ISOLATION AND CHARACTERIZATION OF RABBIT RIBONUCLEASES

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ABSTRACT

Rabbit spleen and pancreatic ribonucleases (RNases) were isolated and purified by a combination of salting-out with ammonium sulfate, gel filtration on Sephadex-75 and ion-exchange chromatography on DEAE-Sephadex A-50, SE-Sephadex C-50 and CM-Sephadex C-50. The isolated enzymes were judged homogenous from the results of analytical ultracentrifugation, electrophoresis and chromatography on Sephadex G-75. The physicochemical properties of these RNases were similar to those of other mammalian RNases.

Injection of the pure rabbit spleen RNase into a sheep elicited 'monospecific' antibodies as revealed by immunodiffusion and immunoelectrophoresis. These antibodies inhibited only partially (i.e. 40-60%) the RNase activity. This incomplete inhibition was proved to be attributable to the formation of soluble RNase-antibody complexes.

Reverse immunosorbents were prepared by insolubilizing the antibodies with ethyl chloroformate or ethylene maleic anhydride. These immunosorbents combined with the rabbit spleen RNase and were shown to be useful and specific reagents for the rapid and simple isolation and purification of rabbit RNase with a yield of 86%. Moreover these immunosorbents were capable of acting as a RNase inhibitor in the isolation of polysomes. However their inhibitory capacity did not exceed that of heparin plus sodium deoxycholate.

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by

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LIST OF ABBREVIATIONS

Å..... Angstrom unit, 10^{-10} meter.

ATP..... adenosine triphosphate

DNA..... deoxyribonucleic acid

DNase..... Deoxyribonuclease

EDTA..... ethylene diamine tetraacetate

EMA..... ethylene maleic anhydride

Enz. act..... enzymatic activity

G..... gravitational unit

gm..... gram

Ig..... immunoglobulin

M..... molar (solution)

mA..... milliampere

mg..... milligram

ml..... milliliter

mM..... millimole

Mol. wt..... molecular weight

m-RNA..... messenger ribonucleic acid

mμ..... millimicron

N..... normal (solution)

O.D..... optical density

%..... percent

PBS..... phosphate-buffered saline

pI..... isoelectric point

RNA..... ribonucleic acid

RNase..... ribonuclease

rpm..... revolutions per minute

S..... sedimentation coefficient in Svedberg unit

S.D. ..... standard deviation

μ..... ionic strength

μg..... microgram

V..... volt

CHAPTER I

INTRODUCTION

Ribonucleases are the best characterized enzymes, so far studied, because of their important roles in nucleic acid metabolism and their relatively small molecular sizes. The extensive studies on bovine pancreatic ribonuclease have led to the elucidation of its complete amino acid sequence (1, 2, 3, 4, 5) and of its three dimensional structure (6, 7). The mechanism of its enzymatic action is well understood (8, 9) and its active site has been shown to involve the three amino acid residues, histidine-12, histidine-119 and lysine-41 (10, 11, 12, 13, 14, 15, 16, 17). Recently the total synthesis of bovine ribonuclease A was accomplished (18, 19) with the Merrifield solid phase method.

Ribonucleases are widely distributed in nature. Numerous ribonucleases have been isolated from diverse sources including different organs of mammals (20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30), invertebrates (31, 32), plants (33) and micro-organisms (34, 35). Rabbit ribonucleases received little attention until recent years because of the relatively low ribonuclease content in the rabbit organs (36). A partially purified ribonuclease from rabbit reticulocytes has been isolated and characterized (37, 38). However, up to the present study rabbit ribonucleases had not been isolated in a pure form. The rabbit reticulocyte ribonuclease was found capable of degrading polyribosomes (37, 38) and it was suggested that its physiological role was to control the progressive breakdown of polyribosomes and ribosomes during the maturation of rabbit reticulocytes to circulating erythrocytes (39, 40).

SCOPE OF THE PRESENT STUDY

This investigation was undertaken with a view to developing physicochemical and immunochemical procedures for the isolation of rabbit spleen and pancreatic ribonucleases in a pure form. Besides the intrinsic biochemical interest of such studies, it was proposed to use these ribonucleases for the production of specific antibodies, in the expectation that these antibodies could in turn be used as effective inhibitors of ribonucleases in investigations designed for the isolation of ribonucleic acids from these tissues. The main reason for the choice of rabbit tissues for this study was that this species has been commonly used for immunological experiments. It was anticipated that anti-rabbit ribonuclease antibodies might be employed as effectors of ribonucleases in the study of biosynthesis of antibodies, in particular as regards the isolation from lymphoid cells of intact m-RNAs from polysomes coding for the amino acid sequence of the heavy and light chains of rabbit immunoglobulins.

Chromatographic procedures were developed in this study for the isolation of ribonucleases from rabbit spleen and pancreas in a pure form. Some physicochemical properties of these ribonucleases, i.e. the optimum pH for enzymatic activity, effects of metal ions on enzymatic activity, Michaelis-Menten constants, isoelectric points, molecular weights, amino acid compositions, were established and found to be similar to those of bovine pancreatic ribonuclease.

The rabbit spleen ribonuclease was used for immunization of a sheep. The resulting antibodies were 'monospecific' inasmuch as they gave rise to only one precipitin band on gel diffusion against

this enzyme. Reverse immunosorbents were prepared by insolubilizing these antibodies with the cross-linking reagents, ethylchloroformate (41) and ethylene maleic anhydride (42, 43). These reverse immunosorbents proved effective for the rapid isolation of rabbit spleen ribonuclease of high purity and high yield. The inhibitory effects of anti-ribonuclease antibodies and of the reverse immunosorbents on the enzymatic activity of rabbit ribonuclease were also examined.

CHAPTER II

LITERATURE REVIEW

(A) Discovery and classification of ribonucleases

As early as 1920, Jones (44) discovered a heat-stable pancreatic enzyme which was capable of hydrolyzing yeast nucleic acids without the liberation of phosphoric acid. This important enzyme is commonly known as ribonuclease (RNase), and systematically as polyribonucleotide 2-oligonucleotidotransferase (cyclizing), EC 2.7.7.16 * (45). Shortly after the discovery of this ribonuclease, a large number of enzymes which catalyzed the hydrolysis of ribonucleic acid through the cleavage of phosphodiester linkages were found to be distributed ubiquitously in nature. These ribonucleases were isolated from various plants (33), micro-organisms (34, 35), and organs of animals among which bovine pancreatic ribonuclease was best characterized. Most of the mammalian ribonucleases were found to be heat-stable (48), the optimum pH range for their enzymatic activities being 7-8, whereas plant ribonucleases (49, 50, 51) possessed an optimum pH between 4.5 and 6.

All the enzymes which are capable of degrading both ribo- and deoxyribonucleic acids (RNA and DNA) are generally known as nucleases. In early years various names, such as ribonuclease, ribonucleo-depolymerase, polynucleotidase, and nucleotidase, were used to specify enzymes capable of degrading RNA (52). However, the

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* The letters EC stand for Enzyme Commission of the International Union of Biochemistry.

term ribonuclease (RNase) is most commonly used for the enzymes which degrade uniquely RNA. In addition to these, Zittle (53) reported on an enzyme different from RNase, also capable of splitting RNA, which they called non-specific phosphodiesterase. In recent years, an increasing number of ribonucleases with diverse specificities and physicochemical properties have been discovered (54). The diversity of ribonucleases led to some confusion in their nomenclature for some years. Recently the enzymes capable of depolymerizing RNA were classified in two major groups (54, 55, 56): (A) RNA phosphotransferases (cyclizing RNases) which have the characteristics of (i) 3'- or 2'-phosphate monoester formers, (ii) specificity towards bases adjacent to the bond cleaved, and (iii) specificity towards macromolecular form of RNA; (B) Phosphodiesterases (noncyclizing RNases and nucleases) which can be divided into two sub-classes: (I) polynucleotide phosphodiesterases which have the characteristics of (i) sugar-specific or sugar-nonspecific, (ii) 3'- or 5'-phosphate monoester formers, (iii) endo- or exonucleases, (iv) specificities towards bases and (v) specificity towards macromolecular form of RNA, and (II) nonspecific phosphodiesterases which hydrolyse both RNA and diesters of phosphates other than nucleotides. The well known bovine pancreatic RNase belongs to group (A). Recently more than eighty RNA-degrading endonucleases have been identified. These ribonucleases are very widely distributed and both classes of ribonucleases are likely to occur together in all species.

(B) Distribution and biological function of ribonucleases

Although ribonucleases are widely distributed in nature, the amount of ribonuclease in each species and for each organ varies greatly. The content of ribonuclease in the pancreas of fifty vertebrate species was determined by Barnard (36). Vertebrates can be divided into three groups (36, 57) with respect to their content of ribonuclease in the pancreas: Group A of high RNase content (200 to 1,200 μg per gram pancreas) including all ungulates, rodents or herbivorous marsupials; Group B of moderate-to-low content (20 to 100 μg per gram pancreas) including pig, turtles, horse, turkey and chicken; Group C of very low content (0 to 20 μg per gram pancreas) which includes snake, frog, pigeon, monkey, man, elephant, rabbit, dog and cat. Barnard (36) reported that the rat and cow have about 300 and 1,200 times more ribonuclease, respectively, in the pancreas than that in man. The amount of ribonuclease in rabbit pancreas is less than that in human pancreas. It has been shown that the content of pancreatic ribonuclease within a given species is fairly constant. The interesting results of Barnard (36) indicated that in several species of animals with low ribonuclease contents, the pancreatic ribonuclease contents did not increase after extensive intake of RNA. This is in contrast to the large increase in total proteases, chymotrypsinogen and trypsinogen, which occur after a high intake of proteins (57, 58, 59). When an animal is fed on starch and protein, respectively, the pancreatic amylase (58, 59) and carboxypeptidase (60) are also readily affected. These results imply that the function of ribonucleases is not simply the digestion of dietary RNA, but that

of regulating the pool of nucleic acids. The biological functions of various ribonucleases can be considered as: (a) elimination of informational RNA molecules, (b) defence against foreign (viral) RNA, (c) a general intracellular digestive function and (d) synthesis of polynucleotides.

Barnard (36) reported that some primates, including man, could metabolize at least small amounts of dietary RNA, although they have only a low content of ribonuclease in their alimentary tract. He showed that low levels of ribonuclease, of the order of 1 μg per gram of pancreas, were sufficient to accumulate in the intestine and hydrolyzed the dietary RNA. The exceptionally high content (1,200 μg per gram of pancreas) of pancreatic ribonuclease in the cow and in other ruminants may suggest a special biological function other than digestion of dietary RNA, such as the utilization of ruminal bacterial RNA (36).

The intracellular localization of ribonucleases in animal tissues has been studied by some workers (61, 62, 63). Schneider (61) separated the mouse-liver homogenate into mitochondria, submicroscopic particulates and soluble fractions and found that the mitochondria fraction contained most of the RNase and DNase activities. Roth (62) also reported that the bulk of RNase activity of rat spleen was localized in mitochondria.

Soon after the discovery of a cellular ribonuclease inhibitor from mammalian tissue (64, 65), this inhibitor has been purified and characterized (65, 66, 67, 68, 69). Ribonuclease inhibitors have been found in the livers of five mammalian species (70). Shortman (71) demonstrated that the levels of ribonuclease inhibitor increased significantly during the regeneration of rat liver, and a maximum increase of 39% was observed at 48 hours after hepatectomy. Marked

increase in RNA concentration occurred 12 to 48 hours after the operation. The amount of acid ribonuclease increased much later when the level of RNA was dropping and there was an apparent decline in the growth rate. Although ribonuclease inhibitors were most commonly found in livers, ribonuclease inhibitor has also been found in rat adrenals as reported by Girija (68). In the presence of rat liver ribonuclease inhibitor, single-stranded m-RNA has been shown to be protected in rat liver cells (72). Complexing of ribonucleases with these inhibitors appears to be the device generally used in mammalian tissues to control the alkaline ribonuclease activity (67, 68, 73, 74).

(C) Structure and activity of bovine pancreatic ribonuclease

The crystallization of bovine pancreatic ribonuclease was first accomplished in 1940 by Kunitz (75). McDonald (76) employed heat treatment to inactivate any residual proteolytic activity due to contaminants in the crystalline preparation. Ribonuclease was treated taking advantage of its heat stability. More recently Hirs et al. (77) isolated two purified preparations of bovine pancreatic ribonuclease by chromatographic fractionation on the carboxylic cation exchanger XE-64, one referred to as RNase A and the other as RNase B. The minor component, RNase B, representing approximately 7 to 10 percent of the total ribonuclease (78), was found to be a glycoprotein. It contained five residues of mannose and two residues of glucosamine with its protein moiety identical to RNase A (21). This finding was supported by the work of Brown (79) who demonstrated that rabbit antibodies to RNase A reacted identically with RNase B.

The elucidation of the amino acid sequence of ribonuclease was of great interest to many workers at the time when purified bovine pancreatic ribonuclease was obtained, because of its relatively small molecular size and homogeneity. The complete amino acid sequence was established by Smyth, Spackman, Hirs, Stein and Moore (1, 2, 3, 4, 5). It was shown to be composed of a single polypeptide chain of 124 amino acid residues (2) forming a relatively compact molecule held by four intra-molecular disulfide bonds (Fig. 1). Further studies on the chemically modified ribonuclease soon revealed the most important portion of the molecule, i.e. its enzymatic active site. The studies on alkylation of histidine residues with iodoacetic or bromoacetic acid (10, 11, 12, 13, 14) indicated that the active site involved two histidine residues at positions 12 and 119. The imidazole groups at positions 12 and 119 were estimated to be 2 to 7 $\mathring{A}$ apart at the active site of the molecule (14). Klee (15) and Anfinsen (16, 17) studied the guanidination of lysine residues and suggested that lysine at position 41 was involved in the active site.

The development of X-ray crystallography led to the elucidation of the conformation of ribonuclease molecule. The crystallographic structure of bovine pancreatic RNase A was determined at 2 $\mathring{A}$ resolution by Kartha et al. (6) and that of ribonuclease S at 3.5 $\mathring{A}$ resolution by Richards and coworkers (7). Interestingly, the results showed that the side chains of histidine-12, histidine-119 and lysine-41 were closely situated with respect to each other, which was in excellent agreement with the prediction derived on the basis of chemical methods. Thus ribonuclease became the best characterized enzyme with a completely established structure and a chemically well

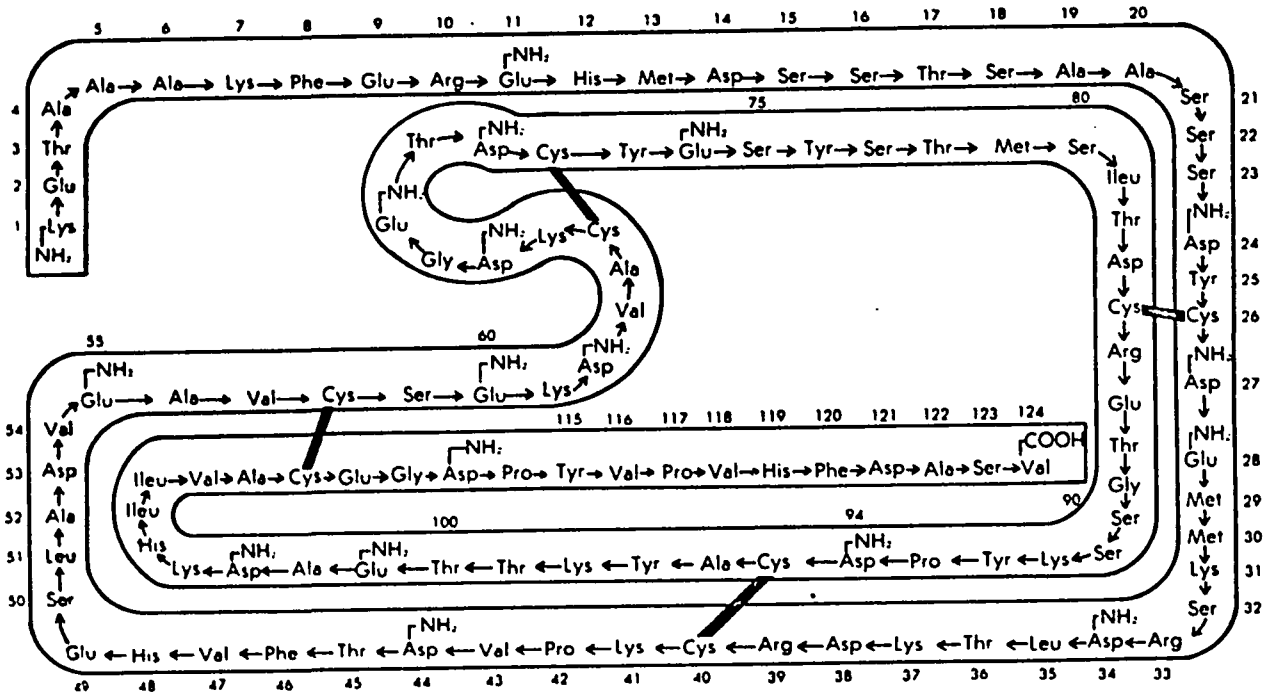


Figure 1. Primary structure of bovine pancreatic ribonuclease A.

defined site responsible for its activity (8, 9). The mode of action of bovine pancreatic ribonuclease (80, 81, 82, 83, 84, 85, 86, 87) appears to involve two consecutive steps: (i) hydrolysis of specific phosphodiester bonds yielding pyrimidine nucleoside 2,3,-cyclic phosphate as an intermediate product, and (ii) hydrolysis of the resulting cyclic phosphate bonds into 3-phosphate end groups. More recently, the total synthesis of ribonuclease A was accomplished (18, 19) following the introduction of the elegant solid phase peptide synthesis method developed by Merrifield (88).

