be dissociated, and then reconstituted to the same active aggregate which would be unlikely in a random association and at the same time give a molecule with exactly the same properties as the original molecule. Random association could be possibly imagined to occur in molecules where sub-units were active, but not in examples such as yeast hexokinase and the E.coli pyruvate dehydrogenation complex. Association structure is thought to be defined by the amino-acid sequence and primary structure together with secondary and tertiary structure factors. The bonding in quaternary or associated structures is discussed below.

Bonding in associated structures

1. Hydrogen bonding

Hydrogen bonding between sub-units is the most frequently evoked force in protein association. The action of urea and guanidinium salts are both assumed to exert their dissociating effects by elimination of hydrogen bonding. Experiments were carried out on a series of oligomeric peptides in a non-polar solution of dioxane (146) and an association of units occured as well as folding. As dioxane interacts very poorly with hydrogen bond acceptors, and does not promote extensive solvation, there would be ample opportunity for the promotion of inter- and intra- molecular hydrogen bonding. Association of the peptide lengths took place and it was proposed that hydrogen bonding was responsible for this.

But since this work was carried out with model systems its value is open to debate with respect to proteins in aqueous systems.

There has been little work done on providing direct proof of hydrogen bonding between sub-units, and most of the work done has involved the use of "denaturants" like urea and in such work there has been no control to prevent destruction of sub-unit structure following sub-unit dissociation or separation. Since hydrogen bonds undoubtedly play a part in stabilizing the helix structure of some proteins, and since this is usually somewhat unfolded to a random coil by the action of urea and guanidinium salts, it is tempting to assume that hydrogen bonds between sub-units have been broken.

However in the case of β-lactoglobulin (147) it is possible to "unfold" polypeptide arrays without concurrent dissociation. Treatment with 6M guanidinium chloride resulted in unfolding of some nature, whereas the monomer-dimer interconversion of β-lactoglobulin was not affected by the presence of 6M guanidinium chloride, indicating other types of bonding than hydrogen bonding are involved.

Hydrophobic or non-polar bonding

If hydrophobic bonding is to play any part in the binding of sub-units into aggregate formations, there must be areas of exposed non-polar groups to interact. For satisfactory hydrophobic bonding a substantial area of non-polar groups would have to be

exposed and this is not reflected in the findings of Kendrew (135) which indicate that in myoglobin most of the non-polar side chains are on the inside of the molecule. None the less the theory is currently held that hydrophobic bonding is thought to be a factor in bonding of associated structures. (148)

It is reasonable to consider all the known non-covalent forces as participating factors in the specific association of sub-units. However as in the maintenance of tertiary structure of a polypeptide chain, the present evidence indicates a more important role for hydrophobic bonding than for H-bonding or for electrostatic forces.

The use of urea as an agent which affects the association-dissociation equilibrium.

The list of agents which can effect dissociation or association is substantial. The effects of dilution, H^+ ion concentrations and electrolyte concentrations were recognized at an early stage. But the agents which seem to be more generally effective and most commonly used are urea, guanidinium salts and detergents.

The extraordinary solvent action of urea has been known for some time and was reported as far back as 1900 by Spiro (149) but as discussed on page 74 its true mode of action continues to remain unexplained. The principal theory is that urea is a competitive breaker of hydrogen bonds (100) and the denaturation of proteins in the presence of large concentrations of urea was ascribed by

Mirsky and Pauling (150) to the ability to "break" hydrogen bonds.

Studies on model compounds in urea solutions indicate that this is not the action of urea. For example, a study by Klotz and Stryker (151) using a model synthetic polymeric compound (a polyvinyl pyrrolidine) which had no hydrogen bonds to be disrupted, showed that this molecule behaved in much the same way as proteins in urea solutions; namely there was a parallel "denaturation" process (or group unmasking) to the denaturation that occurs in protein. However this action of urea on the model compound could not be attributed to a disruption of hydrogen bonds. Klotz and Stryker (151) suggest "solvent - (macromolecular) solute interaction" as a stabilizing influence and further suggest that a perturbation of this interaction by urea results in denaturation. Thus in this hypothesis the action of urea could be more directly concerned with the water than with protein.

Thus the efficacy of urea as a denaturant causing the severance of protein-protein hydrogen bonds with the preferential formation of protein-urea hydrogen bonds has been called into question and the hypothesis that urea breaks hydrophobic bonds and owes at least some of its denaturing capacity to this property has received some experimental support.

There is the possibility that urea has a small direct affect in weakening hydrophobic bonds. Schlenck (152) found that the solubility of n-valearic acid is markedly increased by saturating

the water with urea. The effects of urea on the solubility of non-polar compounds may well be associated with the ability of urea to form crystalline clathrates with hydrocarbons and straight chain aliphatic derivatives. When hydrocarbon material is introduced into an aqueous solution of urea, Waugh (153) visualizes that the urea molecules form a hydrogen bonded cage-like structure which extends in space in such a way as to utilize all the hydrogen bond forming groups on each urea molecule. The urea molecules thus form a hexagonal network, the continuous channels defined by the urea hexagons being large enough to accommodate an extended hydrocarbon chain.

Can the theory above be applied to the effect of urea on proteins? It must be noted that the complexes of hydrocarbons with urea involve compounds having sufficiently long unbranched hydrocarbon chains. Small branched chains and aromatic rings do not form crystalline complexes with urea, and since non-polar side chains of protein fall into this latter category, it might suggest that Waugh's theory (153) is not applicable. But it should be remembered that the side chains are of varied character and are situated closely together. Thus from the evidence of the complexes of hydrocarbons with urea, it can only be taken as suggesting that non-polar residues may be involved.

Whatever the prime action of urea is, the inter-peptide hydrogen bond approach has been the most prolific source of practical

work and research into the influence of various urea concentrations on protein.

Chromatography in the presence of urea

Wilson and Smith 1959 (154) reported using a 3M ⟶ 8M urea gradient elution at a low pH of 1.9 on an IRC-50 chromatographic separation of the alpha and beta peptide chains of horse globin. But the use of urea in chromatographic separation was dealt with more comprehensively by Cole (155) who in 1960 published a paper in which the possibilities and advantages of chromatographic separation of protein were investigated based on assignment to urea of H-bond breaking capabilities. One of the most successful chromatographic systems that had been used with respect to proteins was the carboxylic ion exchange resin Amberlite IRC-50, but elution analysis on this resin was only successfully achieved when applied to neutral and basic proteins. The reason for this was that other proteins of a more acidic nature were bound so tightly to the column, that their removal would mean salt and pH levels of such an order that would result in a serious risk of protein denaturation.

Boardman and Partridge (156) suggest that the relative irreversible binding of non-basic proteins to the ion-exchanger is due in part to hydrogen bonding and they explain that when the pH of the buffer is lowered enough to make the charge on the protein suitable for ion exchange, the buffer causes appreciable protonation of the carboxyl groups of the resin, which then bind each protein

molecule through hydrogen bonds. A buffer of high enough pH to avoid this hydrogen bonding would render the protein unsuitable for reversible ion exchange, while a buffer with a pH low enough for the ion exchange equilibrium would result in the hydrogen bonding problem.

There are two possibilities by which this problem can be overcome. One approach is to use resins containing a lower concentration of carboxyl groups such as carboxymethylcellulose (CM-cellulose), and the alternative approach is to reduce the multiple hydrogen bonding by use of a chemical such as urea, and it is the latter approach that was used by Cole (155) working with a model neutral protein which was available commercially in a pure form.

To ensure that urea did not interfere with the ion exchange process itself the basic protein pancreatic RNAse was also subjected to chromatography on IRC-50 in the presence of 8M urea, and it was found that the chromatographic pattern was essentially the same as the pattern obtained in the absence of urea. Pancreatic ribonuclease was chosen as a control because it was a basic protein and could easily be removed from the resin in a predicted pattern confirmed by previous research. (72) After it had been established that urea had no disturbing effect on ion exchange properties in the case of a basic protein, the study was extended to the neutral protein insulin. Chromatography was carried out using a combination of various pH values, urea concentrations, and buffer ionic strengths. It was found that although ion exchange appears to play an important

role, hydrogen bonding was not eliminated entirely by the presence of urea in the eluting buffer since by varying the pH at constant urea concentration there was an increasing spread of peaks with lowering pH, similarly at constant pH there was an increasing spread of peaks with lowering of urea concentrations.

Thus the chromatography of insulin on IRC-50 in the presence of urea was entirely satisfactory with respect to protein recovery, reproducibility and the resolution achieved was far superior to any previous resolutions of insulin and a third active form of insulin (not previously reported) was separated.

This unexpected high resolution revealed by ion exchange chromatography in the presence of urea was thought (155) to be dependent on the combined action of ion exchange and hydrogen bonding. Consequently there might be added to the usual power of ion exchange an increased power of discrimination among proteins according to their shapes, at least to the extent that molecular shapes determine quality and quantity of hydrogen bonding sites available to the resin. Chromatography in urea containing buffers might then be useful in studying intermediate forms of denatured proteins but it is obvious that proteins to which this method could be applied must be moderately resistant to complete denaturation by urea.

The adaptation of this use of urea in chromatographic separation to the basic RNAse of calf spleen was considered. The

resistance to denaturation by urea of bovine pancreatic RNAse, and its effects, is discussed below.

The effect of urea on Ribonuclease

The possible manners by which urea solution may induce the unfolding of protein molecules has already been discussed (page 170) and included are disruption of hydrophobic bonds, and urea binding at sites required for hydrogen bonding. But regardless of the manner in which urea acts on RNAse, it is not an all or none phenomenon, but it has been shown (157) that varying degrees of unfolding can take place.

Bovine pancreatic ribonuclease consists of a single chain arranged in a compact globular form with four disulphide S-S bridges and an example of evidence of localised denaturation in part of the RNAse molecule is that in the presence of 4M urea, one of the disulphide bonds becomes susceptible to attack, whilst in 8M urea all four disulphide bonds are cleaved in the presence of Na_2SO_3 for example. (158) But Resnick et al. reported (158) that although there was complete inactivation in 8M urea and rupture of all four S-S bonds by Na_2SO_3 , in the presence of 6M urea, 3.9 of the four disulphide bonds were cleaved and considerable activity remained. This pointed to the fact that more than disulphide bonds are involved, and inactivation in 8M urea in the presence of sulphite occurred as a result of subsequent alteration in the structurally weakened

molecule following the cleavage of disulphide bonds. The degree of tolerance to urea concentration, with respect to activity loss is dependent on temperature and pH to some extent (157) and maximal denaturation is attained with 8M urea at pH7.0 and 30°C, but at lower pH ranges or higher temperatures it is reached with lower concentrations of urea.

By recording the character and rate of change of U.V. absorption spectra of bovine pancreatic RNAse in various concentrtions of urea (157), results suggested that at concentrations of urea above 4M, exposure of both phenylalanine and tyrosine residues to the solvent accompanies denaturation. This is in agreement with the work of Harrington and Schellman (159) who found that the changes in the absorption maximum and extinction of ribonuclease in urea solution are compatible with those to be expected for the unmasking of about 2-3 tyrosine residues. The two reports (157 and 159) denote that the secondary structural features of bovine pancreatic RNAse are stabilized by tyrosine—carboxyl group interactions as well as by sulphide bridges, and in the presence of urea (or on denaturation) there is an unfolding of polypeptide chains.

Rotary dispersion studies suggest the presence of an alpha-helix in RNAse A (the main component from IRC-50 fractionation of commercial pancreatic RNAse, ref.160) and it seems reasonable to assume that unfolding in helical regions at least partially contributes to the observed changes in specific rotation at 336 mu by heat

and urea. (161)

Experiments by Anfinsen et al. (162) using rotary dispersion measurements together with viscosity, the rate of exchange of peptide bond hydrogen atoms, and enzymatic activity determinations, suggested that only a relatively small part of the RNAse molecule is directly involved in catalytic activity, and that in the conversion from the native to the extended form, this part, the active centre, may be protected from deleterious unfolding by restricting cross linkages. Indeed Resnick's paper (158) shows that when the S-S cross linkages are reduced in 8M urea, activity is at zero level, whilst under non-reducing conditions ribonuclease retains its activity at 8M urea concentrations.

The use of urea in CM-cellulose chromatography of basic calf spleen RNAse

In adapting the type of chromatography used in the presence of urea as used by Cole (155) to other proteins, several factors are involved. Although the establishemnt of conditions for ion exchange chromatography of proteins is usually at least partly empirical, this type of chromatography is even more so because of the added factor of urea concentration, which results in increased sensitivity of the technique. This effect of the presence of urea will only be realized after running a series of chromatograms at various concentrations of urea.

Although it may generally be predicted that all proteins

will show increased retention with decreasing pH or ionic strength, it may not be safely predicted that all components of the protein mixture will respond to the variation of urea concentration in the same way. For example one component of a protein mixture might be eluted from a column more and more rapidly as the urea concentration is increased, up to a point at which the protein molecule unfolds and is then eluted progressively (or suddenly) more slowly. Another component of that same protein mixture might not undergo any change of molecular folding over the range of urea concentration employed, and so would show a progressive decrease in elution volume as the urea concentration increases. Clearly the relative positions of the two protein peaks and even the order of elution of the two proteins might change with changing urea concentration.

Beard and Razzell (105) in 1964 reported that an isolated alkaline RNAse from liver (also by IRC-50 chromatography) showed stability in urea solutions which is also a property of pancreatic RNAse (104) and it was suggested that this kind of stability of RNAse in urea solutions might serve to facilitate chromatographic separations of purified enzymes from extraneous protein as was shown by Cole (155.)

With the work of Cole in mind, investigation of the action of various concentrations of urea during ion exchange chromatography of basic calf spleen RNAse was investigated using CM-cellulose instead of IRC-50 as a cation exchanger.

Subsequent to the work undertaken and described below, Roth and Hurley (163) using commercial pancreatic RNAse, investigated the effect of urea on the reaction between a RNAse inhibitor and RNAse. RNAse inhibitor is a protein which was discovered by Pirotte and Desreux (164) and partially purified by Roth et al. (106 and 107) and has shown to be widespread in mammalian species. Little is known about its mechanism of action but it seems clear that it combines primarily with RNAse rather than the enzyme substrate RNA.

If the reaction between the inhibitor and ribonuclease depends on the formation of hydrogen bonds between the two molecules, it would be expected that the presence of urea would lessen this association. This was found to be so and in the presence of 8M urea there was little or no inhibitor action. The action of 8M urea causes the unfolding of the RNAse molecule and thus it seems that a prerequisite of inhibitor-enzyme complex formation is the specific RNAse native conformation.

Heat treatment (91) should have destroyed any inhibitor, or alternatively D.E.A.E.-cellulose column chromatography (107) should have removed it but if perchance any enzyme-bound inhibitor is present in the preparation by the stage of CM-cellulose chromatography, the use of urea will encourage its dissociation from the RNAse molecule, thus causing a possible change in position of the enzyme elution peak, and is a possibility to be taken into account

when results are evaluated.

1. Procedure

In extraction - 4, CM-cellulose column chromatography was carried out in the presence of urea at concentrations of 2M, and 7M; and in extraction-3 similar chromatographic analysis was carried out at 6M and 8M urea concentrations. Extraction 5 will provide comparative examples of CM-cellulose chromatography in the absence of urea (fig 28). The use of 7M in preference to 8M urea at cold room temperatures was because of the difficulty of maintaining solution at 5°C or less.

The conditions and details of each column are summarized in the below table (fig. 38) For extra details of, for example, column height relative to reservoir height, see experimental section.

The columns were also run at varying salt gradients in an attempt to obtain a superior separation of activity region over the separation obtained using the standard CMC-I (page128) salt gradient. For example column CMC-4-7M-II had a gradient half as steep as the corresponding CMC-4-7M-I column, and the results are compared below (fig. 38)

The CM-cellulose columns of extraction 3, which are 6M and 8M with respect to urea were run at a constant temperature of about 18°C. Over a period of one week the maximum temperature variation

Column	CMC-4 -2M-I	CMC-4 -2M-II	CMC-4 -7M-I	CMC-4 -7M-II	CMC-3 -6M	CMC-3 -8M
Urea conc.	2M	2M	7M	7M	6M	8M
Fract. size	I0mls	I0	I0	I0	I0	I0
NaCl gradient	0.32M	0.25M	0.25M	0.I5M	0.32M	0.I6M
pH gradient	6.o-8.2	7.0-8.2	6.0-8.2	7.0-8.2	7.0-8.2	7.0-8.2
Position IM NaCl applied	I40	—	93	II0	—	—
Column size	68 x I.7cms	50 x 2.2cms	68 x I.7cms	68 x I.7cms	5I x I.7cms	5I x I.7cms
Protein applied	359 mgs	III mgs	2I8 mgs	200 mgs	I43 mgs	approx. I43 mgs
% retain-ed by CM-cell.	43%	46%	40%	—	—	—
Reserv-oir size	I.5l	I.5l	I.5l	I.5l	I.0l	I.0l
Const. vol.chb. size	0.8l	0.8l	0.8l	0.8l	0.5l	0.5l
Comment	—	—	—	Stirred with 7M urea over night	Chromatography at room temp.	

Fig.38. Table of details of columns run at various urea concentrations. Page I97.

as registered on a thermograph was 3°C. Column chromatography at this temperature was not by design, but due to cold room failure. However the results have been included since the comparison may be of interest as temperature is more than likely to be a factor in the behaviour of a protein at high urea concentrations. Temperature is also an important factor in maintenance of urea solutions free from cyanate contamination. (165)

Cyanate and urea have been long known to comprise an equilibrium pair, and at equilibrium an 8M urea solution may be about 0.02M with respect to cyanate. Under moderate conditions cyanate has the ability to react with amino groups to yield carbamyl derivatives. Exposure of ribonuclease to cyanate in aqueous or urea solutionswas found to lead (165) to a considerable loss of enzymatic activity with a concomitant formation of ε-carbamyl lysine residues, which would thus result in alteration of column adsorption properties and change of chromatographic elution profiles.

Cyanate reacts even more rapidly with -SH groups in proteins than amino groups. When cysteine was treated with an equivalent amount of cyanate, S-carbamylcysteine resulted. Thus when urea is used as a reagent to bring about only physical changes in a protein, special attention should be given to the use of urea as free as possible of cyanate. Pancreatic RNAse however is devoid of -SH groups and notably stable, but inactivation may be possible after long exposure at moderate temperature to high concentrations

of urea.

Thus in the case of the spleen ribonuclease under investigation the following precautions in the use of urea solutions were observed:-

1) Urea solutions (especially those of higher concentration) were made up in fresh batches shortly before use.
2) These solutions were stored at a low temperature ($5^{o}C$)
3) "Analar" grade urea was used and filtered through Whatman No. 1 filter paper in preparation of urea containing buffers.

Starke et al. (165) found that urea solutions made from reagent grade urea even, kept for several weeks in the cold without any appreciable formation of cyanate.

As will be seen from fig. 38, page 197 for reproducibility and comparison the same columns were used for different urea concentrations. This was achieved by eluting with approximately five litres of buffer at the urea concentration to be used. Thus a change of buffer from 2M to 7M urea was achieved without having to repack the column and so giving rise to anomalous results due to possible differences in column packing (although this was avoided as much as possible by a standardized procedure already described - page 44). In extractions 3 and 4, the protein solution to be chromatographed on CM-cellulose was already at 2M urea concentrtion and addition to a 2M urea CM-cellulose column presented no

difficulty. But when columns at higher urea concentrations were being used the protein sample to be applied was brought up to the desired urea concentration by the addition of solid "Analar" urea slowly with constant gentle stirring, and then immediately applied as soon as temperature equilibrium (urea dissolution is endothermic) and complete dissolution had been achieved. One exception to this routine was CMC-4-7M-I (fig. 41) where the protein solution was stirred overnight after the urea concentration had been brought to 7M and then applied to the column head. This procedure was experimental in order to test if the unfolding of peptide chains occurring at high urea concentrations might be a somewhat slow process and also to allow reaction of urea with the protein molecules in the absence of competing effects of the ion exchanger.

3. Discussion of results - a) 2M urea conditions

It should be remembered that enzyme material used in these experiments with urea containing buffers and CM-cellulose chromatography, was prepared from the initial extraction stage in the presence of 2M urea.

As discussed on page I32 , CMC-4-2M-I (fig no. 39) or CMC-4 exhibit the characteristic two principal peaks of activity as shown by CMC-I (page I31). However whether these two peaks obtained by chromatography in the presence of 2M urea, can be equated with the activity regions A and B obtained in the absence of urea (columns CMC-I and CMC-5-I page I33) is a question that can not be unequi-

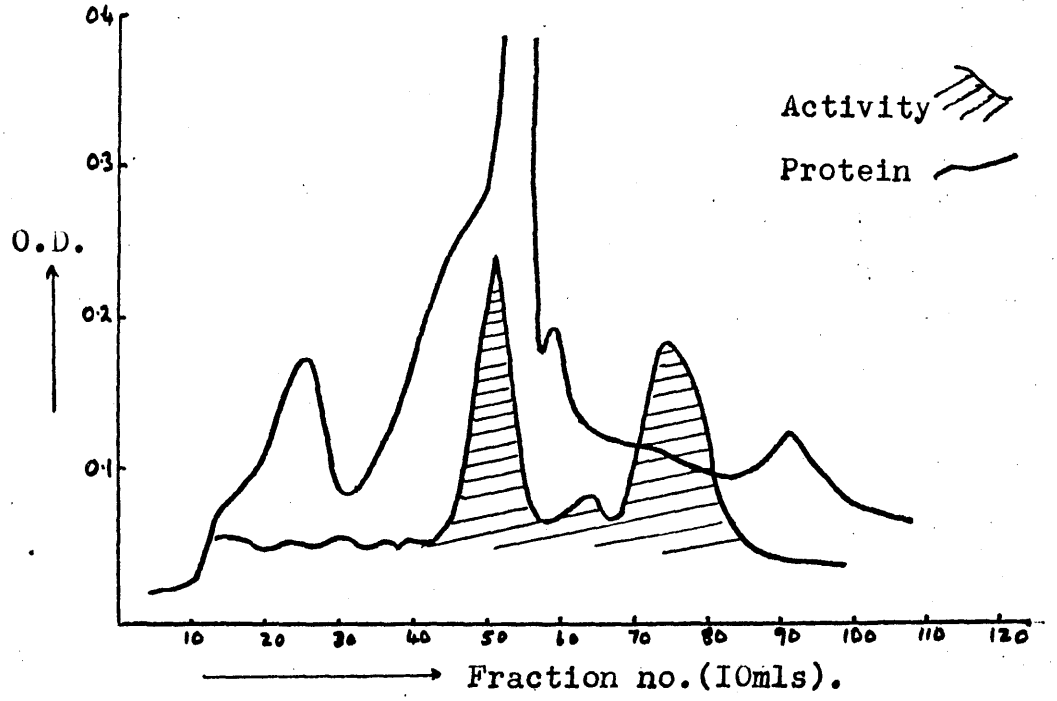


Fig.39. CMC-4-2M-I (see CMC-4,Fig.27.)

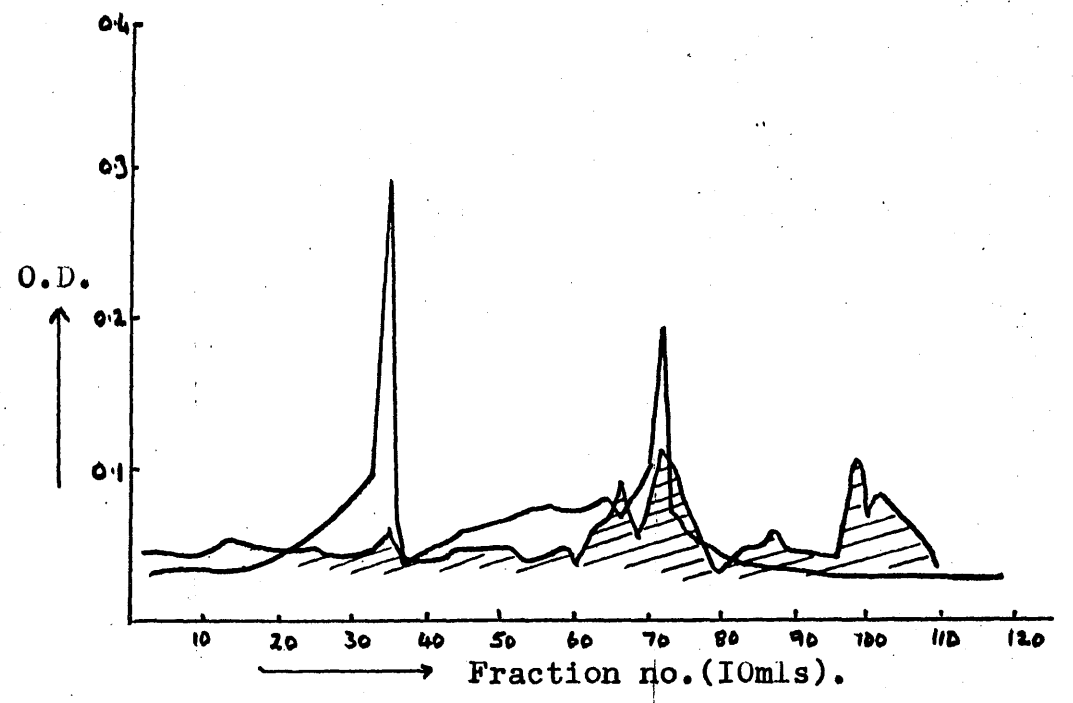


Fig.40. CMC-4-2M-II.

-vocally answered on the evidence available. Whatever the identity of these peaks there has been a shift of the elution profile towards earlier elution of the activity peaks (page 134). This shift could be explained in a number of ways of which perhaps the most straight-forward explanation is that the advancement of the elution position was due to the presence of urea reducing hydrogen bonding between ~~resin~~ exchanger and protein.

The presence of urea at lower levels may contribute to resolution since there is excellent separation of the two activities and as explained previously (page 190) this may be appropriate to the combined action of ion exchange and hydrogen bonding with any increased power of discrimination on elution related to the extent that protein molecular shapes determine quality and quantity of hydrogen bonding sites available to the resin.

There seems no evidence for any dissociation to smaller active units, unless this had taken place in a way as to coincidently remain in such a form as to continue to be eluted as two activity peaks.

The counterpart column CMC-4-2M-II (fig no. 40) presented a somewhat congruous elution profile to CMC-4-2M-I (fig no. 39) with respect to both activity and protein, thus confirming the pattern of the former. The second column (CMC-4-2M-II) differed in that the length of the column was 26.5% shorter, although 22% wider in diameter and also subject to a shallower gradient (see fig 38, page

197). Such an elution-gradient delayed elution and caused spread of activity peaks. The two main activity regions were again apparent but the shallower elution had also resulted in some division of one peak and so it might be more precise to quote there being three main activity peaks.

The initial non-active protein peak of CMC-4-2M-I showed the presence of a small amount of associated activity in the correspondingly eluted peak of CMC-4-2M-II. Protein eluted at this point (fractions 25-40 of CMC-4-2M-II) is unlikely to be very basic and/or be very large.

b) 7M urea conditions

CMC-4-7M-I and II (figs 41, 42, page 204) are directly comparable to CMC-4-2M-I because column sizes and enzyme source were exactly the same.

The outstanding influence is that the presence of higher concentrations of urea have a definite effect on CMC-chromatography of RNAse. Although the amount of protein applied to CMC-4-7M-I was no greater (less in fact) than to CMC-4-2M-I, there was apparently a significantly greater proportion of activity eluted in the presence of 7M urea than 2M urea. The presence of urea may affect the U.V. optical density reading at 280 mu for protein, but the urea concentration present during assay procedure in the final diluted sample read at 260 mu for soluble nucleotides, was negligible and unlikely to account for the relative increase in RNAse activity. A possible explanation is a high concentration of urea acting by reduction of

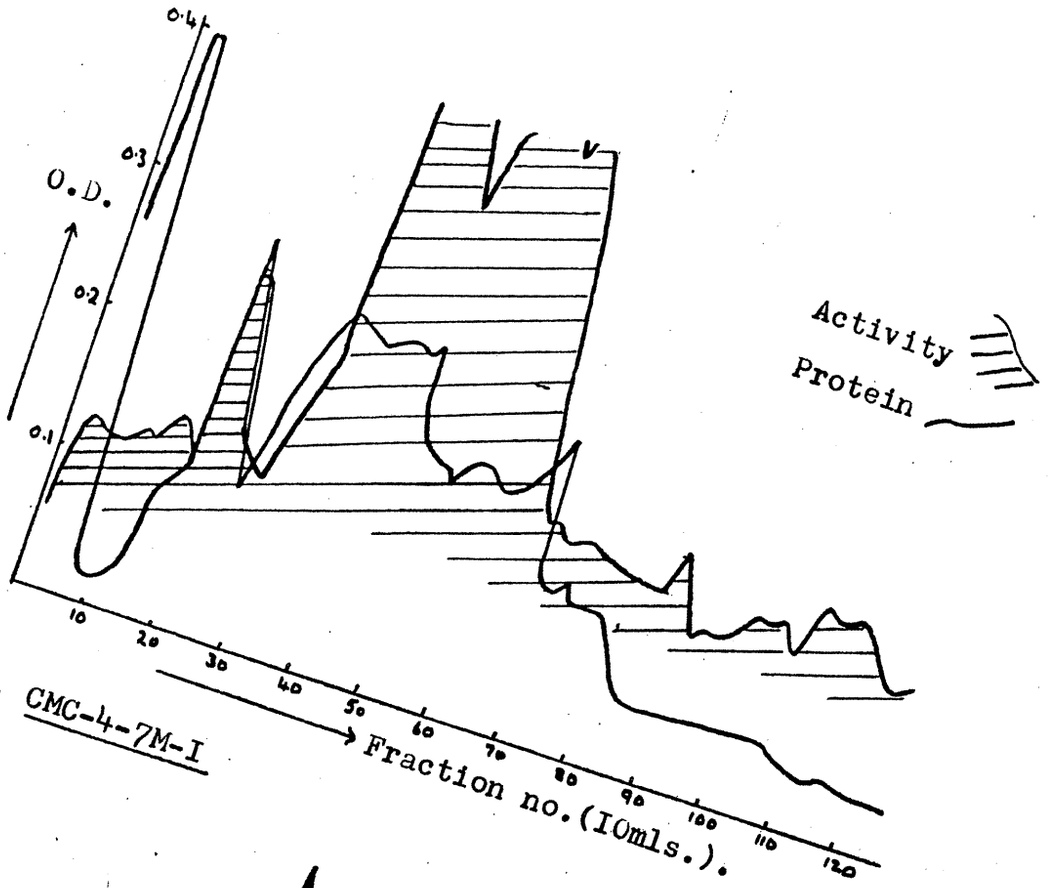


Fig.4I. CMC-4-7M-I

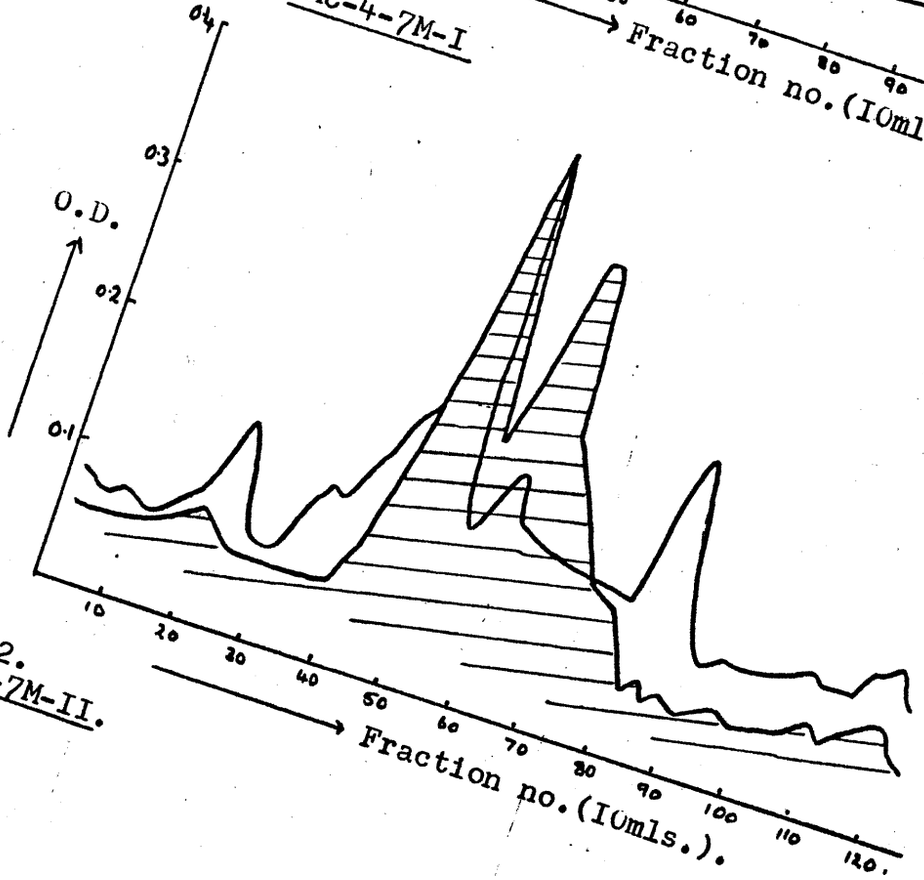


Fig.42.
CMC-4-7M-II.

hydrogen bonding brought about a dissociation of RNAse-inhibitor complexes, and thus giving rise to an increase in RNAse activity (ref 163, page 195). Likewise columns CMC-4-7M-II and CMC-3-6M and 8M exhibit a large activity in relation to the amount of protein applied (see figs. 42, 43, 44 and 38.) as compared with CMC-4-2M-I. However only the column applications from extraction 4 are strictly comparable with respect to this increase in RNAse activity at high concentrations of urea.

A similar stimulation of RNAse activity in 8M urea concentrations using bovine pancreatic RNAse was reported by Klee and Richards 1957 (185). They found that the presence of urea at 8M concentration increased the rate of hydrolysis of RNA twofold, but on the hydrolysis of smaller substrates like U-2', 3'-P and C-2', 3'-P the hydrolysis rate in the presence of 8M urea was actually reduced. This anomaly was not explained but pointed to the fact that the action of urea is a complicated one and not fully understood as yet.

Separation in the presence of 7M urea showed a tendency towards distinct merging of the two main activity peaks, into one bifurcated large activity region spread over forty fractions. Both columns at 7M urea demonstrated this tendency, although there was more separation in CMC-4-7M-II owing to a shallower elution gradient. The initial protein peak eluting between fractions 20-30 or so, present in all of the urea columns from extraction 4, showed a

definite tendency to be associated with more activity at higher urea concentrations. But once again it cannot be assumed that the peak at fraction 20 to 30 eluted at 2M concentration can be equated with those eluted at 7M urea levels.

The treatment given to CMC-4-7M-II of overnight stirring in the presence of 7M urea did not seem to have manifested itself to any significant extent in the results. Presumably the unfolding action of the RNAse molecule that occurs in solutions of high urea concentrations (159) is a reasonably rapid process, at least to the extent defined by the limits of this experiment.

Summary to use of urea as an aid to protein aggregation studies.

CM-cellulose chromatography of calf spleen RNAse at both 2M and 7M urea levels, although different, none the less demonstrated considerable similarity when protein and activity profiles were compared (figs 39,40 cf. 41, 42) The presence of urea did not affect the elution of two main peaks of activity that were normally obtained in absence of urea. (fig 24 cf. 39), but the urea presence did result in earlier elution of activity regions (see page 202). None the less the presence of higher concentrations of urea (7M) resulted in a less defined separation into the two characteristic peaks of activity (fig 39 cf. 41). On the evidence available, it can not be unequivocally stated that the two main peaks of activity eluted in the presence of urea are the same as obtained in its

absence (i.e. peaks A and B of CMC-I fig. 24 for example)

c) 6 and 8M urea conditions - Extraction 3 (figs. 43, 44)

Elution profile from chromatography in the presence of 6 and 8M urea of extraction 3 did not share this same overall likeness shared by those profiles of extraction 4 using high urea concentrations.

No doubt, one of the main contributing reasons was that in the case of extraction 3, chromatography was carried out at a room temperature of 18°C. The higher temperature increased the risk of cyanate formation (page 198 ref 165) with the concomitant adaptation of lysine residues with carbamyl groups and alteration of adsorption properties and change in chromatographic elution profile (165).

The result obtained from column 3-8M (CMC-3-8M) was of no great value because of a failure in fraction collection and the resulting loss of 24 fractions. But it was useful in as much as it showed the effect of 8M urea was somewhat different in that there was a large inactive portion of protein eluted initially. This result could also be caused by cyanate inactivation of enzymatic properties.

B. LEUCINE

Use of DL-leucine in RNAse aggregation studies with CM-cellulose column chromatography

In 1962 Klotz and Franzen (167) studied aggregation by means of the small model compound N-methyl acetamide $CH_3-\overset{O}{\overset{\|}{C}}-NH-CH_3$,

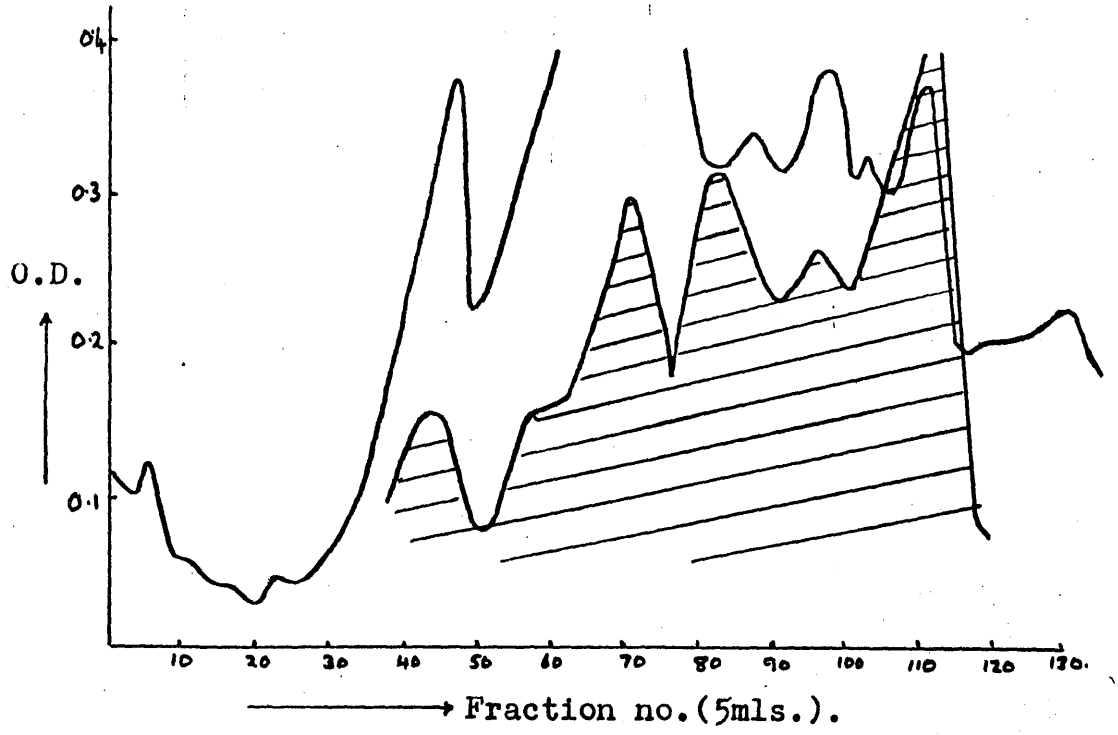


Fig.43. CMC-3-6M.

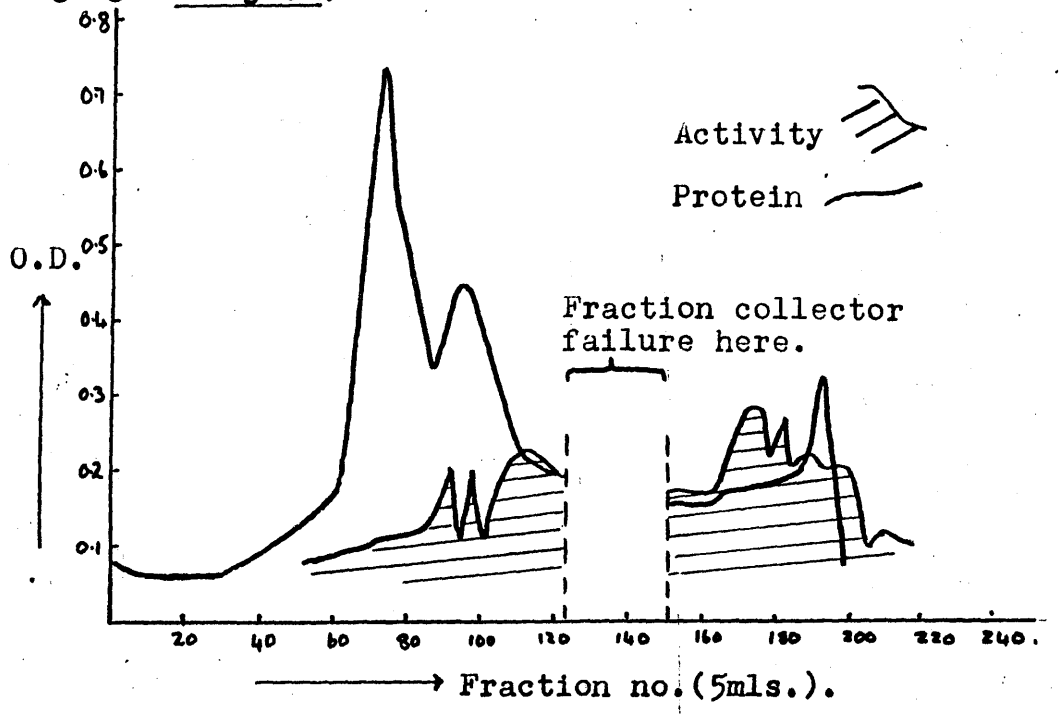


Fig.44. CMC-3-8M.

through the formation of interamide bonds in the presence of various solvents (water, dioxane and carbon tetrachloride), and the conclusion was reached that the intrinsic stability of interpeptide hydrogen bonds in aqueous solution must be very small. Results using this model system indicated that for protein molecules in aqueous solution interpeptide hydrogen bonds cannot contribute significantly to the stabilization of macromolecular organisation, except perhaps in a few regions with a very low dielectric constant due to a specific high concentration of hydrocarbon-like residues. It was shown that the dielectric constant per se did not exert a dominant influence on the stability of amide hydrogen bonds, but rather the crucial feature seemed to be the chemical nature of the solvent. Other forces than hydrogen bonding seem to be primarily responsible for the maintenance of protein configuration in aqueous environments.

Note on dielectric constant

The magnitude of the force acting between two given electric charges placed at a definite distance apart in a uniform medium is determined by a property of the latter (the medium) known as the dielectric constant. Non-polar liquids have very low dielectric constants and polar liquids such as water and hydrocyanic acid have the highest known values for pure liquids, but an aqueous solution saturated with glycine however has a much higher

value still, and other amino-acids and peptides give solutions with a dielectric constant in excess of glycine and water.

Examples of dielectric constants at 20°C (174)

Vacuum	1.00	Acetone	21.4
Hexane	1.87	Methanol	24.0
Octane	1.96		
Benzene	2.28	Water	80.0
Toluene	2.39	Hydrocyanic acid	116.0
Chloroform	5.05	2.5M glycine in water	137.0

Discussion of molecular structure and hydrophobic nature

In an appraisal of the hydrophobic bond on page 175 it was mentioned that after several decades of absolute reliance on the peptide hydrogen bond as the main force in contributing to the stability of folded polypeptide chains in globular proteins, the hydrophobic bond had taken its place as the accepted principal non-covalent attractive force in stabilizing of folded chains. The importance of non-polar side chains, such as alanine, valine and leucine, iso-leucine and phenylalanine is now being appreciated. These side chain molecules tend to cluster together in the interior of the protein molecule where they assume stable native configurations as shown by Kendrew's work on myoglobin. (135, 136)

Kendrew found that three quarters of the peptide chain was in the form of an α-helix with virtually all the polar residues

on the surface and the non-polar residues arranged in the interior of the molecule, thus producing a structure analogous to a soap micelle (97). Polar surface side chains were thought rarely to interact with one another and on general grounds one would expect a polar side chain to be just as content to interact with a water molecule or ion in the ambient solution, as to form a link with one of its neighbours, so that these latter interactions would not effectively contribute to the stability of the structure.

But myoglobin may not be typical of all protein molecules and most proteins are probably less helical than this, for example pepsin, γ-globulin, and carbonic anhydrase scarcely have any helix. Fisher (166) showed that by a mathematical consideration of hydrocarbon areas in relation to polar areas in a protein molecule, that the reversal of both the unfolding of single polypeptide chains and of the dissociation of enzymes into subunits has proved that the primary structure of polypeptide chains can itself constitute the coding of higher order structures of a protein molecule. The equation derived expresses the fact that the ratio of polar to non-polar residues in a peptide chain, taken together with the size of that chain, in itself completely defines the surface area of the tertiary structure and has at least something to say as to whether or not there will be a quaternary structure. The lower limit of the molecular weight, for a possible significant non-polar centre, to define a molecule may be about 7,000.

As long ago as 1935 Edsall (169) noticed the striking effect of hydrocarbon chains in water of increasing heat capacity. In a homologous series of pure organic liquids, alcohols, ketones and fatty acids, each added methylene group ($-CH_2$) increased the heat capacity by about 5 or 6 cal. degree^{-1} mole^{-1}, but when the substances were dissolved in water the effect of an added $-CH_2$ group was 3 or 4 times as great in increasing the apparent molal heat capacity. The introduction of hydrocarbons to water is accompanied by a large evolution of heat and decrease in entropy and therefore the solubility of hydrocarbons is essentially an entropy effect.

The theory of the reaction of hydrocarbon groupings in water was greatly clarified by Frank and Evans (1945) (132) who pointed out that all the evidence favoured the view that the introduction of hydrocarbon groups into water actually promoted hydrogen bonding of water molecules in the immediate vicinity, or led to "iceberg" formation. This essentially means that the water structure close to the hydrocarbon group becomes more ordered in a similar manner as it does in ice formation, and likewise as in freezing, heat is evolved and entropy decreases when hydrocarbon material is introduced to water. The heat capacity increases also as more energy is required to "melt" this ordered structure.

These ideas were further developed but from slightly different directions by Kauzmann (148) and Klotz (170) as discussed in the introduction to this section on page 175 onwards.

Wishnia and Pinder (171) investigated the role of hydrophobic forces in bovine serum albumin (B.S.A.) from the point of view of the solubility of short chain alkanes ($C_2 \longrightarrow C_5$) in a solution of BSA, and the nature of the structure that allows binding of alkanes. The most likely sites of alkane binding in proteins are structures involving apolar amino-acid side chains directly.

The mechanism of binding of the hydrocarbon molecules to the protein can be approached from two view points. Kauzmann would approach the problem from the point of view that the hydrocarbon molecule would require direct contact with some apolar region of the molecule, with the accompaniment of a net decrease in water-hydrocarbon contact. In the second case, as it would be approached by Klotz, a layer of water molecules intervenes, and the interaction is between the alkane and partial clathrate cages surrounding the apolar residues on the protein surface.

Thus the models for these two approaches are respectively

- transfer of alkane from water to detergent micelles (172)
- transfer to crystalline gas hydrates (173)

These models are of course extreme, and the binding of the hydrocarbon is more than likely a combination of these two extremes and the binding might be considered as taking any of the below forms:-

- penetration of the alkane into the protein interior
- penetration into surface clusters

- binding in a surface crevice
- binding on the protein surface with the formation of a more favourable ice cage
- binding with completion of a partially formed clathrate cage.

But Wishnia and Pinder (171) came to the conclusion that binding sites in BSA molecule are for the most part via apolar side chains of interior regions of the molecule.

Cohn, McMeekin and Edsall (174) had shown the systematic influence of added methylene groups or aromatic rings in increasing the relative solubility of molecules in organic solvents as compared to water. If it is assumed that the side chain groups of unfolded peptide chains interact with water in the same way as they do in simpler molecules, then it is clear that the chief stabilizing force that drives peptide chains to assume the compact globular conformation is the tendency of the non-polar side chains to get away from the water and cluster together in the interior of the compact molecule (175)

Thus using this above concept of the importance of hydrophobic bonding and the interpretation of its importance in terms of the presence of molecular centres of an essentially non-polar nature, further strategy with respect to protein aggregation investigations was formulated. If it is assumed (as it was on page 185) that much the same forces are responsible for intermolecular bonding

(association structure) as are for intramolecular bonding, an immediate problem arises with respect to the role of hydrophobic bond in the respect.

For satisfactory hydrophobic bonding, a substantial area of exposed non-polar groups must be available to interact, but this was not reflected in Kendrew's work on the myoglobin molecule. However hydrophobic bonding is none the less thought to be a factor in bonding of associated structures (148). Certainly the interpretation of Klotz of the hydrophobic bond would be a more convenient hypothesis in the consideration of association of protein molecules.