Besides the elucidation of the complete structure of RNase, the studies of partial hydrolysis of ribonuclease by subtilisin led to the interesting developments in the biochemistry of ribonuclease. Richards and colleagues (89) obtained the well-known derivative, RNase S, by enzymatic cleavage of the peptide bond between residues 20 and 21 of bovine pancreatic RNase A with subtilisin. The resulting N-terminal peptide of 20 amino acid residues, termed as S-peptide, can be separated by column chromatography from the rest of the molecule, S-protein. Neither the S-protein nor S-peptide alone is enzymatically active. However, the ribonuclease can be reconstituted non-covalently, with restoration of its full activity, by simply mixing the two fragments in equimolar proportions. Anfinsen et al. (90) demonstrated that the S-protein was stabilized in the presence of S-peptide. Hofmann (91) synthesized several peptides of various lengths corresponding to portions of the S-peptide and found that the combination of S-protein with the synthetic peptide corresponding to residues 1 to 13 of the S-peptide restored 68 to 72 percent of the enzymatic activity. However, the addition of other synthetic peptides of less than 13 residues

showed no enzymatic activity. This is in good agreement with the finding that histidine-12 is involved in the active site (12, 13, 14). Further experiments on the chemically modified S-peptides and its analogues (92, 93, 94) indicated that unmodified histidine at position 12 was essential for restoration of enzymatic activity.

Because of its relatively small molecular weight, ribonuclease has served as a most useful model for studies of the complete structure of a protein as well as for clarification of the relationship between molecular structure of the enzyme and its catalytic activity. Moreover, it has served as a model for the study of the relationship between the enzymatic active site and the site(s) for combination with antibody molecules, as will be discussed in Section (G).

(D) The harmful effect of ribonuclease in the studies of biosynthesis of antibodies in vitro

Ribonucleases, because of their ubiquitousness and their ability to degrade ribonucleic acids over a wide range of conditions, complicate greatly investigations involving polyribosomes in relation to studies of the in vitro biosynthesis of antibodies or other proteins. Since endogenous ribonucleases exist in all the tissues, they are released during the rupture of cells. Polyribosomes, which are active in protein synthesis (95, 96, 97, 98), are inevitably broken down by the RNA-degrading enzymes. Attempts have been made to eliminate the harmful effect of ribonucleases by the addition of non-specific inhibitory agents such as detergents (e.g. sodium dodecyl sulfate (99), sodium cholate (100) and sodium deoxycholate

(98, 101, 100)), bentonite (102, 103), Macaloid (which is a trade name for a purified hectorite, i.e. sodium magnesium lithofluorosilicate) (105, 106) and heparin (100, 104).

Brentani et al. (101) reported that rat-liver ribosomes lost about 50% of their amino acid-incorporating activity when preincubated with bovine pancreatic ribonuclease. They subsequently employed sodium deoxycholate in the isolation of ribosomes. Roth et al. (100) carried out extensive studies on the effects of a variety of reagents on ribonuclease activity in an attempt to discover a system to inhibit or remove the endogenous ribonuclease activity during the isolation of microsomes and ribosomes from rat liver, but failed to abolish the ribonuclease activity completely. They found that treatments with sodium dodecyl sulfate, deoxycholate plus heparin, deoxycholate plus treburon, Amberlite XE-64 resin, histidine, or histidine plus EDTA, removed or inhibited the ribonuclease activity partially or almost completely but, unfortunately, the recovery of microsomal RNA and proteins was reduced considerably. When rat liver was homogenized in the presence of optimal concentration of ATP, the microsomes subsequently obtained showed no ribonuclease activity. However, when such microsomes were treated with 1 percent deoxycholate to obtain ribosomes, they exhibited normal ribonuclease activity. This implies that the use of ATP apparently repressed but did not remove the ribonuclease activity. Recently, the intracellular rat liver ribonuclease inhibitor (64) has been employed to prevent the degradation of polyribosomes during the isolation procedure. Indeed, Gribnau (107) obtained higher yield of intact polyribosomes utilizing the intracellular ribonuclease inhibitor.

(E) Significance of comparative studies of structures of proteins

A far reaching achievement of protein and enzyme chemistry in relation to genetics and evolution is the information provided for a comparison of the amino acid sequences of a particular protein, such as cytochrome C (108), or of an enzyme, such as pancreatic ribonuclease (54), which are isolated from various animal species. The extensive comparison of sequences of similar proteins for large groups of mammals, so far examined (108), has revealed a maximun variation of amino acid sequence of 11 percent for cytochrome C, 20 percent for hemoglobin α , and 26 percent for hemoglobin β . The variation in amino acid sequences for insulins is even less; i.e. for insulins from horse, ox, sheep, pig, sperm whale and sei-whale (109, 110), only the three amino acid residues in position 8-10 of the A chain are variable.

Pancreatic ribonucleases from diverse species have been shown to represent a homogeneous group of similar enzymes having molecular weights close to 14,000 (54). Beintema at al. (111) reported that in spite of the fact that rat and beef ribonucleases differed in 33% of their amino acids, their active sites were identical. Barnard (54) made a comparison of the partial or complete amino acid sequences of ribonucleases from the pancreas of beef, rat, horse, sheep and kangaroo, and found that short segments involving the active sites, which contained histidine-12, histidine-119 and lysine-41, were invariant. The degree of variation in other sequences, beside the active sites, was fairly large. Changes in

the amino acid residues were observed in 35 percent of the positions of cow and rat ribonucleases. The variations of horse and porcine pancreatic ribonucleases is also relatively large.

Although certain similarities are found among pancreatic ribonucleases from different species of mammals, a great number of diverse ribonucleases have been found in animals other than mammals, and also in plants or micro-organisms. All the ribonucleases in mammals so far studied have a molecular weight of approximately 14,000. There are two classes of ribonucleases in plants or micro-organisms: (i) small molecules of 14,000 molecular weight with a high degree of base specificity and (ii) enzymes of molecular weight of 20,000 to 40,000 with low specificity toward the base of RNA. The extracellular ribonuclease from Bacillus Subtilis (112) has almost the same molecular weight as bovine pancreatic ribonuclease, but their amino acid compositions and peptide fingerprints are strikingly different.

The structural similarities of various mammalian ribonucleases have also been examined in terms of their cross-reactivities with antisera against ribonucleases. Roth (113) reported that rabbit anti-bovine pancreatic RNase did not inhibit the activities of alkaline and acid ribonucleases of rat liver. Brown et al. (114) found that antibodies to bovine pancreatic ribonuclease, produced in the first course immunization of rabbit, cross-reacted with ovine ribonuclease but not with porcine ribonuclease. However, some cross-reactivity with porcine ribonuclease was found with antibodies produced during the second-course immunization. The

low cross-reactivity of porcine pancreatic ribonuclease with antibodies against bovine pancreatic ribonuclease has been attributed to the considerable amount of carbohydrate attached to the porcine ribonuclease (115), which may lead to masking of some antigenic determinants. Morikawa (116) reported that rabbit antibodies to bovine alkaline ribonuclease strongly inhibited the alkaline ribonuclease activities of human, rabbit, rat, mouse or guinea pig liver. On the other hand, rabbit antibodies to bovine spleen acid ribonuclease inhibited the acid RNase activities of human, rat or mouse spleen only moderately. The studies of cross-reactivities of rabbit antibodies to rat liver RNase and antibodies to rat pancreatic RNase with rat ribonucleases isolated from different organs revealed the existence of two families of ribonucleases in rat, one comprising the enzymes of kidney and liver, the other those of the pancreas and spleen (23). The amino acid sequences of bovine and ovine pancreatic ribonucleases differ only at three residues (28, 117) and consequently these ribonucleases show a reaction of identity with the antibodies against bovine pancreatic ribonuclease (114). The immunochemical cross-reactivity of various ribonucleases indicates partial or complete similarities of the amino acid sequences and their tertiary structures in the regions of antigenic determinants, and hence provides useful informations for the elucidation of the configuration of related ribonucleases.

(F) Some concepts of immunology and immunochemistry

The introduction of a foreign substance, usually a natural macromolecule such as a protein, a polysaccharide or a conjugated lipid, into an animal induces the production of globular serum proteins which possess the unique property of combining specifically with the foreign substance (118). These serum proteins are known as antibodies and the foreign substances responsible for their production are known as antigens. This property of provoking the synthesis of antibodies is called immunogenicity (119). In contrast, the term antigenicity refers to the capacity of substances to react with homologous antibodies. Although immunogenicity is normally associated with macromolecules foreign to the host, under special circumstances antibody production may be elicited by the host's own proteins; such as those which (i) have undergone slight physical and/or chemical modification so that they were regarded as being foreign; or (ii) proteins such as thyroglobulin, lens protein or spermatozoa, which are normally stored and secreted away from immunologically competent cells but which may reach these cells following trauma or disease of the corresponding organs (thyroid, ocular lens, testis). Similarly, substances which are not normally immunogenic can be rendered so through chemical modification of their structure. Thus, gelatin which is a poor antigen can be made immunogenic by the introduction of tyrosine residues onto the molecule (120).

The reactive portions of the antigen molecule inducing antibody formation are referred to as antigenic determinants or

antigenic sites. Complex immunogenic substances, such as proteins, may possess several antigenic determinants. In studies with enzymatically degraded albumin it was revealed that at least three antigenically distinct fragments reacted with antibodies produced against the native molecule and that each fragment reacted only with its specific antibody (123).

Antibody-antigen reactions are highly specific inasmuch as antibodies will react only with the antigen used for immunization or with molecules possessing groups which are sterically closely related to the determinant groups of the original antigen used. Obviously, a large protein molecule, such as serum albumin or gamma-globulin, will have a complex configuration and may possess various antigenic determinants (123, 124, 125). However, because of the structural complexity of natural antigens, it is rather difficult, if not impossible, to delineate precisely the determinant group(s) of a natural macromolecule. Therefore, in an attempt to elucidate the characteristic features of antibody-antigen reactions, notably Landsteiner (126), Haurowitz (127), Pressman (128), Campbell (129), Karush (130) and their associates used chemically well-defined antigens consisting of a small group, such as nitrobenzene or the benzoate, sulphanilate, arsanilate, or trialkyl anilinium ions, coupled to a protein by covalent bonds. Lansteiner coined the term 'hapten' to describe the small molecule which becomes immunogenic only when coupled to a large carrier molecule, usually a protein. The injection of the hapten by itself does not lead to the production of antibodies, unless the hapten becomes

coupled to the host's own proteins. Furthermore, if free hapten is added to its homologous antiserum, no precipitation results. On the other hand, if hapten-protein conjugate possessing more than one hapten molecule per molecule of conjugate is added to the homologous antihapten antiserum, precipitation of complexes of antibodies with the hapten-protein conjugate occurs according to the laws of the normal precipitin reactions. Moreover, this precipitation can be inhibited or the precipitate formed can be redissolved if the free hapten is added prior to, or after, the addition of the polymeric hapten-conjugate, respectively.

Following stimulation with an immunogen, animals will synthesize globular serum proteins, called antibodies, which possess the unique property of combining specifically with homologous antigens or related materials with similar stereochemical properties. This specificity is a reflection of steric complementarity between the antigenic determinant groups anf the antibody combining sites.

The introduction of electrophoretic techniques (131) led to a partial resolution of the complex spectrum of serum proteins, and to the demonstration that antibody activity was associated with a wide spectrum of globulins (132). By immunoelectrophoresis (133) the globulins have been further resolved into antigenically distinct classes. These antigenic differences of immunoglobulins are also referred to as isotypic specificities, i.e. they are common to all individuals of the same species and differentiate classes and types of immunoglobulins (134, 135). For example human immunoglobulins have been designated as IgG, IgA, IgM, IgD and IgE

(136, 137, 138, 139, 140, 141, 142) which differ in their physico-chemical and immunochemical properties as shown in Table 1 which was compiled by Kisil (143).

The majority (80%) of the immunoglobulins belong to the class of IgG which is characterized by a molecular weight of about 150,000 and a sedimentation coefficient of approximately 7S (144). IgG has a relatively low carbohydrate content of 2% (145). At pH 8.6 it possess the lowest anodic mobility. This property of low net negative charge is exploited for its isolation and purification employing ion-exchange chromatography on DEAE-cellulose (146, 147, 148). Antibodies against gram positive bacteria, viruses, and toxins are associated with IgG.

The abundance of IgG in serum has greatly facilitated structural studies of the immunoglobulin molecule. The IgG molecule was shown to consist of two types of polypeptide chains, termed heavy (H) and light (L) chains (149). Each of the two H chains had a molecular weight of the order of 55,000 and possessed 95% of the total carbohydrate content of intact IgG, and each of the two L chains had a molecular weight of about 20,000 (150).

On the basis of results obtained from enzymatic, chemical and immunochemical studies the structural relationships illustrated in Fig. 2 have been established (151). Thus characteristic antigenic determinants of IgG are located in the Fc portion of the H chains, while the remaining portion of the H chain, known as Fd fragment (152, 153), is located in the Fab fragments contiguous with the L chain. By reduction of Fab, Fd is dissociated from the L chain.