In the association of two protein molecules, it is possible to envisage a mutual shift in the hydrocarbon region of each molecule to form a more central "combined" or "shared" apolar centre. Binding at a molecular level may also place greater emphasis on doubtful or "fringe" amino acids such as proline, tryptophan and tyrosine, which are of a less polar nature, and thus play a more important role in hydrophobic bonding.

Besides the use of urea to bring about a dissociation (and also an unfolding of peptide chains within the molecule) of molecular aggregates by the disruption of interpeptide hydrogen bonds, another approach to dissociation is via disruption of the hydrophobic forces. Urea is however thought to have some effect on hydrophobic forces also (see page 186). In the light of the nature of hydrophobic

forces, the obvious agent is one that will have a solubility in regions of non-polar nature, yet also be soluble in aqueous solution, so allowing a dispersal of the dissociated units. Agents encompassing these properties include the soaps and detergents. But detergents as such have generally speaking, proved unsatisfactory for protein work (page 69). Thus what was wanted was a compound with the detergent type properties of a hydrocarbon, a non-polar moiety that would allow solubility in non-polar areas, and a polar moiety also to allow dissolution in aqueous environments, but without the harsher denaturing tendencies of commercial detergents.

Thus the use of an amino acid with a non-polar side chain was contemplated. Since such side chains are thought to play an integral part in hydrophobic molecular binding forces, solubility in such areas of high concentration of this type of bonding should be easy for a molecule bearing one of these groups.

Amino-acids are also ideal in that they have a water soluble dipolar grouping and so giving the compound a detergent like structure. Size is another characteristic that favours the use of an amino-acid, since large commercial detergents such as sodium dodecyl sulphate may have difficulty in entering some of the smallest restricted protein peptide chain inclusions. In association studies the solute could presumably——

a) Influence the association of protein molecules through direct competition at the binding site.

b) Introduce conformational changes in the protein in the vicinity of the site and reduce or destroy its ability to bind.

c) Modify the properties of the solvent water and bring about changes in association forces on this basis.

It was from the point of view of modification of the properties of the solvent water that the use of an amino acid was also chosen besides its detergent properties.

When a molecule possessing a dipolar nature is dissolved in water the dielectric constant is much higher than that of the pure solvent. For example, the dielectric constant of water is 80 and that of the water made 2.5M with respect to glycine is 137 (see page 210). The dielectric increment caused by glycine in this case is characteristic of the substance and related to its polarity. The choice of amino acid with respect to size of dielectric increment has little bearing on the number of methylene $-CH_2$ groups, as it does in a homologous series of alcohols for example, because amino-acids are dipolar.

Examples of dielectric increments for some amino acids are given below. (177)

Glycine	22.6	β-alanine	25.0	l-α-leucine	25.0
α-Alanine	23.2	dl-α-valine	25.0	l-asparagine	28.4
l-glutamine	20.8				
d-argenine	62.0				

An example of the usefulness of dielectric constant was given by R. B. Rennel (178) in which to the effect of sodium chloride on the solubility of cysteine, is added the effect of another amino acid, - glycine. The higher the glycine concentration rises the smaller the interaction of the salt and cysteine. At sufficiently high glycine concentration, and therefore sufficiently high dielectric constant, interaction due to electrostatic forces substantially vanish (180). Conversely at a low dielectric constant the reverse is true. Thus liquids of low polarity with low dielectric constants such as acetone are often used in protein precipitation to decrease the dielectric constant of the medium, thus increasing the electrostatic forces between charged groups of the same as well as neighbouring molecules, and so aiding protein aggregation and precipitation.

Although the dielectric constants of body fluids and tissues are imperfectly known, they are obviously very high and if the dielectric constant of protein solutions in vitro are raised this will tend to increase stability, Thus it was thought conceivable that the presence of an amino-acid, and therefore a high dielectric constant in solution would, if not actually bring about protein dissociation, certainly would aid the action of other dissociating forces such as urea and the detergent effect of the amino-acid.

On page 209 it was mentioned that hydrogen bonding was possible in regions of low dielectric constant. A rise in the

dielectric constant by the introduction of an amino-acid into the solution in which the protein was dissolved, should reduce any intermolecular hydrogen bonding to a minimum, thus aiding dispersal of molecular units.

If the chief stabilizing force that drives peptide chains to assume compact globular conformation is the tendency of the non-polar side chains to get away from water (175), then the presence of molecules of hydrocarbon nature in the solvent should reduce this tendency. The folding of polypeptide chains due to hydrophobic bonding is brought about by a gain in entropy resulting when the side chains leave the aqueous phase and the water becomes more disordered (entropy being a measure of randomness or disorder). So the introduction of molecules with a hydrocarbon nature to water is accompanied by a decrease in entropy (132) which is the reverse of the force causing folding of peptide chains; and so the addition of an amino-acid with a large hydrocarbon moiety as possible should reduce the tendency to hydrophobic bonding and therefore tend to encourage inter-molecular dissociation (and also unfolding of the polypeptide chain)

In the addition of an amino-acid to the buffer solution during CMC-column chromatography, a dual role was envisaged:-

1. Firstly one of a detergent nature
2. Secondly - a modification of the solvent properties of water by a change of the dielectric constant.

Choice of amino acid

The choice of amino-acid was linked to the following requirements:-

1. It must have aneutral reaction, otherwise complications with respect to salt linkage formation on both the protein and the column absorbent may arise.
2. It must be aliphatic with hydrocarbon side chain as long as possible to confer detergent properties.
3. It must be reasonably soluble in water.
4. Hydroxy- and thio-amino-acids were also avoided because of the specific characteristics of these groups.)

If the above conditions are observed, the amino-acids in fig 45 may be considered for use in chromatographic considerations

The differences in dielectric increments (177) showed very small variation with respect to chain length and were not considered as important with respect to amino-acid choice.

Since nor-leucine had the longest hydrocarbon chain, this amino-acid was chosen, and even though the solubility in water is not very high, it was thought to be sufficient to attain the desired effects.

The nor-leucine source was British Drug Houses, biochemical grade dl-nor-leucine.

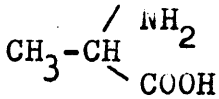
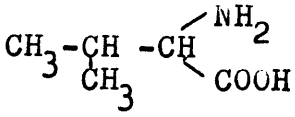
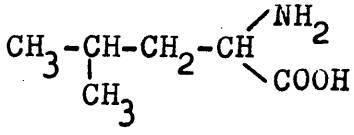
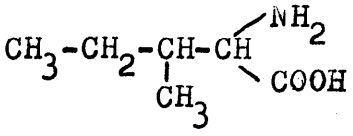
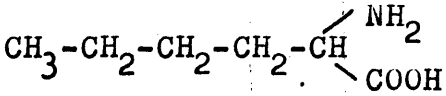
Amino-acid.	Formula.	Solubility in gms/100gm H_2O at 25°C.
DL-alanine		16.72 *
DL-valine		7.09 *
DL-leucine		0.99 *
DL-isoleucine		2.23 *
DL-norleucine		1.15 **

Fig.45. Possible choices of amino-acid. * Ref.181

** Ref.182

Procedure - for the use of nor-leucine with column chromatography on CM-cellulose

Note (i) all references to 'leucine' refer to DL-nor-leucine in the below text.

(ii) Leucine-I and -II refer to columns: CMC-4-2M-leu-I and -II respectively (figs 47, 48)

The source of enzyme used for the column chromatography in the presence of leucine was that from extraction 4 and thus these columns were directly comparable with all other column chromatography results from extraction 4 on CM-cellulose, (CMC-4)-see page197 fig 38. Similarly columns of the same dimensions were used to aid this comparison, and thus detection of any differences in elution profile due to the presence of leucine should be facilitated. The data concerning the chromatography in the presence of leucine is summarized in fig 46.

Columns CMC-4-2M-Leu I and II - General conditions

Throughout all stages of the protein application and elution of the protein on CM-cellulose, the concentration of urea was maintained at 2M. It was decided to retain the omnipresence of urea since it was thought that any molecular dissociation (or unfolding of intramolecular chains) due to leucine action would be stabilized and aided by the presence of urea invoking the theory (page 185) of urea as a competitive breaker of hydrogen bonds (100) . Also as enzyme extraction 4 was carried out entirely in the presence of

Column code number	CMC-4-2M-LeuI	CMC-4-2M-Leu-II
Urea concentration	2M	2M
Fraction size	I0mls.	I0mls.
NaCl gradient	0.25M	0.I5M
pH gradient	pH 7.0 no gradient	pH 7.0-8.2
Position IM NaCl appld.	Fraction I80	Fraction I22
Column size	68 x I.7 cms.	68 x I.7 cms.
Protein applied	359 mgs.	359 mgs.
% protein retained	36%(I28 mgs.)	45%(I62 mgs.)
Fraction at which gradient started	92	I
Volume of reservoir	0.58l.	I.5l.
Volume of const.vol. chamber	0.8l.	0.8l.
Extra comments	Fraction I-9I leucine grad. only,not salt.	

Fig.46. Data for columns CMC-4-2M-Leu I & II.

2M urea and subsequent CM-cellulose chromatography was also carried out in the presence of urea at various concentrations (including 2M), urea was retained with the consideration that the results of chromatography in the presence of leucine should be directly comparable with corresponding results in which leucine did not feature.

As shown on page 221 , DL-nor-leucine has a solubility of 1.15 gms in 100 gms of water (or 11.5 gms/litre) at 25°C. However as all chromatographic work in extraction 4 was conducted at 4-5°C in a cold room, the solubility at this temperature was obtained by drawing a graph from solubility data for other temperatures (183) and reading off the solubility at 5°C.

°C TEMP.	0°	10°	20°	30°	40°	50°
SOLUBILITY in gms/1000 gms H_2O	8.4	9.4	10.7	12.4	14.5	17.3

Solubility of DL-nor-leucine (183)

Solubility at 5°C on the basis of these readings was 8.9 gms/1000 gms water = 0.068M leucine solution or 0.89% solution with respect to leucine. The leucine was maintained at saturation point in solution because firstly, this was not a very water soluble

amino-acid and as strong a solution as possible was wanted, and secondly for the sake of easy reproducibility of leucine concentration. A saturated solution of leucine at 5°C was achieved by dissolving excess leucine in the particular buffer at room temperature and then placing in the cold room and allowing excess leucine to precipitate out and decanting off most of the buffer to obtain a saturated solution of leucine, but not bothering about the presence of a little solid leucine that still remained since this would maintain and ensure a saturated state.

CMC-4-2M-Leu-I :- conditions

The protein in 630 mls of pH7.0, 2M urea buffer, was applied to a CM-cellulose column that had previously been equilibriated with 0.005M Tris, 10^{-4}M EDTA, 2M urea, pH7.0 buffer, and washed with a little of the same buffer after application.

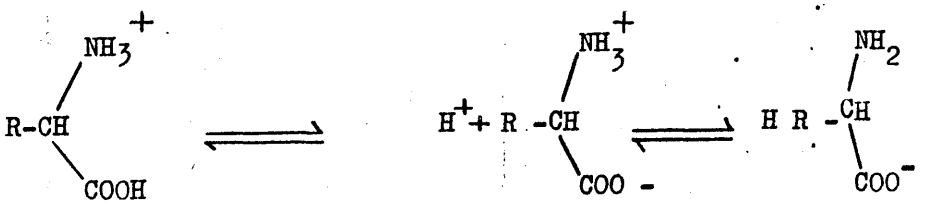
The column was then subjected to a leucine gradient in which the concentration of leucine in the buffer being passed throught the column, was increased from zero to saturation concentration at 5°C (0.068M leucine). Optical density measurements in the U. V. spectrophotometer at 280 mu showed that the increase of leucine concentration alone caused no elution of the applied protein material.

Gradient elution buffers of CMC-4-2M- Leu I

Reservoir 1.5l.		Constant volume mixing chamber 800 mls
0.005 M Tris	LEUCINE GRADIENT →	0.005M Tris
10^{-4}M EDTA		10^{-4}M EDTA
2M urea		2M urea
pH7.0 saturated leucine		pH7.0

In strongly acid solution, all amino-acids are positively charged and act as cations, but in strongly alkaline solutions all are negatively charged and act as anions. But for any amino-acid there is some intermediate pH where the average net charge on the ampholyte molecule is zero; and this pH is known as the iso-electric point (pI) of the amino acid.

For "neutral" amino-acids (i.e. with one amino- and one carboxyl-group) the following equilibrium occurs:-



Amino-acid cation in acid solution — Zwitterion — Amino-acid anion in alkaline solution

The pI for nor-leucine = 6.08 at 25°C (184). But it has been shown that the pI values of many simple amino-acids are not sharp but may extend over a range of several pH units. (184) Michaelis has shown that an amino-acid possesses a sharp iso-electric pH value only if the value of pK1 and pK2 differ by not more than four units. For nor-leucine pK1 = 2.39

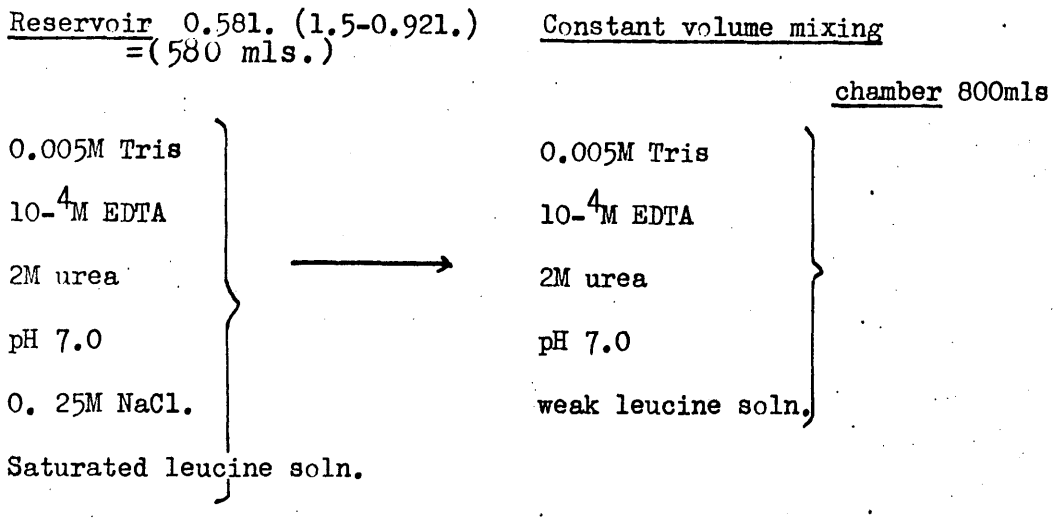
pK2 = 9.76 (184)

and the difference is in excess of four units and therefore there is no sharp pI value for nor-leucine.

Thus to maintain the nor-leucine in a standard ionic form the pH was maintained at a constant level of 7.0 at which pH leucine should be largely in the zwitterion form, but more importantly it will not be in the cationic form which would compete with the protein for column anionic binding sites. In the zwitterion form leucine should cause minimal interference with the normal ion exchange properties of the column with respect to protein. This is in fact shown to be the case since the protein retention of Leu-I column (in the absence of leucine at this stage) was 36% (see fig 46, page 223), whilst in Leu-II the retention in the presence of leucine at saturation level was 45%, and so indicating no derogatory influence by leucine of protein column binding potentiality.

At tube 92 of the leucine gradient elution (i.e. after 920 mls) the reservoir was made 0.25M with respect to NaCl and the elution was continued to produce a salt and leucine gradient. Thus

the gradient consisted of :-



Because of the respective size of the reservoirs, a concentration of 0.25 M NaCl in the constant volume mixing chamber had no chance of being attained, and thus elution was effectively with a solution of a much lower concentration than 0.25M NaCl. But in order to remove any further protein not removed by salt at this concentration, the 530 mls that were present in the mixing chamber were made 1Molar with respect to NaCl at fraction 180 onwards and elution was continued to fraction 253 (see fig 47)

CMC-4-2M-Leu II :- conditions

Conditions differed from the previous leucine column in the following ways:-

1) Instead of applying the 630 mls of protein in 2M urea solution immediately to the column as in Leu I, and applying an

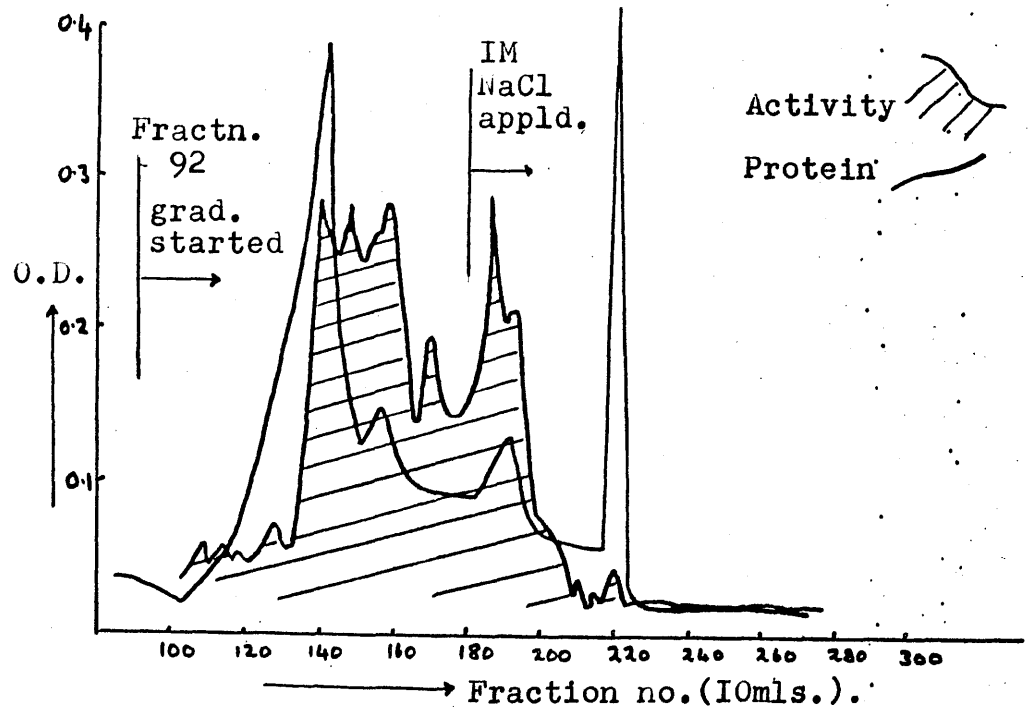


Fig.47. CMC-4-2M-Leu I(Leu I).

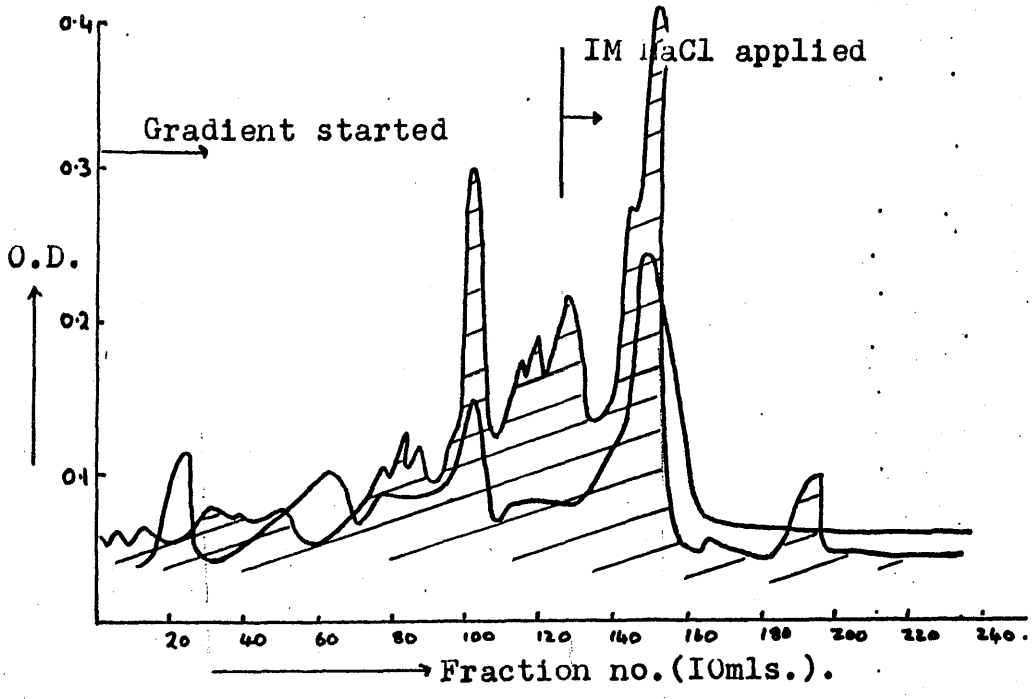


Fig.48. CMC-4-2M-Leu II(Leu II).

initial leucine gradient elution, the protein solution was saturated by the addition of an excess amount of leucine (8gms) and stirred gently by a magnetic stirrer for two days at cold room temperature. The solution was then filtered through Whatman No. 1 filter paper and the protein solution (saturated with respect to leucine) was applied to the CM-cellulose column that had also been equilibriated with saturated leucine.

2) This time a NaCl gradient was applied to the column immediately and also a pH gradient of the type that had been used in all previous and inchoative CM-cellulose chromatography of RNAse enzyme. (see Experimental section EXP. 6,vi).

Gradient elution buffers of CMC-4-2M- Leu II

0.005 M Tris		0.005 M Tris
10^{-4} M EDTA		10^{-4} M EDTA
2M urea	⟶	2M urea
Satd. leucine		Satd. leucine
0.15 M NaCl		pH7.0
pH8.2		

The strength of the NaCl in the reservoir was reduced to aid separation of protein peaks and also to make the gradient elution more comparable to the gradient that was effectively obtained in Leu I.

Therefore Leu II differs from Leu-I in two main respects:-

1) pH gradient applied

2) Pre-treatment of protein material with saturated leucine and a leucine saturated column.

RESULTS see figs 47 and 48.

Discussion

One of the most notable differences between the chromatography carried out in the presence of leucine and the other column results of extraction 4 (figs 39, 40, 41 and 42) after the difference in strength of sodium chloride gradient had been accounted for, was the greatly increased retention of protein (and activity) and late elution of the same.

In all previous chromatography of enzyme material from extraction 4 on CM-cellulose columns whether in the presence of 2M or 7M urea or using the shallowest gradient elution (see page 197 fig 38) the majority of activity had been eluted by about fraction 110. But CMC-4-2M-Leu II (Leu II) showed elution of the majority of activity was not finished until fraction 160.

CMC-4-7M-II (fig 42) and CMC-4-2M-Leu II are directly comparable, since column size and gradient (pH and salt) are identical, although the urea concentrations are different. A study immediately shows that most of the activity in column CMC-4-7M-II (fig 42) was eluted between fractions 40-85, whereas in Leu II most of the activity was eluted between fractions 100-160.

Although chromatography in CMC-4-7M-II was conducted in the presence of 7M urea and not 2M urea as in Leu II, examination of CMC-4-2M and 7M columns (figs 39, 41) shows that this difference does not detract from the importance of this anomalous late elution in the presence of saturated leucine.

The sodium chloride gradient in Leu I was effectively considerably less than the 0.25M stated because of the relative volumes of reservoir, and constant volume chamber of the elution apparatus. In Leu II the ratio of reservoir volume to constant volume chamber capacity was approximately 1.9 : 1, whereas in Leu I this ration was 0.7 : 1. Therefore the gradients of columns Leu I and Leu II would be somewhat similar, even though the respective salt concentrations were different, and if this was so, the retardation of elution was considerably less in Leu I than Leu II. But none the less the retardation of elution in Leu I was appreciably greater than in the chromatography of protein in the absence of leucine. The spread of activity regions extended over a greater area when leucine was present ($\simeq$ 60 fractions) than for all other non-leucine columns of extraction 4 ($\simeq$ 40 fractions)

Another notable feature of chromatography in the presence of leucine was the appearance of three main peaks of activity which was particularly manifested in Leu II, whereas the overall impression of other CMC-4 columns, was one of two main peaks of activity with a tendency towards a merging of these two peaks at higher urea

concentrations.

Possible explanation of results

In Leu I leucine was initially applied as a gradient at pH7.0 to determine whether the amino-acid affected the capacity of the ion exchanger to hold the protein in the presence of a zwitterion. Since no significant amounts of protein or activity were eluted in the first 92 fractions collected of this gradient until the sodium chloride gradient was applied, it was assumed that at pH7.0 leucine did not interfere with the column adsorption properties in a manner that caused premature elution of the adsorbed protein material.

In fact the overall result of leucine presence was one of retention and spread of activity. A protein is retained by a CM-cellulose cation exchanger by means of a salt link between the carboxyl groups of the CM-cellulose and the NH_3^+ groups of the protein. Thus an increased retention of protein by the CM-cellulose above that obtained at the same pH and salt conditions elsewhere, could possibly result from an increased exposure of $-NH_3^+$ groups in the protein.

So to account for this effect of increased column retention in the presence of leucine, the suggestion is put forward that there is an increased exposure of cationic groups due to either a dissociation of protein molecular units (aggregates) and/or an intramolecular unfolding of polypeptide chains due to the detergent

and hydrophobic bond breaking effect of the leucine. The unfolding of polypeptide chains may position more $-NH_3^+$ groups available for salt linkages, since these groups may often be buried or screened internally within the protein's tertiary structure or shielded by areas of hydrophobic nature.

A breakdown of aggregates could, perhaps by attack on intermolecular hydrophobic bonding, equally well lead to earlier or retarded elution because smaller units with a small net charge, or smaller units with a larger net charge than the aggregate structure may result due to exposure of cationic groupings which were masked in the aggregate structure.

Retention may also be influenced by the presence of the dipolar amino-acid and adsorption of the Na^+ eluting ions by the $-COO^-$ groups of the zwitterion. The sodium ions normally bring about the elution of protein by adsorption to the carboxyl groups of the ion exchanger in preference to the protein, causing displacement of the protein. But if the sodium ions form salt links with the amino-acid carboxyl group, a much greater concentration of sodium chloride will be required to elute the protein. This suggested competition for Na^+ ions by leucine will increase as the pH rises due to the increased proportion of leucine present in the carboxyl form rather than the zwitterion form. Experimental results showed that when a pH gradient was used (Leu II) and there was a rise in pH, there was a corresponding increase of protein

retention by the column, thus lending some support to this theory.

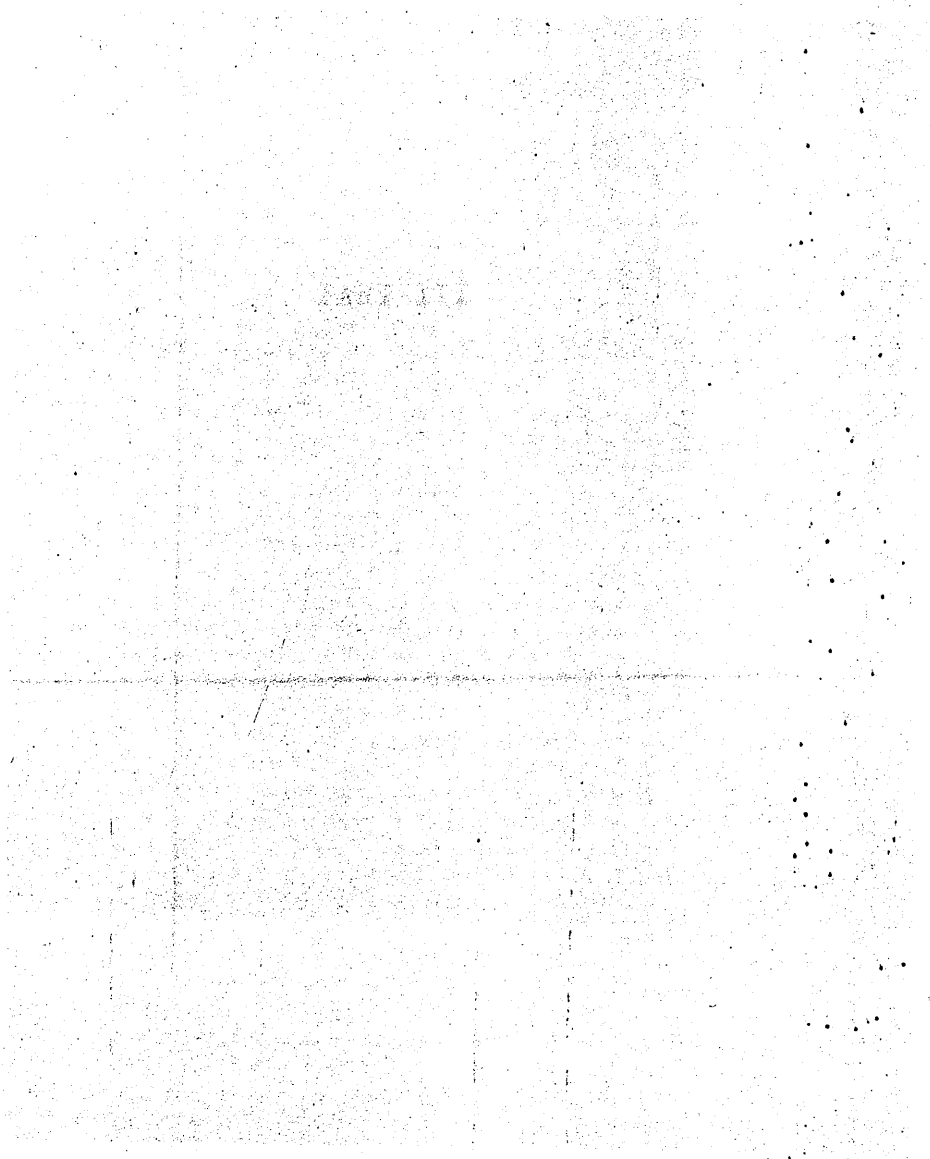
The spread of activity may perhaps be attributed to the formation of several intermediate forms of protein molecules with various degrees of dissociation and/or polypeptide chain unfolding so giving the protein different adsorptive properties and therefore different elution positions.

Summary to investigation of DL-nor-leucine as an aid to protein aggregation studies

This experimental work was intended as a pilot and peripheral investigation to test one or two novel ideas on inter- and intra-molecular bonding in the hope of shedding some light on the importance of hydrophobic bonding. The experimental results obtained in the presence of leucine were distinctive and characteristic enough to warrant further investigation of this amino-acid as a tool in the examination of protein bonding both at inter- and intra - molecular levels.

Ultra-centrifuge studies and Sephadex molecular sieve studies of the molecular weight of active regions eluted in the presence of leucine would be very illuminating with regard to establishing the existence of different molecular weight protein units or not. On the other hand the activity peaks may be simply forms of the same unit at different stages of unfolding of structure. It may be also that there was more than one species of basic RNAse enzyme extracted

from calf spleen and activity peaks need not be related forms of the same enzyme.



PART III

NOTE ON PRESENTATION OF EXPERIMENTAL DETAILS

Because of the nature of the work described and discussed in this section, the text contains more reference to detail of experimental work than is perhaps customary, but for clarity of comparison of experiments which are similar in so many respects this was unavoidable. Thus as a result, the data contained in the experimental section is somewhat curtailed.

Index to Part III. - Specificity Investigations

SECTION 3.

SPECIFICITY INVESTIGATIONS.

Enzyme specificity studies using basic calf spleen ribonuclease.

General Introduction

One of the most striking characteristics of enzymes is their specificity of action, or the strict limitation of action of each enzyme to one substance or to a very small number of closely related substances. Enzyme specificity is an important biological phenomena without which the ordered metabolism of living matter would not be possible.

However not all substances having the necessary combining structure are capable of reacting when they are combined with the enzyme "reactive centre" (see later), thus structural requirements for competitive inhibitors are somewhat less stringent than those for substrates. The size and complexity of the structure required in a substance to make it a substrate is a measure of the specificity of the enzyme; an enzyme requiring in its substrate only a relatively simple structure or small grouping e.g. an ester link, will be relatively unspecific, whereas one requiring a large and complex grouping such as co-enzyme-I is highly

specific. The minimum combining structure deduced from specificity studies differs greatly in chemical nature from one enzyme to another, and the combining groups involved may be of very different natures. Thus many different types of forces may be involved in enzyme substrate combination, and these will include electrostatic attraction, hydrogen bonding, hydrophobic bonding (van der Waals forces) and in a few cases such as the peroxidases, combination is due to co-ordination of the substrate with a metal atom in the enzyme.

The classification and subdivision of enzymes is on the basis of the type of reaction catalysed, and this, together with the name(s) of the substrate(s) provide a foundation for the naming of individual enzymes. Thus the Report of the Commission on Enzymes of the International Union of Biochemistry 1961 (186) recommended that the overall reaction as expressed by a formal equation should be taken as the basis for systematic nomenclature of enzymes and so before an enzyme can be systematically named, the reaction it catalyses and therefore its specificity must be known in advance.

The Commission assigned a number to each enzyme and each of these classification code numbers contained four elements which were as follows:-

(i) The first figure shows to which of the six main

divisions of the enzyme list the particular enzyme belongs. The main divisions are:

1. Oxidoreductases
2. Transferases
3. Hydrolases
4. Lyases
5. Isomerases
6. Ligases (synthetases)

(ii) The second figure indicates the sub-class. For example in the case of transferases, it indicates the nature of the group which is transferred.

(iii) The third figure indicates the sub-sub-class. For transferases the third figure subdivides the types of groups transferred indicating for example, whether the one carbon group is a methyl or a carboxyl group etc.

(iv) The fourth figure is the serial number of the enzyme in its sub-sub-class.

Thus for bovine pancreatic RNAse, the specificity of which is well documented (187), the classification number is EC 2.7.7.16. and this number is made as follows:-

P.T.O.

	EC	-Enzyme Commission.
(i)	2.	Transferase
(ii)	7.	Transferring phosphorus containing groups.
(iii)	7.	Nucleotidyl transferases
(iv)	16.	[Polyribonucleotide 1-Oligonucleotido transferase (cyclizing)] trivial:ribonuclease.

The degree of specificity observed varies with different enzymes. In many cases the enzyme acts as far as is known on one substance only and carries out only one reaction. An example of this type is β-1,4 glucan-4-glucanohydrolase (trivial name -cellulase) EC 3.2.1.4 which specifically hydrolyses β -1,4 glucan links in cellulose. Similarly α-amylase (EC 3.2.1.1.) hydrolyses α-1,4 glucan links in polysaccharide containing three or more α-1,4 linked glucose units. (186) Another example, but of a slightly less specific nature, is the protease trypsin (EC 3.4.4.4. - 186) which is a peptide hydrolase 188) and also hydrolyses amides and esters etc. specifically at bonds involving the carboxyl groups of L-arginine or L-lysine. Many enzymes are less specific and sometimes show a preferential but not absolute specificity to a substrate. An example of this type is chymotrypsin (EC 3.4.4.5) which

hydrolyses peptides, amides and esters etc. (189,190) but with a special preference for bonds involving the carboxyl groups of aromatic L-amino acids. Enzymes such as trypsin and chymotrypsin have proved very useful as tools in selective hydrolysis of proteins (191) since their specificity has been defined and their action predictable.

The specificities of nucleases and ribonucleases in particular, from various sources were extensively discussed in the general introduction (page 1) and also discussed was the rôle of specific ribonucleases in RNA sequence studies. But a knowledge of enzyme specificity, other than for classification purposes, is important to shed some light on the relations between an enzyme and its substrate, and specificity studies form an important part of the experimental evidence on which any theory of enzyme mechanism must be based.

The concept of an enzyme "active-centre" in protein chemistry is a long established one. It is assumed that each molecule has an active catalytic centre of precisely defined chemical structure and that the combination with the substrate occurs at this centre and is responsible for the enzymatic properties

of the molecule. This is a plausible hypothesis but is difficult to examine experimentally because such catalytic centres, if they exist, are parts of complex protein molecules.

Thus the active centre determines both the specificity and catalytic activity. The possibility exists that some enzymes have more than one active centre and that each enzyme molecule can combine with more than one substrate molecule at a time. Recently (30)a 5.5.Å electron density map of bovine pancreatic ribonuclease has been obtained by X-ray diffraction using five isomorphous derivatives. Heavy atom compounds were combined with the protein molecules by diffusion or co-crystallization methods (192) and also by direct chemical reaction. The types of heavy atom compounds used included mercury, iridates and uranyl compounds. By using 2'-cytidylic acid (193) as an inhibitor the resulting electron density distributions showed that the molecule had a "cleft" within which was situated its active site.

But another group also published at almost the same time (194) an X-ray study of bovine pancreatic ribonuclease, but calculated at 2Å resolution level, in which there is disagreement of location of the

active site and also in differences in the electron density maps themselves. Thus much work remains to be done on this pancreatic ribonuclease with respect to its structure and location of active site.

Such work is both slow and costly in man hours and computor time. The importance of this work is that since ribonuclease is such a relatively small active protein or enzyme, an understanding of actually how an enzyme carries out its complex rôle and what groups are involved at the active centre and an understanding of the nature of the enzyme-substrate complex is at last within sight.

Research into the isolation and properties of spleen ribonuclease was initiated and continued in this department partly for the reasons of its low molecular weight and therefore suitability for such X-ray investigations described above. But before an enzyme could be submitted as a basis for X-ray diffraction studies, it would be essential that such basic data as its specificity be available, and it was with this latter object in mind that the following specificity work was inaugurated to form the final section of this dissertation.

Specificity studies were also carried out with the possibility that novel and therefore useful specificity properties might be revealed by this particular RNAse. As discussed in the introduction, there are only two Ribonuclease enzymes with absolute and predictable specificity of attack with respect to RNA as a substrate, and these are Pancreatic RNAse and RNAse T_1 and therefore their action will be discussed in more detail.

Mode of action of pancreatic RNAse (8,9,195)

The action of pancreatic RNAse has been extensively studied and research into the more complex aspects of the mechanism of action at a molecular level still continues.

Pancreatic RNAse catalyses the cleavage of only certain strictly defined internucleotide bonds, and these are the phosphate bonds linking the 3′ position of the sugar (ribose) (see fig. 49) in a pyrimidine nucleotide to the 5′ position of the sugar in an adjacent pyrimidine or purine nucleotide. The phosphate bonds between adjacent purine nucleotides and the phosphate bonds linking pyrimidine nucleotides at the 5′ position with purine nucleotides at the 3′ position, are not hydrolysed. Thus pancreatic ribonuclease

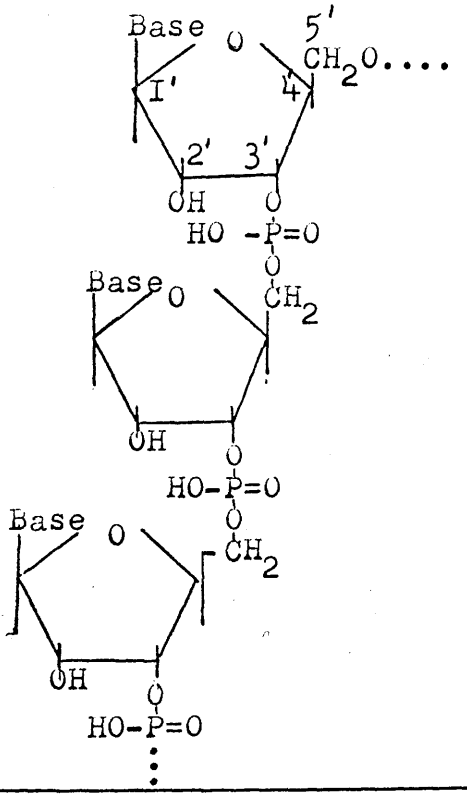
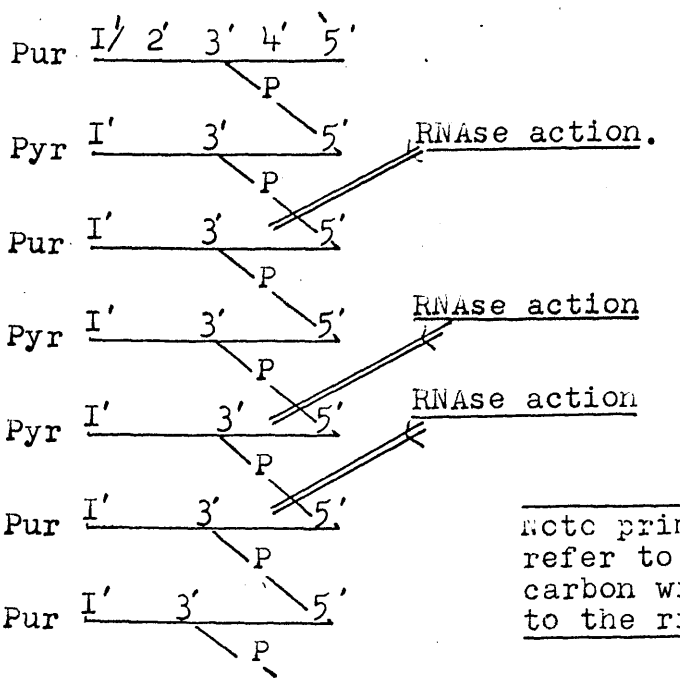


Fig.49. Segment of RNA chain.

Bases=Purine(Adenine or guanine.)
Pyrimidine(Cytosine or uracil.)



Pur=purine base
Pyr=pyrimidine base
P=phosphate linkage

Note primed numbers refer to position of carbon with respect to the ribose ring.

Fig.50. A hypothetical segment of RNA chain and possible points of pancreatic RNAse attack.

splits the phosphodiester bonds of 3' cytidylic and 3' uridylic acid to initially form the pyrimidine nucleoside cylic - 2' 3' phosphate (239)

The action of pancreatic RNAse is a two step reaction and the first of which is a transphosphorylation step in which the phosphate bond to the 5' position of the adjacent pyrimidine or purine is broken, and this released phosphate group is united at the 3' position to form the cyclic phosphate as shown in fig. 52. The 2' 3' cyclic phosphate is then hydrolysed by the enzyme to give the pyrimidine nucleoside 3' phosphate (nucleotide). Pancreatic RNAse does not act upon 2' 3' linkages between nucleotides.

The product of pancreatic RNAse action is a mixture of the free pyrimidine nucleotides, uridine 3' phosphate and cytidine 3' phosphate (abbreviated to U3'P and C3'P or just UMP and CMP), and various oligonucleotides from dinucleotides upwards. A pyrimidine nucleotide is always an end group in the oligonucleotides and has a phosphate attached in the 3' position.

From an investigation of the kinetics of the second step of this reaction (196) in a variety of

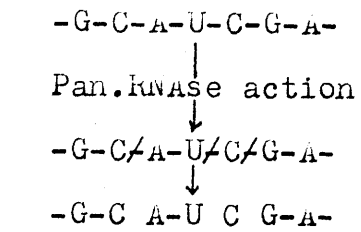


Fig.51. A segment of RNA chain can be represented in a more diagramatic manner as a succession of nucleotides, but remembering the linkage is 3′,5′ internucleotide phosphate linkage as in fig.50. Letters A,G,C, and U represent adenylic, guanylic, cytidylic and uridylic acids respectively.

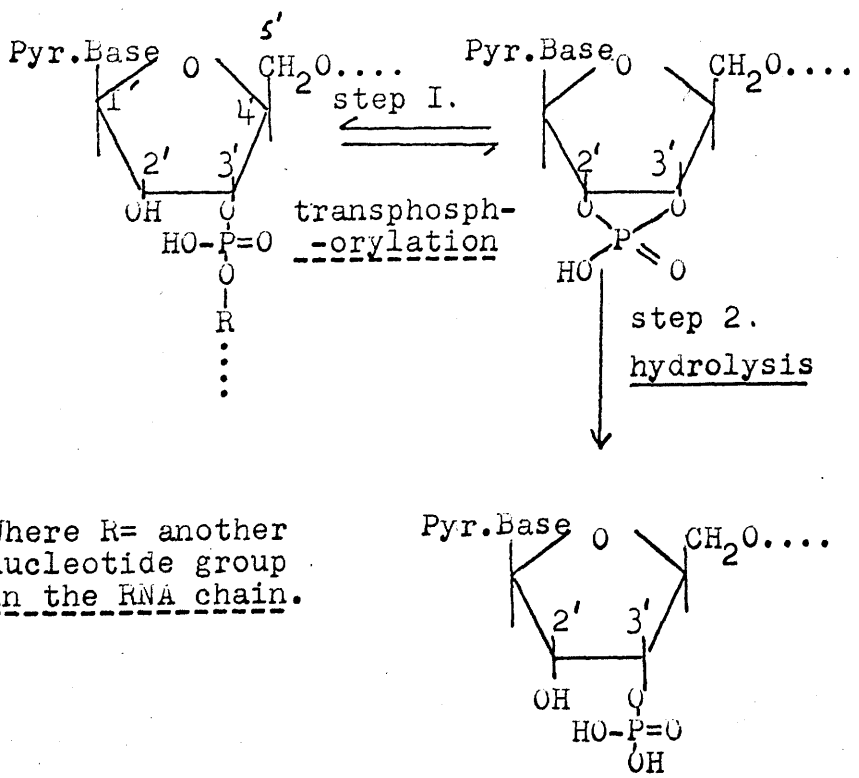


Fig.52. The two stage action of pancreatic RNAse.

solvents it was concluded that two histidine residues, one as an acid and the other as a base form part of the active site of the enzyme. Work by Crestfield, Fructer, Moore and Stein provide further evidence for the postulate that two histidine residues are involved at the catalytic site of the enzyme (197, 198, 199, 200). By the use of inhibitor molecules, recent work (30,201) has shown that the histidine molecules (fig.53) that are involved are those at position 12 and 119 of the enzyme amino-acid sequence. It has been recently proposed (187) that the first step of the pancreatic ribonuclease reaction, the transphosphorylation, requires the interaction of one of the histidine residues (a), in the base form, with the 2' hydroxyl, and the other (b), in the acid form, with the oxygen atom bridging R and P (see fig 52 page 250).

In step 2, residue (a) in the acid form reacts with the 2' oxygen, and the base form of residue (b) binds the attacking H_2O molecule. It was postulated that the proper positioning of the substrate with respect to the catalytic groups of the enzymes was achieved by specific interactions between the protein and the pyrimidine base.

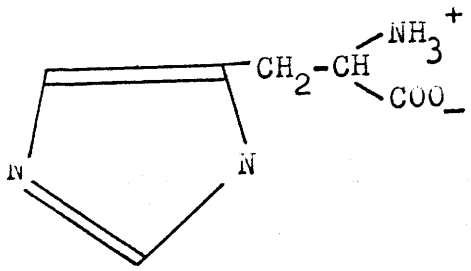


Fig.53. Histidine.

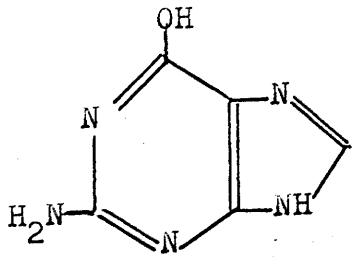


Fig.54. Guanine base.

Mode of Action by RNAse T_1

RNAse T_1 is a ribonuclease isolated from Taka Diastase which is a commercial product of the mould Aspergillus oryzae, and like pancreatic RNAse, is very thermostable. In preliminary experiments with Taka Diastase it was found (11,12) that at least three enzymes attacking RNA could be isolated. One of these enzymes, T_1 (EC 3.1.4.8) was found to have a specificity quite different from pancreatic RNAse but was similar in that specificity was found to be absolute and not preferential as with so many other ribonucleases.

Ribonuclease T_1 was classified in a group quite apart from Pancreatic RNAse (EC 2.7.7.16) by the Commission on Enzymes (186). However Egami et al (12) report that RNAse T_1 like pancreatic RNAse, acts by the formation of nucleoside 2', 3' cyclic phosphates first in an initial transphosphorylation reaction, and these are further hydrolysed to the 3' nucleotides. Thus pancreatic RNAse and RNAse T_1 should be classified in the same group, namely as transferases and the proper nomenclature recommended for RNAse T_1 by Egami et al (12) is Ribonucleate guanine nucleotido - 2' transferase (cyclizing) and thus was adopted in the

revised report of the Commission in 1964 and RNAse was classified along with pancreatic RNAse as EC 2.7.7.26.

RNAse T_1 splits the internucleotide bonds between 3' guanylic acid groups and the 5' hydroxyl groups of the adjacent nucleotides with the intermediary formation of guanosine 2', 3' cyclic phosphate. Hence it may be regarded as a guanylic acid specific endoribonuclease. The mechanism of reaction was deduced from the following experimental evidence:

1) RNAse T_1 digests yeast RNA and produces only 3' guanylic acid as a mononucleotide.

2) The terminal residue of the oligonucleotides produced is exclusively guanylic acid.

3) Guanosine 2', 3' cyclic phosphate is obtained as an intermediate in good yield and it is further hydrolysed to 3' guanylic acid.

Since RNAse T_1 does not split the secondary phosphate ester bonds of adenosine 3' phosphate, the specificity was considered to depend on the substituents of the purine ring. But digests of deaminated RNA showed that it was not the amino group that was responsible for RNAse T_1 specificity (202), and therefore suggested that the essential requirement in the structure of the base for susceptibility to attack may be the hydroxy group at the 6 position of the purine base (fig.54)

Calf spleen ribonuclease specificity reports

Kaplan and Heppel (28) in their original publication on basic calf spleen ribonuclease reported that the specificity appeared to be the same as that reported by others for pancreatic ribonuclease. Cytidine 3' benzyl phosphate was hydrolysed by the spleen enzyme but adenosine 3' benzyl phosphate was not, thus in keeping with the specificity studies of Brown and Todd (8) of pancreatic RNAse. Similarly cytidine-2', 3' phosphate was split by spleen ribonuclease to give cytidine 3' phosphate, but adenosine 2', 3' phosphate was not attacked. Kaplan and Heppel (28) reported that similar to pancreatic RNAse, in the course of digestion of RNA from turnip-yellow mosaic virus, that cytidine 2', 3' phosphate and uridine 2', 3' phosphate were found, and also cyclic dinucleotides containing a pyrimidine nucleoside residue in which the hydroxyl of carbon 5' was involved in the internucleotide phosphodiester bond and the hydroxyls of carbon 2' and 3' were esterified to form the cyclic monohydrogen phosphate ester. No activity against DNA was reported.

Thus the enzyme isolated from calf spleen by Kaplan and Heppel (28) seems to have quite similar catalytic

properties to pancreatic RNAse because both enzymes cause the partial breakdown of RNA and split only secondary phosphate esters of pyrimidine nucleoside 3' phosphates. Cyclic pyrimidine nucleotides are formed from RNA and are themselves cleaved by further action of either enzyme. Both enzymes form only the pyrimidine mononucleotides from RNA. But in spite of these similarities, the two enzymes appear to be distinct proteins.

In 1962 (36) Maver and Greco reported the isolation and separation of both acid and alkaline ribonucleases from bovine spleen. But results differed from those reported by Kaplan and Heppel (28) in that both acid and alkaline ribonuclease were reported to catalyse the hydrolysis of RNA with the formation of cyclic and non-cyclic purine, as well as pyrimidine nucleotides. Guanylic and uridylic acids in both cylic and non-cyclic form were found in the acid and alkaline RNAse digests, but the ratios of adenylic to cytidylic differed, being higher in the acid digest, and lower in the alkaline digest. These results suggest a preferential hydrolysis of nucleotide linkages and the results with respect to the specificity of spleen basic ribonuclease do not coincide with those reported by Kaplan and Heppel (28) in which the specificity of the enzyme was found to be of

the same nature as pancreatic RNAse. One of course must take into account differences in fractionation procedure and consider whether the enzymes isolated are strictly comparable or indeed if one is comparing the same enzyme. Thus to summarize, investigations into the nature of the specificity of calf spleen basic ribonuclease fractions were carried out for the following reasons:-

1. To clarify the nature of the specificity of calf spleen basic ribonuclease fractions isolated, in the light of conflicting reports by Kaplan and Heppel (28), and Maver and Greco (36).
2. Because of the importance of ribonucleases and possible applicability of a new enzyme specificity in RNA sequence determinations.
3. Because a knowledge of enzyme specificity would enable the establishment of the enzyme individuality and identity and thus enable comparisons with other calf spleen basic ribonucleases which have been prepared by different methods, i.e. a knowledge of specificity is of paramount importance in definition of the protein properties.

Conditions of study of enzyme specificity

The satisfactory investigation of the specificity of a given enzyme requires that the enzyme being

investigated should be as pure as possible and if feasible should be quite free from any enzyme acting on similar substances. In many extensive studies of the specificity (and other properties) of enzymes involving much careful chemical work in the preparation of a large number of pure substrates, it is unfortunate that equal care was not taken to work with pure enzymes. Several such studies in fact have been carried out with relatively crude enzymes, and it therefore becomes impossible to be certain that all reactions observed were due to the same enzyme, (possible examples may include refs:- 15, 203, 96).

The substrates should also be as pure as possible and also quite free from any other substances on which the enzyme may act. The enzyme purification was well studied and discussed in Section I and substrate purification and choice will be discussed on the following pages.

Enzymes should also be used at reasonably low concentration in order to avoid reaction due to traces of contaminating enzymes which may be observed even with comparatively pure enzymes if excessive amounts are taken.

Substrate prepartion - Prepartion of RNA

a) General. Nucleic acids occur naturally in association

with proteins and with lipoprotein organelles and it is the separation from these and any polysaccharide in the cell that constitutes the problem of isolation. Also nucleic acids occur in cells which may contain nucleases and these also constitute isolation problems. Practically, the problem is limited by the sensitivity of nucleic acid chains to rupture by changes in pH, by physical damage, and by enzymes present in the cell. It is essential therefore to choose reagents that inhibit, or, preferably put out of commission the cellular nucleases and at the same time leave the native structure of the nucleic acids intact. The problem is further complicated by the presence of two kinds of nucleic acids in the cell; DNA and RNA, with RNA further subdivided into "soluble" or "supernatant" RNA (sRNA), cytoplasmic or ribosomal RNA (rRNA), nuclear RNA (nRNA), and "messenger" RNA (mRNA) species, apart from any nucleic acid that may be present in viruses. The nature of the intermolecular bonding and associations, and the amount of nucleases vary with the organ or organism, so that no general method for isolation and purification of the nucleic acids is to be expected.

Among the cellular organisms yeast is one of the

most satisfactory starting materials for the preparation of practically pure RNA in quantity because its DNA content is so small (less than 2% DNA) that steps in order to obtain separation of the two nucleic acid types are practically superfluous.

Thus yeast was chosen as a source of RNA and was obtained as Baker's yeast (Saccharomyces cerevisiae) in cake form marketed by D.C.L. (via Hubbards Bakeries, Glasgow). In earlier methods of extraction, the separation of RNA from the protein was accomplished by treatment of the yeast cells with alkali (204), but RNA was unstable under these conditions and for a considerable period this method was substituted by one which used high salt concentrations to effect the dissociation of nucleoproteins to their components, but this results in degraded RNA of low biological activity (206). At present the two favoured methods of RNA extraction from yeast cells are by the use of phenol (207) and detergent (208, 209)

The various advantages of each type of extraction technique have been compared and summarized adequately in review articles (205, 210, 211, 212).

The method of RNA isolation from yeast chosen was that of Crestfield, Smith and Allen (208) since "Methods in Enzymology" (213) had recommended this as the best

available method. This method was also used by Beard and Razzell (87) in their studies of an alkaline ribonuclease from mitochondrial and soluble fractions of liver. As a result of previous personal experience in the manipulation of this technique, faster and more efficient extraction would be possible. It may be of interest to quote a Chinese report (214) in which it was suggested that the extraction of RNA by both phenol and sodium dodecyl sulphate procedures are not satisfactory in producing stable RNA. The paper shows that phenol is a reversible inhibitor of RNAse, the degree of inhibition depending on the concentration of phenol. When the phenol concentration is in excess of 6%, the ribonuclease activity is almost completely inhibited and it is for partly this reason that phenol is used in extracting RNA since it inhibits RNAse degradation. When phenol is removed, RNAse recovers normal activity. It was further demonstrated that the distribution of RNAse between phenol and water phases is greatly affected by the presence of RNA. In the absence of RNA, nearly all the RNAse is present in the phenol phase, but a considerable part of the RNAse remains in the water phase when RNA is present. When RNA is

precipitated out from the water phase by ethanol as is used for the IM sodium chloride precipitant procedure, RNAse is precipitated also. Evidently RNA thus obtained will be contaminated with RNAse. This may partly explain the instability of RNA reported in the literature. (RNA instability is also accounted for by the propensity to form the 2', 3' cyclic phosphate in slightly alkaline solutions due to the hydroxyl on the carbon 2' position - unlike DNA which as a result is relatively more stable than RNA). Similarly to phenol, sodium dodecyl sulphate in RNA preparations is also a reversible inhibitor of pancreatic RNAse. The article (214) concludes by suggesting that the danger of RNAse action may be overcome by the use of bentonite which strongly binds RNAse and indeed was used Rees and Southern (250) with success within this department.

The technique and practical details of the extraction procedure are given in detail in "Methods in Enzymology" (212) but briefly, consisted of extraction of the ribonucleate from the yeast cells by a short heating with an aqueous solution of sodium dodecyl sulphate detergent and precipitation by alcohol. Pure ribonucleate settled out from an aqueous solution of

RNA preparation	Prep. I		Prep. II		Prep. III	
	Digest x 2		Digest x 2		Digest x 2	
RNA & enzyme (E)	0.494	0.506	0.290	0.305	0.166	0.179
RNA +TCA + E,cool.	0.300	0.300	0.158	0.149	0.152	0.152
Net O.D.at 260mu.	0.194	0.206	0.141	0.147	0.014	0.027

Where E = enzyme from Extraction 4.

Key. RNA preparation I -Crestfield,Smith & Allan
" " II " " "
--oldest prep.,ref.208.
" " IIICrestfield,Smith & Allan
with growing period
modification.Ref.208.

Note on prep.III

Prep. III showed the least activity and there was also difficulty in obtaining its solution,and as complete solution was not obtained this no doubt would contribute to a low net O.D.as compared with results in which more soluble RNA was used.Gel filtration showed this preparation to have a very high molecular weight and the reason for insolubility may be connected with this.

Fig.56. RNA preparations.

Digest	Yeast RNA source-preparation number.
I	RNA-prep I
II	RNA-prep I
IV	RNA-prep III
V	Commercial RNA
VI	Commercial RNA
VII	Commercial RNA

Fig.57. Summary table of digests carried out during specificity investigations & RNA used.

the alcohol precipitate on addition of sufficient amounts of sodium chloride to bring its concentration to normality and on the subsequent standing of this solution at 0°C. The precipitated material was exhaustively dialysed against deionized water at 4°C. and freeze-dried and stored in a stoppered bottle in the deep freeze (-22°C.). This source of RNA was used for routine assays in the earlier extractions (Nos.1& 2) and also for the initial specificity investigations (see fig. No. 57).

But when the necessity arose to prepare further quantities of RNA for specificity and assay work, one or two variations of technique were experimented with to attempt to increase yields and efficiency of extraction. In a comparative study of different preparations of yeast soluble RNA (205) from Baker's yeast (Saccharomyces cerevisiae) it was found that a necessary preliminary to the isolation of active sRNA in reasonable yield was cultivation of the yeast for one generation (approximately 5 hours) in the laboratory. The reason for this was probably that young and newly formed yeast cells are more susceptible to solubilisation by the sodium dodecyl sulphate, since yeast may tend to become somewhat encapsulated in the

form that it is stored, and also there may be autolysis of the yeast cells which occurs in conditions where cells are in the form of a compact mass depleted of nutrition and under relatively anaerobic conditions as they are in the compressed block form in which Baker's yeast is marketed (215)

b. Procedure for yeast cultivation - method to aid RNA extraction from yeast

Two pounds of D.C.L. Baker's yeast (Saccharomyces cerevisiae) were dispersed in 8 litres of tap water. Other nutrient requirement for a short period of growth were added as follows: (215)

20 gms of glucose initially and another 20 gms after 6 hours.

2 teaspoons of malt extract (Allen and Hanburys proprietary source).

few mls. of 0.2M $Na\ H_2\ PO_4$
" " " 0.2M $Na_2\ HPO_4$
few mgs. $(NH_4)_2\ SO_4$
" " $K_2\ CO_3$
" " $Mg\ Cl_2$

The yeast mixture was stirred and then allowed to grow overnight (about 15 hours) at 18°C. On the addition of the second glucose aliquot the mixture was stirred slowly until the glucose had dissolved and was well distributed. Finally after this overnight growth period the yeast suspension was centrifuged down at 2,300 r.p.m.

(see Exp. I - Experimental Section) - for ten minutes and the extraction of RNA from the yeast was continued in the manner of Crestfield, Smith and Allen (208) using sodium dodecyl sulphate. The final freeze-dried product was stored at - 22°C. in the deep-freeze in a carefully stoppered bottle.

c. Estimation of RNA molecular weight by use of Sephadex G-75 molecular sieving techniques (216)

A sample of RNA prepared by the previously described method and in 2 mls of solution was applied to an earlier prepared (37) Sephadex G-75 column of dimension 56 x 2.5 cms which had previously been equilibriated with 0.05M Tris /HCl at pH 7.0 buffer and was also eluted with the same pH7.0 buffer. This particular column had been prepared and calibrated with respect to elution volumes of various proteins of known molecular weight. The calibrating proteins used were:-

	M.Wt.
Glucagon	3,500
Pancreatic Ribonuclease	13,700
Trypsin inhibitor	20,200
Ovalbumin	45,000
Bovine serum albumin	67,000

and Blue dextran 2000 to determined the exclusion volume. (page 94).

Thus with a knowledge of the elution volume of each of these standard substances, a graph was plotted of

elution volume against log molecular weight which gave a linear relationship from which an approximate estimation of the molecular weight range of the RNA prepared was possible (The column used, together with the relevant data was a personal communication from J. Edmond (37)).

Sixty per cent of the RNA material applied was eluted in a region that corresponded to an approximate molecular weight range, as read from the graph of log. of molecular weight against fraction number compounded for this column, of between 70,000 and 45,000. The other 40% of the eluted material corresponded to a molecular weight limit of 45,000 - 6,000.

However in interpreting these results it must be remembered that the Sephadex G-75 column was calibrated with respect to proteins of a globular nature and the exclusion volume of globular proteins is different from that of dextrans for example of the same molecular weight. Thus molecular shape and conformation is a factor to be considered in gel filtration since dextrans tend to be long chains in contrast to the compact nature of globular proteins. The fractionation ranges for G-75 for both types of molecules are quoted by Pharmacia (Fig.105). (Sephadex manufacturers - information booklet)

	Sephadex G-75 fractionation range	
	Peptides and globular proteins	Dextrans
M.Wt.	3,000-70,000	1,000 - 50,000

Fig. 105 Sephadex fractionation range as quoted by "Pharmacia".

Therefore as the relationship between molecular weight and elution volume is different for different types of molecules, a separate calibration graph should be determined for each type. But the use of a graph calibrated for globular proteins does give some estimate of the molecular weight range of the RNA that was prepared, and so it could be at least stated that 60% of RNA was composed of units with very high molecular weight and probably varying from the exclusion limit (approximately 50,000 if RNA is considered as more of a dextran type molecule than globular protein) to between 20,000 and 40,000. The other 40% of the RNA was of somewhat lower molecular weight. The molecular weight of sRNA according to the most reliable physico-chemical measurements is 25,000-30,000 (217, 218).

The RNA from the above preparation was used in specificity digest number IV (see fig. 57) and also in certain routine column assay procedures. However in

extraction 4, enzyme containing samples were assayed with different yeast RNA preparations that had been prepared at different times and stored in the deep freeze for varying periods. Results (fig. 56) showed considerable inconsistency, but length of storage may contribute to this. However yeast RNA is generally fairly stable over long periods at deep freeze temperatures (-22°C.). Differences in extraction technique may also contribute and therefore for the sake of consistency, commercial yeast RNA was used as a substrate for ribonuclease.

d. Purification of commercial yeast RNA.

Commercial yeast RNA was obtained from L. Light & Co. Ltd., Colnbrook, England, and 25 gms were dissolved in I.25 l. of deionized water. The pH was brought to neutrality by the dropwise addition of Normal sodium hydroxide whilst the solution was stirred mechanically. The solution was then filtered to remove any undissolved RNA and was then exhaustively dialysed against deionized water at 5°C. in stainless steel buckets. The resulting dialysed RNA solution was freeze dried and stored in a tightly capped bottle at deep freeze temperature (-22°C).

Commercial yeast RNA dialysed for 72 hours against running distilled water and concentrated by freeze drying was used by Kaplan and Heppel (28) in their studies of a

basic RNAse from calf spleen.

A consideration of methods of fractionation of the products RNAse-RNA digests.

The object of the specificity studies of calf spleen basic ribonuclease was to acquire a knowledge of the type of mononucleotides produced as a result of this enzyme's action on yeast RNA and so be able to formulate a hypothesis with respect to the enzyme specificity. Therefore the separation of trimers and larger oligonucleotides was of little interest, although dimer separation would be of some interest and might aid as additional evidence to any hypothesis.

Methods of nucleotide separation are now quite extensive and varied and thus it is usually possible to study and confirm fraction identity by several separative techniques.

The polyanionic nature of nucleotides can be utilized for their separation by anion exchange chromatography. It is necessary to find a set of conditions under which each exhibits a different degree of affinity with respect to the ion exchanger. While this affinity is governed by a number of variables, it is reasonable to assume that in a group as closely related as the ribonucleotides, the net charge per ion

will be the most important one. Since these substances possess both acid (phosphate) and basic (amino, except for uridine) groups, the pH of the medium determines the net charge by determining the degree of dissociation of the groups. Using the pK values of Levene (220), calculations can be made of the degrees of dissociation of the mononucleotide groups as a function of pH. This was done by Cohn (221) and presented graphically as in fig.58.

It is apparent from fig.58 that the increase in net negative charge is in the order:-

Cytidylic	C	Increase in net
Adenylic	A	negative charge
Guanylic	G	↓
Uridylic acids	U	

Cytidylic acids and adenylic acids exhibit a net positive charge and hence are cations below pH 2.5, guanylic acid is cationic below pH 1.5, whilst uridylic acid remains anionic down to pH 0. Thus at pH's above 5, all the mononucleotides are strongly anionic and thus are consistent with anion exchange chromatography at pH values of 5 and above. Cohn (221) using this theory carried out a separation of mononucleotides and oligonucleotides by adsorption on to an anion exchanger at pH 6.0 or greater. The mononucleotides

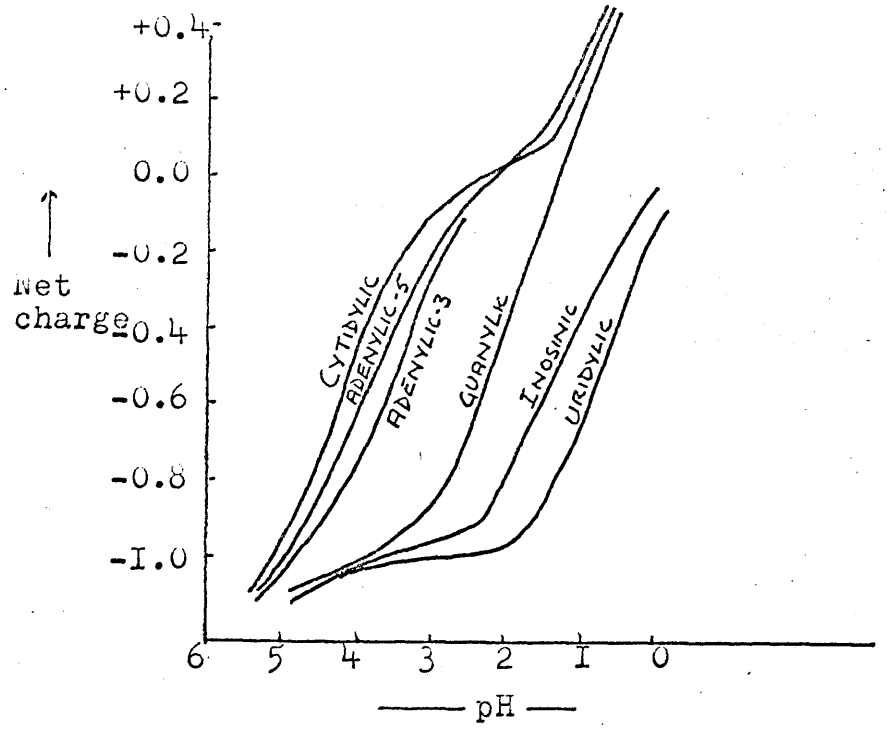


Fig.58. Net charge per molecule as a function of pH (calculated from data of Levene, ref.221).

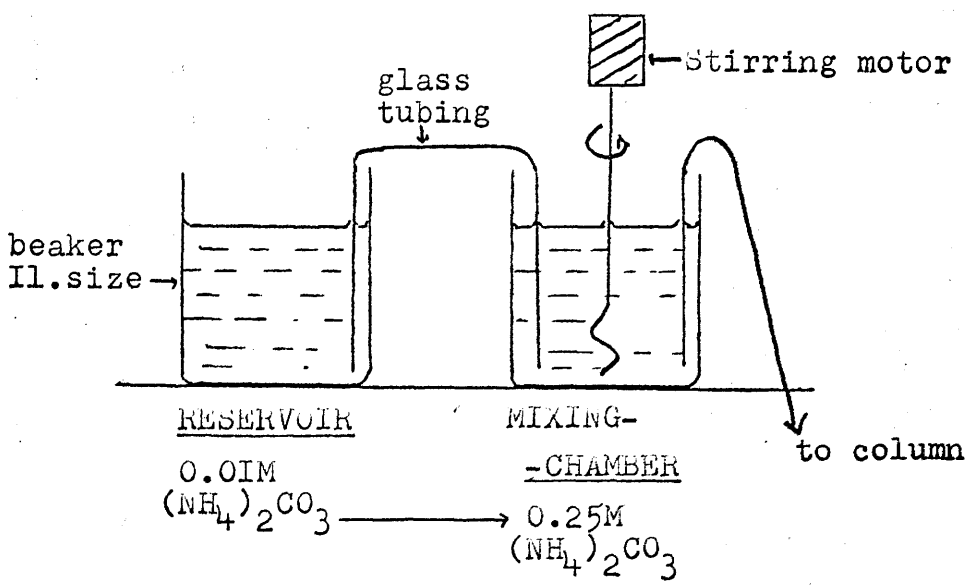


Fig.59. Linear gradient apparatus.

were eluted in the order predicted by Levine of C,A, G and U by lowering the pH in a stepwise manner, and increasing the anion concentration with salt near the end of the elution if necessary to avoid strongly acid conditions which might hydrolyse the compounds. Oligonucleotides will be eluted also according to their net negative charge but the differences in charge will be less distinct than that of mononucleotides. The larger the nucleotide chain the greater will be the net negative charge and therefore the greater the adsorption to an anion exchanger and the greater the salt concentration and the lower the pH required for elution.

Earlier column chromatographic separation methods of nucleotides, such as those of Cohn (221) above, involved the use of polystyrene resins such as Dowex-I or 2 or Amberlite IR-400, but most methods have been developed around Dowex-I because of its early availability in a fine mesh form. The anion exchangers of the strong base quaternary ammonium type are in the form of spherical beads and Dowex-I is of this type.

While a reasonable separation could be obtained up to the trinucleotide level with polystyrene anion exchangers, when complex mixtures were used, considerable

overlapping of peaks occurred. However the anion exchange material found most suitable for oligonucleotide separation has been the amino alkyl subsituted celluloses first described by Peterson and Sober (34) in 1956. Tener et al (222) obtained a satisfactory resolution of oligothymidylic acids on DEAE-cellulose or on ECTEOLA-cellulose. Since then the use of DEAE-cellulose has been widespread and is reviewed by Staehelin(223).

But whilst no difficulty was encountered in the separation of an homologous series of olignucleotides up to the dodecomer (224), the resolution of heterogeneous oligonucleotide mixtures was complicated as a result of secondary binding forces between the cellulose matrix and the purine and pyrimidine bases. Purine rich oligonucleotides are generally more retarded on their passage through the column that pyrimidine rich oligonucleotides. The discovery of the effect of 7M urea in minimising the secondary binding affinities of nucleic acid bases to DEAE-cellulose due to hydrogen bonding probably, has greatly simplified the fractionation of oligonucleotide mixtures (225). In the presence of 7M urea the binding of the oligonucleotides is

mainly of an electrostatic nature, and their order of elution is a function of their net negative charge.

But in the ensuing investigation of calf spleen ribonuclease specificity, it was mainly the mononucleotides that were of interest and it did not matter from the aspect of efficiency of separation which of the two main types of nucleotide chromatographic technique was used, that is whether use was made of polystyrene resins like Dowex-I or substituted celluloses such as DEAE-cellulose. In fact both types were used and will be discussed respectively as their use occurs during the investigation. Similarly other chromatographic techniques will be discussed in the text as they arise and also in the experimental section.

DIGEST I.

Trial digests were carried out to find the best conditions of digest with respect to time, elution conditions, and enzyme quantities etc.

In trial digest I, mononucleotides and small oligonucleotides were separated from large nucleotide units by column chromatography on DEAE-cellulose as used by Rushizky and Sober (228) in their separation of the digestion products of RNAse T_1. In a similar manner to

this report, an ammonium carbonate elution system was used at pH 8.6. The same workers also reported (229) that if the products of an RNAse-RNA digest were applied to a DEAE-cellulose column equilibriated with 0.01M ammonium carbonate, an eluting system - 0.07M with respect to $(NH_4)_2\ CO_3$, would bring about the elution of mononucleotides and small oligonucleotides, but elution of larger oligonucleotides would only be brought about by use of 2M $(NH_4)_2\ CO_3$. Such a system was ideal since it was primarily the mononucleotides that were of interest in this investigation, and by this elution system on DEAE-cellulose these could be separated from the larger products of nucleotide digestion.

Procedure.

a) Column preparation. Whatman DEAE-cellulose (DE50) was prepared as described on page 115 and the column packed by the procedure described on page 44 (refs. 34,74) Column dimensions: 2 x 18 cms.

b) Gradient elution. The column was equilibriated with 0.01M $(NH_4)_2\ CO_3$ which was at pH8.6. A linear gradient was set up for the elution of digest nucleotide products:-

Gradient

1ℓ. of 0.01M $(NH_4)_2\ CO_3$ ⟵ 1ℓ. of 0.25M $(NH_4)_2\ CO_3$ using equal sized reservoirs as fig. 59.

In a linear gradient the concentration of the eluting solution is given by the equation $C_E = Cr - (Cr - CM)(1 - \frac{n}{N})$ where Volume of the mixing chamber = volume of the reservoir

C_E = concentration of the eluting solution

Cr = concentration in the reservoir

n = elution fraction number (or in mls. volume)

N = total fraction number (or total mls. eluted).

CM = concentration in the mixing chamber.

c) Digest. This digest was little more than a trial run and therefore no great attention was paid to detail. RNAse B from extraction 2 was digested with RNA prepared from yeast (RNA preparation number 1 - page 262) at 37°C in a constant temperature water bath. After 30 minutes the digest was terminated by the addition of a 0.25% uranyl acetate /2.5% trichloracetic acid solution (83) which precipitated protein

material and large oligonucleotide components. The precipitated material was centrifuged at 1,500 r.p.m. and the supernatant retained for application to the DEAE-cellulose column for chromatographic separation with the before-mentioned gradient elution system after a tenfold dilution to give a total volume of 112 mls.

O.D. 260 m_u of diluted material before application to column = 0.75

Volume = 112 mls. ∴ Total no. of optical density units applied = 84 units.

No. of optical density units passing straight through the column = 41.9 units.

∴ % retention by the column of nucleotide material = 50.2%

Three ml. fractions were collected using an L.K.B. "Radirac" fraction collector and U.V. optical density at 254 m_u of the column effluent was recorded automatically by an L.K.B. "Uvicord" recorder. Only one peak was registered, and to check, fractions were also read at 260 m_u in a "Unicam" SP500 spectrophotometer.

d) Results. One peak due to U.V. adsorption at 254 and 260 m_u was obtained between fractions 177 and 203.

Fractions 196 and 197 were taken as typical and bulked, and the pH in turn adjusted to 11, 7, and 2 (using concentrated ammonium hydroxide and IM hydrochloric acid respectively) and U.V readings at each pH from 210 to 300 m_u were taken and a graph of optical density against wave length was plotted. Similarly the ratios of optical densities at 250 and 260 m_u were calculated at each pH. The U.V. spectra (graphs of O.D. against wavelength) and optical density ratios were compared with the published values for mononucleotides (226,227) in order to try and identify the product or products eluted in the single peak obtained using a 0.01M to 0.25M $(NH_4)_2 CO_3$ gradient.

However the comparison of results with published values indicated that the curve was not of a nucleotide nature at all. A U.V. scan of the uranyl acetate/ trichloracetic acid solution used to precipitate unwanted protein and high molecular weight RNA fractions was done and it was shown to have U.V absorbance beginning at about 280m_u and rising sharply at 250 m_u as the wavelength decreases. Thus the majority of the peak obtained was probably due to the interfering absorption at 254 and 260 m_u of uranyl acetate/trich-loracetic acid solution, and not, as the results

indicated, to nucleotide material. The lack of any evidence of nucleotidic peak elution was attributed to a very low concentration of RNA and enzyme initially, and also to the fact that the digest plus uranyl acetate/trichloracetic acid solution had stood for two to three days at room temperature before being applied to the DEAE-cellulose column, and no doubt some nucleotide degradation resulted. So in future digests, the use of the uranyl compound was avoided. Further reasons for its avoidance of use was the possible complications that it may produce with respect to nucleotide absorption or displacement on the ion exchanger due to the presence of acetate ions.

DIGEST II.

Using the same column as used in trial digest I, a further trial digest was attempted but the following changes were made:-

i) The digest period was over a period of $2\frac{1}{2}$ days instead of 30 minutes as in digest I.

ii) No uranyl acetate/trichloracetic acid was used.

iii) Larger amounts of enzyme and RNA were used.

a) Digest Procedure

Enzyme source: RNAse B which had been stored at 0° (in a refrigerator.)

RNA source: Yeast RNA preparation No.1 (fig. 56).

RNA and RNAse were incubated together at 37° for 2½ days in a constant temperature water bath, and at pH 6.5 using a 0.1M Sodium succinate buffer made 0.05M with respect to Mg^{++} ions (28,37). The same gradient conditions were used as in digest I. Final elution at fraction 383 was with saturated ammonium carbonate solution. The digest was not terminated using the uranyl solution, but instead the digested material was merely diluted (0.01M $(NH_4)_2\ CO_3$) and applied directly to the DEAE-cellulose column which had been equilibrated with 0.01M $(NH_4)_2\ CO_3$ solution at pH 8.6. Gradient elution was begun immediately and three ml. fractions were collected and the U.V. absorbance (or optical density) recorded by an LKB "Radirac" and "Uvicord" combination.

b) Results

A nucleotide profile as shown in fig. 60 was obtained which was transcribed from the "Uvicord"

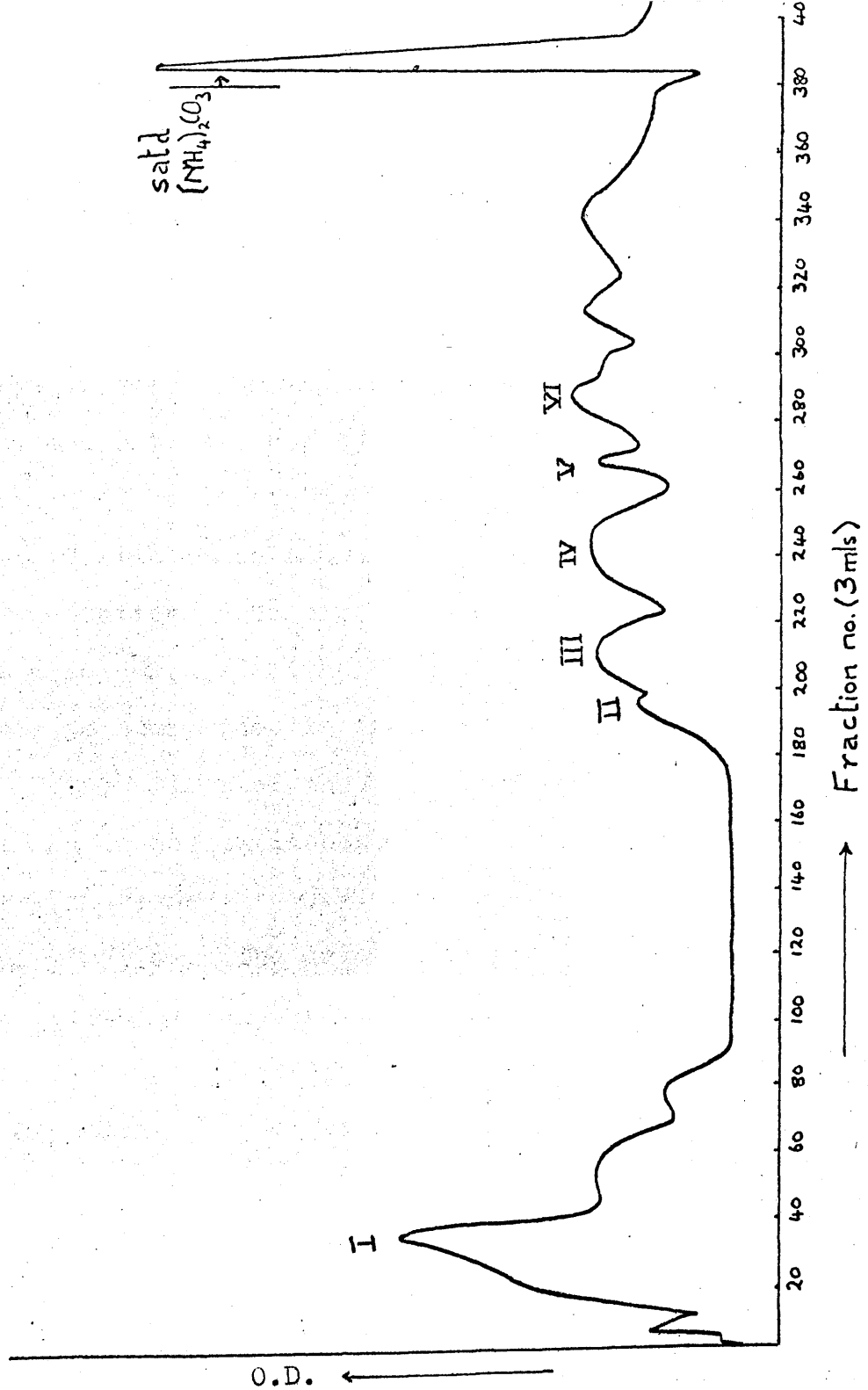


Fig.60. Elution profile (254mu-nucleotide)of digest II.

recorder data print-out sheet. Therefore the scale of optical density at 254 m_u was not absolute and so no scale is given in fig.60.

Two representative fractions from each peak were bulked (total volume 6 mls) and as in digest I, U.V. scans and optical density ratios were read for each peak. U.V. scans of each peak at pH2,7 and 11 were compared with those of published data (227) for mononucleotides, and similarly optical density ratios were also compared with published data (226) and the latter is summarized in fig. 61.

A comparison of the data obtained showed little relation to the published data for mononucleotides both with respect to U.V. spectra and to optical density ratios. The data collected was probably relevant to small oligonucleotides which have, none the less, distinct spectrophotometric properties due to the competing properties of the constituent mononucleotides that make up the oligonucleotide chain but the differences are less discernable. The base is the predominant factor contributing to the U.V. spectrophotometric properties of nucleotides. The members of each series, for example adenine, AMP, ADP and ATP exhibit practically identical U.V. spectra

Ratio optical density	pH	Peak I fraction 36 & 37	II fraction 189 & 190	III fraction 214 & 215	IV fraction 248 & 249	V fraction 271 & 272	VI fraction 289 & 290
250/260	pH 11	0.90	0.99	0.97	0.96	0.95	0.94
280/260		0.47	0.52	0.71	0.52	0.54	0.57
250/260	pH 7	0.87	0.99	1.13	1.07	1.02	1.01
280/260		0.42	0.50	0.68	0.53	0.53	0.56
250/260	pH 2	3.04	0.96	1.96	1.35	1.07	0.95
280/260		0.54	0.67	0.87	0.56	0.63	0.64
		AMP	CMP	GMP	UMP		
250/260	pH 11	0.80	0.84	0.89	0.83		
280/260		0.15	0.98	0.60	0.28		
250/260	pH 7	0.78	0.84	—	0.73		
280/260		0.16	0.98	—	0.39		
250/260	pH 2	0.85	0.45	0.90	0.76		
280/260		0.22	2.00	0.68	0.32		

Lit. ratios for mononucleotides (226)

fig.61 O.D ratios for digest II and known values for mononucleotides

Since no mononucleotides were found, further analysis of peaks obtained was not proceeded with, as mononucleotide separation was the object. It was thought that the initial gradient concentration was too high and that the mononucleotides may have been eluted together in the first peak between tubes 10 - 40 because this peak shows evidence of multiplicity (see fig. 60). Alternately the mononucleotides may not have been held at all at this ammonium carbonate concentration, but according to Rushizky and Sober (229) mononucleotides should be retained on DEAE-cellulose at 0.01M$(NH_4)_2CO_3$. But in the case of further digests it was decided to reduce the initial $(NH_4)_2CO_3$ concentration and equilibriate the column with 0.005M $(NH_4)_2CO_3$.

Although mononucleotide separation does not seem to have been achieved, the column was successful in that a reasonable degree of product separation was obtained and therefore a more accurately defined digest was then carried out and designated digest IV.

DIGEST IV.

Enzyme RNAse B (J. Edmond - personal donation,37) was used and was purified finally by CM-cellulose column chromatography and prepared by an extraction method

virtually identical to that of extraction 1. Prior to use the enzyme had been stored at -22°C in the deep freeze.

Volume of protein solution = 15 mls.
Concentration = 0.703 mgs/ml.

∴ Total concentration of protein = 10.6 mgs.

RNA source: High molecular weight RNA from RNA preparation III (fig. 56) was used and 300 mgs were dissolved in 15 mls of sodium succinate/0.05 M Mg^{++} pH 6.5 buffer.

a) Digest. The fifteen mls. of enzyme solution and 15 mls of RNA solution were mixed to give a total volume of 30 mls. But due to difficulty in obtaining the solution of this RNA (page 263), the combined solution was filtered to remove the small amount of undissolved RNA. The digest was then placed in a constant temperature water bath at 37°C for 16.75 hrs (i.e. overnight). The digest time was not specific, but the length was thought to be adequate to enable digestion to go to completion.

The digest solution was then diluted tenfold with 0.005M $(NH_4)_2 CO_3$ solution (pH 8.6) giving a total of 300 mls of solution which was applied to the DEAE-cellulose

column. The column had been previously washed with saturated $(NH_4)_2 CO_3$ and then equilibriated with 0.005M $(NH_4)_2CO_3$ at pH 8.6. After the application of the digest material, the column was washed with 0.005M $(NH_4)_2 CO_3$ until the effluent had reached an equilibrium U.V. Absorption at 260 m_u. Material passing straight through the column and not held on washing showed strong absorbency with a λ max. at about 262m_u and a λ min. at about 245 m_u. These values for λ max. and λ min. indicate that this material could be of a nucleotide, nucleoside or free base nature, and therefore this was retained for further investigation.

Gradient applied (linear as in digest I & II):-

1ℓ. 0.005 M $(NH_4)_2CO_3$ ⟶ 1ℓ. 0.25M $(NH_4)_2CO_3$

The same column of 2 x 18 cms dimensions was used as for digests I and II. Three ml. fractions were collected using an "LKB Radirac" and "Uvicord" U.V. absorption register at 254 m_u. (See fig.62-elution profile)

RESULTS.

The following fractions were bulked.

fraction	28-42	Peak I
"	43-53	" II
"	54-65	" III
"	94-120	" IV
"	121-128	" V

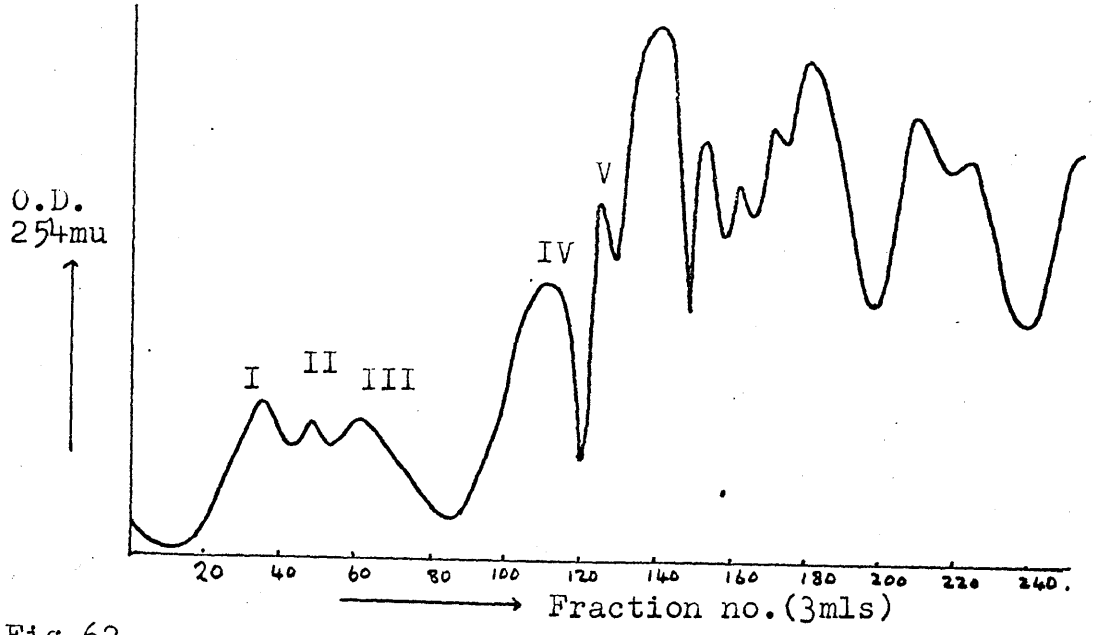


Fig.62.
Elution profile (254mu-nucleotide) of digest IV.

Control	R_{UMP} plate I	2	3	4	Average R_{UMP}
CMP	I.20	I.2I	I.26	I.22	I.22
AMP	0.88	0.89	0.87	0.89	0.88
GMP	0.62	0.60	0.57	0.59	0.60
UMP	I.00	I.00	I.00	I.00	I.00

Fig.63. R_{UMP} for TLC of mononucleotides in Solvent A

AMP Isomer	exampl I	2	3	4	5	6	Average
AMP i	0.77	0.74	0.67	0.74	0.69	0.72	0.72
AMP ii	0.94	0.89	0.82	0.90	0.84	0.87	0.88

Fig.64. R_{UMP} for TLC of AMP isomers in Solvent A -

Since, if mononucleotides were present, one would expect to find them in the initially eluted peaks from DEAE-cellulose, thus the following peaks were examined; - peaks I, II, III, IV, and V and also the material not held by the column when the digest mixture was applied or the "wash-off".

The peaks were vacuum rotary evaporated to reduce volume and then more water was added and the solution re-evaporated to remove as much volatile ammonium carbonate as possible. The residue was dissolved in water and subjected to the below described analytical procedures.

The fractions were examined by thin layer chromatography (T.L.C.), paper chromatography and U.V. spectrophotometry.

a) Thin layer chromatography with respect to digest IV products.

(i) Method of medium preparation - See General Method 10 (G.M. 10).

All solvents in the tanks were allowed to form a saturated atmosphere before used aided by lining the tank sides with filter paper to bring about fast evaporation and attainment of equilibrium with the vapour. Chromatography was carried out at a constant temperature of $18^{o}C \pm 1^{o}$.

(ii) Solvent systems.

The following solvent systems were experimented with using the four mononucleotides (CMP, AMP, GMP and UMP) as controls. These controls were BDH Biochemical grade 2', 3' mixed isomers of mononucleotides.

a) Leloirs solvent at pH 7.5 was initially used (230). The solvent was made up as follows:-

Leloirs solvent
- 3 volumes of 1M ammonium acetate pH 7.5.
- 7½ volumes of 95% ethanol.

However this solvent proved to be of little use when used with T.L.C.

b) A solvent consisting of 0.03N HCl was then used (231) with the result that the four control mononucleotides travelled with the solvent front and therefore since there was no separation, this solvent itself was of no use.

c) The following solvent was also prepared and used to separate the control mononucleotides (231)

- 600 gm "Analar" grade ammonium sulphate in 1ℓ. of disodium hydrogenphosphate (Na_2HPO_4) buffer at pH 6.8, and 200 mls of n-propanol.

The solvent achieved successful separation of all the control mononucleotides except guanylic acid which did not move from the base line.

d). Solvent b). had the characteristic of too rapid mononucleotide movement, whilst solvent c). was a little slow. Thus it was decided to attempt a combination of solvent b). and c). and a solvent was compounded to the following specification:-

Solvent A — 150 mls of 0.03N hydrochloric acid
90 gms of "Analar" grade ammonium sulphate.

Using this solvent (Solvent A), the distance that each entity had moved over the migration period was measured from the base line with reference to the distance travelled on the plate by uridylic acid. Therefore if UMP travelled X cms, and for example GMP travels y cms, then the distance travelled by GMP was expressed as $\frac{y}{X}$ or $R_{ump} = \frac{y}{x}$ cms. - and as all results were expressed relative to UMP (x) then UMP will always be unity. This method of result expression was the manner in which all results from chromatographic procedures, including paper chromatography and paper electrophoresis, were expressed. The solvent front was not used as a reference , since this was frequently not straight due to edge effects of the T.L.C. plate or paper and/or

irregularities in the cellulose medium are often difficult to completely eliminate in manual spreading of plates for thin layer chromatography. It is for these reasons and because small changes in temperature occurred(although these were reduced to a normal maximum variation of 2°C) that R_{ump} values were rarely exactly the same. Because of these variations a practice was made of running controls with nearly every T.L.C. plate to check the control R_{ump} values.

The R_{ump} values in solvent A (0.03N HCl - $(NH_4)_2SO_4$) for the control mononucleotides using data obtained from plates during chromatography of digest IV products are given in fig. 63.

Detection of nucleotide spots was by examination using incident U.V. light ("Hanovia Chromatolite") and the position of the spots was recorded by tracing onto a glassplate and then onto tracing paper. From these results for control mononucleotides it can be seen that good separation of the four components was obtained. However only in the case of AMP and GMP was separation into the individual isomers obtained and then it was not always easy to distinguish the two spots since the overall effect tended to be one elongated spot, especially in the case of GMP isomer separation. In these

difficult cases R_{ump} was calculated using a value for AMP or GMP measured to the centre of the combined spot for that nucleotide. In all cases where the separation of AMP into two isomers was discernable the following values of the R_{ump} for the two isomers were obtained:- fig. 64.

Behaviour of nucleosides on TLC using solvent A.

Control specimens of the four nucleosides (cytidine, adenosine, guanosine and uridine) were subjected to thin layer chromatography in the same manner as the nucleotides (fig. 65(a)) and the results (expressed as R_{ump}) compared with those for nucleotides in fig. 65(b).

Study of R_{ump} data (fig. no 65, page 294) indicates that Solvent A could be of equal use in the separation of nucleosides or nucleotides, but separation and identification of both at the same time would be somewhat confusing.

Results of TLC examination of the products of digest IV.

a) The table in fig. 66 summarizes the Rump's from each bulked peak and the material passing straight through the DEAE-cellulose column. In some cases there was a separation into more than one spot on the plate,

Nucleoside	Plate I R_{UMP}	II	III	Average
Cytosine	0.76	0.76	0.73	0.75
Adenosine	I.I6	I.I6	I.II	I.I5
Guanosine	-	0.45	0.46	0.46
Uridine	0.90	0.90	0.90	0.90

Fig.65a. R_{UMP} for TLC of control nucleosides in Solvent A.

R_{UMP} of:	Nucleosides	Nucleotides
Cytosine	0.75	0.87
Adenosine	I.I5	I.26
Guanosine	0.45	0.57
Uridine	0.90	I.00

Fig.65b. Comparison of R_{UMP} for TLC of control nucleotides and nucleosides in Solvent A,-all run on the same plate and at the same time.

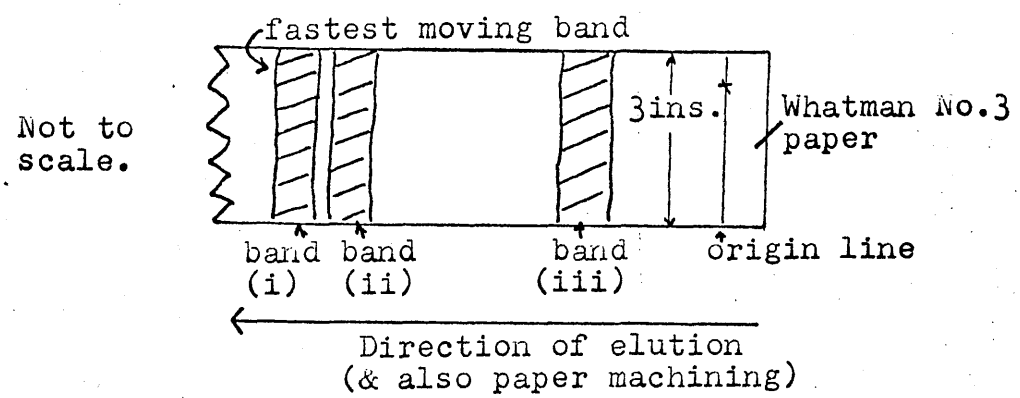


Fig.68. Diagram of 3 bands obtained after elution of combined peaks I,II,&III of digest IV.