TABLE 1

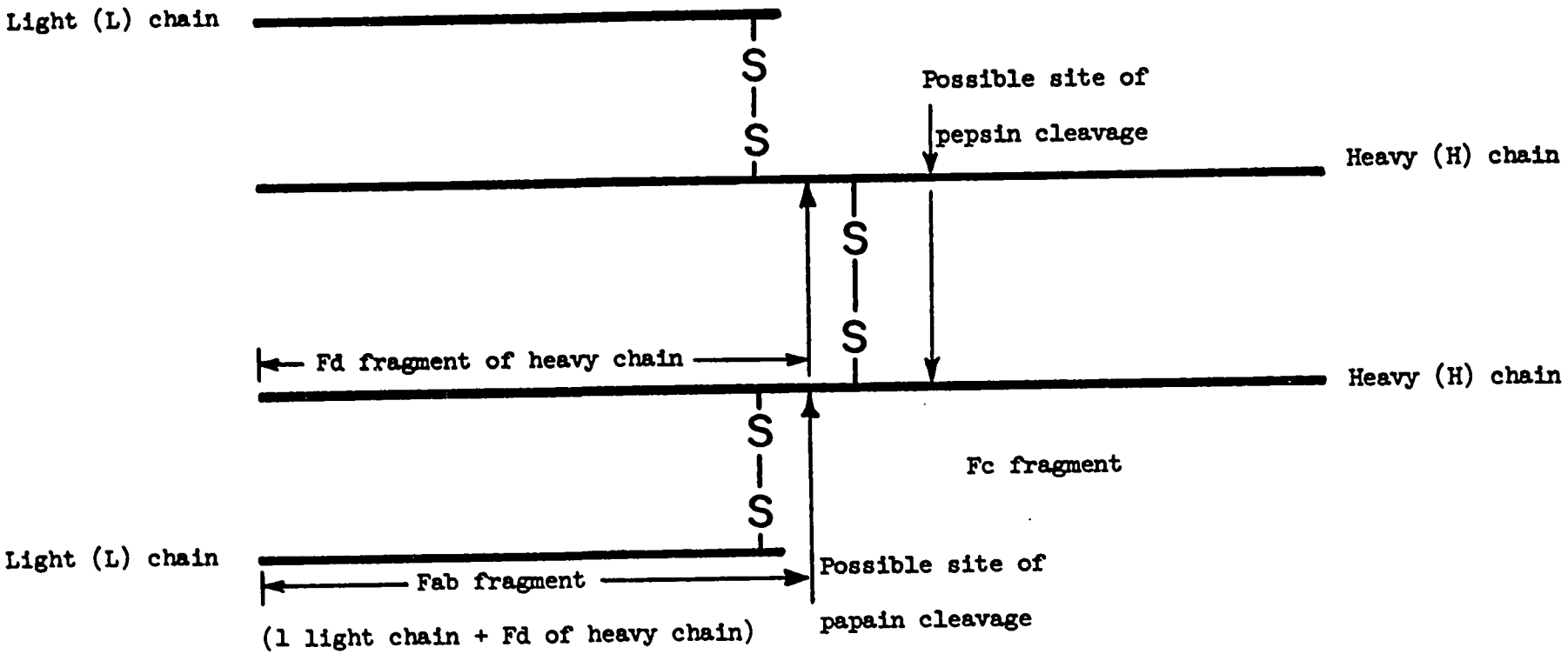
Summary of some properties of human immunoglobulins

	IgG	IgA	IgM	IgD	IgE
Synonyms	γG, $7S\gamma$, γ_2, γ_{ss}	γA, $\beta_2 A$, $\gamma_1 A$	γM, $19S\gamma$, $\beta_2 M$, $\gamma_1 M$	γD	γE, IgND
Physicochemical					
Sedimentation constant	6.5-7.0	7, 10, 13, 15, 17	18-20, 30	6.2-6.8	8
Molecular weight	150,000	180,000: 385,000*	900,000	183,000	196,000
Total carbohydrate (%)	2.9	7.5	11		10
Immunochemical					
Heavy-chain classes	γ	α	μ	δ	ε
Light-chain types	κ, λ	κ, λ	κ, λ	κ, λ	κ, λ
Molecular formula	$\gamma_2\kappa_2$, $\gamma_2\lambda_2$	$\alpha_2\kappa_2$, $\alpha_2\lambda_2$	$(\mu_2\kappa_2)_5$; $(\mu_2\lambda_2)_5$	$\delta_2\kappa_2$, $\delta_2\lambda_2$	$\varepsilon_2\kappa_2$, $\varepsilon_2\lambda_2$
		$(\alpha_2\kappa_2)T^*$ $(\alpha_2\lambda_2)T^*$			
Allotypes					
Gm specificity (γchain)	yes	no	no	no	
Inv specificity (κchain)	yes	yes	yes	?	
Number subclasses known	4	2	2		
Biological					
Serum Conc. (mg%)	800-1680	140-420	50-190	0.3-10	0.0017-0.006
Synthesis rate (mg/kg/d)	20-40	2.7-5.5	3.2-16.9	0.03-14.9	
Catabolic rate (% I.V. pool/d)	4-7	14-34	14-25	18-60	
Distribution (% in I.V. pool)	48-62	40	65-100	63-86	
Antibody activity	yes	yes	yes	yes	yes
Placental passage	yes	no	no	no	no
Presence in cerebrospinal fluid	yes	yes	no		
Skin sensitization					
heterologous species	yes	no	no		yes
homologous species	no	?	no	no	yes
Complement fixation	yes	no	yes		no?

* In exocrine secretions, IgA has a dimeric structure and an attached T chain.

Figure 2

Schematic structure of IgG.


Light (L) chain
S
S
Possible site of
pepsin cleavage
Heavy (H) chain
S
S
Fd fragment of heavy chain
Heavy (H) chain
S
S
Fc fragment
Light (L) chain
Possible site of
papain cleavage
Fab fragment
(1 light chain + Fd of heavy chain)

Enzymatic digestion with papain cleaves the 7S IgG molecule into three 3.5S fragments which can be separated by ion-exchange chromatography on CM-cellulose (152). By this method, two Fab fragments and one Fc fragment were obtained from each IgG molecule. Each of the Fab fragments has a molecular weight of 52,000 and is univalent since it contains only one antibody combining site. On the other hand, the Fc fragment is devoid of antibody activity, but carries most of the antigenic determinants unique to IgG and can be crystallized under appropriate conditions (153).

The injection of a complex antigen into as animal may elicit several antibodies with different specificities, i.e. antibodies formed against different determinant groups of the antigen molecule (154). Thus, for example, antisera prepared by immunization with hapten-protein conjugates contain antibodies directed not only against the haptenic group but also against different regions of the protein carrier. Obviously, as also discussed in Section (H), a spectrum of antibodies with diverse specificities may be expected to be formed against a complex natural antigen possessing different determinant groups. The term monospecific antiserum or antibody will be used throughout this thesis to indicate that the complex population of antibodies directed against different determinants of an antigen, consisting a single polymeric chain, gives rise to only one precipitin band on diffusion in agar gel against the pure antigen or against the crude spleen extract.

In support of the concept that multiple antibodies may be produced against a complex natural antigen, Lapresle (155) demonstrated that with a seemingly homogeneous antigen, such as human serum albumin, the formation of several distinct antibodies directed against different determinant groups of the same molecule was induced. On enzymatic degradation of the antigen molecules, the determinant groups were present on distinct parts of the molecules (155). Thus, he showed that the molecule of human serum albumin contained at least three different antigenic sites (156). Similarly, human serum gamma-globulins were shown to possess at least four antigenic determinants (157). Richter and Sehon (158) demonstrated that as long as the different determinant groups are part of one molecule, i.e. two different haptens (p-aminobenzoic acid and p-sulphanilic acid) coupled to the same protein molecule (human serum albumin), the corresponding antigen-antibody system gives rise to a single precipitin band on immunodiffusion in agar gel thus masking the heterogeneity of the antigen-antibody systems, although in some cases this might be revealed by the more painstaking quantitative precipitation test of Heidelberger in the form of multiple 'optimal zone' of precipitations.

There is a close similarity between antibody-antigen interactions and enzyme-substrate associations. In each of these systems at least one of the reaction partners is a macromolecule, i.e. the antibody or the enzyme molecule, respectively; the second reaction partner (antigen or hapten, or substrate molecule) may be a small or a polymeric molecule. As in most biological systems, the most important physicochemical feature underlying these reactions

is their specificity (159, 160, 161, 202). The specific interaction between antibodies and their appropriate antigens may be manifested in vitro in many ways; below are outlined briefly some of the immunochemical methods used in this study.

(a) Precipitation

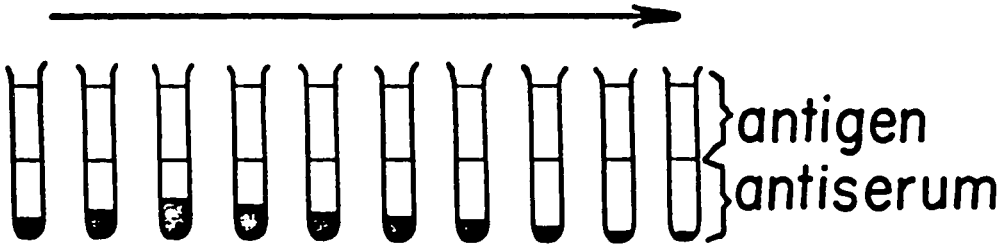
The most commonly detected reaction in vitro between antigen and antibodies is the formation of flocculent precipitate. In general, the amount of precipitate formed on addition of increasing amounts of antigen to a constant amount of antiserum is represented quantitatively by a typical precipitin curve as shown in Fig. 3. The amount of precipitate, consisting of both antigen and antibodies, increases at first in the antibody excess zone, reaches a maximum at the equivalence zone where both reactants are precipitated quantitatively, and decreases in the antigen excess zone where precipitation is progressively inhibited. On the supposition that both antibody and antigen molecules have more than one combining site, the general features of this curve could be explain in terms of the framework theory (162, 163, 164). According to this theory, in the region of antibody excess the precipitate consists of small aggregates composed primarily of antibody molecules cross-linked by a small number of antigen molecules. In the region of maximum precipitation the antibody-antigen complexes are cross-linked into large and more compact aggregates, consisting of an alternating and recurring antibody-antigen pattern. Addition of more antigen than that required to combine with all antibody sites

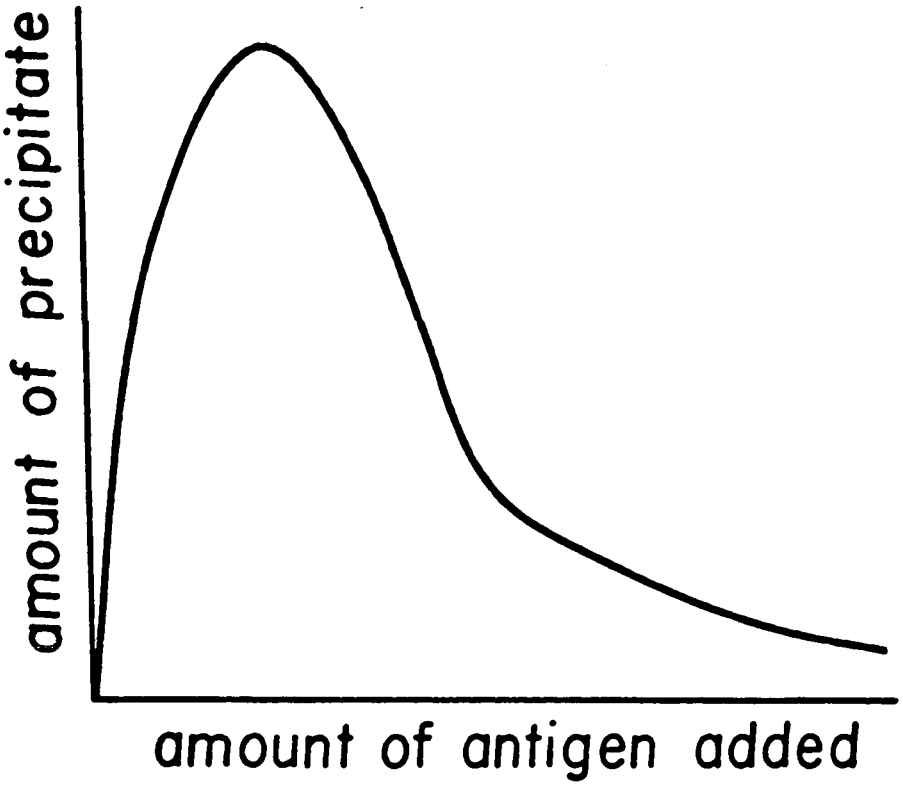
Figure 3

Typical precipitin curve.

TYPICAL PRECIPITIN CURVE

Increasing amounts of antigen added to constant amount of antiserum





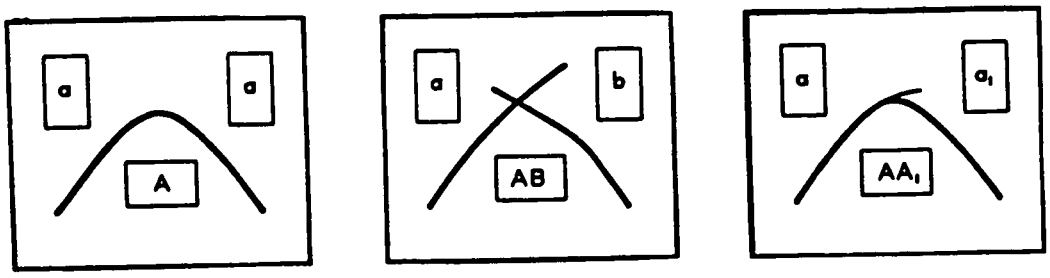
results in the disruption and loosening of this compact, regular framework and in the formation of smaller aggregates. In the limit, in excess antigen, only small complexes are formed consisting of one antibody molecule combined with the number of antigen molecules equivalent to the combining sites of the former; no cross-linking of these can occur and these complexes remain in solution.

This theory has been fully confirmed by experimental data that most antigens were shown to be multivalent; for example the valencies of ovalbumin, thyroglobulin and viviparus hemocyanin were calculated as 5, 40 and 231 respectively (165). and precipitating antibodies were shown to possess two combining sites (166, 167, 168).

(b) Immunodiffusion

Besides an antigen-antibody precipitin reaction in liquid medium, this reaction can also take place in a semi-solid media, such as agar, agarose or polyacrylamide gel. Because of the loose texture of gels, the latter permit the diffusion of antigen and antibodies. Thus, when antigens and antibodies are allowed to diffuse toward each other in a gel, precipitin bands are formed at positions where the optimum concentrations of homologous antigens and antibodies are reached. Furthermore, the gel limits the diffusion of the antigen-antibody precipitate and confines the latter to a narrow region. Many variations of the gel diffusion methods exist. However, the most widely used are simple or double diffusion in tubes (169) and double diffusion in two dimensions using gel on plates (170, 171, 172, 173).

The Ouchterlony's double diffusion method (170, 171) is the simplest and most convenient technique for demonstration of immunochemical relationships between soluble antigens. Precipitates in double diffusion plates are commonly identified by the use of a known reference antigen or antibody in the same plate. As a guide to the interpretation of comparative immune precipitation patterns on double diffusion, Ouchterlony (174, 175, 176, 177) originally described three types of reactions, a fourth one being subsequently added. The basic three patterns are illustrated in Fig. 4 and the terms identity, nonidentity and partial identity were suggested to describe the underlying antibody-antigen interactions. In Type I reaction, a complete fusion of two precipitin bands is observed when two antigens with identical antigenic determinants react with the same antiserum. In Type II reaction, two unrelated antigens react with their specific antibodies respectively. The independence of the precipitating systems characterized by the crossing of the precipitin lines is usually obvious as no deviation or fusion phenomena are produced. In Type III reaction, a spur, on the arc of the partial fusion in the pattern, is produced when serologically related antigens are compared by means of a suitable immune serum. The size and the direction of the spur indicate the degree of the relationship between the antigens compared. If the relationship is close a small spur with a marked deviation is produced. When the similarity is less, the spur is bigger and shows less deviation.



I II III

Fig. 4 Basic precipitation patterns on double diffusion.

Symbols: a and b represent independent antigens.

a and a_1 are cross-reacting antigens.

A, A_1 and B are antibodies against corresponding antigens a, a_1 and b.

(c) Immunoelectrophoresis

By combining electrophoresis with precipitation in agar gel, Grabar and Williams (178, 179) developed a simple but extraordinarily powerful method for identifying antigens of antibodies in complex mixtures. The method consists of first performing electrophoresis of either the antigen or antibody preparation in agar gel and then allowing the homologous antisera or antigen respectively, to diffuse perpendicularly to the electrophoretic migration axis. Using this method antibodies may be exploited with respect to their charge and immunological specificity at the same time.

(G) Immunochemical studies of ribonucleases

Enzymes are capable, as other proteins, of eliciting the production of antibodies in animals into which they are injected and the corresponding antibodies have been reported to inhibit specifically their enzymatic activity. However, recently the production of antibodies having the ability of enhancing enzymatic activity has been reported for some antibodies against amylase (180), penicillinase (181, 182) and ribonuclease (183, 184). This implies that the binding of some antibodies with certain antigenic sites of the enzyme results in conformational changes favouring the accessibility of the active site to the substrate molecules.