	R-UMP								Comment
Plate	I	2	3	4	5	6	7	Av.	
AMP	0.88	0.89		0.87				0.88	nucleosides nucleotides CONTROLS
CMP	I.20	I.2I		I.26				I.24	
GMP	0.62	0.60		0.57				0.60	
UMP	I.00	I.00		I.00				I.00	
Aden.			0.76	0.76	0.73			0.75	
Cyt.			I.I6	I.I8	I.II			I.I5	
Guan.				0.45	0.47			0.46	
Urid.			0.90	0.90	0.90	0.94		0.90	
Peak									
Wash-off (i)	I.03				0.84	0.98			Possibly
(ii)						0.60			UMP
Peak I (i)		I.06			0.93	I.I3		I.04	Poss.UMP
(ii)		0.7I	0.70			0.90		0.75	Poss.AMP
(iii)						0.66		0.66	or dimer
Peak II (i)		I.I4	I.06		I.I6	I.08		I.II	Poss.UMP
(ii)		0.98			0.98	0.9I		0.96	or dimer
(iii)		0.76	0.70		0.7I	0.65		0.7I	or urid.
Peak III (i)	I.05				I.07	I.06	I.04	I.06	Poss.UMP
(ii)	0.97					0.85			?
(iii)									
Peak IV	0.59								? dimer?
Peak V (i)	0.95								UMP?
(ii)	0.70							?	dimers etc

Fig.66. TLC R_{UMP} for peaks I-IV & V and wash-off material -Solvent A. Digest IV.

and in peaks I, II, and III there was a separation into three spots on T.L.C.

Summary of T.L.C. investigation of peaks I - V and Wash-off.

Peaks I - V showed little or no evidence of any CMP but the Wash-off and peaks I, II, III(and IV?) showed evidence of material having R_{ump} very near that of unity indicating therefore UMP or dimers etc. with a very similar value.

b) Paper chromatography of digest IV products.

Descending paper chromatography at room temperature on Whatman No. 1 paper was carried out on peaks I - V and the Wash-off at pH 3.8 and 7.5 with Leloir solvents (230) with nucleotide and nucleoside controls.

Solvent B (Leloir--ref.230).

3 volumes of 1M ammonium acetate pH 7.5 and 3.8
$7\frac{1}{2}$ volumes of 95% Ethanol.

The latter nucleoside controls were only used with respect to the wash off and peaks I→III, since if nucleosides are present it is highly unlikely that they will be present in any peak after peak III. (or even as far as this stage) because of their lack of anionic properties due to the absence of a phosphate group as compared with nucleotides.

Leloir solvent pH 3.8					Leloir solvent pH7.5				Comment
Paper	I	2	3	4	5	6	7	8	
AMP	0.69	0.71	0.65	0.66	0.66	0.65	0.56	0.61	nucleotides
CMP	0.91	0.95	0.88	0.85	0.82	0.83	0.80	0.84	nucleotides
GMP	0.45	0.53	-	-	0.49	0.51	-	-	nucleotides
UMP	I.00	I.00	I.00	I.00	I.00	I.00	I.00	I.00	control
Aden.			I.69	I.55				I.63	nucleosides
Cyt.			I.54				2.70		nucleosides
Guan.				I.20				2.41	nucleosides
Urid.							2.94		control
Peak(i) I		I.30	I.24	I.24			2.40	2.54	nucleoside?
(ii)			I.02	I.09		I.04	I.01	I.09	UMP?
Peak(i) II		I.25	I.21	I.20			2.43	2.46	n'side,dimer?
(ii)		I.06	0.92	I.00		I.01	0.99	0.96	probably UMP
III		0.98	0.95	0.89		0.96	0.97	0.96	" "
IV (i)					I.10				UMP?
(ii)					0.80				CMP?
V (i)	0.34				0.47				dimer etc.?
(ii)					I.00				?
Wash-(i)			I.54				2.69	2.93	n'side,dimer?
off (ii)	I.00		I.02	0.99	I.10		I.15	I.23	UMP?
Time hrs.	47	47	43	43	47	47	43	43	

N.B. n'side = nucleoside, dimer = dinucleotide etc.

Fig.67. Summary of results from paper chromatography of peaks I-V and wash-off from Digest IV.

Location of spots as in T.L.C. was by incident U.V. light and the results are summarized in fig. 67.

Summary of results of the paper chromatography of the products of digest IV.

Peaks I, II, and III and the Wash-off gave a very similar pattern of results, all indicating the presence of UMP, with the wash-off, peaks I and II results also indicated the possible presence of nucleoside material since the solvent used, differentiated very sharply between nucleotides and nucleosides. This was in contrast to T.L.C. separation in solvant A where the nucleotide and nucleoside Rump differences were less distinct. In agreement with T.L.C. findings, paper chromatography using solvent B showed little evidence of any other mononucleotide than UMP, with perhaps the exception of peak V.

c) Further paper chromatography and U.V. spectrophotometric data of the products of digest IV

Because of close similarity of peaks I, II and III as revealed by T.L.C. and paper chromatography, the remaining portions of these peaks were bulked together and applied in a band to a 3" ins. wide strip of Whatman No. 3 paper which had been previously washed according to Connell et al (232). The wash-off fraction was applied in a likewise manner to another strip of Whatman

No.3 paper and both strips were eluted by descending chromatography with Leloir pH 7.5 solvent (230) for 24 hours. The position of the resulting bands that separated during chromatography were marked by viewing via incident U.V. light (fig. no. 68).

The areas of U.V. absorption were then cut out and the small strips of paper were sandwiched between two glass microscope slides and the nucleoside or nucleotide material eluted from the strip by descending elution using water conducted to the strip by a paper wick. U.V. spectra and optical density ratios were obtained for the material so eluted from the bands and compared with the published data for nucleotides (226,227). None of the experimental data obtained corresponded exactly with the published data for uridine, guanosine or adenosine type compounds, but like paper chromatography the U.V. spectra showed no evidence of any characteristic cytosine curves which are diagnostic at pH 7.0 and thus so are the optical density ratio values at $\frac{250}{260}$, and $\frac{280}{260}$ m_u. Similarly adenosine compounds exhibit characteristic spectra, but of which the results showed no evidence.

If the published ratios at $\frac{250}{260}$ and $\frac{280}{260}$ m_u for

uridine compounds are examined, it can be seen that there is a tendency for the ratios at $\frac{250}{260}$ to be approximately double those at $\frac{280}{260}$. This tendency was shown to some extent in the results obtained and all bands eluted from Whatman No. 3 paper including those from the wash-off showed evidence of material of uridine nature but more than likely the spectra incorporated two or more compounds and therefore of hybrid nature. Thus the results were only satisfactory in that they supported the previous T.L.C and paper chromatography in finding no evidence of cytidine compounds.

Summary of digest IV.

A consideration of all the results from T.L.C. paper chromatography and U.V. spectrophotometric data would seem to indicate that RNAse B does not have the same specificity as pancreatic RNAse, namely the production of UMP and CMP as end products since all the available data from digest IV demonstrated that only UMP was produced. It is possible that any CMP present may have been eluted straight through the column and not adsorbed by the DEAE-cellulose at all. However this fraction or wash-off material at the initial 0.005M ammonium carbonate concentration was investigated and

no evidence of any CMP was found. The other alternative was that the CMP was more tightly adsorbed and not eluted until after peak V (fig. 62), which fraction was not investigated. But M. Staehelin (233) in an investigation of nucleotide sequences in RNA also using DEAE-cellulose and ammonium bicarbonate elution at pH 8.6 with a linear gradient from 0.01M to 0.15M ammonium bicarbonate, reported that CMP was eluted first, to be followed by UMP, AMP, and GMP, and then the di- and triphosphate nucleotides in the order CDP,UDP, ADP,CTP,UTP,GDP, ATP and finally GTP. So therefore the pattern of elution under the conditions reported by Staehelin (233), which are very similar to those described for digest IV, follows the rules that, (a) monophosphates are eluted before the corresponding di- and triphosphates, (b) pyrimidine nucleotides are eluted before purine nucleotides and cytosine compounds are eluted before those containing uracil, and adenine containing compounds before those containing guanine.

It will be noted that ammonium carbonate was used in the elution of the products of digest IV from DEAE-cellulose whereas Staehelin (233) used ammonium bicarbonate solution in which the pH was adjusted to pH 8.6 using ammonia. But by treating an aqueous

solution of ammonium bicarbonate with excess ammonia, ammonium carbonate is formed (234). Also the commercial ammonium carbonate used in digest IV (B.D.H. "Analar" grade reagent) was a mixture of ammonium bicarbonate and ammonium carbamate ($NH_4O.CO.NH_2$), and the latter forms $(NH_4)_2CO_3.H_2O$ when dissolved in water. Therefore whether ammonium bicarbonate or ammonium carbonate is used in making up a buffer for DEAE-cellulose separation of nucleotides, the end result is much the same.

The above described work shows that if CMP was a product of RNAse B digestion of yeast RNA, it would have been the first mononucleotide to have been eluted from the DEAE-cellulose anion exchanger.

The results from digest IV seems to suggest that basic calf spleen ribonuclease 'B' appears to produce only UMP as a mononucleotide product of RNA digestion, which would indeed be a unique RNAse specificity, and, so far unreported. On the basis of this result further specificity work was undertaken.

DIGEST V & VI

To achieve an improved separation of nucleotides on DEAE-cellulose an appreciably larger column was used in subsequent chromatography work to that of digest IV

(2 x 18 cms). Various column lengths and digest loads were experimented with before a satisfactory combination of column length with amount of digested material that could be applied to the cellulosic amion exchanger and retained, could be arrived at. The result of the considerable time spent on this initial experimental work was manifested in the conditions used for the DEAE-cellulose chromatographic separation of the products of the following digests - digests V and VI.

Details of digest V.

(a) Enzyme source.

A number of RNAse 'A' sources (Extraction 1 and 2 pages I20,I2I) and some RNAse 'B' donated by J.Edmond (37) were bulked together and reapplied to a CM-cellulose column that had been freshly prepared according to the procedure described on page 43(ref.74).

Chromatography of enzyme samples prior to use in digest V and VI.

Column dimensions 50.5 x 2.2 cms.

The column was prepared at cold room temperature (5^{o}) in the presence of, and equilibriated with, 10^{-4}M-EDTA 0.005M Tris,pH 7.0 buffer which was made 2M with urea.

The bulked enzyme samples were filtered through Whatman No.1 filter paper to remove pieces of cotton

wool for example, that had come to contaminate the enzyme solution during its storage under deep freeze conditions at -22°C. Dialysis was necessary since the samples will have been the products of CM-cellulose concentrating columns (page 57) from which elution was obtained with concentrated NaCl solutions, and if the Na^+ ion concentration is high, the proteins would not be absorbed by the CM-cellulose cation exchanger.

After dialysis the enzyme solution was diluted to 1 l. with pH7.0 buffer and made 2 Molar with respect to urea. This solution was applied to the CM-cellulose column and the resulting column effluent and washing buffer was examined (fig.69)

Volume of protein solution applied = 1 litre

Protein concentration = 0.132 mg/ml

∴ Total protein applied = 1000 x 0.132 mgs
= 0.132 gms.

Volume of effluent + buffer used to wash column after application = 2100 mls

Protein concentration in effluent + wash = 0.023 mgs/ml

∴ Total protein not held by column = 2100 x 0.023 mgs.
= 0.048 gms

∴ % protein retained by CM-cellulose column = 64%.

Gradient elution.

The following gradient was applied using the same apparatus as used in CMC-4 and other columns (page I3o fig.23).

Reservoir 1.5 l.		Constant volume chamber 800 ml.
0.005M Tris/HCl 10^{-4} M EDTA. 2M Urea 0.32M NaCl pH 8.2	←	0.005M Tris/HCl 10^{-4} MEDTA 2M Urea pH 7.0

Results of enzyme chromatography - see fig. 69, page 306.

After elution of all activity, the column was washed with 1100 mls of 1M sodium chloride solution and the washings collected. This eluted material possessed U.V. adsorption at 280 m_u and contained 9.5% of the protein applied to the CM-cellulose column.

Since both RNAse A and B were applied to the column, two peaks of activity were expected as in CMC-4 (page I33) and to some extent this was attained. But the merging of the activity peaks can be explained largely by the fact that the column was eluted at rather a fast rate and so separation of peaks was less distinct. Also some enzyme components of the mixture had been stored for considerable periods (up to two years) at -22°C and aggregation changes could result in the

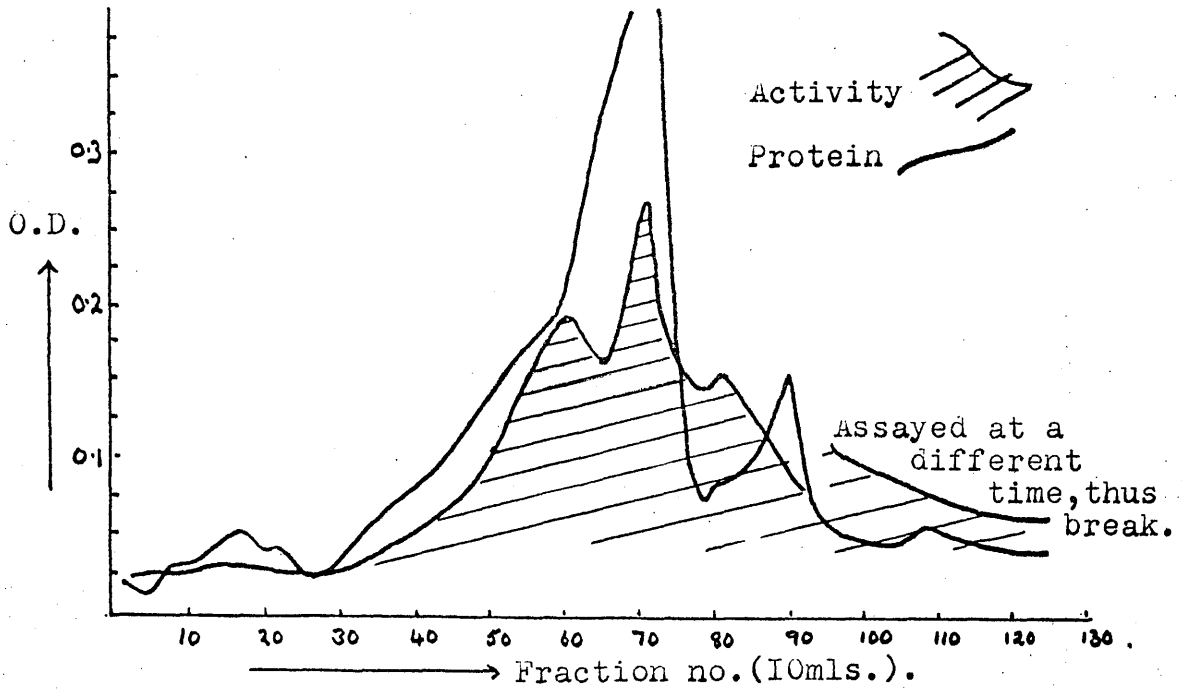


Fig.69. CM-cellulose chromatography of enzyme material for digests V and VI.

tendency of peaks to overlap.

Fractions 40-90 were bulked for use in digest V and VI although this material will contain both RNAse A and B fractions. The bulked fraction was then dialysed against 5ℓ. of 0.005M Tris, 10^{-4} M EDTA, pH 7.0 buffer for three hours.

Volume of solution after dialysis = 480 mls. However only half of this volume of dialysed enzyme solution was used in digest V = 240 mls. Amount of protein used = 0.039 gms.

b) RNA source. Exhaustively dialysed commercial yeast RNA (the preparation of which was described on page 269) was used as an RNA source for digest V at the rate of 3.5 mgs/ml of digest volume. The concentration of RNA in routine digests of column effluent fractions was 2.5 mgs/ml in the final digest. The figure of 3.5 mgs chosen was somewhat arbitrary, since optimum enzyme substrate ratios was not of paramount importance to the results sought after, i.e. specificity of enzyme action.

c) Digest. The digest time was chosen on the basis of what was thought adequate for the reaction to go to completion. Rushizky et al (235) using synthetic nucleotide mixtures reported that the conversion of the

cyclic terminal phosphate to the corresponding 3' phosphate form of either pyrimidine for pancreatic RNAse was not complete after twenty-three hours at room temperature. Thus an arbitrary digest time, well in excess of this figure was chosen for digest V, of about two days.

The enzyme solution, buffered with 0.005M Tris, 10^{-4}M EDTA at pH 7.0, and the RNA were mixed and made 0.025 M with respect to Mg^{++} ions (page 53 ref. 28) in the usual manner. The digest was carried out in a flask at 37°C in a constant temperature water bath. Three drops of Analar grade chloroform were added to the digest mixture before the flask was sealed, to prevent bacterial growth (236)

d) Separation of digest products.

After digestion, the solution containing the products was diluted to 1 litre and allowed to cool to 5°C and ammonium carbonate was added to bring the concentration to 0.005M $(NH_4)_2\ CO_3$ at pH 8.6. The digest solution was then applied to a DEAE-cellulose column which had been prepared previously, by the standard method (page II5,GM3), and washed with saturated $(NH_4)_2\ CO_3$ and then equilibriated with 0.005M

$(NH_4)_2CO_3$ before ready for application of digest products. The column dimensions were 42 x 1.7 cms.

After application of the digest material, the column was washed with 0.005M $(NH_4)_2CO_3$, but even after 4 litres of wash the effluent had not reached an equilibrium U.V. adsorption at 260 m_u. Therefore a very large proportion of the digest products were not held by this DEAE-cellulose column which was probably due to an overloading of the column, since much of the material not adsorbed was held when applied to another DEAE-cellulose column. The overloading problem in subsequent digests could be overcome by either reducing the reactant amounts, or increasing column size. To aid easier product identification, reactant amounts were maintained at the same level, but column size was increased.

Further work on digest V was discontinued because comparison of results with previous and subsequent digests would not be easy if the chromatography was split between two columns and so a further similar digest, which was then applied to a larger DEAE-cellulose column, was undertaken (Digest VI).

DIGEST VI

A) Protein source. The same protein source as in

digest V was used in digest VI, that is mixed calf spleen basic RNAse 'A' and 'B', but a smaller enzyme concentration was used.

Volume of protein solution = 150 mls

Concentration of protein = 0.175 mgs/ml

Total protein concentration for digest VI = $\frac{26.2 \text{ mgs}}{(39.1 \text{ mgs}) \\ (- \text{ digest V})}$

b) RNA source. Exhaustively dialysed commercial yeast RNA as in digest V was used at the same rate of 3.5 mgs/ml of solution in the digest mixture.

c) Digest. The RNA was dissolved in the protein solution which was also made 0.025 M with respect to Mg^{++} ions (page 53 ref. 28). The digest was carried out, in a sealed flask with 4 drops of chloroform to inhibit bacterial action, for 50 hours at 37°C.

d) Separation of digest products.

After digestion the solution containing the products was diluted to 3 litres and made 0.005M with respect to $(NH_4)_2CO_3$ and allowed to cool to 5°C before applying to a DEAE-cellulose column in a coldroom. The column dimensions were 97 x 1.7 cms (x 2.3 that used in digest V). The DEAE-cellulose (prepared by the method on pages II5 and GM3b) had previously been washed

with 2M $(NH_4)_2CO_3$ and then equilibriated with 0.005M $(NH_4)_2CO_3$.

The digest products in 3ℓ. of solutionwere then applied to the column and washed with 0.005M $(NH_4)_2CO_3$. The percentage retention of nucleotide material by the column was ≏ 87%. Thus this column has successfully retained most of the products of digest VI and so the following gradient elution was applied at a flow rate of 0.27 mls/minute:

$$2\ell.\ 0.005M\ (NH_4)_2CO_3 \longrightarrow 2\ell.\ 0.25M\ (NH_4)_2CO_3$$

using equal sized reservoirs.

c) Results

Fractions of 10 mls were collected using "LKB Radirac" fraction collector and the U.V. adsorption of the effluent at 254 m_u was monitored automatically using an "LKB Uvicord" assembly. The optical density at 260m_u was also read manually every alternate fraction in an Unicam SP500 spectrophotometer to enable a more accurate graph of $O.D._{260}$ against fraction number to be drawn (fig. 70).

The 13% of digest not held by DEAE-cellulose and passing straight through was examined by T.L.C., paper chromatography and further column chromatography.

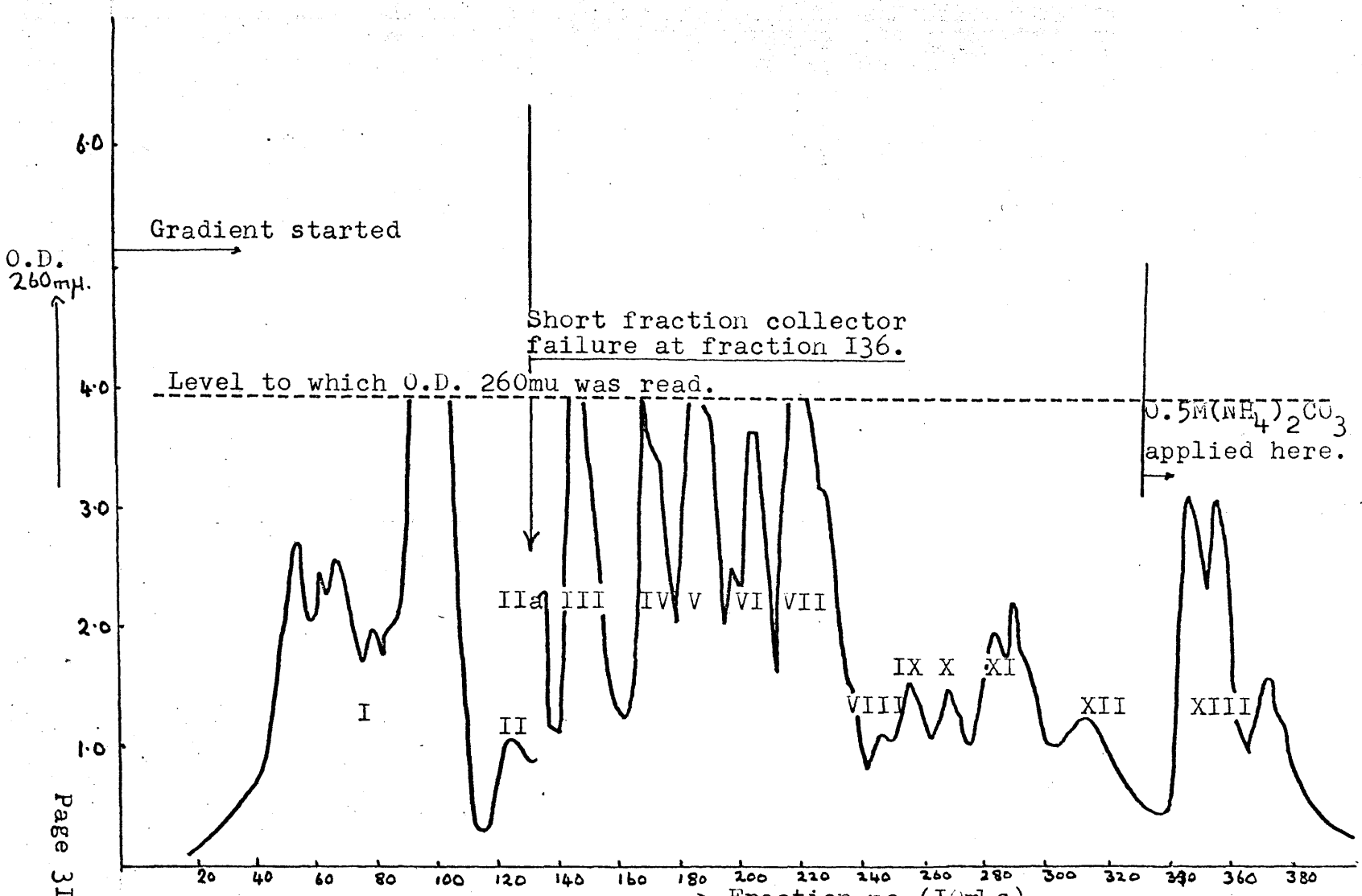


Fig.70. Elution profile of products of digest VI on DEAE-cellulose chromatography.

Fraction no.	Peak no.	No. of tubes
20-116	I	97
120-136	II	17
137-143	IIa	7
145-164	III	20
165-182	IV	18
183-200	V	18
201-216	VI	16
217-246	VII	30
247-254	VIII	8
255-266	IX	12
267-280	X	14
281-307	XI	27
308-329	XII	22

Fig.71. Fractions bulked - digest VI.

Controls	R_{UMP}	Average		Conclusions
AMP	0.91	8	Average factor	Nucleotide controls
CMP	1.25	8		
GMP	0.53	8		
UMP	1.00	8		
Urid.	0.90	8		Nucleoside controls
Cyt.	1.09	8		
Wash-off i)	0.76	3		?
ii)	0.94	3		AMP, UMP ?
iii)	1.14	3		CMP ?

Fig.72. TLC results of digest VI. -Wash-off.

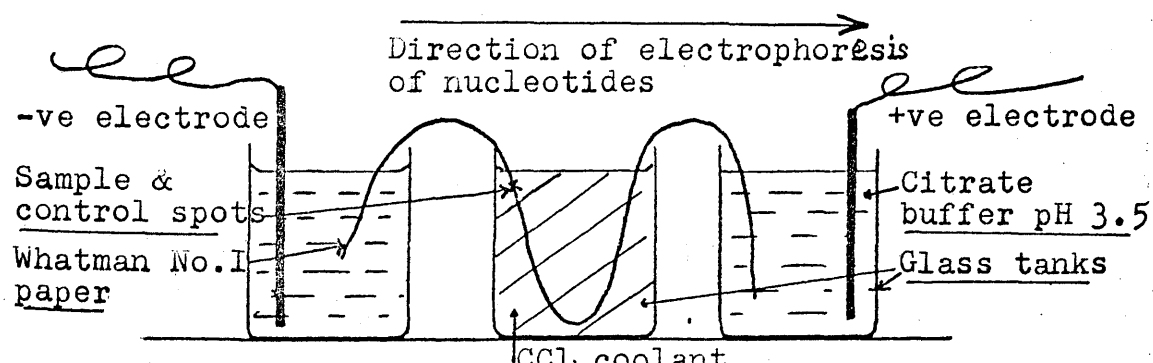


Fig.75. Paper electrophoresis apparatus (ref.239) Page313.

A. Examination of material not absorbed by DEAE-cellulose when digest VI products were applied.

Note. In all future chromatographic procedures in digest product analysis, the methods and solvents will be the same as those used in Digest IV unless otherwise stated. i.e. The T.L.C. solvent and procedure as described on page 289 (G.M. 10), Paper chromatography procedure as described on page 296 (G.M.9), U.V. spectrophotometric data retrieval as described on page 298 . In this and future result tabulations, the T.L.C. and paper chromatography results will be presented as an averaged value (Af.) for a number of separatory runs for that particular sample, and the number of figures from which the average result was calculated will also be tabulated (Af.). Thus for example if a value of 0.91 is the average R_{ump} for control AMP as calculated from 8 T.L.C. plate results, then this is written as 0.91 (8). There are often more than the one separation on each plate or paper, particularly in the case of the unknown compound being subjected to T.L.C. or paper chromatography and therefore the effective number of results from which an average is derived is much larger than just the factor or number of plates or papers that have been run. This has

been done to reduce the sheer mass of chromatographic data to aid interpretation of figures and tables.

a) T.L.C. The results given in fig 72 represent the average R_{ump}'s obtained from three plates, and since each plate contains three spots of the material being analysed, the figure given is really an average of nine chromatographic separations. From these results the wash-off seems to consist of three components which according to the control R_{ump} values may include UMP, U, AMP or CMP.

b) Paper chromatography. Exactly the same conditions were used as for the paper chromatography of products from digest IV and results are given in Fig. 73.

Conclusion from paper chromatography of material not held on DEAE-cellulose. fig.73 and 72.

The results were very inconclusive and T.L.C. suggests possibly AMP or UMP and also a slower running spot somewhere between GMP and AMP. Paper chromatography shows that nucleosides of the pyrimidine bases are not present and also demonstrates little evidence of mononucleotides. Because of the inconclusive nature of these results and since the fraction was not retained by DEAE-cellulose, it was decided to subject this fraction to column chromatography as Dowex-I which is a stronger anion exchanger than DEAE-cellulose.

Leloir buffer pH 7.5				Leloir buffer pH 4.0			
	R_{UMP}	Av.	Comment		R_{UMP}	Av.	Comment
AMP	0.62	6	C	AMP	0.67	2	C
CMP	0.76	6	o	CMP	0.97	2	o
GMP	0.53	5	n	GMP	0.47	2	n
UMP	I.00	6	t	UMP	I.00	2	t
Urid.	3.54	2	r	Urid.	-		r
Cyt.	3.I6	-	o	Cyt.	-		o
			l				l
Wash (i)	0.7I	4	AMP, CMP?	Wash i	0.93	2	CMP, UMP?
-off (ii)	0.96	4	UMP?	-off			
(iii)	I.I6	I	?				

Fig.73. Paper chromatography of wash-off material from digest VI. (The average, average factor or Af, refers to the total number of readings of R_{UMP} taken and on which the average R_{UMP} stated in the result data is based.)

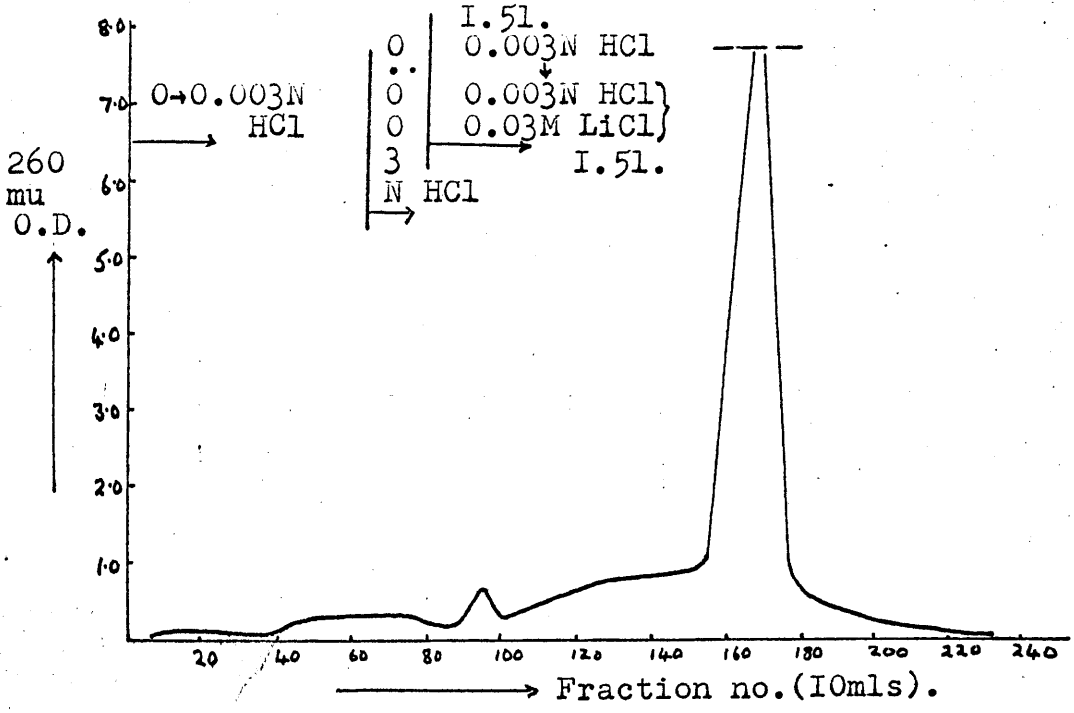


Fig.74. Dowex-(A).Material not held by DEAE-cellulose. (flow rate 0.5mls/min.)

Further ion exchange separation of 'wash-off' on Dowex-I

The nature of Dowex type ion exchange (i.e. a polystyrene resin with a trimethyl substituted quaternary ammonium group as the cationic group) resins was discussed on page 273 (ref. 221). From Cohn's work (221) it can be seen that the optimum separations with hydrochloric acid of ribonucleotide monophosphates have been obtained at pH 2.7 when cytidylic, adenylic, guanylic and uridylic acid are in the anionic form (237). Below pH 2.5 cytidylic and adenylic acids are cationic, and below pH 1.5 guanylic acid is cationic. Thus if nucleotide material is absorbed at pH 6.0 or greater, it will be anionic and then the nucleotides can be removed in sequence by decreasing the pH and/or increasing the concentration of the competing anion. In early ion exchange studies with nucleotides, chloride was favoured as the competing anion. But the use of a formic acid (formate anion) elution system was also favoured in preference to hydrochloric acid (Chloride anion) in the pH 2-3 range for the following reasons:-

1) pH control is more exact.

2) The neutralization of the nucleotides, usually adsorbed as divalent ions is more rapidly achieved, thus eliminating large dead volumes.

3) Simple evaporation may be used to recover the products.

But recent years have favoured the use of the chloride system because it has one very marked advantage in that subsequent concentration by ion exchange, which requires the conversion of the effluent to a solution of pH 8-10 with low (< 0.01M) anion concentration, is possible by the simple addition of a small amount of ammonium hydroxide. Such a solution can readily be stripped of its nucleotide content on tenfold smaller columns, from which the product can be recovered in concentrated solution by elution with 0.02 - 0.1M HCl.

Thus in the examination of the products from digest VI, the chloride system was chosen for gradient elution of nucleotides from the synthetic anion exchange resin Dowex-I. Lithium chloride was chosen as the chloride salt (238), the reason for this being that nucleotide fractions isolated will be in the form of their methanol soluble lithium salts. Thus when excess dry acetone is added, the lithium nucleotide salts precipitate out, whilst the excess lithium chloride remains in solution, and so in the concentration and isolation procedure, a salt-free nucleotide is obtained.

The fresh resin (Dowex-I x 4, 200-400 mesh, Dow Chemical Co. Michigan, U.S.A. and the type used in all future work with Dowex-I) was washed with 1N NaOH and then with water several times in a batchwise fashion (GM3c). "Fines" were removed by stirring and decanting all fines left in suspension after five minutes settling time. The resin was then washed batchwise with 1N HCl and then with water until free of acid, and slurried into a column, and then eluted copiously with 1N HCl followed by deionized water until the effluent was neutral to universal indicator paper ("Johnsons")

Column dimensions: 10 x 1.8 cms.

This column was designated Column Dowex (A) - Digest VI

No. of optical density units applied to Dowex (A) = 1515 units

No. " " " " passing straight through = 127.5 units

% retention by Dowex (A) = 91.5%

After the application of the material not held by DEAE-cellulose to Dowex (A) the column was briefly washed with deionized water and the following gradient elution programme implemented:-

Eluents	Range (10 ml) (fraction)
500 mls H_2O ⟶ 500 mls 0.003N HCl(2.7)	1 - 60
elution with 0.003N HCl	61-74
1.5ℓ. 0.003NHCl ⟶ 1.5ℓ, 0.003N HCl 0.03N LiCl	75-313
Elution with 0.03N HCl 0.05 N LiCl	314 —

Ten ml. fractions were collected and an elution profile obtained as in fig. 74. Fractions 153→185 were bulked as this was the most significant peak of Dowex (A). The pH was adjusted from about 2.7 to 7.0 with 1M lithium hydroxide and then the solution was freeze dried. The dry residue was taken up in the minimum amount of dry methanol and then excess dry acetone was added which caused the precipitation of nucleotide material free of salt. The nucleotide precipitate was removed by centrifugation and dried in a vacuum dessicator and stored at -22° until it was needed for further analysis. (See General Methods GM 6 & 7)

Analysis of the products of Dowex (A). Only the main peak was thought to be significant and so this was examined by the following methods.

1. T.L.C.

	Rump	Af.	Comment
AMP	0.75 0.90	3	Controls
CMP	1.16	3	
GMP	0.51	3	
UMP	1.00	3	
Dowex(A)	0.80	3	AMP?

2. Spectrophotometric data.

Spectra at acid pH. λ max 279 (278) $\frac{250}{260}$ = 0.45 (0.91)

λ min 240 (252) $\frac{280}{260}$ = 2.00 (1.56)

Spectra at alk. pH λ max 270 (270) $\frac{250}{260}$ = 0.86 (1.01)

λ min.255 (255) $\frac{280}{260}$ = 0.93 (0.90)

The values obtained did not coincide with the published figures for any mononucleotide $\begin{pmatrix}226\\227\end{pmatrix}$, but were nearer to the published values for CMP than any mononucleotide (the values for CMP are quoted above in brackets). Of particular significance was the high λ min. at 250 and 240 m_u at alkaline and acid pH's respectively, which is very characteristic of CMP type nucleotides.

3. Paper electrophoresis

Introduction to paper electrophoresis (Also see GM9.)

The experimental conditions and apparatus and also the theoretical considerations are discussed fully by J. D. Smith (241).

Nucleic acids and their components bear a number and variety of ionizable groups, and it is natural that electrophoretic techniques should play an important part in their separation. The method of separation is a very efficient and fast one, and the development of paper electrophoresis has greatly facilitated the separation of nucleic acid derivatives. An important feature of the method is that the relative mobilities of nucleic acid components at any given pH may be predicted quite accurately, thus aiding the identification of unknown components.

Thus a molecule in a fluid subjected to a voltage gradient E, is acted upon by a force equal to (EQ) where Q is the net charge on the molecule and is given by the algebraic sum of the products of the number of ionizing groups and their percentage dissociation. This force is opposed by that due to the resistance of flow of the molecule through the

liquid, which is proportional to the velocity of motion of the molecule (V.) and equal to (KV) where K is a function of the size and shape of the molecule and the retarding effects of other ions in solution. The ions thus migrate at a constant velocity equal to $\frac{(EQ)}{(K)}$. Using the dissociation constants of the ionizable groups in nucleic acid components and making certain approximations as to the nature of the resistance to flow of the charged particles through the fluid, the relative mobility of these substances can be calculated. The relative mobilities of the four mononucleotides at pH 3.5 are:-

decreasing mobility ↓	Uridylic acid	1.00
	Guanylic "	0.95
	Adenlyic "	0.45
	Cytidylic "	0.16

At this pH the degree of dissociation of the NH_2 groups in AMP, GMP and CMP is most advantageous to separation.

Paper electrophoretic separation of Dowex (A) main peak

Electrophoresis was carried out on Whatman No.1 paper strips about 3 inches wide and in 0.1M sodium-citrate/citric acid buffer at pH 3.5. A voltage of 900 v. was used for the time stated below (usually about 50 mins).

The coolant medium was fresh 'Analar' grade carbon-tetrachloride (239) which was renewed each time. The buffer and coolant containers were assembled along with the paper strip as in fig. 75. Using paper strips approximately 38 cms. long, this was equivalent to a voltage gradient of 25 volts per cm.

Results. (Expressing the average of a number of results (Af.) as done for T.L.C. and paper chromatography - page 314)

	R_{ump}	Af.	Comment
AMP	0.49	4	Controls
CMP	0.38	4	
GMP	0.80	4	
UMP	1.00	4	
Dowex (A)	0.29	4	No correln.

Summary of analysis of main peak from Dowex (A) (Material not held by DEAE-cellulose).

T.L.C., paper electrophoresis, and U.V. spectrophotometric data results indicated that this was not a mononucleotide or indeed a nucleoside. The position of the elution from the Dowex resin confirms that the unknown material was not of nucleoside nature since nucleosides would have certainly been eluted in the initial 0.003N HCl gradient (221) and similarly

one would have expected CMP (and possibly AMP), if present, to have been eluted by 0.003N HCl (i.e. before tube 74 in fig. 74) according to the elution order of mononucleotides obtained by Cohn(22I) under similar conditions.

But U.V. spectra showed this fraction to be of nucleotide character and this was substantiated by its behaviour with regard to chromatographic procedures. If this was the case, and this fraction was a nucleotide, it must have the following properties:—

1) Low relative electrophoretic mobility

2) Poor adsorptive properties on DEAE-cellulose but substantial adsorption by Dowex-I resin.

3) Spectral properties akin to CMP types.

These conditions could possibly be fulfilled by a di- or trinucleotide and in particular a di- or —trinucleotide of CMP. If the compound was a di- or trinucleotide (larger oligo-nucleotides were less likely since their properties are unlikely to coincide with the already established properties of the unknown entity) with a common base, the hydrolysis would result in two or more identical constituents

and give rise to one spot by separative chromatography techniques. However although there will be no separation into two detectable spots, properties before and after hydrolysis should be quite different and detectable. If the di- or trinucleotide was composed of two or more different base types, chromatographic separation techniques will exhibit proof of two separate and characteristic entities. Therefore hydrolysis of the main peak from Dowex (A) was carried out to verify the nature of this nucleotide type material.

Hydrolysis of main peak from Dowex (A)

When ribose nucleic acids are treated with mild alkaline reagents, they are rapidly converted into a mixture of their component mononucleotides. The cleavage by alkali is not specific as in the cleavage by pancreatic RNAse, but mononucleotides of all four bases are produced and represent a mixture of nucleoside 2' phosphates and nucleoside 3' phosphates. Markham and Smith (240) showed that alkaline hydrolysis of RNA proceeded via the formation of 2' 3' cyclic phosphate intermediates as was also demonstrated later for enzymatic (pancreatic RNAse) hydrolysis by the same workers (239). However alkaline hydrolysis differs from enzymatic hydrolysis of RNA, in that the final hydrolysis step to the 2' or 3'

phosphate in alkaline hydrolysis is completely random, whereas in pancreatic RNAse the nucleoside 3'-phosphate is produced.

Method.

The material isolated from Dowex (A) was made up to 7 mls. and then 3 mls. of IN lithium hydroxide were added, thus giving a final lithium hydroxide normality of 0.3. The solution was allowed to hydrolyse for 18½ hours at 37° (in a constant temperature water bath). The hydrolysate was then neutralized with 5N hydrochloric acid, diluted to 150 mls and lyophilized.

The isolated material was dissolved in 500 mls of deionized water at pH 7.0 and applied to another Dowex-I column, designated Dowex (B). Column dimensions = 9.5 x 1.8 cms. The retention of this material by the resin was 100%. Dowex (B) was subjected to the following elution programme after initially washing with 215 mls. of deionized water.

Elution programme:-

Elution of Dowex-B.	Fraction range (10 mls)
150 mls. H_2O ⟶ 150 mls. 0.003N HCl	1 — 27
Elution with 0.003N HCl	28 — 70
1 ℓ. 0.003N HCl ⟶ 1 ℓ. 0.003N HCl 0.03M LiCl	71 — 240

Results. The elution profile of Dowex (B) is given in fig. 76 and the following fractions were bulked together:- fractions 32-50 and neutralized with IN Lithium hydroxide and reduced in volume by rotary evaporation.

Analysis of the products of Dowex (B)

a. Position of elution. The only material that was eluted from this column with the gradient sequence that was used, was from fractions 32-50 which was eluted by 0.003N hydrochloric acid. The position of elution indicates that the peak was of a mononucleotide character and probably CMP or AMP in property nature. Prediction of nucleotide character as the basis of position of elution from a Dowex I resin column was based on the work of Cohn (22I) which included the use of hydrochloric acid (0.003N) as an initial eluent. Hydrolysis of the main peak of Dowex (A) has thus

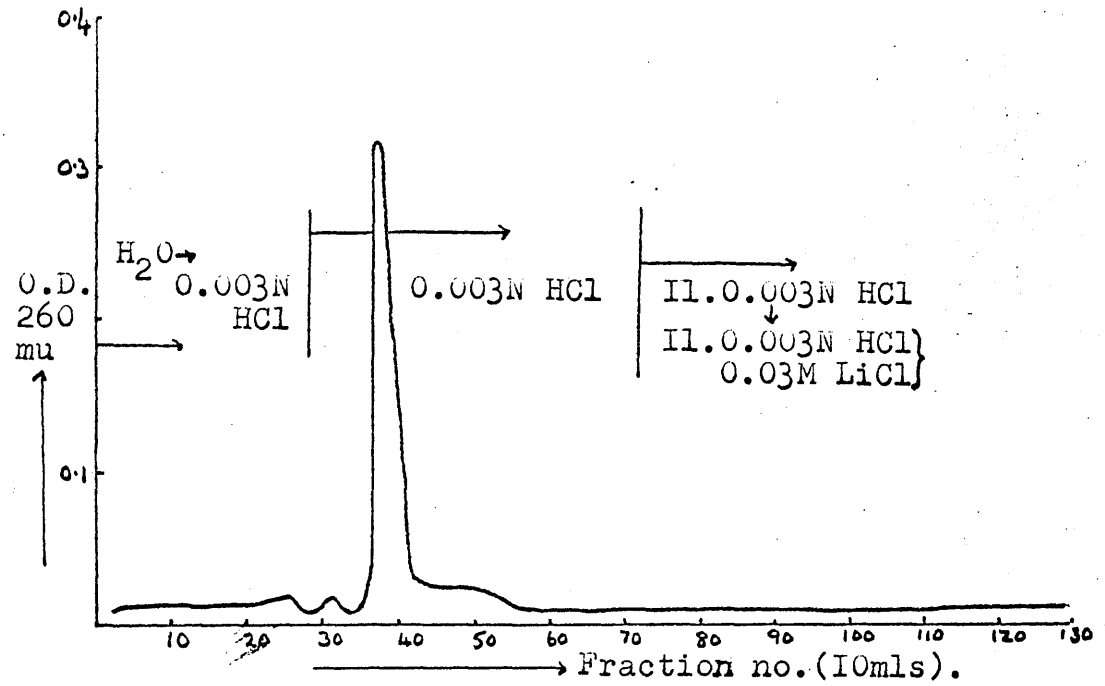


Fig.76. Hydrolysis product of Dowex(A) main peak, —Dowex(B).(Flow rate 0.9mls/min.)

	R_{UMP}	Af.	Comment
AMP	0.88&0.73	5	Controls.
CMP	I.I8	5	
GMP	0.55	5	
UMP	I.00	5	
Dowex(B)	I.20	5	Therefore CMP?

Fig.77. TLC results of material from Dowex(B).

brought about a change in this elution position, indicating (fig. 74 and 76) that Dowex (A) product was of di- or trinucleotide character, and further analysis below was used to confirm this surmise.

b) U.V. Spectrophotometric data.

Spectra at acid pH λ max. 279(279) O.D.$\frac{250}{260}$ = 0.64 (0.45)

λ min. 241(240) O.D.$\frac{280}{260}$ = 1.63 (2.00)

Spectra at alk. pH λ max. 269(272) O.D.$\frac{250}{260}$ = 0.94 (0.86)

λ min. 254 (250) O.D.$\frac{280}{260}$ = 0.89 (0.93)

The data obtained compared favourably with that for the published values of CMP (figures quoted in brackets) (226 ,227).

c) T.L.C. see fig. 77 for a summary of results and also fig. 78.

for a comparison of data for the main peak from Dowex (A) before alkaline hydrolysis,with that of Dowex.(B) after hydrolysis.

Summary of the investigation of material not held when digest VI material was applied to a DEAE-cellulose column. (i.e. Dowex (A) and (B) columns)

Subsequent chromatography of this fraction on Dowex-I resin (Dowex (A)) showed that the compound had affinities

	Dowex(A)		Dowex(B)	
Acid λ max., λ min.	278mu	253mu	279mu	241mu
Alk. λ max., λ min.	270mu	255mu	269mu	254mu
$\frac{250}{260}$ i.acid ii.alk	0.91	1.56	0.64	0.94
$\frac{280}{260}$ i.acid ii.alk	1.01	0.90	1·63	0.89
TLC ....CMP	1.16		1.18	Average
unknown	0.80		1.20	values

Fig.78. Comparison of data of Dowex(A) and (B), before and after alkaline hydrolysis respectively.