The inhibitory capacities of antibodies against different types of enzymes vary from zero to 100 percent (183). The extent of inhibition depends on the molecular weights of the substrates

(183, 185) suggesting that steric hindrance around the active site due to the combining antibodies may be one of the factors responsible for the apparent loss of enzymatic activity. Thus, Cinader (185, 186) reported that bovine pancreatic ribonuclease was inhibited by antibodies to a greater extent if macromolecules, such as RNA, were used as substrate rather than the small 2,3-cyclic-cytidylic acid and suggested that the binding of antibodies with ribonuclease prevented the substrate from reaching the catalytic site. Later, the studies of the inhibitory capacity of univalent and divalent antibodies to ribonuclease (186) and to phosphorylase (187) indicated that aggregate formation did not play a major role in the inhibition of enzymes acting on substrates of low molecular weight.

The variation in the inhibitory capacity of anti-enzyme antibodies is presumably due to the fact that a complex mixture of antibodies directed against different determinants on a protein antigen is always produced (154). Thus, the antibodies with different specificities exert obviously different effects on the activity of the enzymes. It is likely that antibodies combining with determinants close to the catalytic site would inhibit the enzyme more effectively than antibodies directed against determinants remote from this site. However, one may also visualize that combination of enzyme with antibodies may lead to conformational changes resulting in a distortion of their active site. Recently it has been demonstrated by Suzuki et al. (188) that rabbit anti-bovine pancreatic ribonuclease antibodies could be fractionated on DEAE-Sephadex A-50 into twelve fractions; one of these fractions enhanced ribonuclease activity,

whereas the other fractions inhibited the enzymatic activity to vatious extent.

The antigenic properties of ribonuclease has been extensively studied by Brown (79, 114, 189, 190) and others (185, 191). Brown et al. (190) suggested that bovine pancreatic ribonuclease A has two or three antigenic determinants, whereas Stelos et al. (191) reported a value of four. Brown and co-workers (79, 114, 190) studied also the immunochemical reactivity between some chemically modified ribonuclease derivatives and the antibodies against native ribonuclease. They reported that guanidination of ribonuclease led to the decrease of reactivity. Removal or modification of the α -amino group, as well as the deamination of two lysine residues near the N-terminus of the molecules reduced the immunochemical reactivity. However, performic acid oxidized or acetylated ribonucleases did not react with the antibodies against native ribonuclease. Later, Brown (192) demonstrated that certain peptides from bovine pancreatic ribonuclease reacted with antibodies elicited by performic acid oxidized ribonuclease, but not with antibodies against native ribonuclease. The attachment of poly-DL-alanine side chains to native and oxidized ribonucleases produced modified ribonucleases that reacted poorly with antibodies against the unmodified ribonuclease (193). From the results of immunological studies of bovine ribonuclease, Brown (80) reported that the antigenic and enzymatic active sites of the ribonuclease were not identical. Richards et al. (194) demonstrated that ribonuclease S showed weaker precipitin reaction than the unmodified ribonuclease with antibodies against intact ribonuclease, and the S-protein

gave still weaker reaction. The S-peptide which consisted of only 20 amino acid residues did not show, as would be expected, any precipitate at all. However, the absence of precipitate does not imply the non-reactivity between S-peptide and the antibodies. Since a mixture of antibodies directed against different portions of the enzyme molecule is always produced during the immunization (154), a small population of antibodies might be directed against the S-peptide. If only one antigenic determinant is located in the S-peptide which is a small molecule of only 20 amino acid residues, the binding of S-peptide with antibodies would not produce any precipitate. This speculation is supported by the work on antibodies against egg-white lysozyme reported by Arnon (195). A short peptide of amino acid sequence 64-83 obtained from lysozyme digested by pepsin was attached to branched polyalanine to form a conjugate. Immunization of rabbits with this conjugate led to the formation of antibodies which reacted with lysozyme. These antibodies could not give any visible precipitates, as they reacted with only one region within the lysozyme molecule. However, the reaction was demonstrated by the capacity of these antibodies to bind radioactive lysozyme. Moreover, these antibodies could be adsorbed and purified by an immunosorbent prepared by attachment of lysozyme to cellulose covalently.

The study of antigenicity of Staphylococcal nuclease, which possessed both ribonuclease and deoxyribonuclease activities, has recently been reported by Anfinsen (196, 197). The performic acid oxidized Staphylococcal nuclease, which retained only 8% of the

enzymatic activity, cross-reacted identically as the native nuclease with antibodies against the native as well as the oxidized nucleases. This finding contradicts that observed with bovine pancreatic ribonuclease. Since performic acid oxidizes only cystine, cysteine, methionine and tryptophan without altering other amino acids, the discrepancy can be explained by the fact that Staphlococcal nuclease, lacking disulfide bridges and having low helix content, is a highly flexible polypeptide chain. The oxidation of bovine pancreatic ribonuclease with performic acid ruptures the intramolecular disulfide bonds and leads to the disruption of its three dimensional structure. As a result, the oxidized ribonuclease loses the ability to react with the antibodies. On the other hand, performic acid oxidation of Staphylococcal nuclease alters only the methionine and tryptophan residues which involve in a very small portion of the enzyme molecule, and consequently retains the antigenicity. Studies on acetylated and trifluoroacetylated Staphylococcal nuclease (196) showed that the enzymatic and antigenic activities decreased with the increasing extent of substitution.

Anfinsen et al. (197) also studied the antigenicity of some peptide fragments of Staphylococcal nuclease prepared by cyanogen bromide cleavage, by limited tryptic digestion, and also by solid phase synthesis, and found that the antigenic determinants might be located around the carboxy-terminal and the amino-terminal of the polypeptide chain. Brown (192) showed that the peptides of the carboxy-terminal sequence 105-124, as well as the sequence 38-61 of the bovine pancreatic ribonuclease A,

were capable of inhibiting the reaction of performic acid oxidized ribonuclease with its antibodies.

Merigan et al. (198) reported that denaturation of RNase S and S-protein by heat or exposure to urea induced complete loss of precipitin formation between these ribonuclease derivatives and the anti-ribonuclease A serum. Brown et al. (79) showed that alkaline-denatured ribonuclease reacted poorly with the antibodies and also that the disulfide bonds of the ribonuclease molecule were essential for antigenic activity. All these results imply that perservation of the intact three dimensional structure of ribonuclease is essential for the demonstration of antibodies elicited against the native form of the ribonuclease molecule and possibly also for its immunogenicity. This view is further supported by the work of Mills et al. (199) who demonstrated that when the three dimensional structure of ribonuclease was altered by reduction and reoxidation, it became unreactive to antibodies elicited against native ribonuclease.

(H) The use of immunosorbents for the isolation of specific antibodies and antigens

In early studies, antigens adsorbed on various supporting media such as kaolin (200), charcoal (201) and glass beads (202) were used for removing antibodies specifically from the corresponding antisera. More efficient methods by the coupling of diazotized haptens covalently to red-blood-cell stroma were also developed and used for the isolation of anti-hapten antibodies (203, 204, 205, 206, 207). The coupling of protein antigens to polyamino-polystyrene via azo bonds to form polystyrene-antigen conjugates has been employed in many laboratories for the isolation of antibodies (208, 209, 210, 211, 212, 213). However, this method was shown to have the disadvantage of non-specific adsorption through the formation of hydrophobic bonds (214, 215). On general grounds of physical chemistry of proteins, immunosorbents prepared with hydrophilic polymers as insoluble backbones are to be preferred to those involving the use of hydrophobic supporting materials (216).

The isolation of antibodies with specific immunosorbents has been reviewed extensively by Sehon (217), Gurvich (218), and more recently by Campbell et al. (219) and Stelos (220). Cellulose was found to be a good supporting material for the preparation of immunosorbents because of its enormous surface area and its hydrophilic nature (216). Since the first report of a general method for coupling antigens to cellulose for the isolation of anti-protein antibodies by Campbell et al. (221),

a great number of methods have been developed for the covalent attachment of antigens or antibodies to cellulose or its derivatives, such as carboxymethyl cellulose (222), bromoacetyl cellulose (223, 224). Antigens or antibodies, as the case may be, were specifically absorbed and subsequently eluted at pH 2-3 in high yields and high purity.

Immunosorbents have also been produced recently by polymerizing the antibodies or antigens with bi-functional or multi-functional molecules such as ethyl chloroformate (41), glutaraldehyde (225), N-acetyl homocysteine thiolactone (226) and ethylene maleic anhydride copolymer (42, 43). Avrameas et al. (41, 225) reported that insolubilization of protein antigens or antibodies by cross-linking covalently with ethyl chloroformate or glutaraldehyde produced efficient, specific and stable immunosorbents which could be used either in a columnar procedure or in batchwise operation for the isolation of specific antibodies or antigens. Coupling of enzymes such as peroxidase, glucose oxidase, tyrosinase and alkaline phosphatase with glutaraldehyde to some immunoglobulins of man, sheep, or rabbit produced stable conjugates which retained their immunological and enzymatic activity (227). The resulting conjugates were utilized for the intracellular detection of antigens or antibodies and also for the characterization of antibodies after immunoelectrophoresis.

Since immunization of an animal with a protein antigen usually elicits a mixture of serum antibodies against different determinants on the protein antigen (154), several methods have

been developed for the separation of these antibodies (188, 228, 229, 230). When sperm-whale apomyoglobin was digested with α-chymotrypsin, five immunologically active peptides were isolated (230). One of these peptides was shown to be the C-terminal heptapeptide. Givas et al. (228) synthesized the heptapeptide by Merrifield solid-phase method and used this synthetic peptide to elute the corresponding monospecific antibodies from an immunosorbent consisting of myoglobin coupled to ethylene maleic anhydride copolymer. The antibodies eluted with the heptapeptide were restricted to a narrow range of electrophoretic mobilities in polyacrylamide gel, which is indicative of a pure protein. Moreover, while these antibodies (labelled with ^{131}I) could be coprecipitated with the heterogeneous population of antibodies to the whole myoglobin molecule, they alone did not give a precipitate with myoglobin. This observation was considered as evidence for the monospecificity of the antibodies eluted with the heptapeptide, i.e. they were directed solely against the univalent antigenic determinant corresponding to the region of the C-terminal heptapeptide of myoglobin. Recently the native Staphylococcal nuclease or the C-terminal portion (residues 49-149) of this nuclease obtained by limited trypsin digestion, was coupled covalently to Sepharose and the resulting immunosorbents were employed to isolate monospecific antibodies (229). The presence of antibodies directed at the C-terminal portion of the nuclease molecule was demonstrated.

Heterogeneity of anti-hapten antibodies has also been demonstrated with the help of immunosorbents (205, 231, 232, 233). Edelman et al. (233) separated guinea pig anti-DNP antibodies into two fractions by eluting antibodies specifically adsorbed on DNP-fibrinogen. One fraction was eluted with dinitrophenol and the other eluted by N-DNP-ε-lysine. Froese et al. (205) reported that diazotized haptens were coupled to red-blood-cell stroma and the conjugates used for the absorption of anti-arsanilate antibodies which were subsequently eluted with solutions of the hapten. Moreover, they showed that some of the antibody sites possessing high affinity for the hapten had been blocked by the eluting hapten, thus diminishing the apparent purity of the eluted antibodies as measured in terms of their precipitability.

Kreiter et al. (231) reported that rabbit anti-p-azobenzene-arsonate antibodies were separated into a number of fractions, at least six, by adsorption on a specific immunosorbent followed by stepwise elution with increasing concentrations of benzenearsonate over the range of 10^{-6} to 10^{-1} M. It is evident that the use of specific immunosorbents for the isolation of antibodies or antigens has many advantages, i.e. they provide a simple and rapid procedure for the isolation of antibodies in high yield and with high purity.

CHAPTER III

ISOLATION AND PURIFICATION OF RABBIT RIBONUCLEASES BY PHYSICOCHEMICAL METHODS

INTRODUCTION

Extensive chemical and immunochemical studies on ribonucleases have been conducted, since these enzymes play an important role in nucleic acid metabolism and protein biosynthesis. Many types of ribonucleases from various sources have been purified and well characterized. Besides the well known bovine pancreatic ribonuclease (20, 21, 77, 234, 235, 236), ribonucleases have been isolated from organs of other mammals such as man (22, 46, 237), rat (23, 24, 238, 239), mouse (25, 47), guinea pig (26, 240), pig (27, 241, 242), sheep (28), dog (29) and whale (30); from plants (31); from invertebrates, like squid (32) and sea urchin (243, 33), and from micro-organisms, such as yeast (34) and fungi (35). The physicochemical properties and enzymatic specificities of the isolated enzymes have been investigated in great detail. Some of these enzymes especially bovine and rat ribonucleases have also been characterized immunochemically (185, 244, 245). It has been shown that ribonucleases isolated from different organs of the same species may have different immunochemical (244) and physicochemical properties, such as electrophoretic mobility, optimum pH and amino acid composition (244, 246, 247).

At least three types of bovine ribonucleases which differ in physicochemical properties, have been isolated from bovine pancreas, spleen (246) and aorta (247), respectively. Bovine milk ribonuclease was shown to be identical to pancreatic ribonuclease with respect to electrophoretic and chromatographic behaviour, amino acid composition, specific activity and substrate specificity (248, 249). Gordon (244) reported two immunochemically different families of ribonucleases in the rat, one isolated from kidney and liver, the other from pancreas and spleen.

Rabbit ribonucleases received little attention until recent years. Epshtein (250) found that the ribonuclease in rabbit skeletal and heart muscles showed two peaks of activity at pH 5.5 and 7.5. Jagemann et al. (251) studied the activation and inhibition of ribosomal ribonuclease of rabbit reticulocytes and more recently a partially purified ribonuclease from rabbit reticulocytes has been isolated (38, 39). However, up to the present study none of the rabbit ribonucleases had been isolated in a pure form. In the present study, the procedures for the isolation and purification of rabbit spleen and pancreatic ribonucleases by physicochemical methods are described in some detail.

MATERIALS AND METHODS

TISSUES: Rabbit spleens and pancreas, which had been frozen immediately after sacrificing the animals, were purchased from Pel-Freez Biologicals, Inc., Rogers, Arkansas. The tissues originated from adult animals of mixed breeds and mixed sex.

SUBSTRATES: Highly polymerized yeast ribonucleic acid (RNA) and calf thymus deoxyribonucleic acid (DNA) were purchased from Worthington Biochemical Corporation, Freehold, New Jersey. The RNA was further purified by precipitation in a mixture of ethanol and acetic acid as described by Klee (15). The precipitate after two such treatments was sufficiently free of low molecular weight nucleotides. DNA was used without further purification. Calcium $\left(\text{bis (p-nitrophenyl) - phosphate}\right)_2$ was purchased from K & K Laboratories Inc., Plainview, New York. Disodium p-nitrophenyl phosphate was a product of Schwarz BioResearch Inc., Orangburg, New York.

CHEMICALS: All other chemicals used were of reagent grade unless otherwise specified. Sephadex G-75, DEAE-Sephadex A-50, SE-Sephadex C-50 and CM-Sephadex C-50 were products of Phamacia, Uppsala, Sweden. The Folin-Ciocalteu reagent used for protein determination (255) was purchased from Fisher Scientific Company.