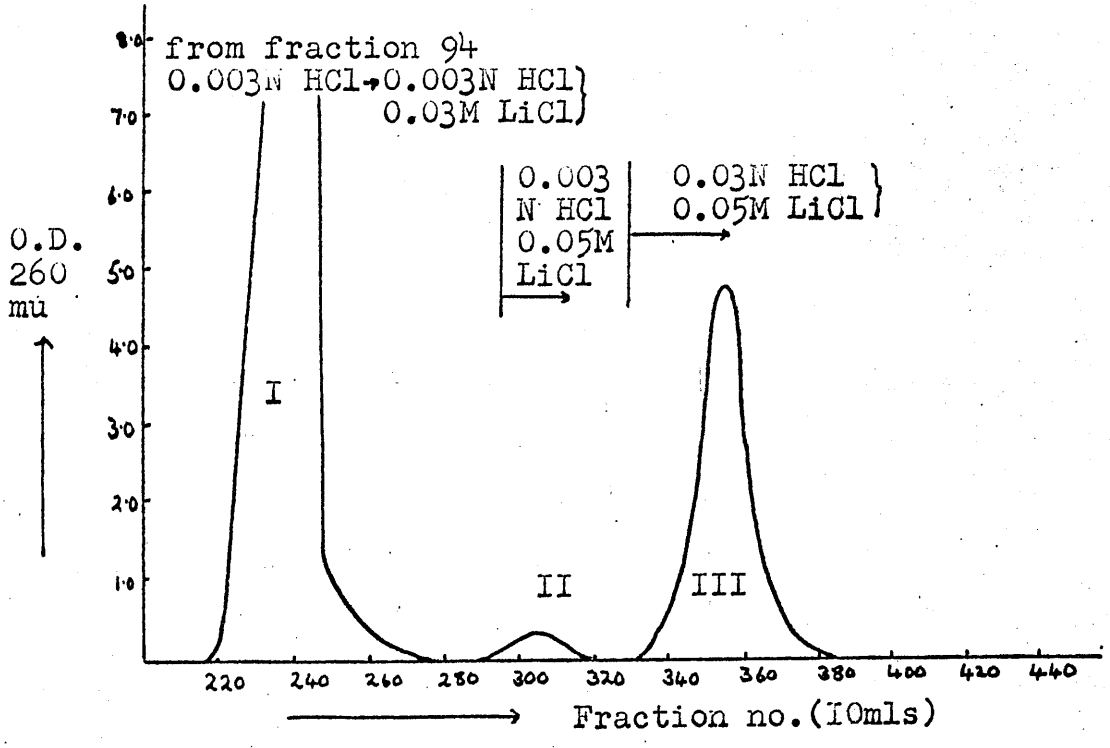


Fig.79. Dowex(C).

with CMP but nonetheless was not CMP. but probably some small oligonucleotide. Hydrolysis and further Dowex-1 ion exchange chromatography (Dowex (B)) produced a single entity which responded to T.L.C. (fig. 76) and U.V. spectrophotometric (fig. 78) investigation in a very similar manner to CMP. Thus the results indicated that the material resulting from digest VI and not held by DEAE - cellulose ion exchanger, was a dimer or trimer etc. of CMP.

B. Examination of peak I of DEAE-cellulose column chromatography of digest VI (fig. No. 70 and 71 page 312).

The bulked fractions 20-116 of peak I were diluted to 1.5 ℓ. with deionized water and applied to a Dowex-1 resin column (designated Dowex (C)) of dimensions 9 x 2 cms. Of the 3000 optical density units at 260 m_u applied, 2966 units were retained by Dowex (C) so giving a 98.9% retention.

The following elution programme was applied:-

Eluent		Fraction range (10 mls)
500 mls H_2O	500 mls 0.003 NHCl	1——49
Elution with 0.003N HCl		50 ——93
1.5 ℓ. 0.003N HCl	1.5 ℓ. 0.003N HCl 0.03 MLiCl.	94——293
Elution with	0.003N HCl 0.05 MLiCl.	294——330
Elution with	0.03N HCl 0.05M LiCl	331——406

a).TLC			
	R_{UMP}	Af.	Comment
AMP	0.79	3	Controls
	0.94		
CMP	I.24	3	
GMP	0.54	3	
	0.75		
UMP	I.00	3	
Dowex(C) (i)	0.56	3	∴GMP?
peak III (ii)	0.74		
b).Paper electrophoresis			
AMP	0.4I	3	Controls
CMP	0.36	3	
GMP	0.8I	3	
UMP	I.00	3	
peak III	0.80	3	∴GMP?
c).Paper chromatography			
AMP	0.7I	2	Controls
CMP	0.90,0.79	2	
GMP	0.56	2	
UMP	I.00	2	
peak III	0.56	2	∴GMP?

Fig.84. TLC,paper electrophoresis,and paper chromatography of Dowex(C) peak III.

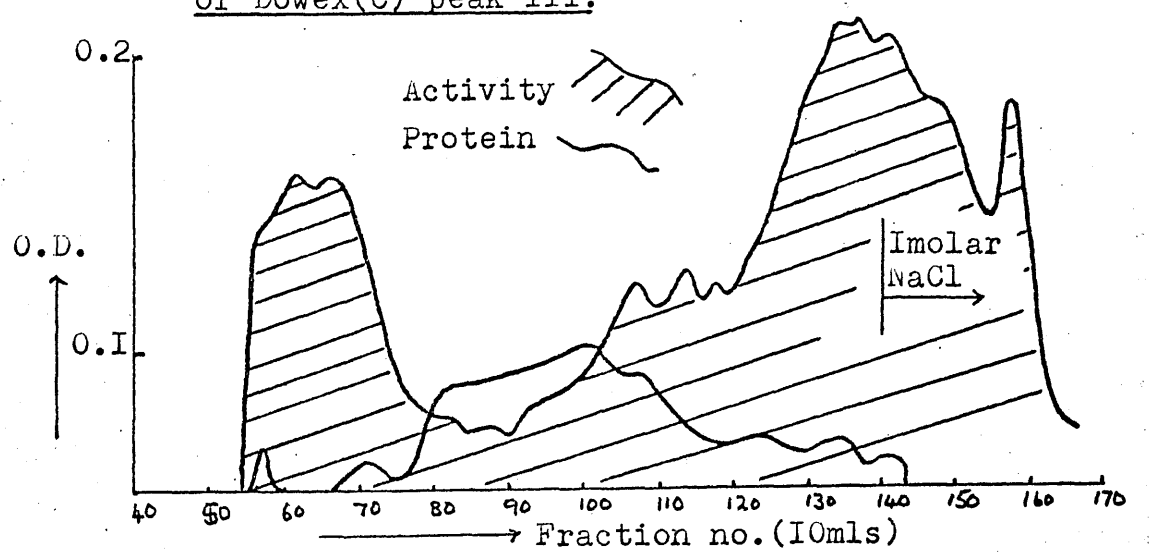


Fig.85. Purification of enzyme on CM-cellulose for digests VII and VIII.

Results: the elution profile of column Dowex (C) is given in fig. 79 and as with previous specificity work and all future such investigations, 10 ml. fractions were collected using an "LKB Radirac" and and "LKB" Uvicord" fraction collector-recording apparatus. The following fractions were bulked together:-

Fraction 222-268 inclusive - Peak I - vol. 570 mls, 2,274 $O.D._{260}$ Units

Fraction 290-330 inclusive - Peak II - vol. 430 mls. 36.5 $O.D._{260}$ Units

Fraction 338-380 inclusive - Peak III - vol. 651 mls. 651 $O.D._{260}$ Units

The pH of the bulked fractions for each peak was adjusted to pH 7.0 with IN Lithium hydroxide and then the solutions were lyophilized (see General Methods - GM 6 and 7).

Analysis of the products of Dowex (C)

Dowex (C) peak I. The various analytical techniques are summarized in fig. 80 and according to these results using T.L.C. and paper electrophoretic techniques, peak I seems fairly certain to contain the three mononucleotides, AMP, CMP and UMP. However for purposes of confirmation, Dowex (C) peak I.was

a).TLC			
	R_{UMP}	Af.	Comment
AMP	0.79	4	Controls
	0.93	4	
CMP	I.20	4	
GMP	0.56	4	
UMP	I.00	4	
Dowex(C) (i)	0.75	4	Therefore AMP?
peak I (ii)	0.98	4	" UMP?
(iii)	I.20	4	" CMP?
b).Paper electrophoresis			
AMP	0.48	3	Controls
CMP	0.38	3	
GMP	0.79	3	
UMP	I.00	3	
peak I (i)	0.38	3	Therefore CMP?
(ii)	0.48	3	" AMP?
(iii)	0.98	3	" UMP?

Fig.80. Analysis of the products of Dowex(C)peakI.

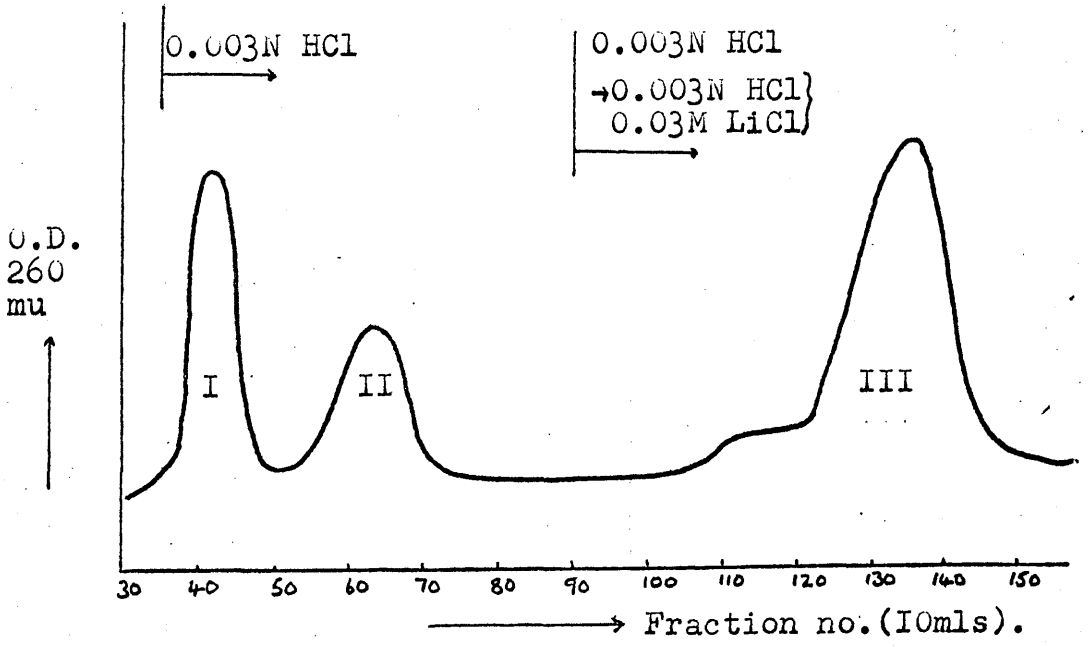


Fig.8I. Dowex(D).Flow-rate=0.35mls/min.

subjected to further Dowex I column separative chromatography.

Further Dowex-I chromatography of Dowex (C) peak I Dowex (D)

The column dimensions (designated Dowex(D).) were 13 x 1 cms, and 1088 optical density units (at 260m_u) in 500 mls of deionized water were applied to Dowex (D) at pH 7.0 and a 91.5% retention of the applied material was obtained after washing with 1350mls of deionized water. Then the following elution programme was inaugurated:-

Eluent.	Fraction range (≃ 10 ml)
300 mls. H_2O ⟶ 300 mls 0.003N HCl	1 — 35
Elution with 0.003N HCl	36 — 88
1 0.003N HCl ⟶ 1 0.003N HCl 0.03M L_iCl	89 — 185
Elution with 0.03N HCl 0.05M L_iCl	186 — 250

RESULTS. The elution profile of Dowex (D) column is given in fig. 81 and the following fractions were bulked together and the pH adjusted to 7.0 with IN lithium hydroxide and freeze dried (GM 6 & 7):

Fraction 36 - 47 inclusive - Peak I, vol. 130 mls, 293 O.D. Units at 260 m_u.

Fraction 50 - 75 inclusive Peak II. vol. 277 mls, 95 O.D. units at 260 m_u.

Fraction 122-150 inclusive Peak III, vol. 310 mls, 515 O.D. units at 260 m_u.

T.L.C., paper electrophoresis, paper chromatography and U.V. spectrophotometric data are summarized in figs. 82 & 83 for peaks I, II, and III.

Prediction of nucleotide character on the basis of elution sequence of Dowex (D)

T.L.C., paper electrophoresis, paper chromatography and U.V. spectrophotometric data (fig. 82,83) indicate that peaks I, II and III correspond to CMP, AMP, and UMP respectively and this is exactly what one would expect under the conditions of elution used, with CMP and AMP (peaks I and II respectively) being eluted by 0.003 N HCl before UMP, which requires a 0.003N HCl and 0.03N lithium chloride salt gradient before it is eluted (221).

Summary of Dowex (D) column chromatography.

Chromatographic separation of peak I of Dowex(C) on Dowex (D) resulted in separation into three nucleotide

a).TLC						
	Peak I	Af.	Peak II	Af.	Peak III	Af.
AMP	0.79 0.90	3	0.7I 0.80	2	0.75 0.90	2
CMP	I.I7	4	I.I3	2	I.I8	2
GMP	0.57	3	0.56 0.70	2	0.66	2
UMP	I.00	3	I.00	2	I.00	2
Unknown	I.09	4	0.88	2	0.99	2
Comment	∴CMP?		∴AMP?		∴UMP?	
b).Paper electrophoresis						
AMP	0.42	2	0.44	2	0.46	2
CMP	0.26	2	0.34	2	0.34	2
GMP	0.76	2	0.78	2	0.79	2
UMP	I.00	2	I.00	2	I.00	2
Unknown	0.25	2	0.4I	2	0.96	2
Comment	∴CMP?		∴AMP?		∴UMP?	
c).Paper chromatography						
			Peaks I,II,&III.			
AMP			0.70	2		
CMP			0.90 0.76	2		
GMP			0.53	2		
UMP			I.00	2		
Unknown	0.75	2	0.7I	2	0.97	2
Comment	∴CMP?		∴AMP?		∴UMP?	

Fig.82. TLC,paper electrophoresis,and paper chromatography of Dowex(D) products.

Peak	λ max.	λ min.	O.D.$\frac{250}{260}$	O.D.$\frac{280}{260}$	Comment
I CMP val.	278mu (279)	24I (240)	0.50 (0.45)	I.0I (2.00)	∴CMP?
II AMP val.	258 (257)	23I (229)	0.85 (0.85)	0.30 (0.22)	∴AMP?
III UMP val.	260 (262)	230 (230)	0.77 (0.80)	0.36 (0.28)	∴UMP?

Fig.83. U.V.data for Dowex(D) products.(The figures in brackets are the published values for mono--nucleotides,ref.226,&227.)

peaks which can beyond reasonable doubt be ascribed to CMP, AMP and UMP in that order of elution on the basis of analysis of the peaks by four methods, namely: T.L.C., paper electrophoresis, paper chromatography, and U.V. spectrophotometric data.

Summary of Dowex (C) peak I investigations.

From the investigation of this peak by T.L.C. and paper electrophoresis, and in the light of further Dowex-I resin column chromatographic separation, it was established that this peak contained CMP, AMP, and UMP. The reason that these three mononucleotides were eluted together from Dowex (C) (fig. 79) instead of the more expected elution and separation that was achieved on Dowex (D) (fig.81), can be explained by the fact that when peak I from the DEAE-cellulose separation of the products of digest VI was applied to Dowex (C) there was no pH adjustment (page 332). Thus the pH would be about 8.6 due to the presence of ammonium carbonate, and elution would be consequently delayed until neutralization had taken place, since normally elution of CMP and AMP would be brought about by eluting with 0.003N HCl alone (as in Dowex(D) - fig.81) where there were no pH and ammonium carbonate

complications. But in Dowex (C), no elution of any mononucleotide occurred until well into the 0.003N HCl → 0.003N HCl / 0.03M L_iCl / gradient. When elution of these mononucleotides did occur, the three (CMP, AMP and UMP) were eluted together.

It may be also conceivable that peak I of Dowex (C) fig. 79. was not composed of three mononucleotides when eluted but at some later stage during analysis, (such as during freeze drying) there may have been some hydrolysis of oligonucleotide material so resulting in the mononucleotides found. But this possibility was thought unlikely since care was taken to see that fractions were neutralized with lithium hydroxide before freeze drying, and so any hydrolysis on concentration during lyophilization was unlikely. Also all column work was conducted at a cold room temperature of 4 - 5°C.

Dowex (C) - peak III.

Fractions 338-380 were bulked as peak III from Dowex (C) (fig. 79 page 331) and neutralized with IN lithium hydroxide and freeze dried (GM 6 & 7). The isolated material was subjected to analytical techniques summarized in fig. 84, on page 333

U.V. spectrophotometric data of Dowex (C) peak III.

Spectra at acid pH λ max. 254 (257) O.D.$\frac{250}{260}$ = 1.13 (0.90)

λ min. 236 (228) O.D.$\frac{280}{260}$ =0.68 (0.68)

Spectra at alk. pH λ max. 257 (256) O.D.$\frac{250}{260}$ = 0.93 (0.89)

λ min. 231 (230) O.D.$\frac{280}{260}$ = 0.64(0.60)

The data obtained compared favourably with that for the published $\binom{226}{227}$ values of GMP (figures in brackets).

Summary of Dowex (C) peak III investigation.

The following analytical methods of T.L.C., paper electrophoresis, paper chromatography and U.V. spectrophotometric data indicate beyond reasonable doubt that this peak was GMP. --See fig.84,page 333.

Dowex (C) peak II.

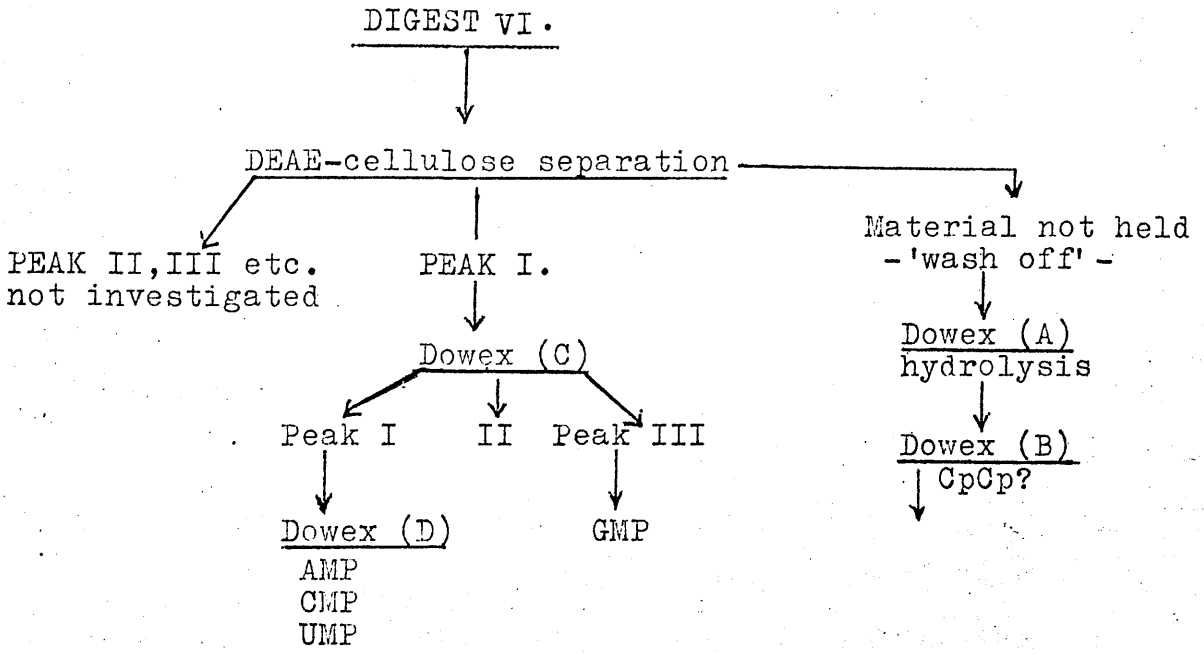
Since all four mononucleotides have been accounted for, as they are present in peaks I and III of Dowex (C), and since to discover the nature of the mononucleotides produced in the digestion of RNA with basic calf spleen enzyme fractions was the primary function of the investigation, peak II was not further investigated. More than likely this was an early eluted dinucleotide such as GpCp for example, because it only represented 1.25% of the

total nucleotide material eluted from Dowex (C).

C. Examination of peak II and III of DEAE-cellulose column chromatography of digest VI.

Because peak I of the DEAE-cellulose effluent had contained all four mononucleotides, investigation of subsequent peaks was not carried out. These peaks probably represent oligonucleotides of increasing size as the elution progresses and at this stage in the present investigation, they were of little interest.

Summary chart of procedures of analysis of digest VI



Conclusions for digest VI.

From the results obtained in digest VI it seems that mixed ribonuclease A and B hydrolyses RNA to produce all four mono-ribonucleotides with the enzyme (or enzymes) showing no specificity of action at all. The mononucleotides were obtained in significant quantities and not merely in trace amounts. This was in complete contrast to the findings of digest IV where UMP was the only mononucleotide found in any significant amount, but digest IV was carried out with RNAse B alone. These results could be interpreted as indicating that in fact RNAse A and B are two different enzymes with different specificities. Thus the separation on CM-cellulose in the final stages of purification into two activity regions A and B, so characteristic of the calf spleen ribonuclease preparation at this stage (page 54), may be due to the presence of two distinct proteins, and not a matter of one protein being eluted in two forms differing in state of aggregation (page 137).

Further specificity work with RNAse B alone was initiated to clarify the nature of the specificity of B and to confirm it is different from that due to the presence of A and B together.

Investigation of exhaustively dialysed commercial RNA as used in digest VI.

The source of RNA used in digest VI was investigated since it was thought possible that substantial amounts of mononucleotides might have been present in it, thus detracting from the results of this digest.

The RNA source was commercial yeast RNA and was exhaustively dialysed and freeze dried as described on page 269 and stored at - 22°C in a stoppered glass bottle. The RNA was white, fluffy and of good appearance.

150.5 mgs of this RNA were dissolved in 1 ℓ. of deionized water to give 1550 optical density units at 260 m_u. This material was then applied to a Dowex-I column (dimensions 7.5 x 1.8 cms) and washed initially with deionized water and then washed with 1ℓ. of 0.003N hydrochloric acid. The effluent stages was monitored at 254 m_u. and no nucleoside or nucleotide material was eluted. The following gradient was then applied:-

$$\underset{0.003N\ H\ Cl}{1.5\ \ell.} \longrightarrow \underset{0.03M\ Li Cl}{1.5\ \ell.\ 0.003N\ H\ Cl}$$

As a result of the above gradient there was no elution of any significant amount of nucleotide material even after 82% of this gradient had passed through the column.

Similarly elution with a solution of 0.03N HCl and 0.05M LiCl produced no significant nucleotide material in the effluent.

Therefore the exhaustively dialysed commercial RNA contains no significant amounts of oligonucleotides or mononucleotides since these, if present, would be easily eluted from Dowex-I anion exchanger by the eluents described. Thus the commercial source of RNA should in no way detract from the validity of the results of digest VI or any future digest in the sense of mononucleotide contamination.

DIGEST VII & VIII.

Introduction. These two digests were carried out using the same enzyme source (RNAse B) and under very similar conditions to give much the same results. In separation of the digest products, the use of DEAE-cellulose as an anion exchanger was dispensed with and Dowex-I used instead. DEAE-cellulose appeared to achieve good separation of oligonucleotides, but the separation of mononucleotides and dinucleotides etc. was somewhat poor since all the mononucleotides from digest VI were eluted in the large peak I from DEAE-cellulose, and subsequent separation of these was by use of Dowex-I. Therefore it was decided to use Dowex-I for the initial

separation of mononucleotides from the larger products of enzyme digestion of RNA, and the former were of little interest to this study anyway.

DIGEST VII.

a) Enzyme source for digest VII.

Having been stored at -22°C, ribonuclease samples from the following sources were bulked together:-

Column source	Fraction	Page No. ref.
CMC-4-2M II	96-120	197
CMC-4-2M-Leu-I	151-164	228
" "	165-179	"
" "	180-198	"
CMC-4-2M-Leu II	133-156	228
CMC-4-2M-I	68-82	197

The RNAses were thought to be mainly of the RNAse 'B' type but especially in the case of those fractions chromatographed in the presence of nor-leucine (page 228) it was not always easy to distinguish the separation into two peaks of activity - A and B (example CMC - 1 page 54). This difficulty was of little consequence since the bulked enzyme samples were to be rechromatographed under the standard conditions which have always been found to result in two peaks of activity and from which the B fraction can be isolated for use in digests VII and VIII.

Procedure for CM-cellulose chromatography of RNAse samples from various sources (listed above)

Volume of bulked protein material = 1255 mls.

Total concentration of bulked material = 44.3 mgs of protein.

The bulked protein samples were diluted to 4 ℓ. with 0.005 M Tris, 10^{-4}M EDTA, pH 7.0 buffer because it was not wanted to dialyse the material at this stage using Visking tubing incase of enzyme loss. (page 89 ref. 105). Thus by dilution, the salt concentration, which will be appreciable in the samples as a result of their previous column separation and elution, would be reduced sufficiently to allow adsorption by the cation exchanger. The presence of leucine and urea should not significantly affect adsorption properties.

The CM-cellulose column used was of 55 x 2.1 cm. dimensions and was thoroughly equilibriated with 0.005M Tris, 10^{-4}M EDTA, pH 7.0 buffer before application of protein material. The diluted protein material was then applied to the column and washed with 1075 mls of pH = 7.0 buffer. All column chromatographic operations were conducted at 4-5^{0}C.

Results. Fig. 85* shows the elution profile obtained with respect to enzyme activity, and protein estimation

*(Note-fig.85 is on page 333).

by U.V. adsorption at 280 m_{μ}, by using the apparatus described previously (fig 23) for CMC-4 and and CMC-5 columns. The gradient elution applied was as follows:-

Constant volume chamber		Reservoir	
800 mls	0.005 M Tris 10^{-4}M EDTA pH 7.0	1600mls	0.005M Tris 10^{-4}M EDTA pH 8.2 0.25M NaCl

Fractions 120-168 were bulked as fraction B and concentrated by use of a small CM-cellulose (described: page 57.). The concentrated protein solution was then dialysed against pH 7.0 buffer to give 42 mls. of dialysed solution containing 7.94 mgs of protein. (GM2).

b) RNA source.

The same RNA source was used as in digest VI, namely exhaustively dialysed commercial RNA at a concentration of 3.75 mgs/ml of solution in the digest mixture. (3.5 mgs/ml in digest VI).

c) Digest.

The RNA was dissolved in the protein solution and the pH checked and adjusted to pH 7.0. The digest was made 0.025 M with respect to Mg^{++} ions using $MgCl_2.6H_2O$ (page53 ref28) and was carried out in a stoppered glass flask with four drops of 'Analar' grade chloroform to inhibit bacterial action. The

digestion time extended over a period of 45 hours and 35 mins. at 37°C. in a constant temperature water bath.

d) Separation of digestion products.

After digestion, the solution containing the products was diluted to 1 l. with deionized water and allowed to cool before applying to a Dowex-I column in the cold room. The column dimensions were 13 x 1.8 cms and the resin had been prepared in the Cl^- form as previously described (page 319). A total of 3990 units were applied to the column (designated Dowex (E)) and retention of this material was virtually 100%. Dowex (E) was then subjected to the elution programme as given in fig. 86 and a separation of products was obtained as shown in fig. 87.

Analysis of peaks of Digest VII eluted from Dowex (E) column (fig. 87)

The material from each peak was bulked, and the pH adjusted to 7.0 with IN lithium hydroxide and then reduced in volume, generally by freeze ——drying (GM6 & 7). The isolated nucleotide products were examined by U.V. spectrophotometry, T.L.C., paper electrophoresis and finally by paper chromatography in Leloir's solvent at pH 3.5. The data and conditions for each technique of analysis used was described for the

Eluent	Fractn.no.	%grad.run	Peaks collected
1l.H_2O→1l.0.003N HCl	I-I34	75%	I,II,III
Elution with 0.003N HCL 2l.	I35-I68	7I%	IV,V,VI
2l.0.003N HCl→2l.0.003N HCl,0.03M LiCl	I69-560	8I%	VII,VIII, IX,XA
Elution with 0.03N HCL 1l. 0.03M LiCl	56I-642	86%	X,XI
Elution with 0.03N HCl 0.05M LiCl	643-792	75%	XII,XIII

Fig.86. Elution programme-Dowex(E)

Method	TLC	Electro--phoresis	Paper chrom--atography
AMP	0.88 0.76	0.59	0.69
CMP	I.I7	0.46	0.82
GMP	0.65	0.80	0.56
UMP	I.00	I.00	I.00
Unknown	I.I7	0.46	0.82
Af.	3	3	2
Comment	∴CMP?	∴CMP?	∴CMP?

Fig.88. Dowex(E),peakII

Method	TLC	Electro--phoresis	Paper chrom--atography
AMP	0.78 0.89	0.64	0.66
CMP	I.I3	0.46	0.84
GMP	0.63	0.85	0.54
UMP	I.00	I.00	I.00
Unknown	I.I6	0.4I	0.84
Af.	3	3	2
Comment	∴CMP?	∴CMP?	∴CMP?

Fig.89. Dowex(E),peak III

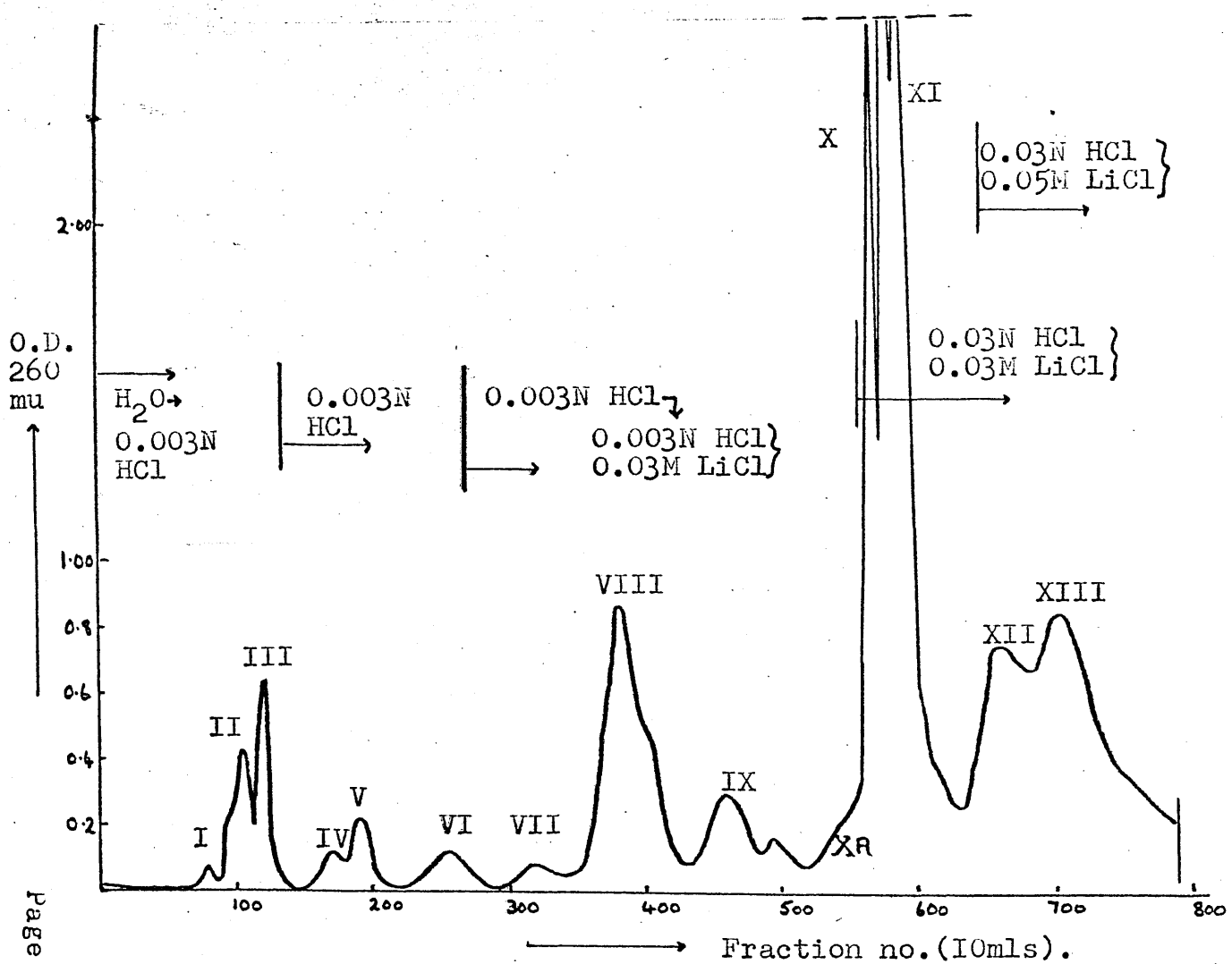


Fig.87. Elution profile of products of Digest VII on Dowex(E) (Flow-rate=0.5mls/min.)

analysis of the products of digest IV and digest VI (electrophoresis). All results with respect to T.L.C., paper electrophoresis and chromatography were expressed relative to those of UMP and the R_{ump} values given in the text are the average R_{ump} values and the number of results they are averaged from is stated and the convention explained in digest VI (page 314). On the basis of these analytical techniques, and also on the position of elution from the Dowex-I anion exchanger, conclusions were drawn as to the nature of each peak.

PEAK I (Fraction 70-76, volume 50 mls, concentration 3.95 O.D. units 260 m_u)

1. U.V. data

Spectra at acid pH λ max 266(280) O.D. $= \frac{250}{260} = 0.83$ (0.44)

λ min 238 (241) O.D. $\frac{280}{260} = 0.82$ (2.09)

λ max. 272 (271) O.D. $\frac{250}{260} = 0.92$ (0.84)

λ min. 246 (249) O.D. $\frac{280}{260} = 1.05$ (0.98)

No definite correlation with any published data could be derived from the above results except the spectra at an alkaline pH resemble those for CMP which are given in brackets.

2) T.L.C.

	Rump	Af.	Comment
AMP	0.90 0.78	3	} Control
CMP	1.15	3	
GMP	0.67	3	
UMP	1.00	3	
Peak I	0.81	3	∴ AMP?

3) No successful electrophoretic or paper chromatographic results were obtained because of the small concentration of nucleotide material available.

Conclusions. Because of the position of this peak in the elution sequence it was definitely not UMP or GMP, and from the results obtained it does not seem to be identifiable with either AMP or CMP, and so with the data available no real conclusion as to the identity of this peak was reached.

PEAK II (Fractions 90-112, volume 180 mls, concentration 50.0.D. units)

1. U.V. spectrophotometric data.

Spectra at acid pH λ max. 278(280) O.D. $\frac{250}{260}$ =0.63 (0.44)

λ min. 242(241) O.D. $\frac{280}{260}$ = 1.78 (2.09)

Spectra at alk pH λ max. 270 (271) O.D.$\frac{250}{260}$ = 0.92 (0.84)

λ min. 252 (249) O.D.$\frac{280}{260}$ = 0.89 (0.98)

There was good correlation with the published data for CMP (which is given in brackets).

2. The results of T.L.C., paper electrophoresis, and paper chromatography are given in fig. 88 and all results indicated that peak II was synonymous with CMP and this was concurrent with the elution of this peak by an hydrochloric acid concentration of less than 0.003N.

PEAK III (Fractions 113-126, Volume 90 mls, concentration 43.7 O.D. units)

1. U.V. Spectrophotometric constants.

Spectra at acid pH λ max. 280 (280) O.D. $\frac{250}{260}$ = 0.56(0.44)

λ min. 244 (241) O.D. $\frac{280}{260}$ = 1.85(2.09)

Spectra at alk pH λ max. 270 (271) O.D. $\frac{250}{260}$ = 0.91(0.84)

λ min. 252 (249) O.D. $\frac{280}{260}$ = 0.93(0.98)

The U.V. data was very similar but not identical to that obtained for peak II in that it was very ———

P.T.O.

characteristic of the published data for CMP (in brackets).

2. The results of T.L.C. etc. of peak III are given in fig. 89 and although not as positive as peak II, these results indicated the probability of the presence of CMP again. Peaks II and III were eluted from the Dowex column in close proximity, and separation on bulking could not possibly be complete and cross contamination of the relative properties would occur. But even so, peaks II and III exhibited very similar properties. It was thought that one of these peaks may be the $2^{1}, 3^{1}$ cyclic phosphate form of CMP. Thus electrophoresis at pH 7.5 with a phosphate buffer was also carried out with peaks II and III, and CMP (non-cyclic) as a control. At pH 7.5 on normal nucleotide 3^{1} phosphates, two of the hydroxyls of the phosphate group are in the ionized form and thus the nucleotide has two negative charges. But at this pH the cyclic phosphate only has one negative change, and so separation of cyclic and non-cyclic forms is made possible by the differential charge at pH 7.5 with paper electrophoresis. Paper electrophoresis was carried out in pH 7.5, 0.2M phosphate buffer at 600 volts for 45 minutes.

Results	R_{CMP}
CMP	1.00
Peak II	1.01
Peak III	0.98

Results showed no significant separation of the materials from peaks II and III, indicating cylic CMP not to be present.

But even if no cyclic nucleotides were found in the isolated peaks II and III, at the time of elution, one of the peaks may have been in the cyclic form, but on isolation it may have been broken down to the non-cyclic form.

Therefore peak III from the results obtained seemed to be CMP or a nucleotide compound with characteristics similar to CMP (such as cylic CMP). The presence of nucleosides was excluded, because these are normally very sharply differentiated by paper chromatography from nucleotide material and in peak III there was no such evidence of these compounds. Nucleosides are normally eluted before any nucleotide material in such a gradient system as was used.(221) Peak III could possibly be a dinucleotide containing as one constituent CMP but at this stage of elution, it is unlikely.

PEAK IV. (Fraction 156-180, volume 270 mls, 20.3 O.D. units concentration)

U.V. Spectrophotmetric data.

1. Spectra at acid pH λ max. 276(280) O.D.$\frac{250}{260}$ = 0.69 (0.44)

λ min. 246(241) O.D.$\frac{280}{260}$ = 1.34 (2.09)

Spectra at alk. pH λ max. 268(271) O.D.$\frac{250}{260}$ = 0.90(0.84)

λ min. 250(249) O.D.$\frac{280}{260}$ = 0.81(0.98)

The U.V. spectrophotometric data showed similarities with peak II and III, but did not exactly coincide with the published values for CMP (given in brackets).

2. The results of T.L.C. etc. for peak IV are given in fig. 90. Paper electrophoresis showed the presence of a very slow moving spot as well as one corresponding to CMP. The slower moving spot was probably due to the presence of a di- or trinucleotide and the three spots obtained by T.L.C. may be due to di-or tri-nucleotides with perhaps hydrolysis products of the same. The overall conclusions as to the nature of peak IV were that possibly CMP and AMP were present and/or a di-or trinucleotide, and that the mononucleotides CMP and AMP were breakdown or hydrolysis products. It should be noticed that good separation between peaks IV and V was not obtained, and therefore overlapping of constituents on bulking was possible.

Method	TLC	Electro-phoresis	Paper chrom-atography
AMP	0.85	0.64	0.69
CMP	I.I5	0.5I	0.82
GMP	0.63 0.77	0.86	0.53
UMP	I.00	I.00	I.00
Unknown	0.75,0.88,I.I9	0.34,0.50	0.73
Af.	4	7	2
Comment	∴CMP,AMP?	CMP,dimer?	∴AMP?

Fig,90. Dowex(E),peak IV.

Method	TLC	Electro-phoresis	Paper chrom-atography
AMP	0.8I 0.9I	0.63	
CMP	I.I4	0.49	
GMP	0.6I	0.83	
UMP	I.00	I.00	
Unknown	0.84,I.I6	0.40,0.55	
Af.	4	6	
Comment	AMP,CMP?	AMP,CMP?	

Fig.9I. Dowex(E),peak V.

Method	TLC	Electro-phoresis	Paper chrom-atography
AMP	0.86	0.4I	0.70
CMP	I.2I	0.3I	0.8I
GMP	0.62	0.76	0.49
UMP	I.00	I.00	I.00
Unknown	0.82	0.40	0.62
Af.	3	3	2
Comment	∴AMP?	∴AMP?	∴AMP?

Fig.92. Dowex(E),peak VI.

PEAK V. (Fraction 181-205, volume 270 mls, concentration 32.9 O.D. units)

1. U.V. Spectrophotometric data

Spectra at acid pH λ max. 268 O.D. $\frac{250}{260}$ = 0.73

λ min. 242 O.D. $\frac{280}{260}$ = 0.98

Spectra at alk. pH λ max. 264 O.D. $\frac{250}{260}$ = 0.87

λ min. 238 O.D. $\frac{280}{260}$ = 0.58

For this peak the results and published data for mononucleotides showed no correlation, although some similarity between the published data of AMP and CMP was discernable.

2. The result T.L.C. etc. for peak V are given in fig. 91. Results indicate the possible presence of CMP and AMP in this peak, but this was by no means certain as the results may have been due to di-or trinucleotides. It is also possible that hydrolysis of a di-or trinucleotide might have given CMP or AMP as products.

PEAK VI. (Fraction 230-280, volume 515 mls, concentration 36.1 O.D. units).

Spectra at acid pH λ max. 258 O.D. $\frac{250}{260}$ = 0.88

λ min. 232 O.D. $\frac{280}{260}$ = 0.41

Spectra at alk. pH λ max. 260 O.D. $\frac{250}{260}$ = 0.86

λ min. 234 O.D. $\frac{280}{260}$ = 0.38

The above results showed no correlation with published data for mononucleotides.

2. The results of T.L.C. etc. for peak VI are given in fig. 92. The results tabulated suggest that this peak may contain AMP, but this is not corroborated by U.V. spectrophotometric data. Thus peak VI is possibly AMP, but more likely an AMP type di- or tri- nucleotide.

PEAK VII (Fraction 300-340, volume 240 mls, concentration 26.5 O.D. units).

U.V. Spectrophotometric data.

Spectra at acid pH λ max 260 O.D.$\frac{250}{260}$ = 0.83

λ min. 238 O.D.$\frac{280}{260}$ = 0.46

Spectra at alk. pH λ max 260 O.D.$\frac{250}{260}$ = 0.82

λ min. 238 O.D.$\frac{280}{260}$ = 0.46

The U.V. spectrophotometric constants and spectra showed no good correlation with any published data for mononucleotides.

2. The results of T.L.C. etc. for peak VII are given in fig. 93 and although these showed strong affinities with data for control AMP, the same affinity was not shown with respect to published U.V. data for AMP. Therefore peak VII was probably an AMP containing di-

or trinucleotide.

PEAK VIII. (Fraction 354-430, volume 505 mls, concentration 340.7 O.D. units).

Peak VIII had a shoulder and thus was divided into two fractions for analysis, peak VIIIA - fractions 372-390.

and VIIIB - fractions 398-404

PEAK VIIIA

1. U.V. spectrophotometric data.

Spectra at acid pH λ max 262(262) $O.D.\frac{250}{260}$ = 0.76(0.73)

λ min 232(230) $O.D.\frac{280}{260}$ = 0.41(0.39)

Spectra at alk. pH λ max 262(261) $O.D.\frac{250}{260}$ = 0.84(0.80)

λ min 242(241) $O.D.\frac{280}{260}$ = 0.39(0.32)

The U.V. constants compared very favourably with the published data for UMP. (given in brackets).

2. The results of T.L.C. etc. for peak VIIIA are given in fig. 94. U.V. spectrophotometric data was in close agreement with the conclusions drawn from other analytical methods, namely that peak VIII A is probably UMP.

Method	TLC	Electro-phoresis	Paper chrom-atography
AMP	0.84	0.41	0.69
CMP	I.I7	0.29	0.82
GMP	0.62	0.79	0.52
UMP	I.00	I.00	I.00
Unknown	0.84	0.38	0.66
Af.	3	3	2
Comment	∴AMP?	∴AMP?	∴AMP?

Fig.93. Dowex(E),peakVII.

Method	TLC	Electro-phoresis	Paper chrom-atography
AMP	0.86	0.49	0.70
CMP	I.I6	0.4I	0.80
GMP	0.63	0.78	0.49
UMP	I.00	I.00	I.00
Unknown	I.03	I.0I	0.97
Af.	4	4	2
Comment	∴UMP?	∴UMP?	∴UMP?

Fig.94. Dowex(E),peak VIIIA.

	O.D.$\frac{250}{260}$	O.D.$\frac{280}{260}$	λ max.	λ min.
Acid spectra				
peak VIIIA	0.76	0.4I	262	232
peak VIIIB	0.80	0.43	26I	244
UMP	0.73	0.39	262	230
Alkaline spectra				
VIIIA	0.84	0.39	262	242
VIIIB	0.84	0.43	262	242
UMP	0.80	0.32	26I	24I

Fig.95. Comparison of U.V.spectrophotometric constants for Dowex(E),peaks VIIIA & B.Published values for UMP are also given,references 226,227.

Peak VIIIB gave virtually identical results although the U.V. spectra were a little different (see fig. 95), and as with the relationship between peaks II and III, the small shoulder in the elution profile of peak VIII may have been due to the cyclic form of UMP, which has since broken down to give the non-cyclic mononucleotide of UMP. Alternatively peak VIIIB might have resulted due to the presence of a UMP containing di- or tri-nucleotide, thus altering the spectrophotometric constants. Thus peak VIII as a whole was taken on the basis of the above information, to be UMP.

PEAK IX. (Fraction 430-480, volume 505 mls. concentration 93.4 O.D. units).

1. U.V. Spectrophotometric data

Spectra at acid pH λ max.268 $\quad$ O.D.$\frac{250}{260}$ = 0.71

λ min.237 $\quad$ O.D.$\frac{280}{260}$ = 0.89

Spectra at alkaline pH λ max.264 O.D.$\frac{250}{260}$ = 0.88

λ min.244 O.D.$\frac{280}{260}$ = 0.63

The above data exhibited little in common with published U.V. data for mononucleotides.

2) The results of T.L.C. etc. for peak IX are given in fig. 96. T.L.C. results indicate the presence of UMP

Method	TLC	Electro--phoresis	Paper chrom--atography
AMP	0.63	0.44	0.68
CMP	I.I9	0.32	0.75
GMP	0.58	0.8I	0.50
UMP	I.00	I.00	I.00
Unknown	0.96	0.83 0.95	0.3I
Af.	3	8	2
Comment	∴UMP?	∴UMP +...?	Di-or tri-nucleotide?

Fig.96. Dowex(E),peak IX.

Method	TLC	Electro--phoresis	Paper chrom--atography
AMP	0.86	0.49	0.68
CMP	I.I5	0.35	0.80
GMP	0.59	0.83	0.50
UMP	I.00	I.00	I.00
Unknown	0.58	0.76	0.53
Af.	3	5	2
Comment	∴GMP?	∴GMP?	∴GMP?

Fig.97. Dowex(E),peak XA.

Peak	O.D.$_{260}$units	Results of analysis
I	4	Identity not established.
II	50	CMP } both taken
III	44	Probably CMP } as CMP.
IV	20	CMP & AMP,probably a dimer or trinucleotide of these.
V	33	Same as above.i.e.peak IV.
VI	36	AMP or more probably a dinucleotide or tri- etc.
VII	27	Same conclusion as peak VI
VIII	34I	UMP.
IX	93	Dimer or oligonucleotide.
X	87	Possibly GMP?

Fig.98. Summary chart of analysis of digest VII products.

in peak IX also, but this is not supported by U.V. data and paper chromatography. The results from paper electrophoresis could be interpreted as indicating the presence of UMP and GMP whereas both T.L.C. and paper chromatography demonstrated the existence of one nucleotide constituent. By this stage in the elution sequence it was probably that the constituent of peak IX was of di- or tri- or of oligonucleotide nature, and may be the two constituents separated using paper electrophoresis were hydrolysis products of small oligonucleotides.

PEAK XA (Fraction 520-567, Volume 495 mls, concentration 87.0 O.D. units)

1. U.V. Spectrophotometric data.

Spectra at acid pH	λ max. 258(257)	O.D.$\frac{250}{260}$	= 0.88 (0.90)
	λ min. 241(228)	O.D.$\frac{280}{260}$	= 0.80 (0.68)
Spectra at alkali pH	λ max. 260(256)	O.D.$\frac{250}{260}$	= 0.93 (0.89)
	λ min. 239(230)	O.D.$\frac{280}{260}$	= 0.62 (0.60)

As can be seen from the figures quoted in brackets which are the published figures for GMP, this peak has very similar U.V. spectrophotometric characteristics. The

similarity to GMP is similarly reflected in results from T.L.C., paper electrophoresis and paper chromatography.

2. The results of T.L.C., paper electrophoresis and paper chromatography are given in fig. 97 and the data does seem to indicate the presence of GMP but the U.V. data does not correspond exactly and therefore there is always the possibility that peak XA may be a GMP containing di- or trinucleotide.

PEAK X (Fraction 568-574, volume 84 mls, concentration 137 O.D. units)

Peak X was analysed by the same techniques as used for all the previous peaks and was not found to be of a mononucleotide character. Analysis of subsequent peaks to peak X was not attempted since any mononucleotides would have been eluted by this stage in the salt gradient (22I)anyway.

Conclusions and summary of digest VII

The object of these specificity investigations has been to ascertain the nature of the mononucleotides produced on digestion of yeast RNA with calf spleen ribonuclease enzyme in order to be able to formulate a theory as to the nature of the specificity of the particular enzyme fraction used. Fig. 98 summarizes

as far as was feasible the nature of the fractions separated from digest VII. The only mononucleotides that were found to be present with any degree of certainty were CMP and UMP with also the possibility of the presence of GMP, but the latter was of a more doubtful degree of certainty. It will be observed that the quantities of UMP separated were far in excess of those of CMP and the ratio of UMP; CMP isolated in digest VII was 3.8:1. A number of explanations could be compounded for these results with respect to specificity of enzyme action.