ASSAY FOR RIBONUCLEASE ACTIVITY: The ribonuclease activity was measured in terms of the amount of acid-soluble oligonucleotides produced from highly polymerized yeast ribonucleic acid as described

by Kalnitsky et al. (252) with some modifications. The reaction mixture consisted of 1.0 ml of 0.15 M phosphate buffer, pH 7.3, 1.6 mg of yeast ribonucleic acid in 0.4 ml of the phosphate buffer and 0.2 ml of diluted enzyme solution. After incubation of the reaction mixture for 30 minutes at 37° C, 0.4 ml of the uranyl reagent, consisting of a solution of 0.75% uranyl acetate in 10% trichloroacetic acid, was added to stop the reaction and for precipitation of the undegraded ribonucleic acid. The precipitate was centrifuged off at 1,000 x G for 10 minutes at 4° C. A portion (0.2 ml) of the supernatant was promptly diluted to a volume of 3.0 ml with distilled water, and the optical density at 260 mμ was read against a blank processed in the same manner except that 0.2 ml of buffer was used in place of the diluted enzyme solution. Thus, one enzyme unit is defined as the amount of enzyme causing an increase in the optical density at 260 mμ of 1.0, which is due to the amount of acid-soluble oligonucleotides liberated under the assay conditions described.

ASSAY FOR DEOXYRIBONUCLEASE ACTIVITY: The assay procedure was the same as described above for the assay of ribonuclease activity except that highly polymerized calf thymus deoxyribonucleic acid was used as a substrate in place of RNA. In addition, the buffer (0.1 M acetate buffer, pH 4.5) contained 0.01 M magnesium chloride. The unit of DNase activity was defined as for RNase.

ASSAY FOR ACID AND ALKALINE PHOSPHATASE ACTIVITIES: The method described by Koerner et al. (253) was followed. A reaction mixture of a total volume of 1.5 ml containing 1.0 ml of 0.002 M disodium p-nitrophenylphosphate, 0.1 ml of 2 M buffer (acetate buffer at pH 5.5 was used for acid phosphatase, whereas Tris buffer at pH 9.0 was employed for alkaline phosphatase) and 0.4 ml of suitably diluted enzyme solution (0.1-1.5 mg/ml) was incubated at 37°C for 30 minutes. The reaction was stopped by addition of 1.5 ml of 1 M ammonium hydroxide. The absorbancy at 440 mμ, read against the reagent blank which had been treated in the same way but without the enzyme, was defined as the total phosphatase unit in the sample.

ASSAY FOR PHOSPHODIESTERASE: The method of Koerner et al. (253) was employed. A mixture of 0.1 ml of 2 M buffer (acetate buffer at pH 4.5 for acid phosphodiesterase and Tris buffer at pH 9.0 for alkaline phosphodiesterase), 1.0 ml of 0.001 M calcium $\left[\text{bis (p-nitrophenyl) phosphate}\right]_2$, and 0.4 ml of a diluted enzyme solution (0.1-1.5 mg protein/ml) was incubated for 12 hours at 37°C. The reaction was stopped by the addition of 1.5 ml of 1 M ammonium hydroxide. The optical density was measured at 440 mμ against a blank without enzyme but had been treated identically. The optical density obtained at 440 mμ was defined as the number of phosphodiesterase units in the sample.

DETERMINATION OF PROTEIN CONTENT: The protein concentration was estimated with the Folin-Ciocalteu reagent (254) following the procedure of Lowry (255).

The following reagents were used for determination of the protein concentration:

Reagent A: 2.0% $NaCO_3$ in 0.1 N NaOH.

Reagent B: 0.5% $CuSO_4 \cdot 5H_2O$ in 1.0% sodium citrate.

Reagent C: Alkaline copper solution. It was prepared by mixing 50 ml of reagent A with 1.0 ml of reagent B, and was discarded after one day.

Reagent D: two fold diluted Folin-Ciocalteu reagent with distilled water.

A volume (1.0 ml) of the suitably diluted protein solution (0.01-0.1 mg protein/ml) was mixed well with 5.0 ml of reagent C. After standing at room temperature for 10 minutes, 0.5 ml of reagent D was added. The optical density was measured in a Unicam spectrophotometer after 30 minutes which was sufficient for the full development of the color. For the range of 5 to 25 μg of protein per ml, the optical density was read at 750 mμ. For more concentrated solutions, the reading was made at 500 mμ.

COLUMN CHROMATOGRAPHY: Gel filtration on Sephadex G-75 was carried out at 4° C in a column of 8 x 100 cm. The column was equilibrated and eluted with 0.15 M sodium phosphate buffer at pH 7.3. The effluent was collected in 20-ml fractions at a flow rate of 2.5 ml per minute. Samples of 2 to 5 gm of proteins in a 100-300 ml volume were fractionated in each experiment. The optical density of the effluent was recorded by an LKB UVicord automatic recorder. The fractions containing RNase activity were pooled.

Chromatography on DEAE-Sephadex A-50 was carried out at 4 °C with a column of 2.5 x 100 cm, equilibrated with 0.01 M Tris buffer at pH 7.4. Samples of 1 gm of protein in 20 ml of the buffer were applied to the column. This buffer was used for elution until the first peak emerged from the column; thereafter, elution was continued with the same buffer admixed with NaCl so as to give a linear gradient with respect to NaCl from zero to 1 M NaCl. The linear gradient was prepared by placing 500 ml of 0.01 M Tris buffer, pH 7.4, in one bottle and 500 ml of 1 M NaCl in the same buffer in another bottle of exactly the same size. These two bottles were connected by glass tubing filled with the buffer. The solution were mixed with a magnetic stirrer in the first bottle. Fractions (10 ml) of the effluent were collected at a flow rate of 1 ml per minute.

Chromatography on SE-Sephadex C-50 was carried out in a 4 x 70 cm column at 4° C. The column was equilibrated and eluted with 0.1 M acetate buffer at pH 4.5. In each experiment 0.5 to 1 gm of proteins in 30 ml of the acetate buffer was applied to the column. After the first protein peak emerged from the column, elution was continued with the same buffer to which NaCl was added to produce a linear gradient up to 1 M NaCl as indicated in Fig. 8. The column effluent was collected in 15-ml fractions at a flow rate of 1.5 ml per minute.

Column chromatography on CM-Sephadex C-50 was carried out in a 2.5 x 70 cm column at 4° C. The column was equilibrated with 0.05 M acetate buffer at pH 4.5. After application of the sample (1 gm of proteins in 20 ml of the acetate buffer), the same buffer was used

to elute the proteins until the first peak emerged from the column. At this point, as above, NaCl was added to the buffer to produce a linear gradient up to 1 M NaCl. Fractions of 20 ml were collected at a flow rate of 1.2 ml per minute.

ELECTROPHORESIS: Cellulose acetate electrophoresis was carried out in barbital buffer of 0.075 ionic strength at pH 8.6 in a Beckman microzone electrophoresis cell, Model R-101. A solution of 1% protein in the same buffer was applied on the membrane. A voltage of 250 V was applied for 30 minutes; the current varying from 3.5 to 5.8 mA during electrophoresis. After the experiment, the membrane was fixed and stained with a solution composed of 0.2% Ponceau-S stain, 3.0% trichloroacetic acid and 3.0% of sulfo-salicylic acid in distilled water. The membrane was rinsed with 5% acetic acid until clear and dried at room temperature.

ANALYTICAL ULTRACENTRIFUGATION: All sedimentation velocity measurements were performed in a Spinco model E optical ultracentrifuge. The enzyme preparation was dissolved in 0.15 M phosphate buffer at pH 7.3 to give a 1% solution. The ultracentrifugation was performed at 59,780 rpm and the rotor temperature was maintained at 20^{o} C.

RESULTS

(A) ISOLATION AND PURIFICATION OF RABBIT SPLEEN RIBONUCLEASE

The methods used for the purification of spleen ribonuclease are summarized in the flowsheet given in Fig. 5. Rabbit spleens (100 pieces) were trimmed and rinsed in cold saline at 4° C. Surgical gloves were employed whenever it was necessary to handle the spleens, in order to avoid contamination of the preparation by 'finger nucleases' (256). After cutting the spleens into small pieces, they were homogenized in a Waring blendor with 3.5 volumes (w/v) of cold 0.25 N sulfuric acid for two minutes. The homogenate was stirred for 24 hours at 4° C and was subsequently centrifuged in a IEC B-20 centrifuge at 13,000 rpm for 30 minutes to remove insoluble materials. After adjusting the pH of the supernatant to 6.0, the resulting precipitate was removed by centrifugation at 13,000 rpm for 30 minutes. This clear solution was subjected to gel filtration on a column (8 x 100 cm) of Sephadex G-75, which was prepared as described by Gelotte (257). The elution profile is shown in Fig. 6; the fractions which contained RNase activity were pooled and dialyzed overnight against distilled water at 4° C to remove salts. Lyophilization of this solution yielded preparation II which contained 5.5 gm of proteins.

A portion of Preparation II (900 mg) was dissolved in 20 ml of 0.01 M Tris buffer at pH 7.4 and applied to a column (2.5 x 100 cm) of DEAE-Sephadex A-50. In Fig. 7 the elution profile is shown; the first peak containing the enzymatic activity (Preparation III) was pooled, dialyzed and lyophilized.

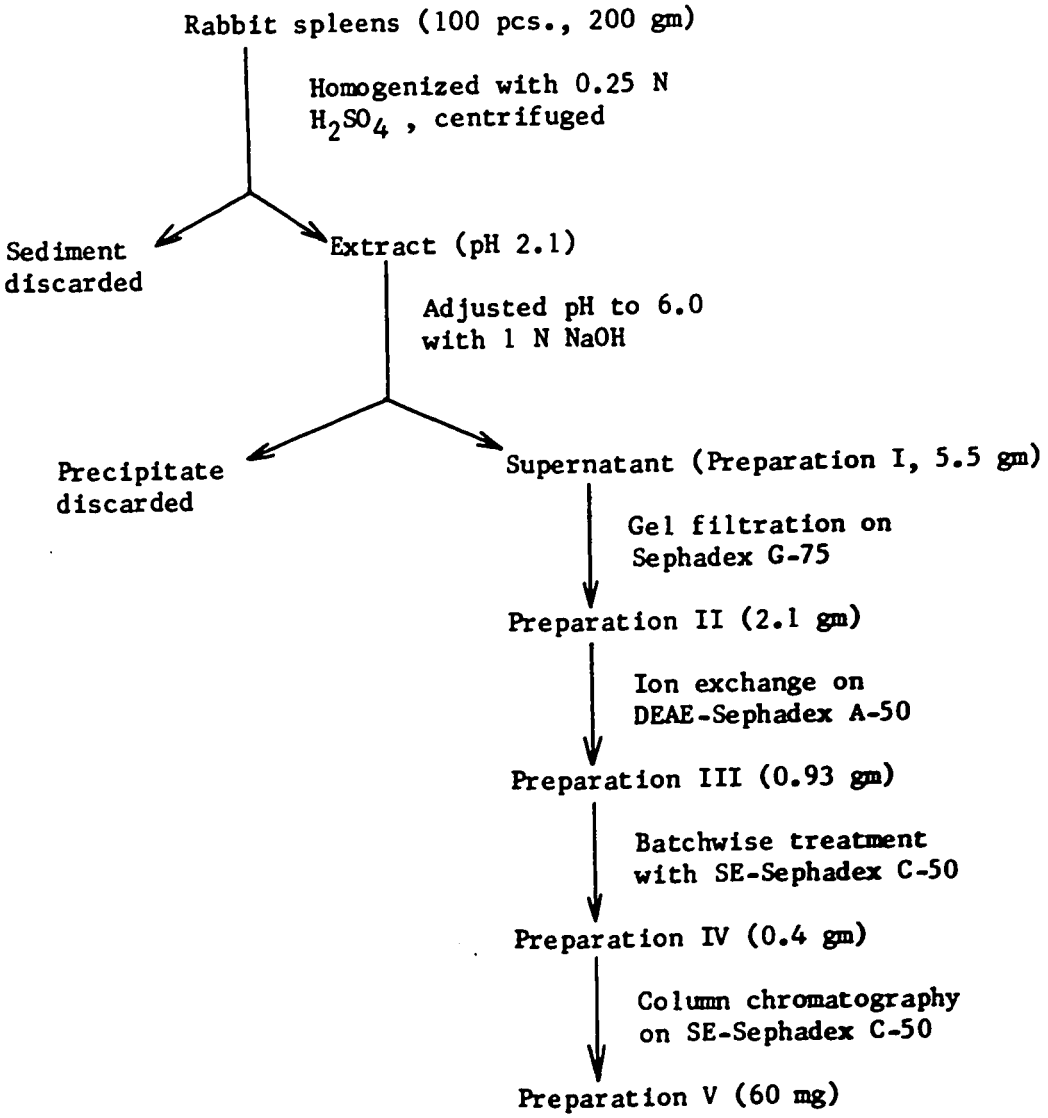


Fig. 5. Flowsheet of purification of rabbit spleen ribonuclease.

Figure 6

Gel filtration of rabbit spleen ribonuclease on Sephadex G-75 column (8 x 100 cm).