If the enzyme used had the same specificity as bovine pancreatic RNAse, UMP and CMP would be the only mononucleotides produced as a result of the enzyme action. If the RNA used in such a digest had approximately equal CMP and UMP content, one would expect in all probability that CMP and UMP produced as a result of enzymatic hydrolysis would be present in very approximately equal quantities also. But however this was not the case in digest VII and UMP was present in considerably greater quantities than CMP and this may have been due to preferential specificity on the part of the ribonuclease enzyme, or due to the use of RNA which contained a higher proportion of UMP as compared

with CMP in its nucleotide structure. But the published values for nucleotide and base ratios of yeast RNA (since it was yeast RNA used in digest VII) are:

	AMP	GMP	CMP	UMP	(Ref.)
1) Nucleotide ratios of yeast (no type of yeast quoted).	1.24	1.38	1.00	1.23	(246)
2) baker's yeast. (Saccharomyces cerevisiae)	1.39	1.65	1.00	1.58	(248)

Therefore presuming the base compositions of baker's yeast does not vary except perhaps slightly due to chain length being of a somewhat random nature, the predominance of UMP found, as opposed to CMP in the digest, can not be interpreted in terms of RNA used entirely, since although there is a greater preponderance of UMP over CMP in naturally occurring baker's yeast (1.58:1.00 respectively), it is not sufficient to account for the ratio of 3.8:1 obtained in digest VII. The predominance of UMP as a product of the digestion of RNA is akin to the results obtained in digest IV where no CMP at all was found and UMP was the only mononucleotide isolated. Both digest VII and IV were carried out using Ribonuclease activity 'B' fractions and so one would expect the same results. Possibly in

digest IV the CMP concentration produced was not present in sufficient concentration to be detected and thus giving rise to the impression that the specificity was unique to UMP.

If this RNAse 'B' fraction isolated from calf spleen was specific in its action to result in UMP alone on digestion of RNA, or if it had merely preferential but not absolute specificity as digest VII results indicated, then further confirmation of these important findings was needed. Thus a final digest was carried out using the same RNA source, the same enzyme source, and using the same technique of isolation and product identification as used in digest VII.

DIGEST VIII.

a) Enzyme source.

The same enzyme source was used in digest VIII as was used in digest VII, namely the RNAse B fraction 120-168, of the CM-cellulose column chromatography preparation described on page 346 (fig. 85). This material was dialysed against pH 7.0 Tris buffer prior to use and 41 mls of the resulting protein solution containing 8.25 mgs. of protein was used in the digest.

b) RNA source.

Exhaustively dialysed RNA, as used in digest VII was dissolved to the extent of 173 mgs in the enzyme solution and made 0.025M with respect to Mg^{++} ions as in digest VII and the digest was incubated at 37°C in a constant temperature water bath for 36.75 hours.

c) Separation of the digest products.

After digestion the solution containing the digest products was diluted to 1 ℓ. with deionized water and applied to a 15 x 1.8 cm. Dowex-I column (designated Dowex (F)). An almost identical elution profile (fig. 100) to that of Digest VII was obtained and any differences could be accounted for by differences in elution procedure which is described in fig. 99.

d) Results.

Analysis of each peak was undertaken using the same methods as in digest VII and very similar results were obtained. Because of this similarity, experimental results are not quoted below in detail but merely the final findings as regards the nature of the individual peaks is presented (fig. 101.) A comparison of the results of digest VII and VIII (fig. 102) shows the results to be virtually identical, namely that the

Eluent	Fraction	%gradient run	Peaks
Material coming straight though column	I-87		
Elution with H_2O 0.5l.	88-129		
1l.H_2O→1l.0.003N HCl	130-293	92%	I,II,III
Elution with 0.003N HCl 1.5l.	294-427		IV,V
2l.0.003N HCl→ 2l.0.003N HCl) 0.03M LiCl)	428-725	82.5%	VI,VII, VIII,IX
1l.0.003N HCl) 0.03M LiCl)→ 1l.0.03N HCl) 0.03M LiCl)	726-878	82%	X
Elution with 0.03N HCl 0.05M LiCl	879-965		XI.

Fig.99. Elution programme for digest VIII,Dowex(F).

Peak	0.D.units 260mu	Results of analysis
I	35	Possibly CMP.
II	41	CMP
III	79	Possibly CMP.
IV	23	Di- or trinucleotide?
V	59	Possibly AMP or AMP dimer or oligonucleotide?
VI	59	Dimer or oligonucleotide?
VII	41	Possibly UMP & also a dimer or oligonucleotide.
VIII	425	UMP,& another two minor constitu--ents,which are not of mono--nucleotide nature.
IX	98	Some GMP characteristics,probably dimer etc.

Fig.101. Results of digest VIII,Dowex(F).

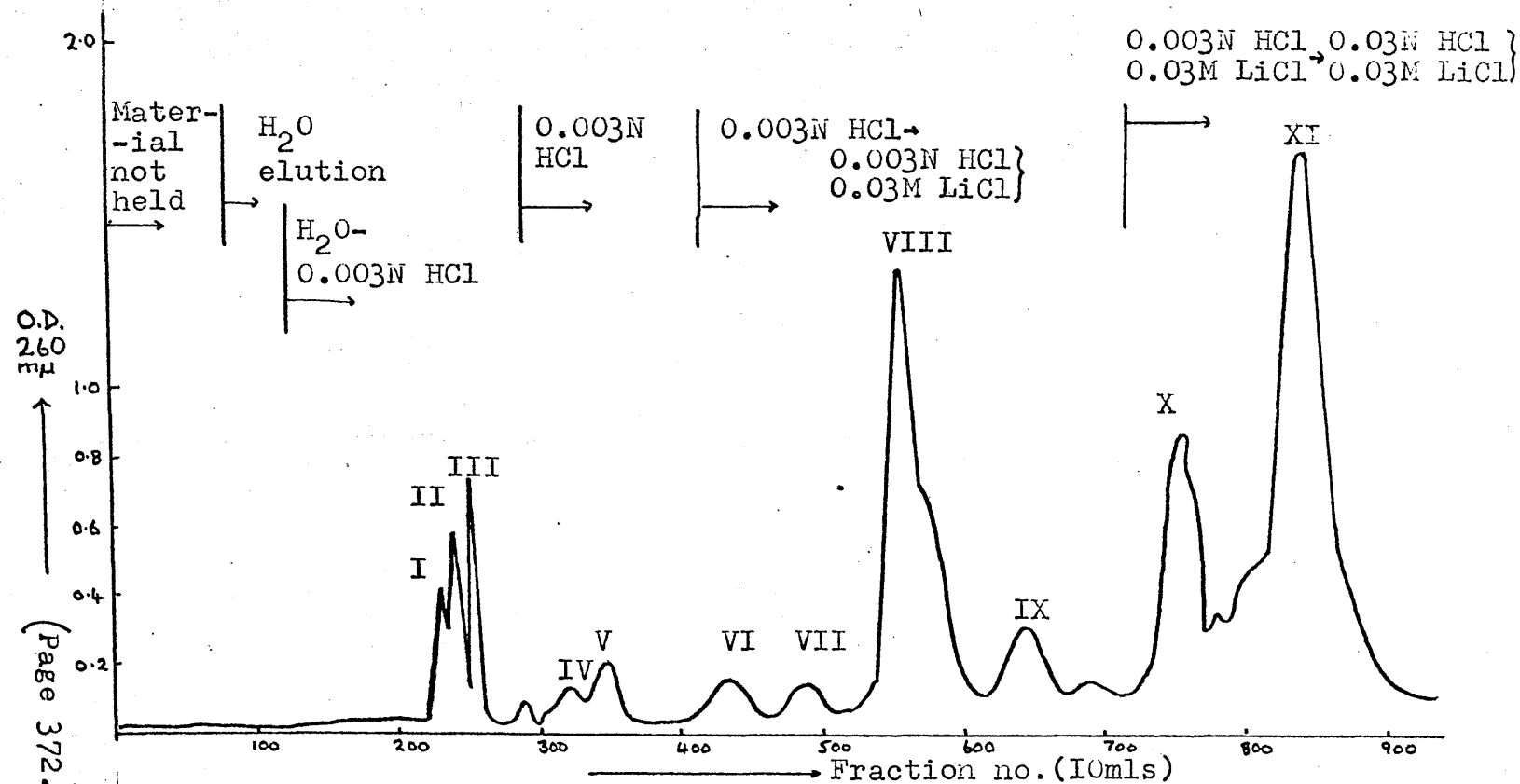


Fig.I00. Elution profile of the products of digest VIII on Dowex(F) (flow-rate=0.5mls/min.)

DIGEST VII				DIGEST VIII			
Column length I3xI.8cms.4,200 units				Column length I5xI.8cms.46I7 units			
Peak	O.D. units	Identity	Eluent	Peak	O.D. units	Identity	Eluent
I	4	?	H_2O→	I	35	CMP?	H_2O→
II	50	CMP	0.003N HCl	II	4I	CMP	0.003N HCl
III	44	CMP?		III	79	CMP?	
IV	20	CMP, AMP dimer?		IV	23	di or tri nucleotide	0.003N HCl
V	33	"	0.003N HCl	V	59	AMP or oligomer?	
VI	36	AMP or di,tri--mer etc.		VI	59	"	0.003N HCl→
VII	27	"	0.003N HCl→	VII	4I	UMP? or dimer etc.	0.003N HCl } 0.03M LiCl }
VIII	34I	UMP	0.003N HCl } 0.03M LiCl }	VIII	425	UMP+oli--gomer	
IX	93	di- or oligomer?		IX	98	GMP?	
XA	87	GMP?	0.03N HCl } 0.03M LiCl }				

Fig.I02. Comparison of imformation from digests VII and VIII, Dowex(E) & (F).

enzyme action on RNA has produced CMP and UMP mononucleotides with UMP being the predominant product.

Summary of Part III - summary of all specificity investigations.

The most notable and significant finding that these specificity investigations have yielded, has been that RNAse A and B fractions, seem to have differing specificities. Of course fuller investigation, which time did not allow, would be needed to confirm and elucidate further the nature of the respective activities.

The RNAse fractions arise in the final stage of the isolation of basic calf spleen ribonuclease by fractionation into two activity peaks on CM-cellulose under the conditions described on page 54 . The nature and inter-relation of these two activity regions has been discussed at various stages (pages 59,137,138,151, 206) and was discussed in the final sectional summaries. If the findings of these somewhat rudimentary and iniatory specificity investigations are accepted, then it does seem that calf spleen ribonuclease A and calf spleen ribonuclease B may be two separate protein entities and not related forms by way of any differences in state of aggregation or similar association.

Evidence from digests IV, VII and VIII suggests

that RNAse B has a similar specificity to pancreatic RNAse, in that the pyrimidine nucleotides are the mononucleotide products of digestion with yeast RNA. However, unlike pancreatic RNAse, calf spleen RNAse B seems to act preferentially with the appearance of UMP as a hydrolysis product in far greater amounts than CMP. Further research would have to be done to obtain data as to the intermediary stages of hydrolysis, such as a knowledge of whether hydrolysis is via a 2', 3' cyclic stage. Information of this latter type would be essential before a correct classification of the enzyme could be attempted.

In digest VI the enzyme source was largely of RNAse A type and since all four mononucleotides were characterized among the hydrolysis products of yeast RNA, an enzyme of no mononucleotide specificity at all was indicated. As the action of RNAse B on yeast RNA resulted in the release of only pyrimidine mononucleotides, the non-specificity of action in digest VI was attributed to RNAse A, even though small amounts of RNAse B activity was present in the enzyme source used in this investigation. It could be theorized that the production of the purine mononucleotides was due to RNAse A and the pyrimid-

ine mononucleotides, was due to the presence of RNAse B. Whatever the exact specificity of RNAse A fraction, the results obtained show it to be different from RNAse B, which is the essential feature, and further investigation would be needed to clarify the exact mechanism of enzymatic hydrolysis.

GENERAL METHODS (GM)

GM I. Nessler determination of ammonium nitrogen.

The reagent was prepared as follows (65): 3.5g. of gum acacia (B.D.H.) were dissolved in 750 mls. of water. To this was added 4g. of potassium iodide and 4g. of mercuric iodide dissolved in 25mls.water. The resulting solution was made up to I l.with deionized water.

Qualitive analyses for ammonia were carried out by adding 2mls. of this reagent together with 3mls. of 2N sodium hydroxide solution to Iml. of the test solution.The yellow to orange produced after I5 minutes was estimated by reading the absorbance at 490 mu in a "Unicam SP 500" spectrophotometer or a photoelectric colorimeter (E.E.L.-Evans Electroselenium Ltd.-Model B colorimeter with a number 603 filter)

GM 2 Dialysis

Dialysis was carried out using "HMC" visking tubing which was rinsed inside and tested for puncture holes with deionized water prior to use. The dialysis bag, formed by knotting the ends of the soaked visking tubing, was never more than two thirds filledwith material to be dialysed to allow for water uptake due to osmosis. Dialysis took place against frequent changes of the relevant buffer in 5 l. glass beakers. The dialysing

solution was agitated by means of a slowly rotating magnetic stirrer ("Gallenkamp" magnetic stirrer) on the bottom of the beaker.

GM 3. Preparation of commercial adsorbants and ion exchangers.

a). Preparation of CM-cellulose(74) ·

Material:- Whatman carboxymethylcellulose powder CM-70, cation exchanger. Manufactured by W.& R.Balston Ltd.

The dry powder was suspended in a solution of 0.5N sodium hydroxide made 0.5M with respect to sodium chloride and mechanically stirred for about ten minutes. It was then lightly centrifuged at 1500 rpm in an MSE "Magnum" centrifuge with a four place, 600ml. cup swing out head ('g' at 1500 rpm = 1160x g.) for 5 minutes and the supernatant discarded. The residual CM-cellulose was then repeatedly washed with deionized water until free of base (as indicated by Johnsons "Universal" indicator paper). Fines were also removed at this stage by decantation of all material remaining in suspension after 15 minutes settling time. The cellulosic cation exchanger was then thoroughly equilibrated in the appropiate buffer and was ready for use in the Na^+ ionic form.

b). Preparation of DEAE-cellulose

Material:- Whatman diethylaminoethyl cellulose powder-DE-50 anion exchanger. Manufactured by W.& R. Balston Ltd.

The dry powder was suspended in a solution of 0.5N sodium hydroxide and mechanically stirred for ten minutes, and then after washing free of base using deionized water, it was washed with 0.5N hydrochloric acid solution. After freedof acid with deionized water, and removing fines as described in GM 3(a),the DEAE-cellulose was in the Cl^- form and ready for use after equilibrating in the appropiate buffer.

c). Polstyrene anion exchange resin.

Material:- Dowex-I x 4,200-400 mesh, manufactured by the Dow Chemical Company,U.S.A.

The resin was initially suspended in IN sodium hydroxide and stirred for a few minutes and then the alkali was removed by washing repeatedly with deionized water. "Fines" were removed by decanting all material retained in suspension five minutes after stirring. The resin was then suspended in IN hydrochloric acid and stirred for a few minutes and then washed free of acid with deionized water to be ready for use in the Cl^- form.

GM 4. Routine assay for RNAse enzyme location.(28).

Materials:- RNA - exhaustively dialysed and lyophilized yeast commercial RNA. The RNA was made up in the following buffer at a rate of 10mgs. per ml. The buffer used was 0.1M sodium succinate buffer at pH 6.5 and 0.05M with respect to magnesium ions.

MacFadyens reagent:- 0.25% uranyl acetate and 2.5% trichloracetic acid solution.

Procedure Aliquots of 0.25ml. of RNA in sodium succinate buffer were added to 0.25ml. of the test solution and incubated at 37°C in a constant temperature water bath for 30 minutes. Digestion was terminated by the addition of 0.5ml. of MacFadyens reagentand the digest tube was immersed in an ice-cold water bath. After cooling for a period of 30 minutes, each digest tube was centrifuged at 2,000 x g.for five minutes, and 0.1ml. of the resulting supernatant was withdrawn and added to a boiling tube and diluted to 4mls. with de-ionized water. Estimation of the nucleotide content of each tube was by reading, at 260_{mu}, the optical density in a Unicam SP 500 spectrophotometer.

GM 5. General chromatographic column filling technique.

The glass chromatographic column to be used was thoroughly washed and decreased by steeping in the industrial detergent "Pyroneg", and rinsed by the passage of running water for 10 minutes. The column was then

filled with the starting buffer and the base plugged with glass wool which had also been soaked in the starting buffer to ensure air bubbles had been excluded as much as possible. The column was assembled into a vertical position (checked by a spirit level) as in fig.I. The reservoir funnel was filled to about half capacity with buffer and the stirring motor set into action. The material with which the column was to be filled was added at intervals to the reservoir funnel as the column filled up. After an initial 2-3cms. of material had settled under gravity, a slow flow of buffer was maintained, by partially opening the basal tap, to ensure good and efficient packing of the column. On completion of the filling procedure, the column was copiously eluted with the initial buffer.

GM 6. Isolation of nucleotide material from Dowex-I column elution.

The bulked material from each peak believed to contain nucleotide material was neutralized with IN lithium hydroxide and freeze - dried. The dry material was then taken up in the minimum volume of dry methanol. Excess dry acetone was added and nucleotide material was precipitated free of salt. The precipitate was collected by centrifugation and dried in a dessicator over silica gel with a constantly applied vacuum to remove the

organic solvent vapour.

GM 7. Lyophilization or freeze drying.

Apparatus:- Edwards High Vacuum Ltd.-Freeze drier model IC P serial no.198,with a two stage 28C50B Edwards "Speedivac"pump.

Procedure:- The material to be freeze dried was placed in a an appropiately sized flask (round-bottomed "Quick-fit"B24 neck to fit the freeze drier) and the flask was filled to no more than a quarter full. The flask plus contents was then rotated by hand in a "dry-ice" and acetone freezing mixture in such a way as to obtain the freezing of the contents in a thin layer over a large as area as possible on the inside of the flask, The flask was quickly connected up to the freeze drier which had been previously filled with a "dry-ice" and methanol mixture and the whole system subjected to a vacuum. Low temperature distillation then takes place. An ice coat quickly forms on the outside of the flask in which the contents are being freeze dried,due to air moisture condensation and subsequent freezing,and when this ice had completely cleared it was an indication that the freeze drying process was finished. The vacuum was carefully released and the flask containing the freeze dried material detached and stoppered.

GM 8. Disc gel electrophoresis -see page

The experimental details and procedure are well described in references II6,II7,& II8

Materials:

a).Staining solution I% naphthol blue (Kodak ASI498) in 7% acetic acid.

b).Tray buffer solution

3I.2gms. of β-alanine(BDH) were dissolved in 8.0mls. of glacial acetic acid and this was made up to Il.with deionized water.

c).The following stock solutions were made up and stored in brown bottles in a refrigerator at 5°C.

Solution A. 4.0mls of N,N,N',N',tetramethylethylenediamine (Kodak A92462 or 8I78),48mls of N potassium hydroxide, I7.2mls of glacial acetic acid. This was made up to I00mls with deionized water.

Solution B. 0.46mls of N,N,N',N',tetramethylethylenediamine, 48mls potassium hydroxide, 2.87mls glacial acid. This was made up to I00mls with deionized water. (The Potassium hydroxide was of Normal concentration)

Solution C. 60gms of acrylamide (Kodak552I),0.4 gm N,N',methylenebisacrylamide (Kodak 8383) was made up to I00mls with deionized water.

Solution D. I0gms of acrylamide,2.5gms N,N',-methylenebisacrylamide was made up to I00mls with deionized water.

Solution E. 4.0mgs of riboflavin in 100ml of deionized water.

d). Destaining polyacrylamide solution

6.0gms of acrylamide, 0.5mgs of riboflavin were made up to 100mls with deionized water

From the above stock solutions, small and large pore gel solutions were compounded in the following proportions:-

Small pore solution

I part A
2 parts C
I part H_2O
} Small pore solution at pH 4.3

The small pore solution above was mixed just before use with an equal volume of freshly prepared ammonium persulphate (0.56gms /100mls water)

Large pore solution

I part B
2 parts D
I part E
4 parts H_2O
} Large pore solution at pH 6.8

Procedure The procedure and use of these materials was described in detail in part I of this thesis and also by R.A.Reisfeld et al (118). Recording of results was by photography against a translucent screen illuminated from behind by fluorescent lighting

GM 9. Chromatography and electrophoresis on paper; detection of compounds on paper.

Whatman No.1 chromatography paper was used unless otherwise stated. The method was the descending technique

used by Martin (249). The composition of the solvents used was that used by Leloir (230) namely: 3 volumes of IM ammonium acetate and 7.5 volumes of 95% ethanol at either pH 7.5 or 3.6. The time of development was approximately 48 hours at room temperature (± 18°C).

Paper electrophoresis was carried in an apparatus similar to that described by Markham and Smith (240) using Whatman No.I paper strips of 6 x 38cms dimensions unless otherwise stated. The buffers used, the voltage applied, together with the times of run are mentioned in the text in the appropiate places.

Nucleotides were located on paper by inspection under ultra-violet light. Distances moved by components were measured to their centres and relative to the distance moved by control UMP.

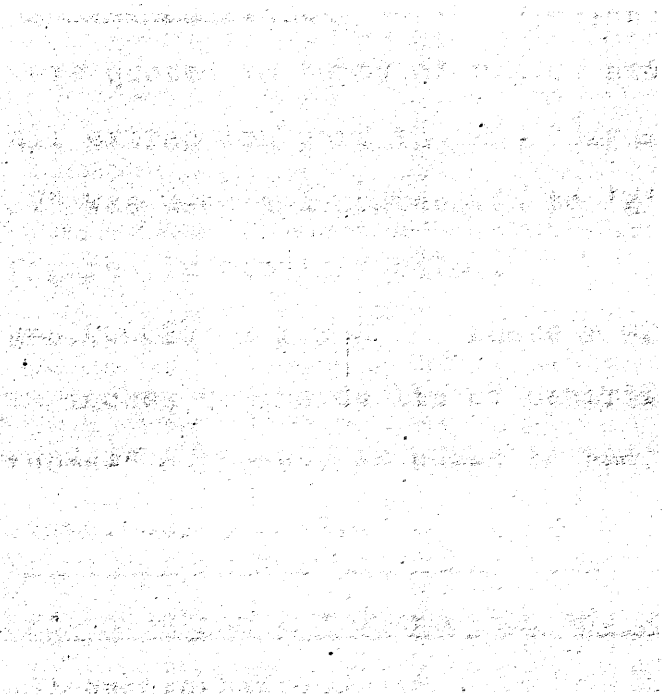
GM IO. Thin layer chromatography (TLC) and detection of compounds

Materials: Whatman "Chromedia" cellulose powder CC4I (cataloque no.I24I2) with a mean particle size passing 200 BSS.

Procedure: The cellulose powder was mixed with water in a ratio of I:2.09 (w/v) and the resulting suspension spread, to a thickness of 0.3mm on clean grease free glass 20 x 20cms plates, by means of a Shandon "Unoplan" plate making apparatus. Further details of preparation and

solvents used in the ascending chromatographic separation on thin layer plates are mentioned in the text.(Part III).

Detection: of nucleotides was by inspection under U.V. light. The method of detection was aided by spraying the plate with a 0.005% rhodamine-6G spray which made the background pale yellow, thus accentuating the areas of U.V. absorbance.



EXPERIMENTAL SECTION

Note: Not all experiments that have been carried out are detailed below, since they are adequately detailed in the text and to list them under the experimental section would entail mere repetition.

EXP I. Spleen extraction I

The extraction was carried out as described in the text and each stage of extraction of calf spleen ribonuclease, with respect to details of amounts of substances used etc. is given below, as this was not described in the text.

Note: Centrifuge speeds. In the text, speeds were quoted in terms of r.p.m. and in all extraction work the MSE "Highspeed I7" was used and conversion to 'g' figures is obtained below:

$g = 0.0000284 \times r \times N^2$ where r = radius in inches to inside lip of centrifuge tubes = 5.6 inches. N = speed in rpm.

$\therefore g = 0.000159 \times N^2$.

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a). Stage I - Homogenisation and extraction and ammonium sulphate (AS) precipitation.

25 spleen were homogenised in 0.05M sodium acetate, 10^{-3}M EDTA, pH 7.2 buffer giving a total of 8.19l. of homogenate. The pH was adjusted to 3.5 with IN hydro-

-chloric acid giving a total volume of 8.741. of acid homogenate (in aliquots of 3.2,3.2 and 2.341.). A total of I39.4gms of ammonium sulphate were added (in 5I2, 5I2,and 374.4gm aliquots respectively at the rate of I60gms/l.) to bring the saturation level to 0.3*,and left to stand over night. The precipitate was centrifuged at I6,000 x g at 2-3°C for 8 minutes in 250ml polypropylene bottles and the residue discarded. The total volume of the supernatant was 4.9I 1. [* 0.3 & 30% = same]

b).Stage II - 0.6 AS saturation.

4.9I 1. of supernatant (in 31. and I.9.1. aliquots) were made 0.6 saturated with I,748gms of ammonium sulphate(I,068 and 680 gms respectively). After standing overnight the material was centrifuged at I6,000 x g at 2-3°C for 8 minutes. The precipitate collected was dissolved in I.I71. of pH 7.2 buffer.

c). Stage III - Heat treatment.

The I.I71. of dissolved material were adjusted to pH 3.5 with IN hydrochloric acid, giving a total volume of I.5I51. for heat treatment. Then in aliquots of 700, 500 and 315 mls, the material was subjected to heat treatment as described in the text for I0 minutes at 60°C and then the pH was re-adjusted to 7.0 with IN sodium hydroxide. The material precipitated was centrifuged at 26,000 x g and the supernatant retained.

d). Stage IV - Second acid ammonium sulphate precipitation (0.85 AS satn.).

The pH of the supernatant from the previous stage was adjusted to 2 with IN hydrochloric acid, thus giving a total volume of I.87l. and 374gms of ammonium sulphate were added to bring the solution to 0.4 - 0.5 saturation level . The precipitate formed was removed by centrifugation at I6,000 x g for 30 minutes at 2-3°C and the 2.05l. of supernatant were then brought to 0.85 saturation by the addition of 656gms of ammonium sulphate. The precipitate formed after standing over night was centrifuged at 26,000 x g for I5 minutes at 2-3°C and dissolved in 300mls of 0.005M Tris/HCl, 10^{-4}M EDTA, pH 5.5 buffer and solution was aided also by the presence of IM sodium acetate in the buffer.

e). Stage V. Dialysis of 0.8 AS precipitated protein.

Dialysis of the above dissolved material was in I inch visking tubing against 0.005M Tris/HCl, 10^{-4}M EDTA,pH 5.5 buffer with three changes of 4½-5l. over a period of 24 hours. The dialysed material was centrifuged at I6,000 x g for I5 minutes at 0°C to give 360 mls of solution.

f). Stage VI. CM-cellulose chromatography.

The protein solution (360mls at a concentration of I.67 mgs/ml protein) was applied to a 64 x I.7cm CM-cellulose

column and the following gradient elution was applied:

Reservoir 5l.(+ 5l. later).		Constant volume chamber 3.75l.
0.005M Tris/HCl 10^{-4}M EDTA 0.32M NaCl pH 8.2	⟶	0.005M Tris/HCl 10^{-4}M EDTA pH 5.5

Column data.

Buffer level in constant vol. chamber above floor level	=113cms
Buffer level in reservoir above floor level	=162cms
Top of column above floor level	=165cms
Flow rate	=175mls /hour
Fraction size collected	=50mls

The fractions were read at 200 mu in a Unicam SP 500 spectrophotometer and assayed for enzyme activity by GM -4.

EXP 2. Concentration of peak B from extraction I

Peak B (fraction 107-115) from extraction I was dialysed and then concentrated by means of adsorption onto a $3\frac{1}{2}$ x $\frac{1}{2}$ cm column of CM-cellulose and subsequently eluted with buffer that was IM with respect to sodium chloride. The concentrated protein fraction B was stored at -22°C in the deep freeze.

EXP.3. CM-cellulose chromatography of peak A from extraction I

Peak A protein material (fractions 90-105) was applied to a 17.5 x 2cm column of CM-cellulose and subjected to the following gradient elution:

Reervoir I l.		Constant vol.chamber 400mls
0.005M Tris/HCl 10^{-4}M EDTA pH 8.2 0.32M NaCl	⟶	0.005M Tris/HCl 10^{-4}M EDTA pH 8.2

A100 fractions were collected of 10mls each and at fraction 74, I l. of buffer made IM with respect to sodium chloride was added. Protein estimation was by U.V. absorption at 280mu and enzyme activity estimated by GM 4. Fractions 10-30 were bulked, dialysed, and concentrated by means of adsorption on to a 6 x ½cm column of CM-cellulose and subsequent elution with IM sodium chloride containing buffer. Storage of the enzyme was at -22°C.

EXP.4. Spleen extraction 2

Summary charts to this extraction and all others are given in figs.17-21 and relevant differences in the procedure in this extraction as compared to previous extraction I, and future extractions are described and discussed in the pertinent portions of the text.

a). Stage I - Homogenisation and extraction and ammonium sulphate precipitation.

2I2 spleen were homogenised and extracted as described in the text for extraction I.

b). Stage II - 0.6 AS saturation

c). Stage III Heat treatment at pH 3.5

The material was subjected to heat treatment at pH 3.5 at 67°C for I0 minutes and then centrifuged at 26,000 x g for I5 minutes at 2-3°C after adjusting the pH to 7.0

d). Stage IV The second acid ammonium sulphate precipitation was excluded.

e). Stage V - Dialysis

Dialysis of the supernatant from stage III was against 0.005M Tris/HCl, $I0^{-4}$M EDTA, pH 5.5 buffer with three changes of 4½-5l. over a period of 24 hours. The dialysed material was centrifuged at I6,000 x g for I5 minutes at 2-3°C, and the supernatant retained.

f). Stage VI - CM-cellulose chromatography.

The protein solution from stage V was applied to a 5I x I.7cm CM-cellulose column and subjected to the below gradient elution:

Reservoir, 5l. (+5l. Later)		Constant vol. chamber, 3.75l.
0.005M Tris/HCl		0.005M Tris/HCl
$I0^{-4}$M EDTA	⟶	$I0^{-4}$M EDTA
0.32M NaCl		pH 5.5
pH 8.2		

50ml fractions were collected and read at 280mu in a u.v.

spectrophotometer and assayed for enzyme by GM 4.

EXP 5. Spleen extraction 3

a). Stage I - Homogenisation and extraction and ammonium sulphate precipitation.

A total of 24 spleen (3 fresh and 21 deep freeze stored at -22°C) were homogenized in 0.05M sodium acetate, 10^{-3}M EDTA, 2M urea, pH 7.2 buffer. The urea used was BDH Analar grade and all urea solutions were filtered through Whatman No.1 filter paper before use. The pH of the homogenate was adjusted to pH 3.5 with 1N HCl giving a total volume of 6.6l. The homogenate was then made 0.3 saturated by the addition of 160gms/l. of ammonium sulphate, and left to stand over night. The resulting precipitate was re-extracted with twice its volume of the above pH 7.2 buffer after centrifuging at 16,000 x g. The buffer (also 2M w.r.t.urea) and precipitate were stirred for 30 minutes and similarly made 0.3 saturated with ammonium sulphate, and, after centrifugation, the re-extracted and the original supernatants were combined.

b). Stage II - 0.8 AS saturation.

The combined supernatants were brought up to 0.8(i.e.80%) saturation by the addition of 356gms/l. of ammonium sulphate. After standing over night and centrifuging at 16,000 x g at 2-3°C for 15 minutes, the precipitate obtained was dissolved in 1l. of 0.005M sodium acetate,

10^{-4}M EDTA, 2M urea, pH 7.2 buffer

c). Stage III - Heat treatment at pH 3.5.

The protein solution was adjusted to pH 3.5 with IN HCl and subjected to a temperature of 60°C for 10 minutes in aliquots of about 500mls at a time, and then the pH was adjusted to pH 7.0 before centrifuging at 26,000 x g for 15 minutes at 2-3°C.

d). Stage IV - Second AS precipitation (at neutral pH)

The ammonium sulphate concentration was initially raised to about 0.4 saturation by the addition of 190gms/l. The small precipitate produced was removed by centrifugation of the solution at 26,000 x g. The ammonium sulphate concentration of the supernatant was then raised to almost saturation point and left to stand over night. Centrifugation was at 26,000 x g for 15 minutes at 2-3°C and the precipitate obtained was dissolved in 0.005M Tris/HCl, 10^{-4}M EDTA, 2M urea, pH 7.5 buffer.

e). Stage V - Desalting using Sephadex G-75 molecular sieve.

Materials: Sephadex G-75, particle size 40-120μ, water regain 7.5 ± 0.5 g/g, in bead form.

Desalting was carried out by 9 separate applications and elutions of approximately 5.5ml aliquots of protein solution from stage IV to a G-75 Sephadex preparative column of 75.5 x 4.8cms dimensions and eluting with

0.005M Tris/HCl, 10^{-4}M EDTA, 2M urea, pH 7.5 buffer at a rate of about 84mls/hour. Fractions of 10mls were collected using a "Towers" automatic fraction collector and the fractions were assayed for RNAse activity by GM 4 to ascertain the position of elution of active RNASe protein, and this was correlated with protein elution profile from optical density readings at 280mu in the "Unicam" SP500 spectrophotometer in order that the necessity of assaying every G-75 column elution may be bypassed.

f). Stage VI - Application of protein extract to a DEAE-column.

The enymically active material from stage V was applied to a 13 x 1.8cms DEAE-cellulose column (Cl^-form) which was equilibriated with 0.005M Tris/HCl, 10^{-4}M EDTA, 2M urea, pH 7.0 buffer and eluted withthe same. Collection of effluent was in bulk and this material which passed straight through the DEAE-cellulose adsorbant was retained for stage VII.

g). Stage VII - CM-cellulose chromatography.

The above material at pH 7.0 and 2M w.r.t. urea was subjected to the following column chromatography below: The columns were equilibriated with urea at the concentration stated and the protein solution applied, and eluting buffers were also adjusted to this same urea

concentration.

i). CM-cellulose column no. CMC-3.- This column was not equilibriated with urea and neither were the eluting buffers. The below gradient elution was used and 5ml fractions were collected.

Reservoir I l.		Constant vol. chamber 500 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA	⟶	10^{-4}M EDTA
pH 8.2		pH 7.0
0.32M NaCl		

ii). CM-cellulose column no.CMC-3-6M -in the presence of 6M urea

Reservoir I l.		Constant vol. chamber 500 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA	⟶	10^{-4}M EDTA
pH 8.2		pH 7.0
6M urea		6 M urea
0.32M NaCl		

5ml fractions were collected.

iii). CM-cellulose column no.CMC-3-8M -in the presence of 8M urea

Reservoir I l.		Constant vol. chamber 500 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA	⟶	10^{-4}M EDTA
pH 8.2		pH 7.0
8M urea		8M urea
0.I6M NaCl		

5ml fractions were collected.

EXP 6. Spleen extraction 4.

Like extraction-/ p.t.o:

3 the processing of 36 spleen in extraction 4 was also carried through entirely in the presence of 2M urea using exactly the same basic procedure (stages I-VII as in EXP 5) except for various extra re-extraction techniques which are described adequately in the text. The following conditions were used for CM-cellulose chromatography at stage VII

a). CM-cellulose chromatography

i). CMC-4-2M-I. Chromatography of RNAse enzyme was carried out in the presence of 2M urea on a column of 68 x I.7 cm dimensions. An LKB "Radirac" fraction collector and "Uvicord" assembly was used for all column chromatography in extraction 4 (and also in extraction 5), and similarly for both extractions 4 and 5, the fraction size collected was I0mls.

The following gradient elution was used:

Reservoir I600mls.		Constant vol. chamber 800 mls.
0.005M Tris/HCl		0.005M Tris/HCl
$I0^{-4}$M EDTA		$I0^{-4}$M EDTA
pH 8.2	⟶	pH 6.0
2M urea		2M urea
0.32M NaCl		

Column data:

Flow rate	0.5-0.7mls/min.
Height of column above bench level	90cms

Height of liquid in constant vol.chamber above bench level 79cms

Height of reservoir above constant vol. chamber(i.e.difference in liquid levels) I4.5cms

Height of outlet of siphon on fraction collector above bench level 2o.5cms

This data was approximately same for all extraction 4 CM-cellulose chromatography and likewise so was the method of enzyme location (GM 4) and protein estimation by reading the optical density at 280mu in a Unicam SP500 spectrophotometer.

ii).CMC-4-2M-II. Column dimensions: 50 x 2.2cms The following gradient elution was used:

Reservoir I600mls		Constant vol. chamber 800 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA		10^{-4}M EDTA
pH 8.2	⟶	pH 7.0
2M urea		2M urea
0.25M NaCl		

iii).CMC-4-7M-I. Column dimensions: 68 x I.7cms and chromatography was carried out entirely in the presence of 7M urea.

Reservoir I600mls		Constant vol.chamber 800 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA		10^{-4}M EDTA
pH 8.2	⟶	pH 6.0
7M urea		7M urea
0.25M NaCl		

iv). CMC-4-7M-II. Column dimensions: 68 x I.7cms

Reservoir I600mls		Constant vol.chamber 800 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA		10^{-4}M EDTA
pH 8.2	⟶	pH 7.0
7M urea		7M urea
0.I5M NaCl		

v). CMC-4-2M-Leu I, The protein solution which was made 2M w.r.t. urea was applied to a CM-cellulose column of 68 x I.7cms dimensions and also 2M w.r.t. urea. A DL nor-leucine (BDH Biochemical grade) gradient was then applied as below:

Reservoir I500mls.		Constant vol.chamber 800 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA		10^{-4}M EDTA
pH 7.0	⟶	pH 7.0
2M urea		2M urea
saturated DL nor-leucine		

After fraction 92 (10ml fractions were collected) the reservoir was made 0.25M w.r.t. sodium chloride and the elution continued to give a salt and DL nor-leucine gradient combined. Thus the gradient from fraction 92 onwards was:

Reservoir 580mls		Constant vol.chamber 800 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA		10^{-4}M EDTA
pH 7.0		pH 7.0

2M urea 0.25M NaCl Saturated nor-leucine	⟶	2M urea weak solution w.r.t.nor-leucine

vi). CMC-4-2M-Leu II. This column differed from CMC-4-2M-Leu I in that there was no nor-leucine gradient, but nor-leucine was present at saturation level throughout all stages of the below gradient elution:

Reservoir 1500mls		Constant vol.chamber 800 mls
0.005M Tris/HCl 10^{-4}M EDTA pH 8.2 2M urea 0.15M NaCl Saturated nor-leucine	⟶	0.005M Tris/HCl 10^{-4}M EDTA pH 7.0 2M urea Saturated nor-leucine

EXP 7. Spleen extraction 5

The extraction and processing of 30 spleen was carried through all stages in the absence of urea unless otherwise stated. The average weight of calf spleen was 68gms and this weight was in accordance withthe spleen used in all previous extractions.

The procedure used was basically the same as that used for extraction 4(EXP 6) with the ommission of the presence of urea. Any differences in procedure are described and explained in the relevant portions of the text and these are mainly with respect to the large amount of material discarded at stage I of the extraction

process.

a). CM-cellulose chromatography

Chromatography was carried out in the absence of urea except for column CMC-5-III. Fraction collection was by means of an LKB "Radirac" and "Uvicord" assembly, collecting 10ml fractions.

i). CMC-5-I Column dimensions 49 x 2.5cms.

Reservoir 1600mls		Constant vol.chamber 800 mls.
0.005M Tris/HCl 10^{-4}M EDTA pH 8.2 0.25M NaCl	⟶	0.005M Tris/HCl 10^{-4}M EDTA pH 7.0

ii). CMC-5-II Column dimensions 46 x 2.5cms

Reservoir 1600mls		Constant vol. chamber 800 mls.
0.005M Tris/HCl 10^{-4}M EDTA pH 8.2 0.32M NaCl	⟶	0.005M Tris/HCl 10^{-4}M EDTA pH 6.0

iii). CMC-5-III. Column dimensions 20 x 2.2 cms. This column was equilibriated with 2M urea and the protein solution applied to this column was also made 2M with respect to urea. The following gradient elution was carried out:

Reservoir 1600mls		Constant vol.chamber 800 mls
0.005M Tris/HCl 10^{-4}M EDTA pH 8.2,2M urea 0.32M NaCl.	⟶	0.005M Tris/HCl 10^{-4}M EDTA pH 6.0, 2M urea

iv). CMC-5-IV. Column dimensions 18 x 2.2 cms.

Reservoir 1600mls.	Constant vol.chamber 800 mls.
0.005M Tris/HCl	0.005M Tris/HCl
10^{-4}M EDTA	10^{-4}M EDTA
pH 8.2	pH 8.2
0.32M NaCl	

Index to journal name abbreviations used in references.

Acta Chem.Scand.	Acta Chemica Scandinavia
Adv.in Enzymology	Advances in Enzymology
Adv.in Prot.Chem.	Advances in Protein Chemistry
Ann.	Annalen der Chemie, Justus Liebigs.
Ann.N.Y.Acad.Sci.	Annals of the New York Academy of Sciences
Ann.Rev.Biochem.	Annual Review of Biochemistry
Archivs.Biochem.Biophys.	Archives of biochemistry and biophysics
BBA	Biochimica et Biophysica Acta
BBRC	Biochemical,biophysical research communications
BJ	Biochemical Journal
Biochem.	Biochemistry(American Chemical Society)
Brit.J.Expertl.Pathol.	British Journal of Experimental Pathology
Brit.Med.Bull.	British Medical Bulletin
Bull.Soc.Chim.Belg.	Bulletin des Societes Chimiques Belges
Can.J.Biochem.& Pathol.	Canadian Journal of Biochemistry and Physiology
Chem.Revs.	Chemical Reviews(American Chemical Society)

Comp.Rend.Trav.Lab. Carlsberg,ser Chim.	Comptes Rendus des Travaux du Laboratoire Carlsberg, serie Chimique.
Fed.Proc.	Federation Proceedings
JACS	Journal of the American Chemical Society
JBC	Journal of Biological Chemistry
J.Biochem (Tokyio)	Journal of Biochemistry (TOKYIO)
J.Chem.Phys.	Journal of Chemical Physics
J.Chromat.	Journal of Chromatography
J.MoL.Biol.	Journal of Molecular Biology
J.Phys.Chem.	Journal of Physical Chemistry
J.Natl.Inst.Cancer,U.S.	Journal of the National Cancer Institute,U.S.A.
J.Pathol.Bacteriol.	Journal of Pathology and Bacteriology
Kolloid.Z.	Kolloidnyi Zhurnal
Photochem ,photobiol.	Photochemistry and photobiology
Proc.Natl.Acad.Sciences,US.	Proceedings of the National Academy of Sciences of the U.S.A.
Ukr.Biokhim.Zhur.	Ukrains'kii Biokhimich-eskii Zhurnal

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	Rump	Af.	Comment
AMP	0.75 0.90	3	Controls
CMP	1.16	3	
GMP	0.51	3	
UMP	1.00	3	
Dowex(A)	0.80	3	AMP?

2. Spectrophotometric data.

Spectra at acid pH. λ max 279 (278) $\frac{250}{260}$ = 0.45 (0.91)

λ min 240 (252) $\frac{280}{260}$ = 2.00 (1.56)

Spectra at alk. pH λ max 270 (270) $\frac{250}{260}$ = 0.86 (1.01)

λ min.255 (255) $\frac{280}{260}$ = 0.93 (0.90)

The values obtained did not coincide with the published figures for any mononucleotide $\begin{Bmatrix}226\\227\end{Bmatrix}$, but were nearer to the published values for CMP than any mononucleotide (the values for CMP are quoted above in brackets). Of particular significance was the high λ min. at 250 and 240 m_u at alkaline and acid pH's respectively, which is very characteristic of CMP type nucleotides.

3. Paper electrophoresis

Introduction to paper electrophoresis (Also see GM9.)

The experimental conditions and apparatus and also the theoretical considerations are discussed fully by J. D. Smith (241).

Nucleic acids and their components bear a number and variety of ionizable groups, and it is natural that electrophoretic techniques should play an important part in their separation. The method of separation is a very efficient and fast one, and the development of paper electrophoresis has greatly facilitated the separation of nucleic acid derivatives. An important feature of the method is that the relative mobilities of nucleic acid components at any given pH may be predicted quite accurately, thus aiding the identification of unknown components.

Thus a molecule in a fluid subjected to a voltage gradient E, is acted upon by a force equal to (EQ) where Q is the net charge on the molecule and is given by the algebraic sum of the products of the number of ionizing groups and their percentage dissociation. This force is opposed by that due to the resistance of flow of the molecule through the

liquid, which is proportional to the velocity of motion of the molecule (V.) and equal to (KV) where K is a function of the size and shape of the molecule and the retarding effects of other ions in solution. The ions thus migrate at a constant velocity equal to $\frac{(EQ)}{(K)}$. Using the dissociation constants of the ionizable groups in nucleic acid components and making certain approximations as to the nature of the resistance to flow of the charged particles through the fluid, the relative mobility of these substances can be calculated. The relative mobilities of the four mononucleotides at pH 3.5 are:-

decreasing mobility ↓	Uridylic acid	1.00
	Guanylic "	0.95
	Adenlyic "	0.45
	Cytidylic "	0.16

At this pH the degree of dissociation of the NH_2 groups in AMP, GMP and CMP is most advantageous to separation.

Paper electrophoretic separation of Dowex (A) main peak

Electrophoresis was carried out on Whatman No.1 paper strips about 3 inches wide and in 0.1M sodium-citrate/citric acid buffer at pH 3.5. A voltage of 900 v. was used for the time stated below (usually about 50 mins).

The coolant medium was fresh 'Analar' grade carbon-tetrachloride (239) which was renewed each time. The buffer and coolant containers were assembled along with the paper strip as in fig. 75. Using paper strips approximately 38 cms. long, this was equivalent to a voltage gradient of 25 volts per cm.

Results. (Expressing the average of a number of results (Af.) as done for T.L.C. and paper chromatography - page 314)

	Rump	Af.	Comment
AMP	0.49	4	Controls
CMP	0.38	4	
GMP	0.80	4	
UMP	1.00	4	
Dowex (A)	0.29	4	No correln.

Summary of analysis of main peak from Dowex (A) (Material not held by DEAE-cellulose).

T.L.C., paper electrophoresis, and U.V. spectrophotometric data results indicated that this was not a mononucleotide or indeed a nucleoside. The position of the elution from the Dowex resin confirms that the unknown material was not of nucleoside nature since nucleosides would have certainly been eluted in the initial 0.003N HCl gradient (221) and similarly

one would have expected CMP (and possibly AMP), if present, to have been eluted by 0.003N HCl (i.e. before tube 74 in fig. 74) according to the elution order of mononucleotides obtained by Cohn(22I) under similar conditions.

But U.V. spectra showed this fraction to be of nucleotide character and this was substantiated by its behaviour with regard to chromatographic procedures. If this was the case, and this fraction was a nucleotide, it must have the following properties:-

1) Low relative electrophoretic mobility

2) Poor adsorptive properties on DEAE-cellulose but substantial adsorption by Dowex-I resin.

3) Spectral properties akin to CMP types.

These conditions could possibly be fulfilled by a di- or trinucleotide and in particular a di- or -trinucleotide of CMP. If the compound was a di- or trinucleotide (larger oligo-nucleotides were less likely since their properties are unlikely to coincide with the already established properties of the unknown entity) with a common base, the hydrolysis would result in two or more identical constituents

and give rise to one spot by separative chromatography techniques. However although there will be no separation into two detectable spots, properties before and after hydrolysis should be quite different and detectable. If the di- or trinucleotide was composed of two or more different base types, chromatographic separation techniques will exhibit proof of two separate and characteristic entities. Therefore hydrolysis of the main peak from Dowex (A) was carried out to verify the nature of this nucleotide type material.

Hydrolysis of main peak from Dowex (A)

When ribose nucleic acids are treated with mild alkaline reagents, they are rapidly converted into a mixture of their component mononucleotides. The cleavage by alkali is not specific as in the cleavage by pancreatic RNAse, but mononucleotides of all four bases are produced and represent a mixture of nucleoside 2' phosphates and nucleoside 3' phosphates. Markham and Smith (240) showed that alkaline hydrolysis of RNA proceeded via the formation of 2' 3' cyclic phosphate intermediates as was also demonstrated later for enzymatic (pancreatic RNAse) hydrolysis by the same workers (239). However alkaline hydrolysis differs from enzymatic hydrolysis of RNA, in that the final hydrolysis step to the 2' or 3'

phosphate in alkaline hydrolysis is completely random, whereas in pancreatic RNAse the nucleoside 3'-phosphate is produced.

Method.

The material isolated from Dowex (A) was made up to 7 mls. and then 3 mls. of IN lithium hydroxide were added, thus giving a final lithium hydroxide normality of 0.3. The solution was allowed to hydrolyse for 18½ hours at 37° (in a constant temperature water bath). The hydrolysate was then neutralized with 5N hydrochloric acid, diluted to 150 mls and lyophilized.

The isolated material was dissolved in 500 mls of deionized water at pH 7.0 and applied to another Dowex-I column, designated Dowex (B). Column dimensions = 9.5 x 1.8 cms. The retention of this material by the resin was 100%. Dowex (B) was subjected to the following elution programme after initially washing with 215 mls. of deionized water.

Elution programme:-

Elution of Dowex-B.	Fraction range (10 mls)
150 mls. H_2O ——→ 150 mls. 0.003N HCl	1 —— 27
Elution with 0.003N HCl	28 —— 70
1 ℓ. 0.003N HCl ——→ 1 ℓ. 0.003N HCl 0.03M LiCl	71 —— 240

Results. The elution profile of Dowex (B) is given in fig. 76 and the following fractions were bulked together:- fractions 32-50 and neutralized with IN Lithium hydroxide and reduced in volume by rotary evaporation.

Analysis of the products of Dowex (B)

a. Position of elution. The only material that was eluted from this column with the gradient sequence that was used, was from fractions 32-50 which was eluted by 0.003N hydrochloric acid. The position of elution indicates that the peak was of a mononucleotide character and probably CMP or AMP in property nature. Prediction of nucleotide character as the basis of position of elution from a Dowex I resin column was based on the work of Cohn (221) which included the use of hydrochloric acid (0.003N) as an initial eluent. Hydrolysis of the main peak of Dowex (A) has thus

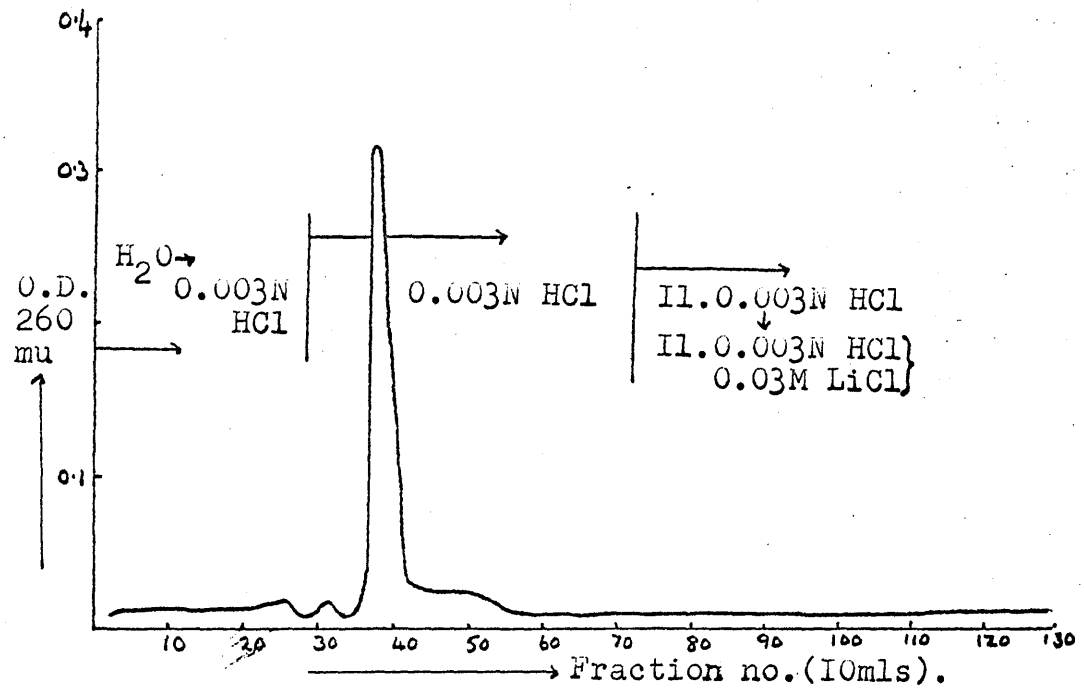


Fig.76. Hydrolysis product of Dowex(A) main peak, -Dowex(B).(Flow rate 0.9mls/min.)

	R_{UMP}	Af.	Comment
AMP	0.88&0.73	5	Controls.
CMP	I.I8	5	
GMP	0.55	5	
UMP	I.00	5	
Dowex(B)	I.20	5	Therefore CMP?

Fig.77. TLC results of material from Dowex(B).

brought about a change in this elution position, indicating (fig. 74 and 76) that Dowex (A) product was of di- or trinucleotide character, and further analysis below was used to confirm this surmise.

b) U.V. Spectrophotometric data.

Spectra at acid pH λ max. 279(279) O.D.$\frac{250}{260}$ = 0.64 (0.45)

λ min. 241(240) O.D.$\frac{280}{260}$ = 1.63 (2.00)

Spectra at alk. pH λ max. 269(272) O.D.$\frac{250}{260}$ = 0.94 (0.86)

λ min. 254 (250) O.D.$\frac{280}{260}$ = 0.89 (0.93)

The data obtained compared favourably with that for the published values of CMP (figures quoted in brackets) (226 , 227).

c) T.L.C. see fig. 77 for a summary of results and also fig. 78.

for a comparison of data for the main peak from Dowex (A) before alkaline hydrolysis, with that of Dowex (B) after hydrolysis.

Summary of the investigation of material not held when digest VI material was applied to a DEAE-cellulose column. (i.e. Dowex (A) and (B) columns)

Subsequent chromatography of this fraction on Dowex-I resin (Dowex (A)) showed that the compound had affinities

	Dowex(A)		Dowex(B)	
Acid λ max., λ min.	278mu	253mu	279mu	241mu
Alk. λ max., λ min.	270mu	255mu	269mu	254mu
$\frac{250}{260}$ i.acid ii.alk	0.91	1.56	0.64	0.94
$\frac{280}{260}$ i.acid ii.alk	1.01	0.90	1·63	0.89
TLC ....CMP	1.16		1.18	Average
unknown	0.80		1.20	values

Fig.78. Comparison of data of Dowex(A) and (B), before and after alkaline hydrolysis respectively.

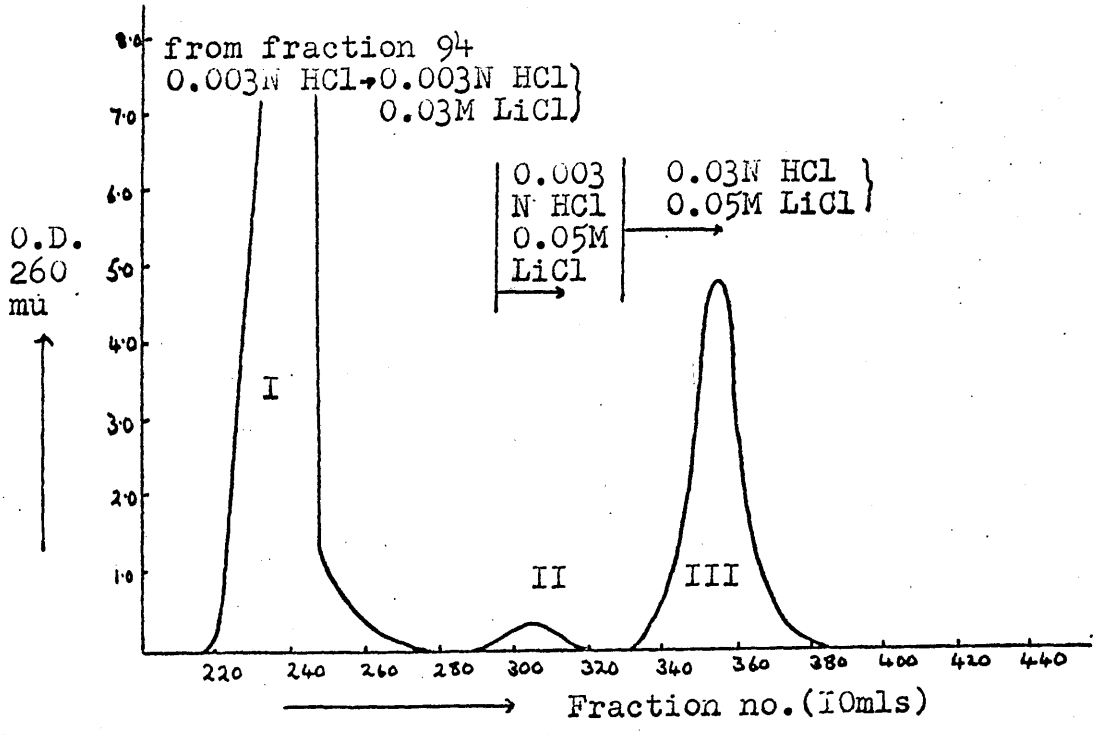


Fig.79. Dowex(C).

with CMP but nonetheless was not CMP. but probably some small oligonucleotide. Hydrolysis and further Dowex-I ion exchange chromatography (Dowex (B)) produced a single entity which responded to T.L.C. (fig. 76) and U.V. spectrophotometric (fig. 78) investigation in a very similar manner to CMP. Thus the results indicated that the material resulting from digest VI and not held by DEAE - cellulose ion exchanger, was a dimer or trimer etc. of CMP.

B. Examination of peak I of DEAE-cellulose column chromatography of digest VI (fig. No. 70 and 71 page 312).

The bulked fractions 20-116 of peak I were diluted to 1.5 ℓ. with deionized water and applied to a Dowex-I resin column (designated Dowex (C)) of dimensions 9 x 2 cms. Of the 3000 optical density units at 260 m_u applied, 2966 units were retained by Dowex (C) so giving a 98.9% retention.

The following elution programme was applied:-

Eluent		Fraction range (10 mls)
500 mls H_2O	500 mls 0.003 NHCl	1——49
Elution with 0.003N HCl		50 ——93
1.5 ℓ. 0.003N HCl	1.5 ℓ. 0.003N HCl 0.03 MLiCl.	94 ——293
Elution with	0.003N HCl 0.05 MLiCl.	294 ——330
Elution with	0.03N HCl 0.05M LiCl	331——406

a).TLC			
	R_{UMP}	Af.	Comment
AMP	0.79	3	Controls
	0.94		
CMP	I.24	3	
GMP	0.54	3	
	0.75		
UMP	I.00	3	
Dowex(C) (i)	0.56	3	∴GMP?
peak III (ii)	0.74		
b).Paper electrophoresis			
AMP	0.4I	3	Controls
CMP	0.36	3	
GMP	0.8I	3	
UMP	I.00	3	
peak III	0.80	3	∴GMP?
c).Paper chromatography			
AMP	0.7I	2	Controls
CMP	0.90,0.79	2	
GMP	0.56	2	
UMP	I.00	2	
peak III	0.56	2	∴GMP?

Fig.84. TLC,paper electrophoresis,and paper chromatography of Dowex(C) peak III.

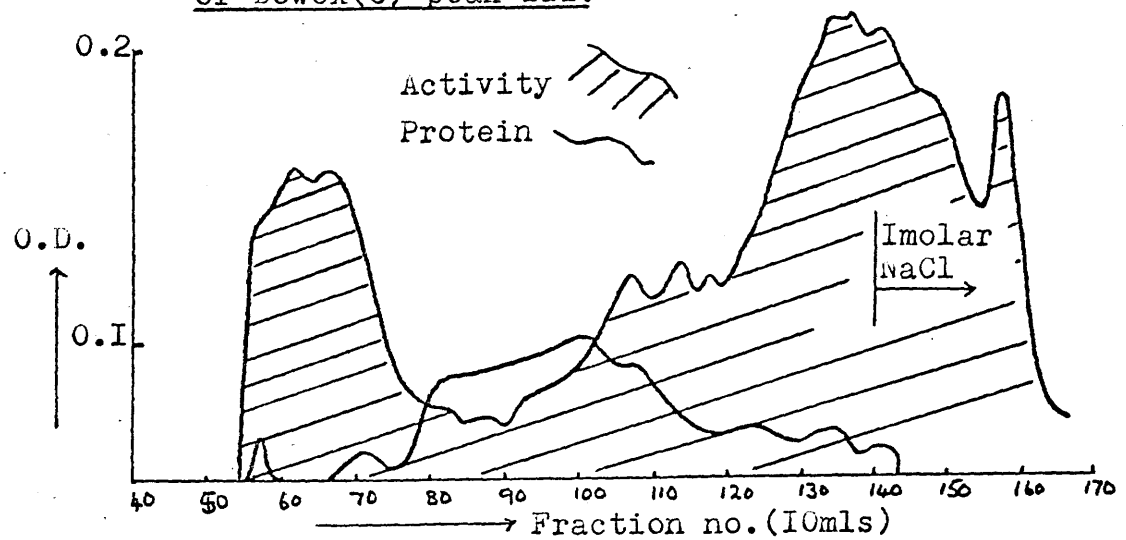


Fig.85. Purification of enzyme on CM-cellulose for digests VII and VIII.

Results: the elution profile of column Dowex (C) is given in fig. 79 and as with previous specificity work and all future such investigations, 10 ml. fractions were collected using an "LKB Radirac" and and "LKB" Uvicord" fraction collector-recording apparatus. The following fractions were bulked together:-

Fraction 222-268 inclusive - Peak I - vol. 570 mls, 2,274 O.D. $_{260}$ Units

Fraction 290-330 inclusive - Peak II - vol. 430 mls. 36.5 O.D.$_{260}$ Units

Fraction 338-380 inclusive - Peak III - vol. 651 mls. 651 O.D$_{260}$ Units

The pH of the bulked fractions for each peak was adjusted to pH 7.0 with IN Lithium hydroxide and then the solutions were lyophilized (see General Methods - GM 6 and 7).

Analysis of the products of Dowex (C)

Dowex (C) peak I. The various analytical techniques are summarized in fig. 80 and according to these results using T.L.C. and paper electrophoretic techniques, peak I seems fairly certain to contain the three mononucleotides, AMP, CMP and UMP. However for purposes of confirmation, Dowex (C) peak I.was

a).TLC			
	R_{UMP}	Ref.	Comment
AMP	0.79	4	Controls
	0.93	4	
CMP	I.20	4	
GMP	0.56	4	
UMP	I.00	4	
Dowex(C) (i)	0.75	4	Therefore AMP?
peak I (ii)	0.98	4	" UMP?
(iii)	I.20	4	" CMP?
b).Paper electrophoresis			
AMP	0.48	3	Controls
CMP	0.38	3	
GMP	0.79	3	
UMP	I.00	3	
peak I (i)	0.38	3	Therefore CMP?
(ii)	0.48	3	" AMP?
(iii)	0.98	3	" UMP?

Fig.80. Analysis of the products of Dowex(C)peakI.

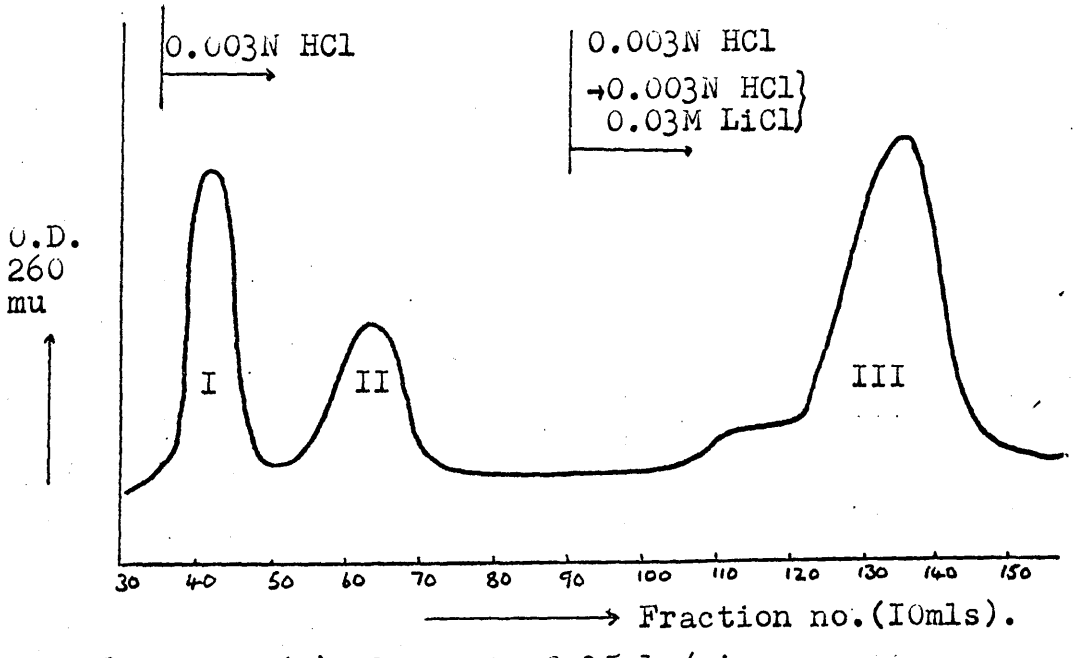


Fig.81. Dowex(D).Flow-rate=0.35mls/min.

subjected to further Dowex I column separative chromatography.

Further Dowex-I chromatography of Dowex (C) peak I Dowex (D)

The column dimensions (designated Dowex(D).) were 13 x 1 cms, and 1088 optical density units (at $260m_u$) in 500 mls of deionized water were applied to Dowex (D) at pH 7.0 and a 91.5% retention of the applied material was obtained after washing with 1350mls of deionized water. Then the following elution programme was inaugurated:-

Eluent.	Fraction range (≙ 10 ml)
300 mls. H_2O ⟶ 300 mls 0.003N HCl	1 — 35
Elution with 0.003N HCl	36 — 88
1 0.003N HCl ⟶ 1 0.003N HCl 0.03M L_iCl	89 — 185
Elution with 0.03N HCl 0.05M L_iCl	186 — 250

RESULTS. The elution profile of Dowex (D) column is given in fig. 81 and the following fractions were bulked together and the pH adjusted to 7.0 with IN lithium hydroxide and freeze dried (GM 6 & 7):

Fraction 36 - 47 inclusive - Peak I, vol. 130 mls, 293 O.D. Units at 260 m_u.

Fraction 50 - 75 inclusive Peak II. vol. 277 mls, 95 O.D. units at 260 m_u.

Fraction 122-150 inclusive Peak III, vol. 310 mls, 515 O.D. units at 260 m_u.

T.L.C., paper electrophoresis, paper chromatography and U.V. spectrophotometric data are summarized in figs. 82 & 83 for peaks I, II, and III.

Prediction of nucleotide character on the basis of elution sequence of Dowex (D)

T.L.C., paper electrophoresis, paper chromatography and U.V. spectrophotometric data (fig. 82,83) indicate that peaks I, II and III correspond to CMP, AMP, and UMP respectively and this is exactly what one would expect under the conditions of elution used, with CMP and AMP (peaks I and II respectively) being eluted by 0.003 N HCl before UMP, which requires a 0.003N HCl and 0.03N lithium chloride salt gradient before it is eluted (221).

Summary of Dowex (D) column chromatography.

Chromatographic separation of peak I of Dowex(C) on Dowex (D) resulted in separation into three nucleotide

a).TLC						
	Peak I	Af.	Peak II	Af.	Peak III	Af.
AMP	0.79 0.90	3	0.7I 0.80	2	0.75 0.90	2
CMP	I.I7	4	I.I3	2	I.I8	2
GMP	0.57	3	0.56 0.70	2	0.66	2
UMP	I.00	3	I.00	2	I.00	2
Unknown	I.09	4	0.98	2	0.99	2
Comment	∴CMP?		∴AMP?		∴UMP?	
b).Paper electrophoresis						
AMP	0.42	2	0.44	2	0.46	2
CMP	0.26	2	0.34	2	0.34	2
GMP	0.76	2	0.78	2	0.79	2
UMP	I.00	2	I.00	2	I.00	2
Unknown	0.25	2	0.4I	2	0.96	2
Comment	∴CMP?		∴AMP?		∴UMP?	
c).Paper chromatography						
			Peaks I,II,&III.			
AMP			0.70	2		
CMP			0.90	2		
GMP			0.76 0.53	2		
UMP			I.00	2		
Unknown	0.75	2	0.7I	2	0.97	2
Comment	∴CMP?		∴AMP?		∴UMP?	

Fig.82. TLC,paper electrophoresis,and paper chromatography of Dowex(D) products.

Peak	λ max.	λ min.	O.D.$\frac{250}{260}$	O.D.$\frac{280}{260}$	Comment
I CMP val.	278mu (279)	24I (240)	0.50 (0.45)	I.0I (2.00)	∴CMP?
II AMP val.	258 (257)	23I (229)	0.85 (0.85)	0.30 (0.22)	∴AMP?
III UMP val.	260 (262)	230 (230)	0.77 (0.80)	0.36 (0.28)	∴UMP?

Fig.83. U.V.data for Dowex(D) products.(The figures in brackets are the published values for mono--nucleotides,ref.226,&227.)

peaks which can beyond reasonable doubt be ascribed to CMP, AMP and UMP in that order of elution on the basis of analysis of the peaks by four methods, namely: T.L.C., paper electrophoresis, paper chromatography, and U.V. spectrophotometric data.

Summary of Dowex (C) peak I investigations.

From the investigation of this peak by T.L.C. and paper electrophoresis, and in the light of further Dowex-I resin column chromatographic separation, it was established that this peak contained CMP, AMP, and UMP. The reason that these three mononucleotides were eluted together from Dowex (C) (fig. 79) instead of the more expected elution and separation that was achieved on Dowex (D) (fig.81), can be explained by the fact that when peak I from the DEAE-cellulose separation of the products of digest VI was applied to Dowex (C) there was no pH adjustment (page 332). Thus the pH would be about 8.6 due to the presence of ammonium carbonate, and elution would be consequently delayed until neutralization had taken place, since normally elution of CMP and AMP would be brought about by eluting with 0.003N HCl alone (as in Dowex(D) - fig.81) where there were no pH and ammonium carbonate

complications. But in Dowex (C), no elution of any mononucleotide occurred until well into the 0.003N HCl → 0.003N HCl / 0.03M L_iCl gradient. When elution of these mononucleotides did occur, the three (CMP, AMP and UMP) were eluted together.

It may be also conceivable that peak I of Dowex (C) fig. 79. was not composed of three mononucleotides when eluted but at some later stage during analysis, (such as during freeze drying) there may have been some hydrolysis of oligonucleotide material so resulting in the mononucleotides found. But this possibility was thought unlikely since care was taken to see that fractions were neutralized with lithium hydroxide before freeze drying, and so any hydrolysis on concentration during lyophilization was unlikely. Also all column work was conducted at a cold room temperature of 4 - 5°C.

Dowex (C) - peak III.

Fractions 338-380 were bulked as peak III from Dowex (C) (fig. 79 page 331) and neutralized with IN lithium hydroxide and freeze dried (GM 6 & 7). The isolated material was subjected to analytical techniques summarized in fig. 84, on page 333

U.V. spectrophotometric data of Dowex (C) peak III.

Spectra at acid pH λ max. 254 (257) O.D.$\frac{250}{260}$ = 1.13 (0.90)

λ min. 236 (228) O.D.$\frac{280}{260}$ =0.68 (0.68)

Spectra at alk. pH λ max. 257 (256) O.D.$\frac{250}{260}$ = 0.93 (0,89)

λ min. 231 (230) O.D.$\frac{280}{260}$ = 0.64(0.60)

The data obtained compared favourably with that for the published $\begin{pmatrix}226\\227\end{pmatrix}$ values of GMP (figures in brackets).

Summary of Dowex (C) peak III investigation.

The following analytical methods of T.L.C., paper electrophoresis, paper chromatography and U.V. spectrophotometric data indicate beyond reasonable doubt that this peak was GMP. --See fig.84,page 333.

Dowex (C) peak II.

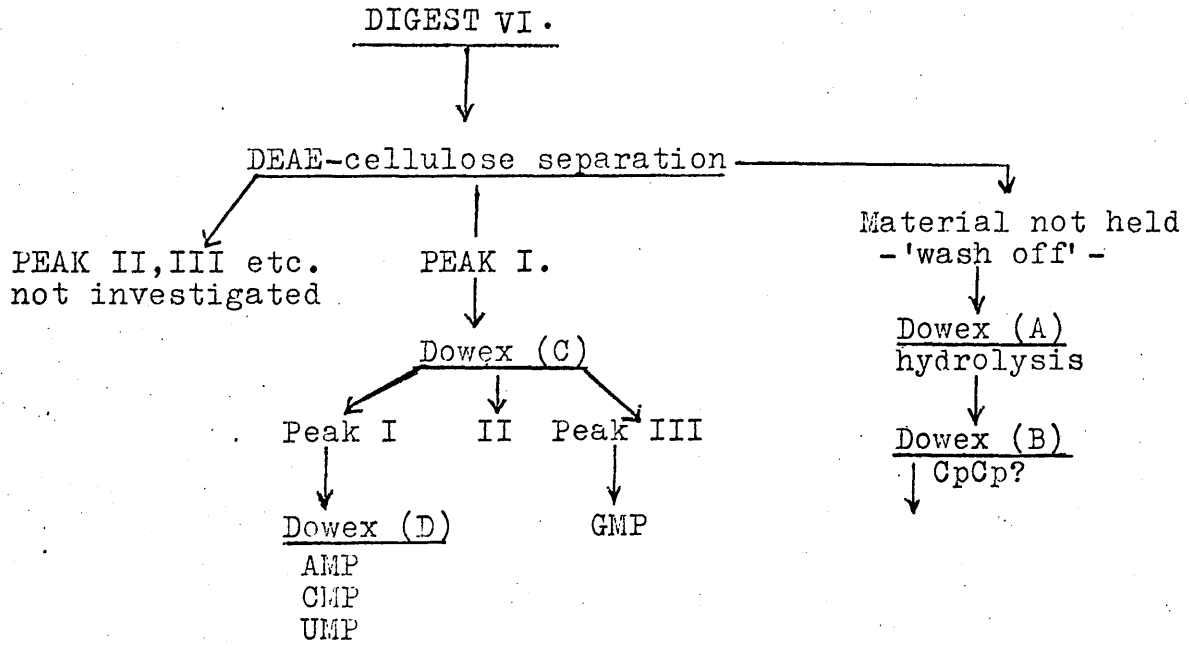
Since all four mononucleotides have been accounted for, as they are present in peaks I and III of Dowex (C), and since to discover the nature of the mononucleotides produced in the digestion of RNA with basic calf spleen enzyme fractions was the primary function of the investigation, peak II was not further investigated. More than likely this was an early eluted dinucleotide such as GpCp for example, because it only represented 1.25% of the

total nucleotide material eluted from Dowex (C).

C. Examination of peak II and III of DEAE-cellulose column chromatography of digest VI.

Because peak I of the DEAE-cellulose effluent had contained all four mononucleotides, investigation of subsequent peaks was not carried out. These peaks probably represent oligonucleotides of increasing size as the elution progresses and at this stage in the present investigation, they were of little interest.

Summary chart of procedures of analysis of digest VI



Conclusions for digest VI.

From the results obtained in digest VI it seems that mixed ribonuclease A and B hydrolyses RNA to produce all four mono-ribonucleotides with the enzyme (or enzymes) showing no specificity of action at all. The mononucleotides were obtained in significant quantities and not merely in trace amounts. This was in complete contrast to the findings of digest IV where UMP was the only mononucleotide found in any significant amount, but digest IV was carried out with RNAse B alone. These results could be interpreted as indicating that in fact RNAse A and B are two different enzymes with different specificities. Thus the separation on CM-cellulose in the final stages of purification into two activity regions A and B, so characteristic of the calf spleen ribonuclease preparation at this stage (page 54), may be due to the presence of two distinct proteins, and not a matter of one protein being eluted in two forms differing in state of aggregation (page 137).

Further specificity work with RNAse B alone was initiated to clarify the nature of the specificity of B and to confirm it is different from that due to the presence of A and B together.

Investigation of exhaustively dialysed commercial RNA as used in digest VI.

The source of RNA used in digest VI was investigated since it was thought possible that substantial amounts of mononucleotides might have been present in it, thus detracting from the results of this digest.

The RNA source was commercial yeast RNA and was exhaustively dialysed and freeze dried as described on page 269 and stored at - 22°C in a stoppered glass bottle. The RNA was white, fluffy and of good appearance.

150.5 mgs of this RNA were dissolved in 1 l. of deionized water to give 1550 optical density units at 260 m_u. This material was then applied to a Dowex-I column (dimensions 7.5 x 1.8 cms) and washed initially with deionized water and then washed with 1 l. of 0.003N hydrochloric acid. The effluent stages was monitored at 254 m_u. and no nucleoside or nucleotide material was eluted. The following gradient was then applied:-

1.5 l. 0.003N H Cl ⟶ 1.5 l. 0.003N H Cl / 0.03M LiCl

As a result of the above gradient there was no elution of any significant amount of nucleotide material even after 82% of this gradient had passed through the column.

Similarly elution with a solution of 0.03N HCl and 0.05M LiCl produced no significant nucleotide material in the effluent.

Therefore the exhaustively dialysed commercial RNA contains no significant amounts of oligonucleotides or mononucleotides since these, if present, would be easily eluted from Dowex-I anion exchanger by the eluents described. Thus the commercial source of RNA should in no way detract from the validity of the results of digest VI or any future digest in the sense of mononucleotide contamination.

DIGEST VII & VIII.

Introduction. These two digests were carried out using the same enzyme source (RNAse B) and under very similar conditions to give much the same results. In separation of the digest products, the use of DEAE-cellulose as an anion exchanger was dispensed with and Dowex-I used instead. DEAE-cellulose appeared to achieve good separation of oligonucleotides, but the separation of mononucleotides and dinucleotides etc. was somewhat poor since all the mononucleotides from digest VI were eluted in the large peak I from DEAE-cellulose, and subsequent separation of these was by use of Dowex-I. Therefore it was decided to use Dowex-I for the initial

separation of mononucleotides from the larger products of enzyme digestion of RNA, and the former were of little interest to this study anyway.

DIGEST VII.

a) Enzyme source for digest VII.

Having been stored at -22°C, ribonuclease samples from the following sources were bulked together:-

Column source	Fraction	Page No. ref.
CMC-4-2M II	96-120	197
CMC-4-2M-Leu-I	151-164	228
" "	165-179	"
" "	180-198	"
CMC-4-2M-Leu II	133-156	228
CMC-4-2M-I	68-82	197

The RNAses were thought to be mainly of the RNAse 'B' type but especially in the case of those fractions chromatographed in the presence of nor-leucine (page 228) it was not always easy to distinguish the separation into two peaks of activity - A and B (example CMC - 1 page 54). This difficulty was of little consequence since the bulked enzyme samples were to be rechromatographed under the standard conditions which have always been found to result in two peaks of activity and from which the B fraction can be isolated for use in digests VII and VIII.

Procedure for CM-cellulose chromatography of RNAse samples from various sources (listed above)

Volume of bulked protein material = 1255 mls.

Total concentration of bulked material = 44.3 mgs of protein.

The bulked protein samples were diluted to 4 ℓ. with 0.005 M Tris, 10^{-4}M EDTA, pH 7.0 buffer because it was not wanted to dialyse the material at this stage using Visking tubing incase of enzyme loss. (page 89 ref. 105). Thus by dilution, the salt concentration, which will be appreciable in the samples as a result of their previous column separation and elution, would be reduced sufficiently to allow adsorption by the cation exchanger. The presence of leucine and urea should not significantly affect adsorption properties.

The CM-cellulose column used was of 55 x 2.1 cm. dimensions and was thoroughly equilibriated with 0.005M Tris, 10^{-4}M EDTA, pH 7.0 buffer before application of protein material. The diluted protein material was then applied to the column and washed with 1075 mls of pH = 7.0 buffer. All column chromatographic operations were conducted at 4-5^{0}C.

Results. Fig. 85* shows the elution profile obtained with respect to enzyme activity, and protein estimation

*(Note-fig.85 is on page 333).

by U.V. adsorption at 280 m_{μ}, by using the apparatus described previously (fig 23) for CMC-4 and and CMC-5 columns. The gradient elution applied was as follows:-

Constant volume chamber		Reservoir	
800 mls	0.005 M Tris 10^{-4}M EDTA pH 7.0	1600mls	0.005M Tris 10^{-4}M EDTA pH 8.2 0.25M NaCl

Fractions 120-168 were bulked as fraction B and concentrated by use of a small CM-cellulose (described: page 57.). The concentrated protein solution was then dialysed against pH 7.0 buffer to give 42 mls. of dialysed solution containing 7.94 mgs of protein. (GM2).

b) RNA source.

The same RNA source was used as in digest VI, namely exhaustively dialysed commercial RNA at a concentration of 3.75 mgs/ml of solution in the digest mixture. (3.5 mgs/ml in digest VI).

c) Digest.

The RNA was dissolved in the protein solution and the pH checked and adjusted to pH 7.0. The digest was made 0.025 M with respect to Mg^{++} ions using $MgCl_2.6H_2O$ (page53 ref28) and was carried out in a stoppered glass flask with four drops of 'Analar' grade chloroform to inhibit bacterial action. The

digestion time extended over a period of 45 hours and 35 mins. at 37°C. in a constant temperature water bath.

d) Separation of digestion products.

After digestion, the solution containing the products was diluted to 1 l. with deionized water and allowed to cool before applying to a Dowex-I column in the cold room. The column dimensions were 13 x 1.8 cms and the resin had been prepared in the Cl^- form as previously described (page 319). A total of 3990 units were applied to the column (designated Dowex (E)) and retention of this material was virtually 100%. Dowex (E) was then subjected to the elution programme as given in fig. 86 and a separation of products was obtained as shown in fig. 87.

Analysis of peaks of Digest VII eluted from Dowex (E) column (fig. 87)

The material from each peak was bulked, and the pH adjusted to 7.0 with IN lithium hydroxide and then reduced in volume, generally by freeze ——drying (GM6 & 7). The isolated nucleotide products were examined by U.V. spectrophotometry, T.L.C., paper electrophoresis and finally by paper chromatography in Leloir's solvent at pH 3.5. The data and conditions for each technique of analysis used was described for the

Eluent	Fractn.no.	%grad.run	Peaks collected
1l.H_2O→1l.0.003N HCl	I-I34	75%	I,II,III
Elution with 0.003N HCL 2l.	I35-I68	7I%	IV,V,VI
2l.0.003N HCl→2l.0.003N HCl,0.03M LiCl	I69-560	8I%	VII,VIII, IX,XA
Elution with 0.03N HCL 1l. 0.03M LiCl	56I-642	86%	X,XI
Elution with 0.03N HCl 0.05M LiCl	643-792	75%	XII,XIII

Fig.86. Elution programme-Dowex(E)

Method	TLC	Electro--phoresis	Paper chrom--atography
AMP	0.88 0.76	0.59	0.69
CMP	I.I7	0.46	0.82
GMP	0.65	0.80	0.56
UMP	I.00	I.00	I.00
Unknown	I.I7	0.46	0.82
Af.	3	3	2
Comment	∴CMP?	∴CMP?	∴CMP?

Fig.88. Dowex(E),peakII

Method	TLC	Electro--phoresis	Paper chrom--atography
AMP	0.78 0.89	0.64	0.66
CMP	I.I3	0.46	0.84
GMP	0.63	0.85	0.54
UMP	I.00	I.00	I.00
Unknown	I.I6	0.4I	0.84
Af.	3	3	2
Comment	∴CMP?	∴CMP?	∴CMP?

Fig.89. Dowex(E),peak III

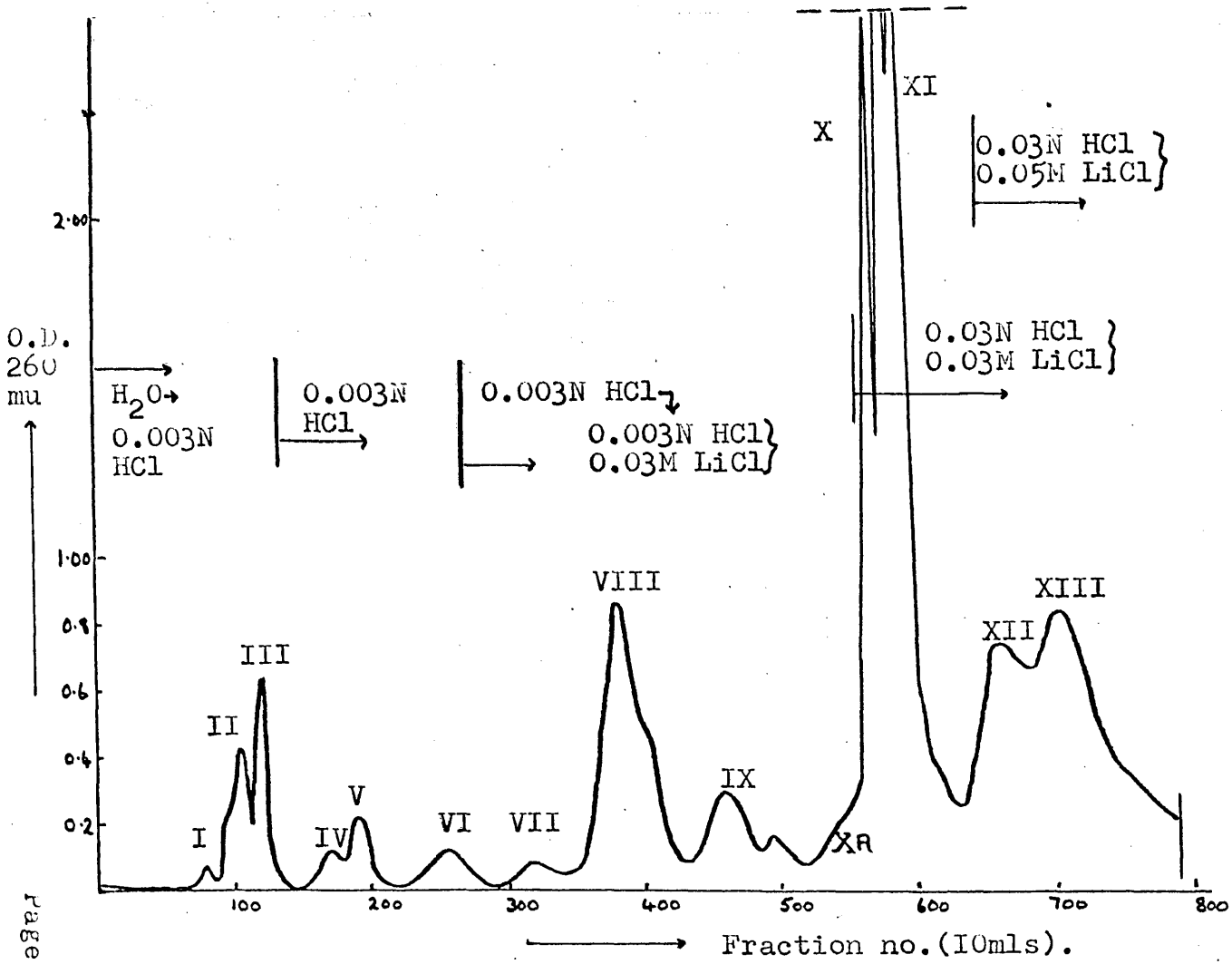


Fig.87. Elution profile of products of Digest VII on Dowex(E) (Flow-rate=0.5mls/min.)

analysis of the products of digest IV and digest VI (electrophoresis). All results with respect to T.L.C., paper electrophoresis and chromatography were expressed relative to those of UMP and the R_{ump} values given in the text are the average R_{ump} values and the number of results they are averaged from is stated and the convention explained in digest VI (page 314). On the basis of these analytical techniques, and also on the position of elution from the Dowex-I anion exchanger, conclusions were drawn as to the nature of each peak.

PEAK I (Fraction 70-76, volume 50 mls, concentration 3.95 O.D. units 260 m_u)

1. U.V. data

Spectra at acid pH λ max 266(280) O.D. $= \frac{250}{260} = 0.83$ (0.44)

λ min 238 (241) O.D. $\frac{280}{260} = 0.82$ (2.09)

λ max. 272 (271) O.D. $\frac{250}{260} = 0.92$ (0.84)

λ min. 246 (249) O.D. $\frac{280}{260} = 1.05$ (0.98)

No definite correlation with any published data could be derived from the above results except the spectra at an alkaline pH resemble those for CMP which are given in brackets.

2) T.L.C.

	Rump	Af.	Comment
AMP	0.90 0.78	3	} Control
CMP	1.15	3	
GMP	0.67	3	
UMP	1.00	3	
Peak I	0.81	3	∴ AMP?

3) No successful electrophoretic or paper chromatographic results were obtained because of the small concentration of nucleotide material available.

Conclusions. Because of the position of this peak in the elution sequence it was definitely not UMP or GMP, and from the results obtained it does not seem to be identifiable with either AMP or CMP, and so with the data available no real conclusion as to the identity of this peak was reached.

PEAK II (Fractions 90-112, volume 180 mls, concentration 50.O.D. units)

1. U.V. spectrophotometric data.

Spectra at acid pH λ max. 278(280) O.D. $\frac{250}{260}$ =0.63 (0.44)

λ min. 242(241) O.D. $\frac{280}{260}$ = 1.78 (2.09)

Spectra at alk pH λ max. 270 (271) O.D.$\frac{250}{260}$ = 0.92 (0.84)

λ min. 252 (249) O.D.$\frac{280}{260}$ = 0.89 (0.98)

There was good correlation with the published data for CMP (which is given in brackets).

2. The results of T.L.C., paper electrophoresis, and paper chromatography are given in fig. 88 and all results indicated that peak II was synonymous with CMP and this was concurrent with the elution of this peak by an hydrochloric acid concentration of less than 0.003N.

PEAK III (Fractions 113-126, Volume 90 mls, concentration 43.7 O.D. units)

1. U.V. Spectrophotometric constants.

Spectra at acid pH λ max. 280 (280) O.D. $\frac{250}{260}$ = 0.56(0.44)

λ min. 244 (241) O.D. $\frac{280}{260}$ = 1.85(2.09)

Spectra at alk pH λ max. 270 (271) O.D. $\frac{250}{260}$ = 0.91(0.84)

λ min. 252 (249) O.D. $\frac{280}{260}$ = 0.93(0.98)

The U.V. data was very similar but not identical to that obtained for peak II in that it was very ———

P.T.O.

characteristic of the published data for CMP (in brackets).

2. The results of T.L.C. etc. of peak III are given in fig. 89 and although not as positive as peak II, these results indicated the probability of the presence of CMP again. Peaks II and III were eluted from the Dowex column in close proximity, and separation on bulking could not possibly be complete and cross contamination of the relative properties would occur. But even so, peaks II and III exhibited very similar properties. It was thought that one of these peaks may be the $2^{1}, 3^{1}$ cyclic phosphate form of CMP. Thus electrophoresis at pH 7.5 with a phosphate buffer was also carried out with peaks II and III, and CMP (non-cyclic) as a control. At pH 7.5 on normal nucleotide 3^{1} phosphates, two of the hydroxyls of the phosphate group are in the ionized form and thus the nucleotide has two negative charges. But at this pH the cyclic phosphate only has one negative change, and so separation of cyclic and non-cyclic forms is made possible by the differential charge at pH 7.5 with paper electrophoresis. Paper electrophoresis was carried out in pH 7.5, 0.2M phosphate buffer at 600 volts for 45 minutes.

Results	R_{CMP}
CMP	1.00
Peak II	1.01
Peak III	0.98

Results showed no significant separation of the materials from peaks II and III, indicating cylic CMP not to be present.

But even if no cyclic nucleotides were found in the isolated peaks II and III, at the time of elution, one of the peaks may have been in the cyclic form, but on isolation it may have been broken down to the non-cyclic form.

Therefore peak III from the results obtained seemed to be CMP or a nucleotide compound with characteristics similar to CMP (such as cylic CMP). The presence of nucleosides was excluded, because these are normally very sharply differentiated by paper chromatography from nucleotide material and in peak III there was no such evidence of these compounds. Nucleosides are normally eluted before any nucleotide material in such a gradient system as was used.(221) Peak III could possibly be a dinucleotide containing as one constituent CMP but at this stage of elution, it is unlikely.

PEAK IV. (Fraction 156-180, volume 270 mls, 20.3 O.D. units concentration)

U.V. Spectrophotmetric data.

1. Spectra at acid pH λ max. 276(280) O.D.$\frac{250}{260}$ = 0.69 (0.44)

λ min. 246(241) O.D.$\frac{280}{260}$ = 1.34 (2.09)

Spectra at alk. pH λ max. 268(271) O.D.$\frac{250}{260}$ = 0.90(0.84)

λ min. 250(249) O.D.$\frac{280}{260}$ = 0.81(0.98)

The U.V. spectrophotometric data showed similarities with peak II and III, but did not exactly coincide with the published values for CMP (given in brackets).

2. The results of T.L.C. etc. for peak IV are given in fig. 90. Paper electrophoresis showed the presence of a very slow moving spot as well as one corresponding to CMP. The slower moving spot was probably due to the presence of a di- or trinucleotide and the three spots obtained by T.L.C. may be due to di-or tri-nucleotides with perhaps hydrolysis products of the same. The overall conclusions as to the nature of peak IVwere that possibly CMP and AMP were present and/or a di-or trinucleotide, and that the mononucleotides CMP and AMP were breakdown or hydrolysis products. It should be noticed that good separation between peaks IV and V was not obtained, and therefore overlapping of constituents on bulking was possible.

Method	TLC	Electro--phoresis	Paper chrom--atography
AMP	0.85	0.64	0.69
CMP	I.I5	0.5I	0.82
GMP	0.63	0.86	0.53
	0.77		
UMP	I.00	I.00	I.00
Unknown	0.75,0.88,I.I9	0.34,0.50	0.73
Af.	4	7	2
Comment	∴CMP,AMP?	CMP,dimer?	∴AMP?

Fig,90. Dowex(E),peak IV.

Method	TLC	Electro--phoresis	Paper chrom--atography
AMP	0.8I	0.63	
	0.9I		
CMP	I.I4	0.49	
GMP	0.6I	0.83	
UMP	I.00	I.00	
Unknown	0.84,I.I6	0.40,0.55	
Af.	4	6	
Comment	AMP,CMP?	AMP,CMP?	

Fig.9I. Dowex(E),peak V.

Method	TLC	Electro--phoresis	Paper chrom--atography
AMP	0.86	0.4I	0.70
CMP	I.2I	0.3I	0.8I
GMP	0.62	0.76	0.49
UMP	I.00	I.00	I.00
Unknown	0.82	0.40	0.62
Af.	3	3	2
Comment	∴AMP?	∴AMP?	∴AMP?

Fig.92. Dowex(E),peak VI.

PEAK V. (Fraction 181-205, volume 270 mls, concentration 32.9 O.D. units)

1. U.V. Spectrophotometric data

Spectra at acid pH λ max. 268 O.D. $\frac{250}{260}$ = 0.73

λ min. 242 O.D. $\frac{280}{260}$ = 0.98

Spectra at alk. pH λ max. 264 O.D. $\frac{250}{260}$ = 0.87

λ min. 238 O.D. $\frac{280}{260}$ = 0.58

For this peak the results and published data for mononucleotides showed no correlation, although some similarity between the published data of AMP and CMP was discernable.

2. The result T.L.C. etc. for peak V are given in fig. 91. Results indicate the possible presence of CMP and AMP in this peak, but this was by no means certain as the results may have been due to di-or trinucleotides. It is also possible that hydrolysis of a di-or trinucleotide might have given CMP or AMP as products.

PEAK VI. (Fraction 230-280, volume 515 mls, concentration 36.1 O.D. units).

Spectra at acid pH λ max. 258 O.D. $\frac{250}{260}$ = 0.88

λ min. 232 O.D. $\frac{280}{260}$ = 0.41

Spectra at alk. pH λ max. 260 O.D. $\frac{250}{260}$ = 0.86

λ min. 234 O.D. $\frac{280}{260}$ = 0.38

The above results showed no correlation with published data for mononucleotides.

2. The results of T.L.C. etc. for peak VI are given in fig. 92. The results tabulated suggest that this peak may contain AMP, but this is not corroborated by U.V. spectrophotometric data. Thus peak VI is possibly AMP, but more likely an AMP type di- or tri- nucleotide.

PEAK VII (Fraction 300-340, volume 240 mls, concentration 26.5 O.D. units).

U.V. Spectrophotometric data.

Spectra at acid pH λ max 260 O.D.$\frac{250}{260}$ = 0.83

λ min. 238 O.D.$\frac{280}{260}$ = 0.46

Spectra at alk. pH λ max 260 O.D.$\frac{250}{260}$ = 0.82

λ min. 238 O.D.$\frac{280}{260}$ = 0.46

The U.V. spectrophotometric constants and spectra showed no good correlation with any published data for mononucleotides.

2. The results of T.L.C. etc. for peak VII are given in fig. 93 and although these showed strong affinities with data for control AMP, the same affinity was not shown with respect to published U.V. data for AMP. Therefore peak VII was probably an AMP containing di-

or trinucleotide.