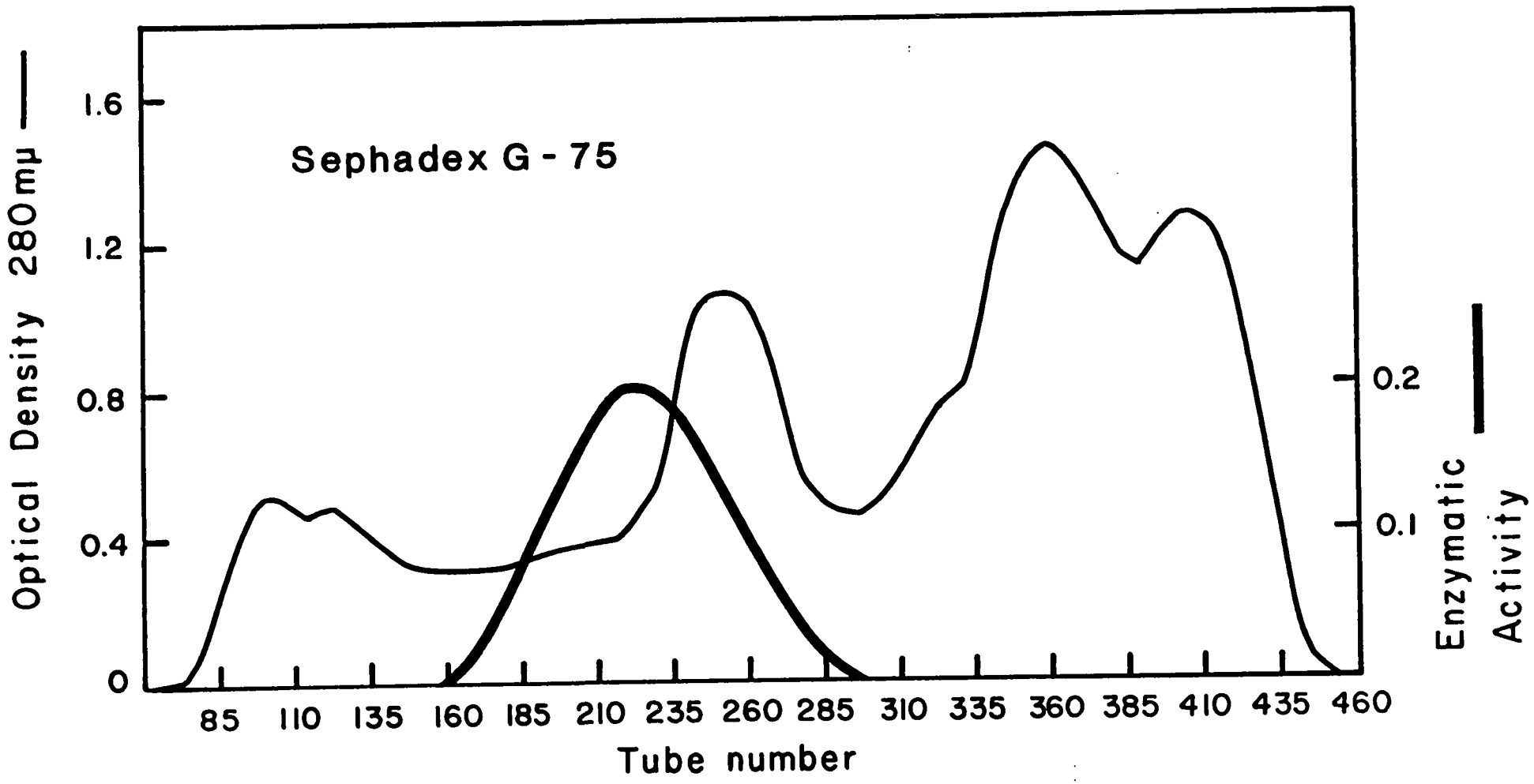
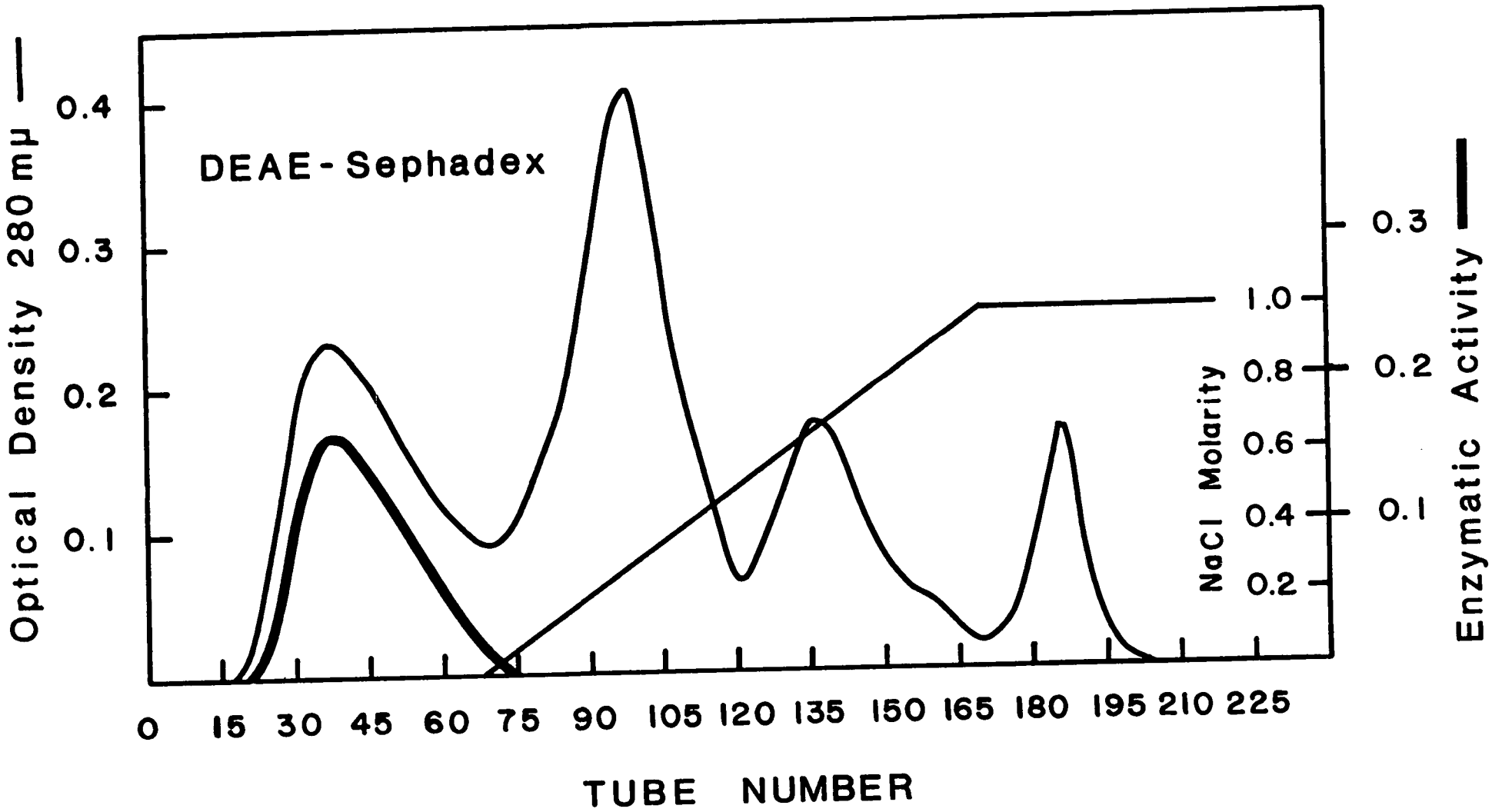

Sephadex G - 75
Optical Density 280mμ
1.6
1.2
0.8
0.4
0
0.2
0.1
Enzymatic Activity
85 110 135 160 185 210 235 260 285 310 335 360 385 410 435 460
Tube number

Figure 7

Chromatography of rabbit spleen ribonuclease on DEAE-Sephadex A-50 column (2.5 x 100 cm).


DEAE - Sephadex
Optical Density 280 mμ
0.4
0.3
0.2
0.1
NaCl Molarity
1.0
0.8
0.6
0.4
0.2
Enzymatic Activity
0.3
0.2
0.1
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225
TUBE NUMBER

The enzyme preparation was further purified by batchwise treatment with SE-Sephadex C-50. A portion (500 mg) of Preparation III was dissolved in 100 ml of 0.1 M acetate buffer at pH 4.5 and was then equilibrated with the gel prepared from 10 gm of SE-Sephadex C-50 by stirring at 4° C for two hours. The supernatant which contained negligible RNase activity was filtered off through a Buchner funnel. The gel was equilibrated with 200 ml of 0.4 M NaCl in 0.1 M acetate buffer. After stirring for two hours at 4° C, the supernatant was filtered off and discarded. A volume of 200 ml of 1 M NaCl in 0.1 M acetate buffer was added to the gel with stirring and the suspension was incubated for two hours at 4° C. The supernatant which contained the enzymatic activity was collected by filtration through a Buchner funnel and the gel was further treated with 200 ml of 1 M NaCl in 0.1 M acetate buffer. The enzymatically active supernatant was collected and combined with the previous solution, dialyzed and lyophilized to yield Preparation IV. Finally, this preparation was dissolved in 40 ml of 0.1 M acetate buffer, pH 4.5, and subjected to column chromatography on SE-Sephadex C-50 (4 x 70 cm). The elution profile is shown in Fig. 8; again, the fractions containing enzymatic activity were pooled, dialyzed and lyophilized (Preparation V).

The specific activities of the preparations isolated after each purification step are listed in Table 2 from which it is evident that an overall 50-fold purification of the enzyme had been attained. The percent recovery of enzymatic activity was calculated with respect to the crude extract (Preparation I),

Figure 8

Chromatography of rabbit spleen ribonuclease on SE-Sephadex C-50 column (4 x 70 cm).

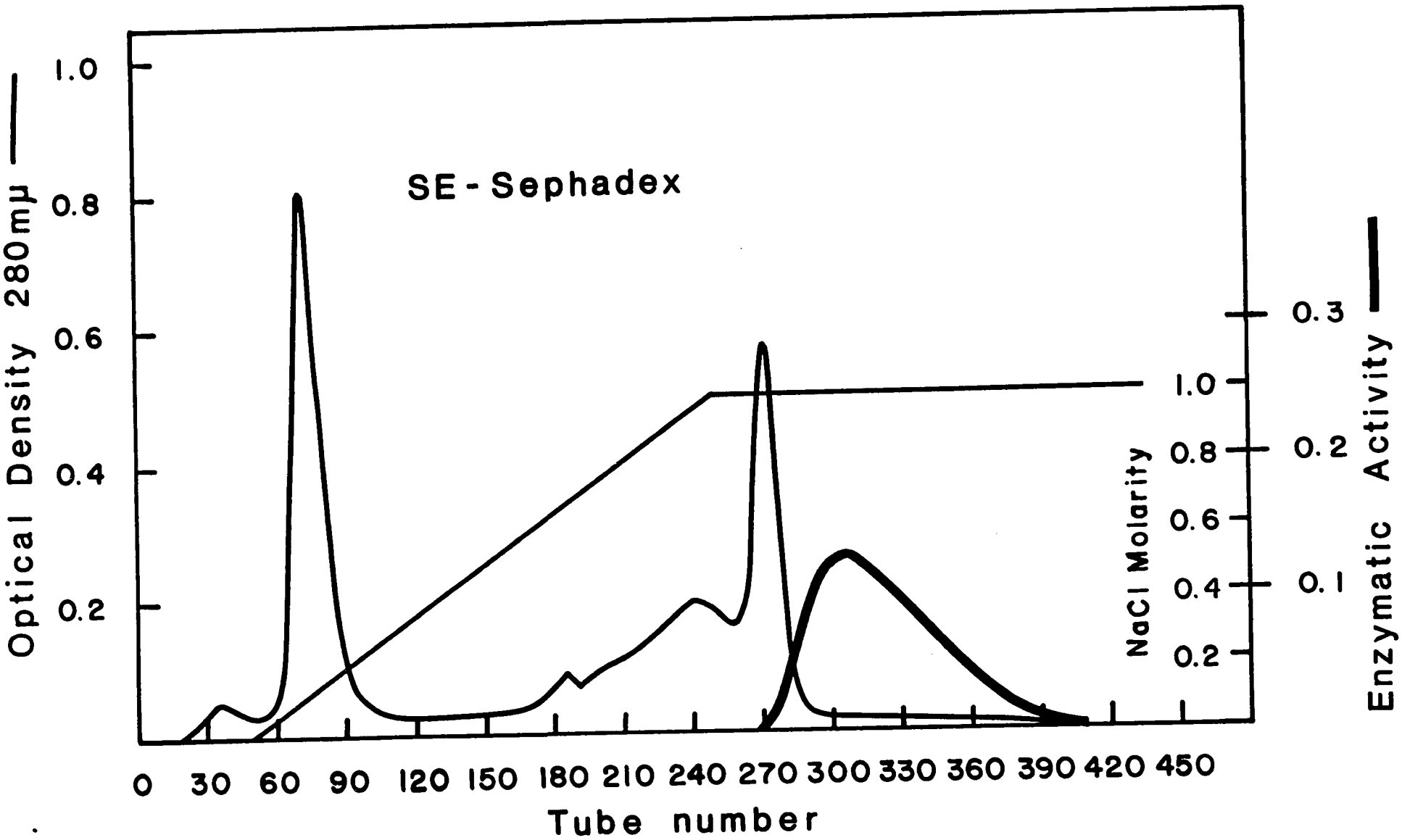

SE - Sephadex
Optical Density 280mμ
1.0
0.8
0.6
0.4
0.2
NaCl Molarity
1.0
0.8
0.6
0.4
0.2
Enzymatic Activity
0.3
0.2
0.1
0 30 60 90 120 150 180 210 240 270 300 330 360 390 420 450
Tube number

Table 2. Specific activities of rabbit spleen RNase preparations at each purification Step.

Sample	Volume, ml	Total activity, unit	Total protein, mg	Specific activity, units/mg	% recovery *
Preparation I	720	57,750	5,500	10.5	100.0
Preparation II	3,658	51,128	2,076	24.6	88.5
Preparation III	460	43,960	926	42.1	76.2
Preparation IV	350	40,080	376	106.5	69.4
Preparation V	1,670	31,260	62	505.1	54.2

* The recovery of enzymatic activity was calculated with respect to that present in the crude extract (Preparation I).

and was found to be of the order of 54%.

Electrophoresis on cellulose acetate membrane in veronal buffer at pH 8.6 (μ =0.075) was employed to examine the purity of the preparations at each purification step (Fig. 9). As can be seen, numerous electrophoretically discrete components were present in the crude extract. The enzymatically non-active proteins were removed progressively in sucessive purification steps, the final preparation V containing only one protein component with RNase activity.

For the ultracentrifugal pattern of the purified spleen RNase (Preparation V) shown in Fig. 10, it is obvious that this preparation was ultracentrifugally homogeneous; its sedimentation coefficient was 1.6S.

The purified ribonuclease preparation at a concentration of 0.5 mg/ml was assayed for RNase, DNase, acid or alkaline phosphatase and phosphodiesterase activities. It was found that this preparation had RNase activity corresponding to 50 units of pure enzyme and less than 0.01 unit of DNase activity. No other enzymatic activities were detected.

Figure 9

Electrophorogram of rabbit spleen ribonuclease on cellulose acetate.

Preparation V

Preparation IV

Preparation III

Preparation II

Preparation I

Figure 10

Ultracentrifugal pattern of the purified rabbit spleen ribonuclease in Preparation V.

Pictures were taken at intervals of 4 minutes.

(B) ISOLATION AND PURIFICATION OF RABBIT PANCREATIC RIBONUCLEASE

The rabbit pancreas were rinsed in cold saline at 4° C, cut into small pieces and homogenized with three volumes (w/v) of cold sulfuric acid (0.25 N) in a Waring blendor for two minutes. After stirring the homogenate for 24 hours at 4° C, the mixture was squeezed through cheese-cloth. The use of surgical gloves was essential for preventing the contamination by 'finger nucleases' (256). The homogenate was next clarified by centrifugation at 13,000 rpm in an IEC, B-20 centrifuge for 30 minutes. Some precipitate formed when the pH of the supernatant was adjusted to 6.0. Since this precipitate was devoid of ribonuclease activity, it was discarded. The procedures used for purification of the rabbit pancreatic ribonuclease are illustrated schematically in Fig. 11.

The supernatant (Preparation I) was fractionated by precipitation with ammonium sulfate at 4° C. A saturated solution of ammonium sulfate (at pH 6.0, 4° C) was added dropwise from a separatory funnel to Preparation I until 40% saturation had been attained. This step was carried out at 4° C with constant stirring. The precipitate was removed by centrifugation and discarded, since 90% of the ribonuclease activity remained in the supernatant. Solid ammonium sulfate was added to make the solution 95% saturated with respect to ammonium sulfate. The precipitate was collected and dissolved in 300 ml of 0.15 M phosphate buffer, pH 7.3 (Preparation II).

Preparation II was fractionated by gel filtration on a column (8 x 100 cm) of Sephadex G-75 (Fig. 12). The fractions

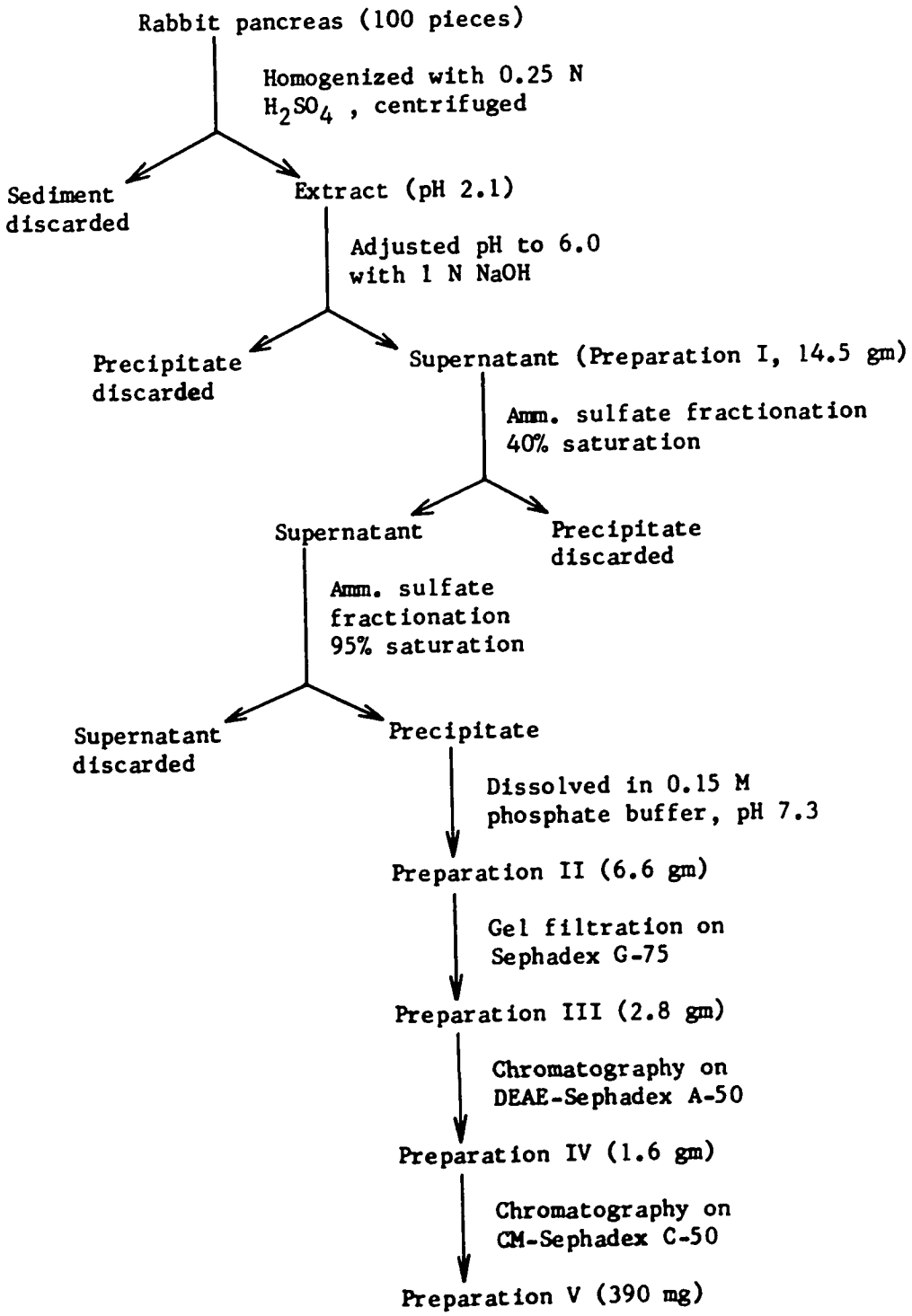
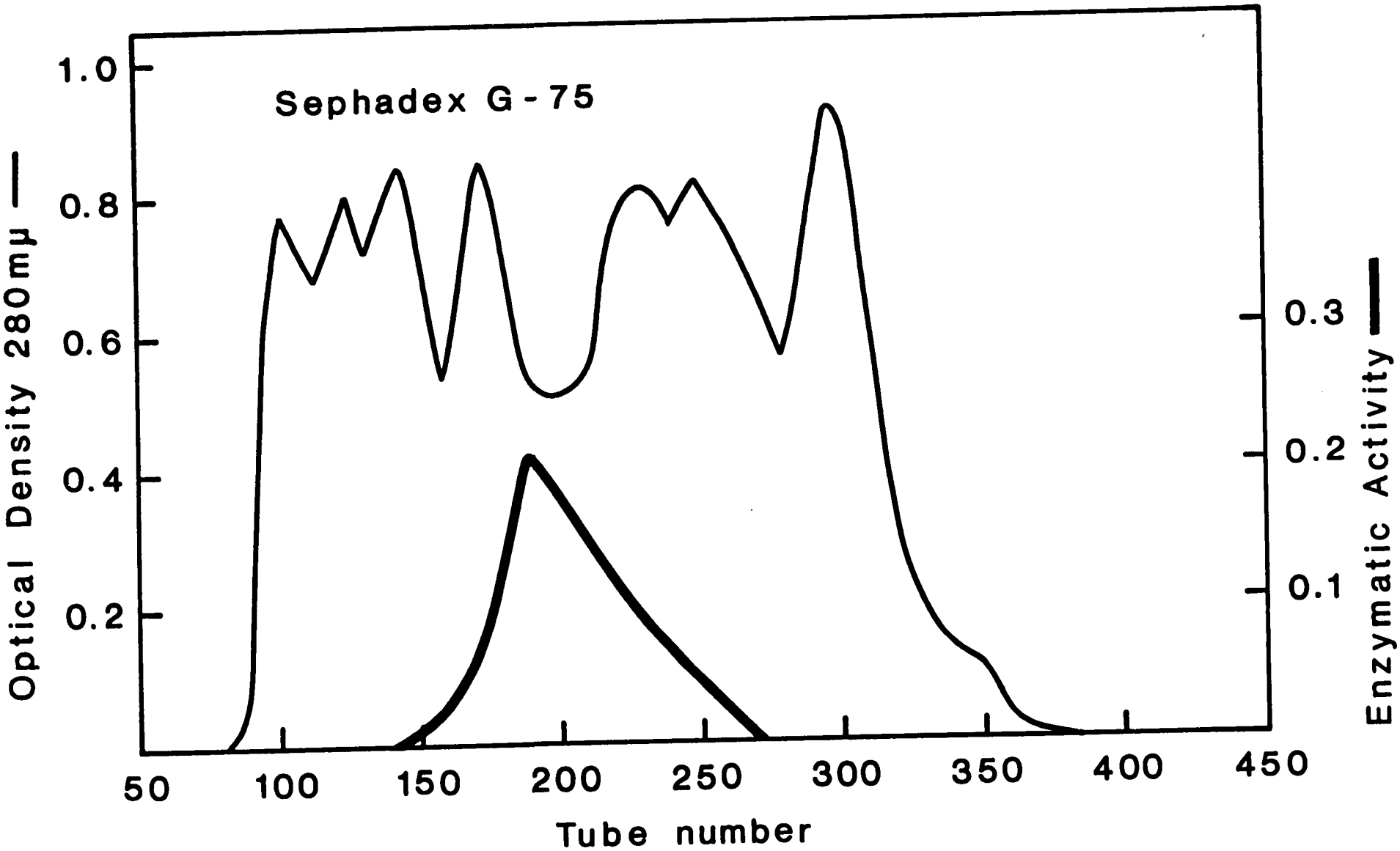


Figure 11. Flowsheet of purification of rabbit pancreatic ribonuclease.

Figure 12

Gel filtration of rabbit pancreatic ribonuclease on Sephadex G-75 column (8 x 100 cm).


Sephadex G-75
Optical Density 280mμ
Enzymatic Activity
Tube number
1.0
0.8
0.6
0.4
0.2
0.3
0.2
0.1
50
100
150
200
250
300
350
400
450

which contained ribonuclease activity were pooled, dialyzed and lyophilized (Preparation III).

A portion (900 mg) of Preparation III was dissolved in 20 ml of 0.01 M Tris buffer, pH 7.4, and applied to a column (2.5 x 100 cm) od DEAE-Sephadex A-50 (Fig. 13). The fractions which were enzymatically active were pooled and dialyzed against distilled water at 4° C for five hours. After lyophilization (Preparation IV) it was dissolved in 20 ml of 0.05 M acetate buffer at pH 4.5 and applied to a column (2.5 x 70 cm) of CM-Sephadex C-50 (Fig. 14). The active ribonuclease fractions were pooled, dialyzed and lyophilized (Preparation V).

The specific activities of the preparations at each purification step are shown in Table 3. As can be seen, the overall purification of rabbit pancreatic ribonuclease was 23-fold, with a recovery of about 60%.

The preparations of rabbit pancreatic ribonuclease obtained at each purification step, were examined by electrophoresis on cellulose acetate membrane in veronal buffer at pH 8.6, $\mu = 0.075$. In Fig. 15 the corresponding electrophorograms are shown from which it can be deduced that non-active proteins were removed in successive purification steps, the final preparation having only a single electrophoretically separable component. The electrophoretic mobilities of the isolated rabbit spleen and pancreatic ribonucleases are different from that of the bovine pancreatic RNase A as shown in Fig. 16.

Moreover this Preparation V, containing the purified rabbit pancreatic ribonuclease, gave rise in the analytical ultracentrifuge

Figure 13

Chromatography of rabbit pancreatic ribonuclease on DEAE-Sephadex A-50 column (2.5 x 100 cm).

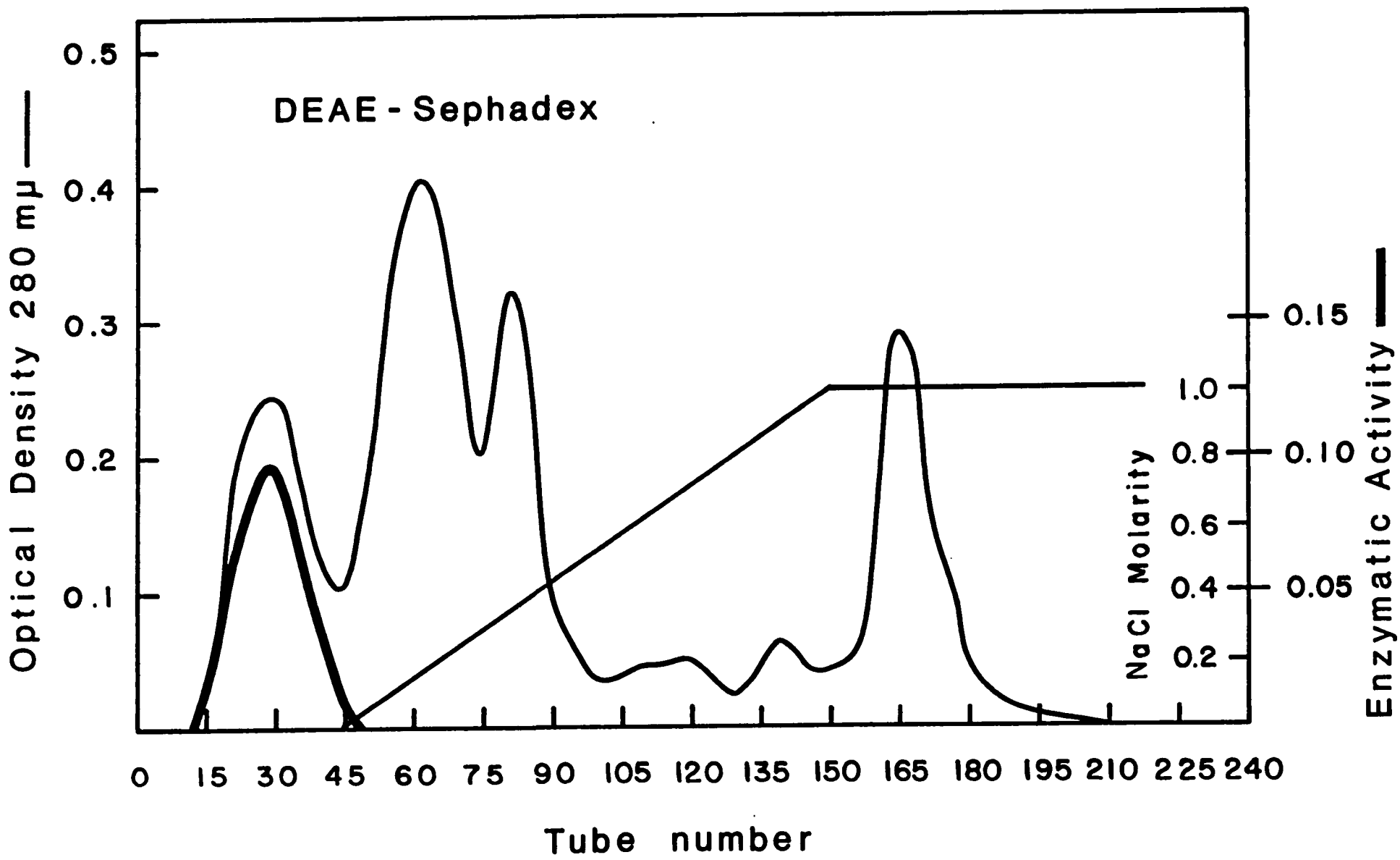

DEAE - Sephadex
Optical Density 280 mμ
0.5
0.4
0.3
0.2
0.1
NaCl Molarity
1.0
0.8
0.6
0.4
0.2
Enzymatic Activity
0.15
0.10
0.05
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240
Tube number

Figure 14

Chromatography of rabbit pancreatic ribonuclease on CM-Sephadex C-50 column (2.5 z 70 cm).

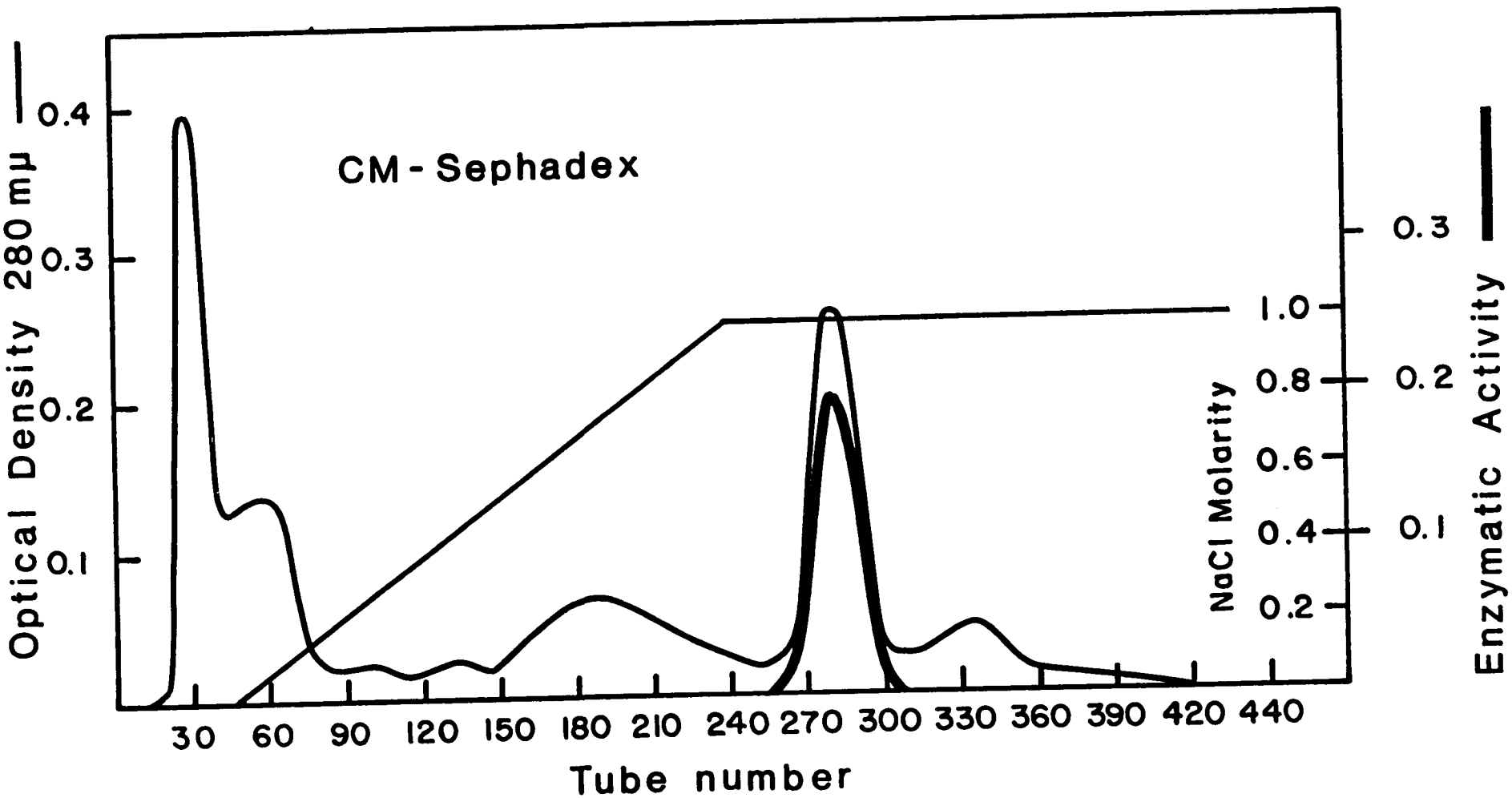

CM - Sephadex
Optical Density 280 mμ
0.4
0.3
0.2
0.1
NaCl Molarity
1.0
0.8
0.6
0.4
0.2
Enzymatic Activity
0.3
0.2
0.1
30 60 90 120 150 180 210 240 270 300 330 360 390 420 440
Tube number

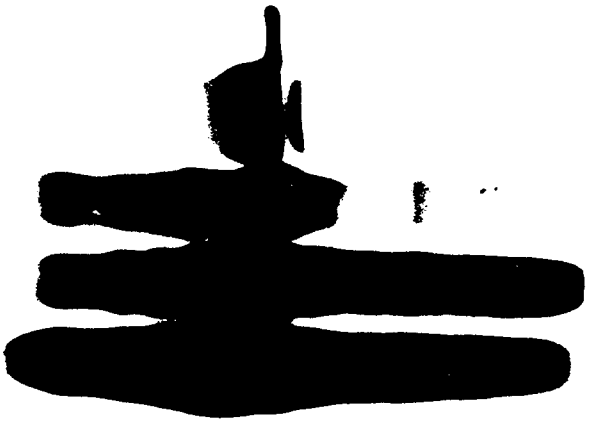
Table 3 Specific activities of rabbit pancreatic ribonuclease preparations at each purification step.