PEAK VIII. (Fraction 354-430, volume 505 mls, concentration 340.7 O.D. units).

Peak VIII had a shoulder and thus was divided into two fractions for analysis, peak VIIIA - fractions 372-390.

and VIIIB - fractions 398-404

PEAK VIIIA

1. U.V. spectrophotometric data.

Spectra at acid pH λ max 262(262) $O.D.\frac{250}{260} = 0.76(0.73)$

λ min 232(230) $O.D.\frac{280}{260} = 0.41(0.39)$

Spectra at alk. pH λ max 262(261) $O.D.\frac{250}{260} = 0.84(0.80)$

λ min 242(241) $O.D.\frac{280}{260} = 0.39(0.32)$

The U.V. constants compared very favourably with the published data for UMP. (given in brackets).

2. The results of T.L.C. etc. for peak VIIIA are given in fig. 94. U.V. spectrophotometric data was in close agreement with the conclusions drawn from other analytical methods, namely that peak VIII A is probably UMP.

Method	TLC	Electro-phoresis	Paper chrom-atography
AMP	0.84	0.41	0.69
CMP	I.I7	0.29	0.82
GMP	0.62	0.79	0.52
UMP	I.00	I.00	I.00
Unknown	0.64	0.38	0.66
Af.	3	3	2
Comment	∴AMP?	∴AMP?	∴AMP?

Fig.93. Dowex(E),peakVII.

Method	TLC	Electro-phoresis	Paper chrom-atography
AMP	0.86	0.49	0.70
CMP	I.I6	0.4I	0.80
GMP	0.63	0.78	0.49
UMP	I.00	I.00	I.00
Unknown	I.03	I.0I	0.97
Af.	4	4	2
Comment	∴UMP?	∴UMP?	∴UMP?

Fig.94. Dowex(E),peak VIIIA.

	O.D.$\frac{250}{260}$	O.D.$\frac{280}{260}$	λ max.	λ min.
Acid spectra				
peak VIIIA	0.76	0.4I	262	232
peak VIIIB	0.80	0.43	26I	244
UMP	0.73	0.39	262	230
Alkaline spectra				
VIIIA	0.84	0.39	262	242
VIIIB	0.84	0.43	262	242
UMP	0.80	0.32	26I	24I

Fig.95. Comparison of U.V.spectrophotometric constants for Dowex(E),peaks VIIIA & B.Published values for UMP are also given,references 226,227.

Peak VIIIB gave virtually identical results although the U.V. spectra were a little different (see fig. 95), and as with the relationship between peaks II and III, the small shoulder in the elution profile of peak VIII may have been due to the cyclic form of UMP, which has since broken down to give the non-cyclic mononucleotide of UMP. Alternatively peak VIIIB might have resulted due to the presence of a UMP containing di- or tri-nucleotide, thus altering the spectrophotometric constants. Thus peak VIII as a whole was taken on the basis of the above information, to be UMP.

PEAK IX. (Fraction 430-480, volume 505 mls. concentration 93.4 O.D. units).

1. U.V. Spectrophotometric data

Spectra at acid pH λ max.268 $\quad$ O.D.$\frac{250}{260}$ = 0.71

λ min.237 $\quad$ O.D.$\frac{280}{260}$ = 0.89

Spectra at alkaline pH λ max.264 O.D.$\frac{250}{260}$ = 0.88

λ min.244 O.D.$\frac{280}{260}$ = 0.63

The above data exhibited little in common with published U.V. data for mononucleotides.

2) The results of T.L.C. etc. for peak IX are given in fig. 96. T.L.C. results indicate the presence of UMP

Method	TLC	Electro--phoresis	Paper chrom--atography
AMP	0.63	0.44	0.66
CMP	I.I9	0.32	0.75
GMP	0.58	0.8I	0.50
UMP	I.00	I.00	I.00
Unknown	0.96	0.83 0.95	0.3I
Af.	3	8	2
Comment	∴UMP?	∴UMP +...?	Di-or tri-nucleotide?

Fig.96. Dowex(E),peak IX.

Method	TLC	Electro--phoresis	Paper chrom--atography
AMP	0.86	0.49	0.66
CMP	I.I5	0.35	0.80
GMP	0.59	0.83	0.50
UMP	I.00	I.00	I.00
Unknown	0.58	0.76	0.53
Af.	3	5	2
Comment	∴GMP?	∴GMP?	∴GMP?

Fig.97. Dowex(E),peak XA.

Peak	O.D.$_{260}$units	Results of analysis
I	4	Identity not established.
II	50	CMP } both taken
III	44	Probably CMP } as CMP.
IV	20	CMP & AMP,probably a dimer or trinucleotide of these.
V	33	Same as above.i.e.peak IV.
VI	36	AMP or more probably a dinucleotide or tri- etc.
VII	27	Same conclusion as peak VI
VIII	34I	UMP.
IX	93	Dimer or oligonucleotide.
X	87	Possibly GMP?

Fig.98. Summary chart of analysis of digest VII products.

in peak IX also, but this is not supported by U.V. data and paper chromatography. The results from paper electrophoresis could be interpreted as indicating the presence of UMP and GMP whereas both T.L.C. and paper chromatography demonstrated the existence of one nucleotide constituent. By this stage in the elution sequence it was probably that the constituent of peak IX was of di- or tri- or of oligonucleotide nature, and may be the two constituents separated using paper electrophoresis were hydrolysis products of small oligonucleotides.

PEAK XA (Fraction 520-567, Volume 495 mls, concentration 87.0 O.D. units)

1. U.V. Spectrophotometric data.

Spectra at acid pH	λ max. 258(257)	O.D.$\frac{250}{260}$	= 0.88 (0.90)
	λ min. 241(228)	O.D.$\frac{280}{260}$	= 0.80 (0.68)
Spectra at alkali pH	λ max. 260(256)	O.D.$\frac{250}{260}$	= 0.93 (0.89)
	λ min. 239(230)	O.D.$\frac{280}{260}$	= 0.62 (0.60)

As can be seen from the figures quoted in brackets which are the published figures for GMP, this peak has very similar U.V. spectrophotometric characteristics. The

similarity to GMP is similarly reflected in results from T.L.C., paper electrophoresis and paper chromatography.

2. The results of T.L.C., paper electrophoresis and paper chromatography are given in fig. 97 and the data does seem to indicate the presence of GMP but the U.V. data does not correspond exactly and therefore there is always the possibility that peak XA may be a GMP containing di- or trinucleotide.

PEAK X (Fraction 568-574, volume 84 mls, concentration 137 O.D. units)

Peak X was analysed by the same techniques as used for all the previous peaks and was not found to be of a mononucleotide character. Analysis of subsequent peaks to peak X was not attempted since any mononucleotides would have been eluted by this stage in the salt gradient (22I)anyway.

Conclusions and summary of digest VII

The object of these specificity investigations has been to ascertain the nature of the mononucleotides produced on digestion of yeast RNA with calf spleen ribonuclease enzyme in order to be able to formulate a theory as to the nature of the specificity of the particular enzyme fraction used. Fig. 98 summarizes

as far as was feasible the nature of the fractions separated from digest VII. The only mononucleotides that were found to be present with any degree of certainty were CMP and UMP with also the possibility of the presence of GMP, but the latter was of a more doubtful degree of certainty. It will be observed that the quantities of UMP separated were far in excess of those of CMP and the ratio of UMP; CMP isolated in digest VII was 3.8:1. A number of explanations could be compounded for these results with respect to specificity of enzyme action.

If the enzyme used had the same specificity as bovine pancreatic RNAse, UMP and CMP would be the only mononucleotides produced as a result of the enzyme action. If the RNA used in such a digest had approximately equal CMP and UMP content, one would expect in all probability that CMP and UMP produced as a result of enzymatic hydrolysis would be present in very approximately equal quantities also. But however this was not the case in digest VII and UMP was present in considerably greater quantities than CMP and this may have been due to preferential specificity on the part of the ribonuclease enzyme, or due to the use of RNA which contained a higher proportion of UMP as compared

with CMP in its nucleotide structure. But the published values for nucleotide and base ratios of yeast RNA (since it was yeast RNA used in digest VII) are:

	AMP	GMP	CMP	UMP	(Ref.)
1) Nucleotide ratios of yeast (no type of yeast quoted).	1.24	1.38	1.00	1.23	(246)
2) baker's yeast. (Saccharomyces cerevisiae)	1.39	1.65	1.00	1.58	(248)

Therefore presuming the base compositions of baker's yeast does not vary except perhaps slightly due to chain length being of a somewhat random nature, the predominance of UMP found, as opposed to CMP in the digest, can not be interpreted in terms of RNA used entirely, since although there is a greater preponderance of UMP over CMP in naturally occurring baker's yeast (1.58:1.00 respectively), it is not sufficient to account for the ratio of 3.8:1 obtained in digest VII. The predominance of UMP as a product of the digestion of RNA is akin to the results obtained in digest IV where no CMP at all was found and UMP was the only mononucleotide isolated. Both digest VII and IV were carried out using Ribonuclease activity 'B' fractions and so one would expect the same results. Possibly in

digest IV the CMP concentration produced was not present in sufficient concentration to be detected and thus giving rise to the impression that the specificity was unique to UMP.

If this RNAse 'B' fraction isolated from calf spleen was specific in its action to result in UMP alone on digestion of RNA, or if it had merely preferential but not absolute specificity as digest VII results indicated, then further confirmation of these important findings was needed. Thus a final digest was carried out using the same RNA source, the same enzyme source, and using the same technique of isolation and product identification as used in digest VII.

DIGEST VIII.

a) Enzyme source.

The same enzyme source was used in digest VIII as was used in digest VII, namely the RNAse B fraction 120-168, of the CM-cellulose column chromatography preparation described on page 346 (fig. 85). This material was dialysed against pH 7.0 Tris buffer prior to use and 41 mls of the resulting protein solution containing 8.25 mgs. of protein was used in the digest.

b) RNA source.

Exhaustively dialysed RNA, as used in digest VII was dissolved to the extent of 173 mgs in the enzyme solution and made 0.025M with respect to Mg^{++} ions as in digest VII and the digest was incubated at 37°C in a constant temperature water bath for 36.75 hours.

c) Separation of the digest products.

After digestion the solution containing the digest products was diluted to 1 l. with deionized water and applied to a 15 x 1.8 cm. Dowex-I column (designated Dowex (F)). An almost identical elution profile (fig. 100) to that of Digest VII was obtained and any differences could be accounted for by differences in elution procedure which is described in fig. 99.

d) Results.

Analysis of each peak was undertaken using the same methods as in digest VII and very similar results were obtained. Because of this similarity, experimental results are not quoted below in detail but merely the final findings as regards the nature of the individual peaks is presented (fig. 101.) A comparison of the results of digest VII and VIII (fig. 102) shows the results to be virtually identical, namely that the

Eluent	Fraction	%gradient run	Peaks
Material coming straight though column	I-87		
Elution with H_2O 0.5l.	88-129		
1l.H_2O→1l.0.003N HCl	130-293	92%	I,II,III
Elution with 0.003N HCl I.5l.	294-427		IV,V
2l.0.003N HCl→ 2l.0.003N HCl) 0.03M LiCl)	428-725	82.5%	VI,VII, VIII,IX
1l.0.003N HCl) 0.03M LiCl)→ 1l.0.03N HCl) 0.03M LiCl)	726-878	82%	X
Elution with 0.03N HCl 0.05M LiCl	879-965		XI.

Fig.99. Elution programme for digest VIII,Dowex(F).

Peak	O.D.units 260mu	Results of analysis
I	35	Possibly CMP.
II	41	CMP
III	79	Possibly CMP.
IV	23	Di- or trinucleotide?
V	59	Possibly AMP or AMP dimer or oligonucleotide?
VI	59	Dimer or oligonucleotide?
VII	41	Possibly UMP & also a dimer or oligonucleotide.
VIII	425	UMP,& another two minor constitu--ents,which are not of mono--nucleotide nature.
IX	98	Some GMP characteristics,probably dimer etc.

Fig.101. Results of digest VIII,Dowex(F).

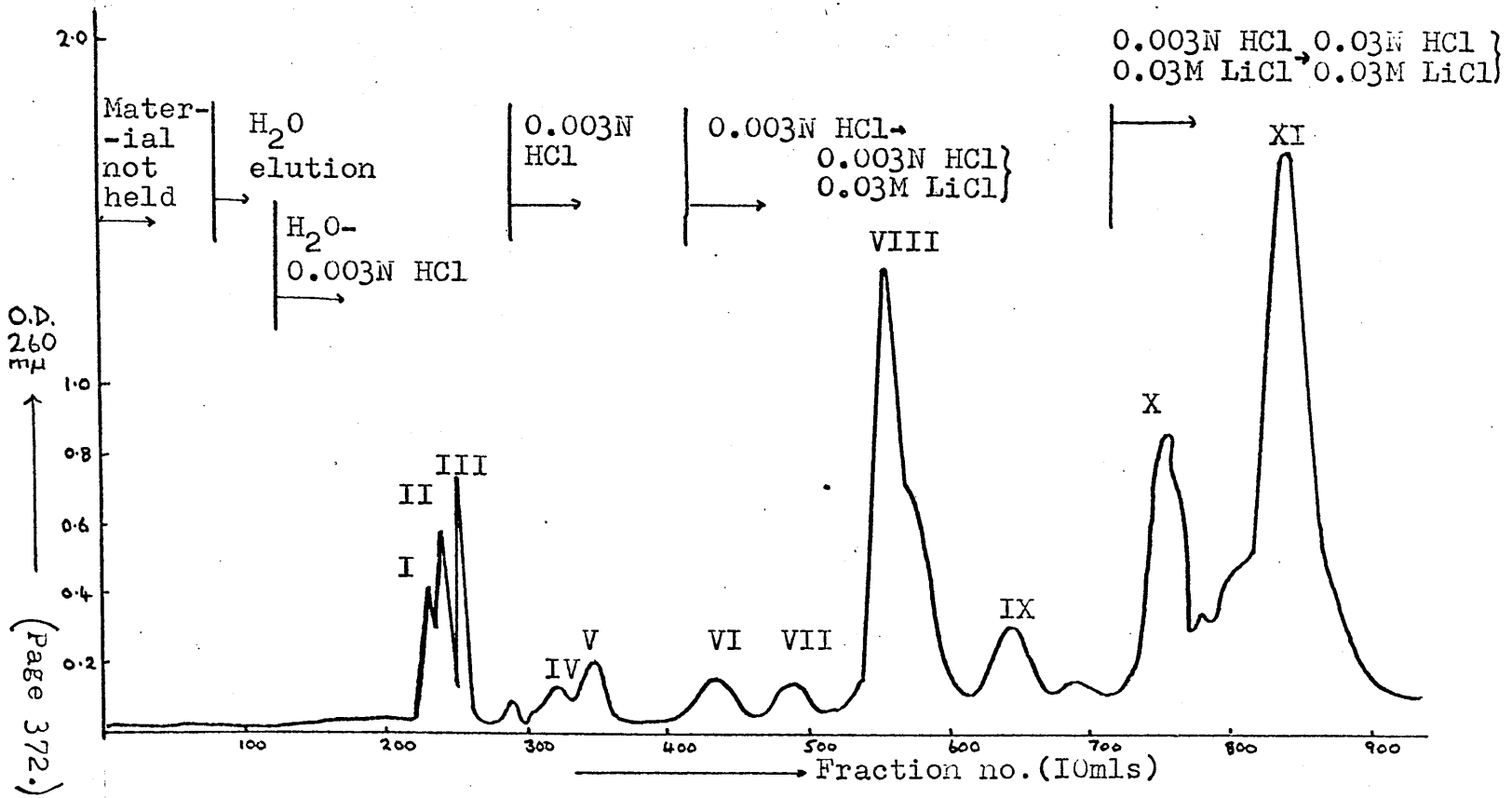


Fig.100. Elution profile of the products of digest VIII on Dowex(F) (flow-rate=0.5mls/min.)

(Page 372.)

DIGEST VII				DIGEST VIII			
Column length I3xI.8cms.4,200 units				Column length I5xI.8cms.4817 units			
Peak	O.D. units	Identity	Eluent	Peak	O.D. units	Identity	Eluent
I	4	?	H_2O→	I	35	CMP?	H_2O→
II	50	CMP	0.003N HCl	II	41	CMP	0.003N HCl
III	44	CMP?		III	79	CMP?	
IV	20	CMP, AMP dimer?		IV	23	di or tri nucleotide	0.003N HCl
V	33	"	0.003N HCl	V	59	AMP or oligomer?	
VI	36	AMP or di,tri--mer etc.		VI	59	"	0.003N HCl→
VII	27	"	0.003N HCl→	VII	41	UMP? or dimer etc.	0.003N HCl } 0.03M LiCl }
VIII	341	UMP	0.003N HCl } 0.03M LiCl }	VIII	425	UMP+oli--gomer	
IX	93	di- or oligomer?		IX	98	GMP?	
XA	87	GMP?	0.03N HCl } 0.03M LiCl }				

Fig.I02. Comparison of imformation from digests VII and VIII, Dowex(E) & (F).

enzyme action on RNA has produced CMP and UMP mononucleotides with UMP being the predominant product.

Summary of Part III - summary of all specificity investations.

The most notable and significant finding that these specificity investigations have yielded, has been that RNAse A and B fractions, seem to have differing specificities. Of course fuller investigation, which time did not allow, would be needed to confirm and elucidate further the nature of the respective activities.

The RNAse fractions arise in the final stage of the isolation of basic calf spleen ribonuclease by fractionation into two activity peaks on CM-cellulose under the conditions described on page 54 . The nature and inter-relation of these two activity regions has been discussed at various stages (pages 59,137,138,151, 206) and was discussed in the final sectional summaries. If the findings of these somewhat rudimentary and iniatory specificity investigations are accepted, then it does seem that calf spleen ribonuclease A and calf spleen ribonuclease B may be two separate protein entities and not related forms by way of any differences in state of aggregation or similar association.

Evidence from digests IV, VII and VIII suggests

that RNAse B has a similar specificity to pancreatic RNAse, in that the pyrimidine nucleotides are the mononucleotide products of digestion with yeast RNA. However, unlike pancreatic RNAse, calf spleen RNAse B seems to act preferentially with the appearance of UMP as a hydrolysis product in far greater amounts than CMP. Further research would have to be done to obtain data as to the intermediary stages of hydrolysis, such as a knowledge of whether hydrolysis is via a 2', 3' cyclic stage. Information of this latter type would be essential before a correct classification of the enzyme could be attempted.

In digest VI the enzyme source was largely of RNAse A type and since all four mononucleotides were characterized among the hydrolysis products of yeast RNA, an enzyme of no mononucleotide specificity at all was indicated. As the action of RNAse B on yeast RNA resulted in the release of only pyrimidine mononucleotides, the non-specificity of action in digest VI was attributed to RNAse A, even though small amounts of RNAse B activity was present in the enzyme source used in this investigation. It could be theorized that the production of the purine mononucleotides was due to RNAse A and the pyrimid-

ine mononucleotides, was due to the presence of RNAse B. Whatever the exact specificity of RNAse A fraction, the results obtained show it to be different from RNAse B, which is the essential feature, and further investigation would be needed to clarify the exact mechanism of enzymatic hydrolysis.

GENERAL METHODS (GM)

GM I. Nessler determination of ammonium nitrogen.

The reagent was prepared as follows (65): 3.5g. of gum acacia (B.D.H.) were dissolved in 750 mls. of water. To this was added 4g. of potassium iodide and 4g. of mercuric iodide dissolved in 25mls.water. The resulting solution was made up to I l.with deionized water.

Qualitive analyses for ammonia were carried out by adding 2mls. of this reagent together with 3mls. of 2N sodium hydroxide solution to Iml. of the test solution.The yellow to orange produced after I5 minutes was estimated by reading the absorbance at 490 mu in a "Unicam SP 500" spectrophotometer or a photoelectric colorimeter (E.E.L.-Evans Electroselenium Ltd.-Model B colorimeter with a number 603 filter)

GM 2 Dialysis

Dialysis was carried out using "HMC" visking tubing which was rinsed inside and tested for puncture holes with deionized water prior to use. The dialysis bag, formed by knotting the ends of the soaked visking tubing, was never more than two thirds filledwith material to be dialysed to allow for water uptake due to osmosis. Dialysis took place against frequent changes of the relevant buffer in 5 l. glass beakers. The dialysing

solution was agitated by means of a slowly rotating magnetic stirrer ("Gallenkamp" magnetic stirrer) on the bottom of the beaker.

GM 3. Preparation of commercial adsorbants and ion exchangers.

a). Preparation of CM-cellulose(74) ·

Material:- Whatman carboxymethylcellulose powder CM-70, cation exchanger. Manufactured by W.& R.Balston Ltd.

The dry powder was suspended in a solution of 0.5M sodium hydroxide made 0.5M with respect to sodium chloride and mechanically stirred for about ten minutes. It was then lightly centrifuged at 1500 rpm in an MSE "Magnum" centrifuge with a four place, 600ml. cup swing out head ('g' at 1500 rpm = 1160x g.) for 5 minutes and the supernatant discarded. The residual CM-cellulose was then repeatedly washed with deionized water until free of base (as indicated by Johnsons "Universal" indicator paper). Fines were also removed at this stage by decantation of all material remaining in suspension after 15 minutes settling time. The cellulosic cation exchanger was then thoroughly equilibrated in the appropiate buffer and was ready for use in the Na^+ ionic form.

b). Preparation of DEAE-cellulose

Material:- whatman diethylaminoethyl cellulose powder-DE-50 anion exchanger. Manufactured by W.& R. Balston Ltd.

The dry powder was suspended in a solution of 0.5N sodium hydroxide and mechanically stirred for ten minutes, and then after washing free of base using deionized water, it was washed with 0.5N hydrochloric acid solution. After freedof acid with deionized water, and removing fines as described in GM 3(a),the DEAE-cellulose was in the Cl^- form and ready for use after equilibrating in the appropiate buffer.

c). Polstyrene anion exchange resin.

Material:- Dowex-I x 4,200-400 mesh, manufactured by the Dow Chemical Company,U.S.A.

The resin was initially suspended in IN sodium hydroxide and stirred for a few minutes and then the alkali was removed by washing repeatedly with deionized water. "Fines" were removed by decanting all material retained in suspension five minutes after stirring. The resin was then suspended in IN hydrochloric acid and stirred for a few minutes and then washed free of acid with deionized water to be ready for use in the Cl^- form.

GM 4. Routine assay for RNAse enzyme location.(28).

Materials:- RNA - exhaustively dialysed and lyophilized yeast commercial RNA. The RNA was made up in the following buffer at a rate of 10mgs. per ml. The buffer used was 0.1M sodium succinate buffer at pH 6.5 and 0.05M with respect to magnesium ions.

MacFadyens reagent:- 0.25% uranyl acetate and 2.5% trichloracetic acid solution.

Procedure Aliquots of 0.25ml. of RNA in sodium succinate buffer were added to 0.25ml. of the test solution and incubated at 37°C in a constant temperature water bath for 30 minutes. Digestion was terminated by the addition of 0.5ml. of MacFadyens reagentand the digest tube was immersed in an ice-cold water bath. After cooling for a period of 30 minutes, each digest tube was centrifuged at 2,000 x g. for five minutes, and 0.1ml. of the resulting supernatant was withdrawn and added to a boiling tube and diluted to 4mls. with de-ionized water. Estimation of the nucleotide content of each tube was by reading, at 260_{mu}, the optical density in a Unicam SP 500 spectrophotometer.

GM 5. General chromatographic column filling technique.

The glass chromatographic column to be used was thoroughly washed and decreased by steeping in the industrial detergent "Pyroneg", and rinsed by the passage of running water for 10 minutes. The column was then

filled with the starting buffer and the base plugged with glass wool which had also been soaked in the starting buffer to ensure air bubbles had been excluded as much as possible. The column was assembled into a vertical position (checked by a spirit level) as in fig.I. The reservoir funnel was filled to about half capacity with buffer and the stirring motor set into action. The material with which the column was to be filled was added at intervals to the reservoir funnel as the column filled up. After an initial 2-3cms. of material had settled under gravity, a slow flow of buffer was maintained, by partially opening the basal tap, to ensure good and efficient packing of the column. On completion of the filling procedure, the column was copiously eluted with the initial buffer.

GM 6. Isolation of nucleotide material from Dowex-I column elution.

The bulked material from each peak believed to contain nucleotide material was neutralized with IN lithium hydroxide and freeze - dried. The dry material was then taken up in the minimum volume of dry methanol. Excess dry acetone was added and nucleotide material was precipitated free of salt. The precipitate was collected by centrifugation and dried in a dessicator over silica gel with a constantly applied vacuum to remove the

organic solvent vapour.

GM 7. Lyophilization or freeze drying.

Apparatus:- Edwards High Vacuum Ltd.-Freeze drier model IC P serial no.I98,with a two stage 2SC50B Edwards "Speedivac"pump.

Procedure:- The material to be freeze dried was placed in a an appropiately sized flask (round-bottomed "Quick-fit"B24 neck to fit the freeze drier) and the flask was filled to no more than a quarter full. The flask plus contents was then rotated by hand in a "dry-ice" and acetone freezing mixture in such a way as to obtain the freezing of the contents in a thin layer over a large as area as possible on the inside of the flask, The flask was quickly connected up to the freeze drier which had been previously filled with a "dry-ice" and methanol mixture and the whole system subjected to a vacuum. Low temperature distillation then takes place. An ice coat quickly forms on the outside of the flask in which the contents are being freeze dried,due to air moisture condensation and subsequent freezing,and when this ice had completely cleared it was an indication that the freeze drying process was finished. The vacuum was carefully released and the flask containing the freeze dried material detached and stoppered.

GM 8. Disc gel electrophoresis -see page

The experimental details and procedure are well described in references II6,II7,& II8

Materials:

a).Staining solution I% naphthol blue (Kodak ASI498) in 7% acetic acid.

b).Tray buffer solution

3I.2gms. of β-alanine(BDH) were dissolved in 8.0mls. of glacial acetic acid and this was made up to Il.with deionized water.

c).The following stock solutions were made up and stored in brown bottles in a refrigerator at 5°C.

Solution A. 4.0mls of N,N,N',N',tetramethylethylenediamine (Kodak A92462 or 8I78),48mls of N potassium hydroxide, I7.2mls of glacial acetic acid. This was made up to I00mls with deionized water.

Solution B. 0.46mls of N,N,N',N',tetramethylethylenediamine, 48mls potassium hydroxide, 2.87mls glacial acid. This was made up to I00mls with deionized water. (The Potassium hydroxide was of Normal concentration)

Solution C. 60gms of acrylamide (Kodak552I),0.4 gm N,N',methylenebisacrylamide (Kodak 8383) was made up to I00mls with deionized water.

Solution D. I0gms of acrylamide,2.5gms N,N',-methylenebisacrylamide was made up to I00mls with deionized water.

Solution E. 4.0mgs of riboflavin in 100ml of deionized water.

d). Destaining polyacrylamide solution

6.0gms of acrylamide, 0.5mgs of riboflavin were made up to 100mls with deionized water

From the above stock solutions, small and large pore gel solutions were compounded in the following proportions:-

I part A
2 parts C
I part H_2O
} Small pore solution at pH 4.3

The small pore solution above was mixed just before use with an equal volume of freshly prepared ammonium persulphate (0.56gms /100mls water)

Large pore solution

I part B
2 parts D
I part E
4 parts H_2O
} Large pore solution at pH 6.8

Procedure The procedure and use of these materials was described in detail in part I of this thesis and also by R.A.Reisfeld et al (118). Recording of results was by photography against a translucent screen illuminated from behind by fluorescent lighting

GM 9. Chromatography and electrophoresis on paper; detection of compounds on paper.

Whatman No.1 chromatography paper was used unless otherwise stated. The method was the descending technique

used by Martin (249). The composition of the solvents used was that used by Leloir (230) namely: 3 volumes of IM ammonium acetate and 7.5 volumes of 95% ethanol at either pH 7.5 or 3.6. The time of development was approximately 48 hours at room temperature (± 18°C).

Paper electrophoresis was carried in an apparatus similar to that described by Markham and Smith (240) using Whatman No.I paper strips of 6 x 36cms dimensions unless otherwise stated. The buffers used, the voltage applied, together with the times of run are mentioned in the text in the appropiate places.

Nucleotides were located on paper by inspection under ultra-violet light. Distances moved by components were measured to their centres and relative to the distance moved by control UMP.

GM IO. Thin layer chromatography (TLC) and detection of compounds

Materials: Whatman "Chromedia" cellulose powder CC4I (cataloque no.I24I2) with a mean particle size passing 200 BSS.

Procedure: The cellulose powder was mixed with water in a ratio of I:2.09 (w/v) and the resulting suspension spread, to a thickness of 0.3mm on clean grease free glass 20 x 20cms plates, by means of a Shandon "Unoplan" plate making apparatus. Further details of preparation and

solvents used in the ascending chromatographic separation on thin layer plates are mentioned in the text.(Part III).

Detection: of nucleotides was by inspection under U.V. light. The method of detection was aided by spraying the plate with a 0.005% rhodamine-6G spray which made the background pale yellow, thus accentuating the areas of U.V. absorbance.

EXPERIMENTAL SECTION

Note: Not all experiments that have been carried out are detailed below, since they are adequately detailed in the text and to list them under the experimental section would entail mere repetition.

EXP I. Spleen extraction I

The extraction was carried out as described in the text and each stage of extraction of calf spleen ribonuclease, with respect to details of amounts of substances used etc. is given below, as this was not described in the text.

Note: Centrifuge speeds. In the text, speeds were quoted in terms of r.p.m. and in all extraction work the MSE "Highspeed I7" was used and conversion to 'g' figures is obtained below:

$g = 0.0000284 \times r \times N^2$ where r = radius in inches to inside lip of centrifuge tubes = 5.6 inches. N = speed in rpm.

$\therefore g = 0.000159 \times N^2$.

---

a). Stage I - Homogenisation and extraction and ammonium sulphate (AS) precipitation.

25 spleen were homogenised in 0.05M sodium acetate, 10^{-3}M EDTA, pH 7.2 buffer giving a total of 8.1901. of homogenate. The pH was adjusted to 3.5 with IN hydro-

-chloric acid giving a total volume of 8.741. of acid homogenate (in aliquots of 3.2,3.2 and 2.341.). A total of I39[illegible].4gms of ammonium sulphate were added (in 5I2, 5I2,and 374.4gm aliquots respectively at the rate of I60gms/1.) to bring the saturation level to 0.3*,and left to stand over night. The precipitate was centrifuged at I6,000 x g at 2-3°C for 8 minutes in 250ml polypropylene bottles and the residue discarded. The total volume of the supernatant was 4.9I 1. [* 0.3 & 30% = same]

b).Stage II - 0.6 AS saturation.

4.9I 1. of supernatant (in 31. and I.9.1. aliquots) were made 0.6 saturated with I,748gms of ammonium sulphate(I,068 and 680 gms respectively). After standing overnight the material was centrifuged at I6,000 x g at 2-3°C for 8 minutes. The precipitate collected was dissolved in I.I71. of pH 7.2 buffer.

c). Stage III - Heat treatment.

The I.I71. of dissolved material were adjusted to pH 3.5 with IN hydrochloric acid, giving a total volume of I.5I51. for heat treatment. Then in aliquots of 700, 500 and 3I5 mls, the material was subjected to heat treatment as described in the text for I0 minutes at 60°C and then the pH was re-adjusted to 7.0 with IN sodium hydroxide. The material precipitated was centrifuged at 26,000 x g and the supernatant retained.

d). Stage IV - Second acid ammonium sulphate precipitation (0.85 AS satn.).

The pH of the supernatant from the previous stage was adjusted to 2 with IN hydrochloric acid, thus giving a total volume of I.87l. and 374gms of ammonium sulphate were added to bring the solution to 0.4 - 0.5 saturation level . The precipitate formed was removed by centrifugation at I6,000 x g for 30 minutes at 2-3°C and the 2.05l. of supernatant were then brought to 0.85 saturation by the addition of 656gms of ammonium sulphate. The precipitate formed after standing over night was centrifuged at 26,000 x g for I5 minutes at 2-3°C and dissolved in 300mls of 0.005M Tris/HCl, 10^{-4}M EDTA, pH 5.5 buffer and solution was aided also by the presence of IM sodium acetate in the buffer.

e). Stage V. Dialysis of 0.8 AS precipitated protein.

Dialysis of the above dissolved material was in I inch visking tubing against 0.005M Tris/HCl, 10^{-4}M EDTA,pH 5.5 buffer with three changes of 4½-5l. over a period of 24 hours. The dialysed material was centrifuged at I6,000 x g for I5 minutes at 0°C to give 360 mls of solution.

f). Stage VI. CM-cellulose chromatography.

The protein solution (360mls at a concentration of I.67 mgs/ml protein) was applied to a 64 x I.7cm CM-cellulose

column and the following gradient elution was applied:

Reservoir 5l.(+ 5l. later). Constant volume chamber 3.75l.

0.005M Tris/HCl
10^{-4}M EDTA
0.32M NaCl
pH 8.2

⟶

0.005M Tris/HCl
10^{-4}M EDTA
pH 5.5

Column data.

Buffer level in constant vol. chamber above floor level	=113cms
Buffer level in reservoir above floor level	=162cms
Top of column above floor level	=165cms
Flow rate	=175mls /hour
Fraction size collected	=50mls

The fractions were read at 200 mu in a Unicam SP 500 spectrophotometer and assayed for enzyme activity by GM -4.

EXP 2. Concentration of peak B from extraction I

Peak B (fraction 107-115) from extraction I was dialysed and then concentrated by means of adsorption onto a $3\frac{1}{2}$ x $\frac{1}{2}$ cm column of CM-cellulose and subsequently eluted with buffer that was IM with respect to sodium chloride. The concentrated protein fraction B was stored at -22°C in the deep freeze.

EXP.3. CM-cellulose chromatography of peak A from extraction I

Peak A protein material (fractions 90-105) was applied to a 17.5 x 2cm column of CM-cellulose and subjected to the following gradient elution:

Reervoir I l.		Constant vol.chamber 400mls
0.005M Tris/HCl 10^{-4}M EDTA pH 8.2 0.32M NaCl	⟶	0.005M Tris/HCl 10^{-4}M EDTA pH 8.2

A100 fractions were collected of 10mls each and at fraction 74, I l. of buffer made IM with respect to sodium chloride was added. Protein estimation was by U.V. absorption at 280mu and enzyme activity estimated by GM 4. Fractions 10-30 were bulked, dialysed, and concentrated by means of adsorption on to a 6 x ½cm column of CM-cellulose and subsequent elution with IM sodium chloride containing buffer. Storage of the enzyme was at -22°C.

EXP.4. Spleen extraction 2

Summary charts to this extraction and all others are given in figs.17-21 and relevant differences in the procedure in this extraction as compared to previous extraction I, and future extractions are described and discussed in the pertinent portions of the text.

a). Stage I - Homogenisation and extraction and ammonium sulphate precipitation.

2I2 spleen were homogenised and extracted as described in the text for extraction I.

b). Stage II - 0·6 AS saturation

c). Stage III Heat treatment at pH 3.5

The material was subjected to heat treatment at pH 3.5 at 67°C for I0 minutes and then centrifuged at 26,000 x g for I5 minutes at 2-3°C after adjusting the pH to 7.0

d). Stage IV The second acid ammonium sulphate precipitation was excluded.

e). Stage V - Dialysis

Dialysis of the supernatant from stage III was against 0.005M Tris/HCl, $I0^{-4}$M EDTA, pH 5.5 buffer with three changes of 4½-5l. over a period of 24 hours. The dialysed material was centrifuged at I6,000 x g for I5 minutes at 2-3°C, and the supernatant retained.

f). Stage VI - CM-cellulose chromatography.

The protein solution from stage V was applied to a 5I x I.7cm CM-cellulose column and subjected to the below gradient elution:

Reservoir, 5l. (+5l. Later)		Constant vol. chamber, 3.75l.
0.005M Tris/HCl		0.005M Tris/HCl
$I0^{-4}$M EDTA	⟶	$I0^{-4}$M EDTA
0.32M NaCl		pH 5.5
pH 8.2		

50ml fractions were collected and read at 280mu in a u.v.

spectrophotometer and assayed for enzyme by GM 4.

EXP 5. Spleen extraction 3

a). Stage I - Homogenisation and extraction and ammonium sulphate precipitation.

A total of 24 spleen (3 fresh and 2I deep freeze stored at -22°C) were homogenized in 0.05M sodium acetate, 10^{-3}M EDTA, 2M urea, pH 7.2 buffer. The urea used was BDH Analar grade and all urea solutions were filtered through Whatman No.I filter paper before use. The pH of the homogenate was adjusted to pH 3.5 with IN HCl giving a total volume of 6.6l. The homogenate was then made 0.3 saturated by the addition of I60gms/l. of ammonium sulphate, and left to stand over night. The resulting precipitate was re-extracted with twice its volume of the above pH 7.2 buffer after centrifuging at I6,000 x g. The buffer (also 2M w.r.t.urea) and precipitate were stirred for 30 minutes and similarly made 0.3 saturated with ammonium sulphate, and, after centrifugation, the re-extracted and the original supernatants were combined.

b). Stage II - 0.8 AS saturation.

The combined supernatants were brought up to 0.8(i.e.80%) saturation by the addition of 356gms/l. of ammonium sulphate. After standing over night and centrifuging at I6,000 x g at 2-3°C for I5 minutes, the precipitate obtained was dissolved in Il. of 0.005M sodium acetate,

10^{-4}M EDTA, 2M urea, pH 7.2 buffer

c). Stage III - Heat treatment at pH 3.5.

The protein solution was adjusted to pH 3.5 with IN HCl and subjected to a temperature of 60°C for 10 minutes in aliquots of about 500mls at a time, and then the pH was adjusted to pH 7.0 before centrifuging at 26,000 x g for 15 minutes at 2-3°C.

d). Stage IV - Second AS precipitation (at neutral pH)

The ammonium sulphate concentration was initially raised to about 0.4 saturation by the addition of 190gms/l. The small precipitate produced was removed by centrifugation of the solution at 26,000 x g. The ammonium sulphate concentration of the supernatant was then raised to almost saturation point and left to stand over night. Centrifugation was at 26,000 x g for 15 minutes at 2-3°C and the precipitate obtained was dissolved in 0.005M Tris/HCl, 10^{-4}M EDTA, 2M urea, pH 7.5 buffer.

e). Stage V - Desalting using Sephadex G-75 molecular sieve.

Materials: Sephadex G-75, particle size 40-120μ, water regain 7.5 ± 0.5 g/g, in bead form.

Desalting was carried out by 9 separate applications and elutions of approximately 5.5ml aliquots of protein solution from stage IV to a G-75 Sephadex preparative column of 75.5 x 4.8cms dimensions and eluting with

0.005M Tris/HCl, 10^{-4}M EDTA, 2M urea, pH 7.5 buffer at a rate of about 84mls/hour. Fractions of 10mls were collected using a "Towers" automatic fraction collector and the fractions were assayed for RNAse activity by GM 4 to ascertain the position of elution of active RNASe protein, and this was correlated with protein elution profile from optical density readings at 280mu in the "Unicam" SP500 spectrophotometer in order that the necessity of assaying every G-75 column elution may be bypassed.

f). Stage VI - Application of protein extract to a DEAE-column.

The enzymically active material from stage V was applied to a 13 x 1.8cms DEAE-cellulose column (Cl^-form) which was equilibriated with 0.005M Tris/HCl, 10^{-4}M EDTA, 2M urea, pH 7.0 buffer and eluted withthe same. Collection of effluent was in bulk and this material which passed straight through the DEAE-cellulose adsorbant was retained for stage VII.

g). Stage VII - CM-cellulose chromatography.

The above material at pH 7.0 and 2M w.r.t. urea was subjected to the following column chromatography below: The columns were equilibriated with urea at the concentration stated and the protein solution applied, and eluting buffers were also adjusted to this same urea

concentration.

i). CM-cellulose column no. CMC-3.- This column was not equilibriated with urea and neither were the eluting buffers. The below gradient elution was used and 5ml fractions were collected.

Reservoir I 1.		Constant vol. chamber 500 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA	⟶	10^{-4}M EDTA
pH 8.2		pH 7.0
0.32M NaCl		

ii). CM-cellulose column no.CMC-3-6M -in the presence of 6M urea

Reservoir I 1.		Constant vol. chamber 500 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA	⟶	10^{-4}M EDTA
pH 8.2		pH 7.0
6M urea		6 M urea
0.32M NaCl		

5ml fractions were collected.

iii). CM-cellulose column no.CMC-3-8M -in the presence of 8M urea

Reservoir I 1.		Constant vol. chamber 500 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA	⟶	10^{-4}M EDTA
pH 8.2		pH 7.0
8M urea		8M urea
0.16M NaCl		

5ml fractions were collected.

EXP 6. Spleen extraction 4.

Like extraction-/ p.t.o:

3 the processing of 36 spleen in extraction 4 was also carried through entirely in the presence of 2M urea using exactly the same basic procedure (stages I-VII as in EXP 5) except for various extra re-extraction techniques which are described adequately in the text. The following conditions were used for CM-cellulose chromatography at stage VII

a). CM-cellulose chromatography

i). CMC-4-2M-I. Chromatography of RNAse enzyme was carried out in the presence of 2M urea on a column of 68 x I.7 cm dimensions. An LKB "Radirac" fraction collector and "Uvicord" assembly was used for all column chromatography in extraction 4 (and also in extraction 5), and similarly for both extractions 4 and 5, the fraction size collected was I0mls.

The following gradient elution was used:

Reservoir I600mls.		Constant vol. chamber 800 mls.
0.005M Tris/HCl		0.005M Tris/HCl
$I0^{-4}$M EDTA		$I0^{-4}$M EDTA
pH 8.2	⟶	pH 6.0
2M urea		2M urea
0.32M NaCl		

Column data:

Flow rate	0.5-0.7mls/min.
Height of column above bench level	90cms

Height of liquid in constant vol.chamber above bench level 79cms

Height of reservoir above constant vol. chamber(i.e.difference in liquid levels) I4.5cms

Height of outlet of siphon on fraction collector above bench level 26.5cms

This data was approximately same for all extraction 4 CM-cellulose chromatography and likewise so was the method of enzyme location (GM 4) and protein estimation by reading the optical density at 280mu in a Unicam SP500 spectrophotometer.

ii).CMC-4-2M-II. Column dimensions: 50 x 2.2cms The following gradient elution was used:

Reservoir I600mls		Constant vol. chamber 800 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA		10^{-4}M EDTA
pH 8.2	⟶	pH 7.0
2M urea		2M urea
0.25M NaCl		

iii).CMC-4-7M-I. Column dimensions: 68 x I.7cms and chromatography was carried out entirely in the presence of 7M urea.

Reservoir I600mls		Constant vol.chamber 800 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA		10^{-4}M EDTA
pH 8.2	⟶	pH 6.0
7M urea		7M urea
0.25M NaCl		

iv). CMC-4-7M-II. Column dimensions: 68 x I.7cms

Reservoir I600mls		Constant vol.chamber 800 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA		10^{-4}M EDTA
pH 8.2	⟶	pH 7.0
7M urea		7M urea
0.I5M NaCl		

v). CMC-4-2M-Leu I, The protein solution which was made 2M w.r.t. urea was applied to a CM-cellulose column of 68 x I.7cms dimensions and also 2M w.r.t. urea. A DL nor-leucine (BDH Biochemical grade) gradient was then applied as below:

Reservoir I500mls.		Constant vol.chamber 800 mls.
0.005M Tris/HCl		0.005M Tris/HCl
10^{-4}M EDTA		10^{-4}M EDTA
pH 7.0	⟶	pH 7.0
2M urea		2M urea
saturated DL nor-leucine		

After fraction 92 (10ml fractions were collected) the reservoir was made 0.25M w.r.t. sodium chloride and the elution continued to give a salt and DL nor-leucine gradient combined. Thus the gradient from fraction 92 onwards was:

Reservoir 580mls	Constant vol.chamber 800 mls.
0.005M Tris/HCl	0.005M Tris/HCl
10^{-4}M EDTA	10^{-4}M EDTA
pH 7.0	pH 7.0

2M urea 0.25M NaCl Saturated nor-leucine	⟶	2M urea weak solution w.r.t.nor-leucine

vi). CMC-4-2M-Leu II. This column differed from CMC-4-2M-Leu I in that there was no nor-leucine gradient, but nor-leucine was present at saturation level throughout all stages of the below gradient elution:

Reservoir 1500mls		Constant vol.chamber 800 mls
0.005M Tris/HCl 10^{-4}M EDTA pH 8.2 2M urea 0.15M NaCl Saturated nor-leucine	⟶	0.005M Tris/HCl 10^{-4}M EDTA pH 7.0 2M urea Saturated nor-leucine

EXP 7. Spleen extraction 5

The extraction and processing of 30 spleen was carried through all stages in the absence of urea unless otherwise stated. The average weight of calf spleen was 68gms and this weight was in accordance withthe spleen used in all previous extractions.

The procedure used was basically the same as that used for extraction 4(EXP 6) with the ommission of the presence of urea. Any differences in procedure are described and explained in the relevant portions of the text and these are mainly with respect to the large amount of material discarded at stage I of the extraction

process.

a). CM-cellulose chromatography

Chromatography was carried out in the absence of urea except for column CMC-5-III. Fraction collection was by means of an LKB "Radirac" and "Uvicord" assembly, collecting 10ml fractions.

i). CMC-5-I Column dimensions 49 x 2.5cms.

Reservoir 1600mls		Constant vol.chamber 800 mls.
0.005M Tris/HCl 10^{-4}M EDTA pH 8.2 0.25M NaCl	⟶	0.005M Tris/HCl 10^{-4}M EDTA pH 7.0

ii). CMC-5-II Column dimensions 46 x 2.5cms

Reservoir 1600mls		Constant vol. chamber 800 mls.
0.005M Tris/HCl 10^{-4}M EDTA pH 8.2 0.32M NaCl	⟶	0.005M Tris/HCl 10^{-4}M EDTA pH 6.0

iii). CMC-5-III. Column dimensions 20 x 2.2 cms. This column was equilibriated with 2M urea and the protein solution applied to this column was also made 2M with respect to urea. The following gradient elution was carried out:

Reservoir 1600mls		Constant vol.chamber 800 mls
0.005M Tris/HCl 10^{-4}M EDTA pH 8.2,2M urea 0.32M NaCl.	⟶	0.005M Tris/HCl 10^{-4}M EDTA pH 6.0, 2M urea

iv). CMC-5-IV. Column dimensions I8 x 2.2 cms.

Reservoir I600mls.

0.005M Tris/HCl
$I0^{-4}$M EDTA
pH 8.2
0.32M NaCl

Constant vol.chamber 800 mls.

0.005M Tris/HCl
$I0^{-4}$M EDTA
pH 8.2

Index to journal name abbreviations used in references.

Acta Chem.Scand.	Acta Chemica Scandinavia
Adv.in Enzymology	Advances in Enzymology
Adv.in Prot.Chem.	Advances in Protein Chemistry
Ann.	Annalen der Chemie, Justus Liebigs.
Ann.N.Y.Acad.Sci.	Annals of the New York Academy of Sciences
Ann.Rev.Biochem.	Annual Review of Biochemistry
Archivs.Biochem.Biophys.	Archives of biochemistry and biophysics
BBA	Biochimica et Biophysica Acta
BBRC	Biochemical,biophysical research communications
BJ	Biochemical Journal
Biochem.	Biochemistry(American Chemical Society)
Brit.J.Expertl.Pathol.	British Journal of Experimental Pathology
Brit.Med.Bull.	British Medical Bulletin
Bull.Soc.Chim.Belg.	Bulletin des Societes Chimiques Belges
Can.J.Biochem.& Pathol.	Canadian Journal of Biochemistry and Physiology
Chem.Revs.	Chemical Reviews(American Chemical Society)

Comp.Rend.Trav.Lab. Carlsberg,ser Chim.	Comptes Rendus des Travaux du Laboratoire Carlsberg, serie Chimique.
Fed.Proc.	Federation Proceedings
JACS	Journal of the American Chemical Society
JBC	Journal of Biological Chemistry
J.Biochem (Tokyio)	Journal of Biochemistry (TOKYIO)
J.Chem.Phys.	Journal of Chemical Physics
J.Chromat.	Journal of Chromatography
J.MoL.Biol.	Journal of Molecular Biology
J.Phys.Chem.	Journal of Physical Chemistry
J.Natl.Inst.Cancer,U.S.	Journal of the National Cancer Institute,U.S.A.
J.Pathol.Bacteriol.	Journal of Pathology and Bacteriology
Kolloid.Z.	Kolloidnyi Zhurnal
Photochem ,photobiol.	Photochemistry and photobiology
Proc.Natl.Acad.Sciences,US.	Proceedings of the National Academy of Sciences of the U.S.A.
Ukr.Biokhim.Zhur.	Ukrains'kii Biokhimich--eskii Zhurnal

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