Sample	Volume, ml	Total activity, unit	Total protein, mg	Specific activity, unit/mg	% Recovery *
Preparation I	1,780	50,580	14,430	3.5	100.0
Preparation II	630	44,980	6,614	6.8	89.0
Preparation III	1,676	43,400	2,858	15.2	85.0
Preparation IV	964	39,520	1,602	24.7	77.4
Preparation V	500	30,430	390	78.1	59.6

* The recovery of enzymatic activity was calculated with respect to that present in the crude extract (Preparation I).

Figure 15

Electrophorogram of rabbit pancreatic ribonuclease preparations on cellulose acetate.



Preparation V

Preparation IV

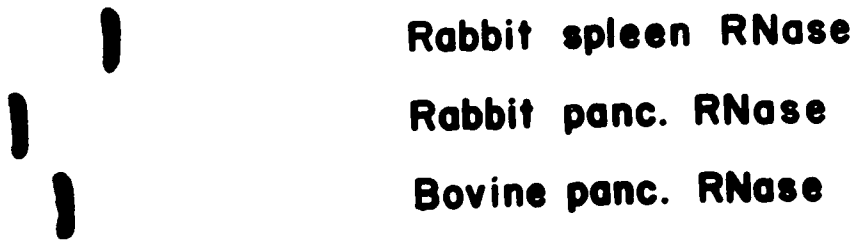
Preparation III

Preparation II

Preparation I

Figure 16

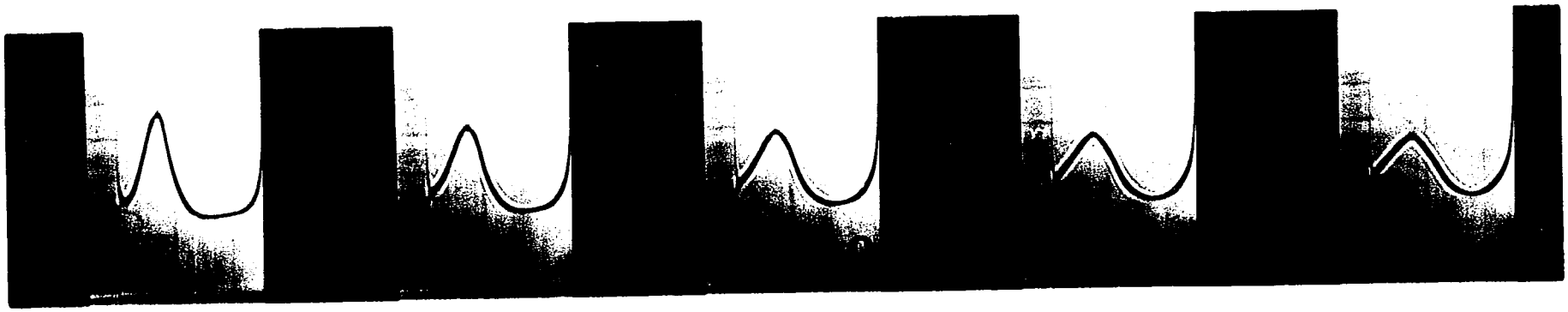
Comparison of electrophoretic mobilities of rabbit and bovine ribonucleases.


Rabbit spleen RNase
Rabbit panc. RNase
Bovine panc. RNase

to a single symmetrical peak (Fig. 17) having a sedimentation coefficient of 1.6S. The pure pancreatic ribonuclease (Preparation V) was found devoid of DNase, acid and alkaline phosphatase and phosphodiesterase activities.

Figure 17

Ultracentrifugal pattern of the purified rabbit pancreatic ribonuclease in Preparation V. Pictures were taken at intervals of 4 minutes.



DISCUSSION

It would appear that rabbit ribonucleases had not been isolated in a pure form up to present study because of their relatively low concentration in rabbit tissues (244). The methods described in the present study were designed for the purification of rabbit ribonucleases according to their respective molecular size by gel filtration, and in terms of their net surface charges by ion exchange chromatography.

Extraction of ribonucleases from various tissues has been carried out with different solvents such as distilled water (25, 247), 0.25 M sucrose solution (25, 27, 240) and 0.25 N sulfuric acid (25, 23, 28, 258, 259). The use of dilute sulfuric acid (0.25 N) has been commonly employed, as in the present study, because of the stability of ribonucleases at low pH, in contrast to numerous contaminating enzymes which are destroyed under this condition.

Column chromatography on different supporting media proved useful in the main purification steps for rabbit ribonucleases. Of the different grades of Sephadex, in agreement with the findings of Gordon (23), Sephadex G-75 gave the best results for the separation of ribonucleases from other contaminating proteins. However the rabbit ribonucleases, unlike the rat liver ribonuclease as reported by Gordon (23), were not retained by Sephadex G-25. From the elution profiles shown in Figures 6 and 12, it is evident that both rabbit spleen and pancreatic ribonucleases emerged from the column of Sephadex G-75 within the same volume range, i.e. in fractions 150-280, from which it may be inferred that both the spleen and pancreatic ribonucleases have similar molecular weights.

The elution profiles obtained by chromatography on DEAE-Sephadex A-50 which is an anion exchanger (Figures 7 and 13) indicate that rabbit ribonucleases were not retained by the gel at pH 7.4, from which it may be inferred that rabbit ribonucleases are positively charged at this pH. This is supported by the results of column chromatography on cation exchangers, which indicate that spleen ribonuclease was strongly adsorbed on SE-Sephadex C-50 (Fig. 8) whereas pancreatic ribonuclease was strongly adsorbed by CM-Sephadex C-50 (Fig. 14). The ribonucleases were not eluted with sodium chloride solution below 1 M in concentration.

In general, the purification of proteins by ion exchange column chromatography is more efficient than the use of gel filtration. In the present study a two- to five-fold increase in specific activity of ribonucleases was achieved in each step of ion exchange chromatography, whereas only a two-fold increase in ribonuclease activity was achieved on gel filtration. On ion exchange chromatography, higher resolution of protein components is achieved as compared to gel filtration. Thus, bovine pancreatic ribonuclease which was considered homogeneous in early years, was resolved into two components, namely RNase A and RNase B, with ion exchange chromatography on XE-64 (77). Ribonucleases from different sources have also been purified on ion exchange chromatography. Bernardi et al. (260) purified hog spleen acid ribonuclease by column chromatography on DEAE-Sephadex A-50 and CM-Sephadex C-50. He obtained a four-fold purification on each step of the chromatography. Three components of T. pyriformis intracellular ribonuclease have been separated chromatographically on SE-Sephadex C-50 (258). Recently sea-urchin ribonuclease has been

highly purified by chromatography on SE-Sephadex C-50 and CM-Sephadex C-50 (33). All these results imply that ion exchange column chromatography is capable of leading to homogeneous preparations of ribonucleases from various sources. In the present study rabbit spleen and pancreatic ribonucleases appeared to be homogeneous after passing successively through the anion and cation exchange columns. In addition, the final preparations of rabbit ribonucleases were judged to be mono-disperse by electrophoresis (Figures 9 and 15) and by optical ultracentrifugation (Figures 10 and 17). The homogeneity of rabbit spleen ribonuclease was further demonstrated by the fact that 'monospecific' antibodies to this enzyme were produced when it was injected into a sheep as described in Chapter V.

In an attempt to separate ribonuclease from other proteins in terms of the relative difference in surface charges of these molecules, zone electrophoresis in Sephadex G-25 was used. However, because of the relatively small molecular weight of ribonucleases, their diffusion coefficients are rather high and consequently the latter technique was not satisfactory.

Column chromatography on Amberlite IRC-50 (XE-64) had been most commonly used by many workers (15, 77) to purify the well known bovine pancreatic ribonuclease. However, IRC-50 was found unsuitable for the purification of rabbit ribonucleases in the present study. Still, unlike the bovine pancreatic ribonuclease, rabbit ribonucleases were eluted together with some inactive proteins.

In the present study, an effective way of desalting the rabbit ribonucleases was found by employing a column (4 x 80 cm) of

Sephadex G-25 fine grade using distilled water as eluant. This method of desalting is preferable to dialysis, because some losses of ribonucleases were occasionally found during prolonged dialysis.

The results of electrophoresis on cellulose acetate (Figures 9 and 15) show that both rabbit spleen and pancreatic ribonucleases moved toward the cathode at pH 8.6, as did the bovine pancreatic ribonuclease. This implies that rabbit ribonucleases are also basic proteins, which is supported by the finding that rabbit spleen and pancreatic ribonucleases showed an isoelectric point at pH 10.84 and 9.93, respectively, as described later in Chapter IV. Rabbit spleen ribonuclease having a much higher electrophoretic mobility is expected to have a higher content of basic amino acids. The results of amino acid composition of these ribonucleases agree with these findings. By comparison, the electrophoretic mobility of bovine pancreatic ribonuclease was found lower than that of the rabbit spleen ribonuclease, but slightly higher than that of the rabbit pancreatic ribonuclease (Fig. 16). The difference in electrophoretic mobilities of rabbit ribonucleases is further supported by the difference in the positions of the precipitin bands formed on immunoelectrophoresis (Chapter V, Fig. 26).

Although the purification procedures described were highly effective leading to a 50- and 23-fold increase in activity of rabbit spleen and pancreatic ribonucleases, respectively, the enzymes were recovered in relatively low yields. Thus, from 200 gm of rabbit spleens, collected from 100 rabbits, only 60 mg of purifiedribonuclease was obtained. Similarly from 100 rabbit pancreas only 390 mg of ribonuclease was isolated.

CHAPTER IV

PHYSICOCHEMICAL CHARACTERIZATION OF RABBIT SPLEEN AND PANCREATIC RIBONUCLEASES

INTRODUCTION

Ribonucleases with various physicochemical properties from different animal and plant sources have been isolated and characterized. Their physicochemical characteristics led to the generalizations that mammalian pancreatic ribonucleases are more or less similar, and can be regarded as a class of homologous proteins which are basic in nature and with a molecular weight of approximately 14,000 (54) whereas ribonucleases from invertebrates, plants or micro-organisms have diverse characteristics which differ in molecular weights, pH optimum, heat stability, substrate specificity and amino acid compositions.

Rabbit reticulocyte ribonuclease has been partially purified and characterized (37, 38). The physicochemical characterization of this ribonuclease showed that it differed from the bovine pancreatic ribonuclease in base specificity, pH optimum and stability. Since rabbit spleen and pancreatic ribonucleases had been obtained, in this study, in a highly purified state as described previously in Chapter III, it was deemed worth studying the physicochemical properties of these ribonucleases on a comparative basis. The characterization of these rabbit spleen and pancreatic ribonucleases

not only reveal the intrinsic biochemical nature of these ribonucleases, but also provide further understanding for the comparative studies of mammalian ribonucleases.

In the present phase of the study, the pH optimum, Michaelis-Menten constants, isoelectric points, effects of ions on enzymatic activities, molecular weights as well as amino acid compositions of rabbit spleen and pancreatic ribonucleases were determined. The properties of these ribonucleases were similar to those of bovine pancreatic ribonuclease A, which is the best characterized prototype of this class of enzymes.

MATERIALS AND METHODS

REAGENTS: All chemicals were reagent grade available from various commercial sources. Sephadex G-75 and blue dextran were purchased from Phamacia Co., Uppsala, Sweden. Bovine pancreatic ribonuclease A, 4x crystallized, was obtained from Worthington Biochemical Corp., New Jersey. Cytochrome C from equine heart, A grade, and myoglobin of equine heart, 2x crystallized, were products of CalBiochem. Company. Trypsin (beef pancreas, 2x crystallized), pepsin (2x crystallized) and α-chymotrypsin (beef pancreas, 3x crystallized) were purchased from Mann Research Laboratories, Inc.

RABBIT RIBONUCLEASES: Rabbit spleen and pancreatic ribonucleases were purified as described in Chapter III.

ELECTROPHORESIS: Electrophoresis was carried out on cellulose acetate membrane in Tris-HCl and Glycine-NaOH buffers of ionic strength 0.1, at different pH values, in a Beckman Microzone electrophoresis apparatus, Model R-101. direct current at 250 volts was applied for one hour.

GEL FILTRATION ON SEPHADEX G-75: Sephadex G-75 gel was equilibrated with 0.15 M phosphate buffer at pH 7.3 and packed into a column of 2.5 x 160 cm. All chromatographic experiments were performed under the same hydrostatic pressure, and the fractions were collected by an LKB drop-count fraction collector at 4° C.

ASSAY FOR RIBONUCLEASE ACTIVITY: Yeast RNA was used as substrate and the assay was carried out as described in Chapter III.

AMINO ACID ANALYSIS: The amino acid analyses were performed in the Beckman automatic amino acid analyzer, Model 120 B.

RESULTS

(A) OPTIMUM pH FOR ENZYMATIC ACTIVITIES OF RABBIT RIBONUCLEASES

The method for assay of ribonuclease activity was the same as described in Chapter III, except that buffer solutions at various pH values were used. The buffer solutions were prepared as described by Gomori (261). Acetate buffer solutions (0.2 M) with a pH range of 3.6 to 5.6, phosphate buffers (0.2 M) with a pH range of 6.0 to 8.0 and Tris buffers (0.2 M) over the range of pH 8.4 to 9.0 were employed. A stock solution of yeast RNA in distilled water at a concentration of 8 mg per ml was diluted with an equal volume of the appropriate buffer.

Portions of 0.2 ml of the purified rabbit ribonuclease (20 µg of spleen RNase or 80 µg of pancreatic RNase per ml of distilled water) were placed in a series of test tubes, to which were added 1.0 ml volumes of the different buffers; the tubes were then incubated in a water bath at 37° C for 30 minutes. Volumes of 0.4 ml of the RNA solutions at the appropriate pH were added to the corresponding tubes. After incubation at 37° C for 30 minutes, 0.4 ml of uranyl reagent, composed of 0.75% uranyl acetate in 10% trichloroacetic acid, was added to stop the reaction. The precipitate was removed by centrifugation at 1,000 x G for 10 minutes at 4° C. A 0.2 ml volume of the supernatant was diluted to 3.0 ml with distilled water. The enzymatic activities of rabbit spleen and pancreatic ribonucleases

(which were measured in terms of the optical density at 260 mμ) at different pH are listed in Tables 4 and 5 respectively and plotted in Figures 18 and 19, from which it can be concluded that both ribonucleases exhibited optimum activity at pH 7.0. A comparison of optimum pH for enzymatic activities of ribonucleases from diverse sources is listed in Table 6.

pH	Enzymatic activity, unit
3.6	0
4.0	0.05
4.6	0.12
5.0	0.23
5.6	0.56
6.0	1.07
6.6	1.52
7.0	1.84
7.6	1.42
8.0	0.90
8.4	0.43
9.0	0

Table 4

OPTIMUM pH FOR ENZYMATIC ACTIVITY OF RABBIT SPLEEN RIBONUCLEASE.

pH	Enzymatic activity, unit
3.6	0
4.0	0
4.6	0.10
5.0	0.21
5.6	0.56
6.0	0.85
6.6	1.13
7.0	1.25
7.6	0.97
8.0	0.65
8.4	0.34
9.0	0

Table 5

OPTIMUM pH FOR ENZYMATIC ACTIVITY OF RABBIT PANCREATIC RIBONUCLEASE.

Figure 18

OPTIMUM pH FOR ENZYMATIC ACTIVITY OF RABBIT SPLEEN RIBONUCLEASE.

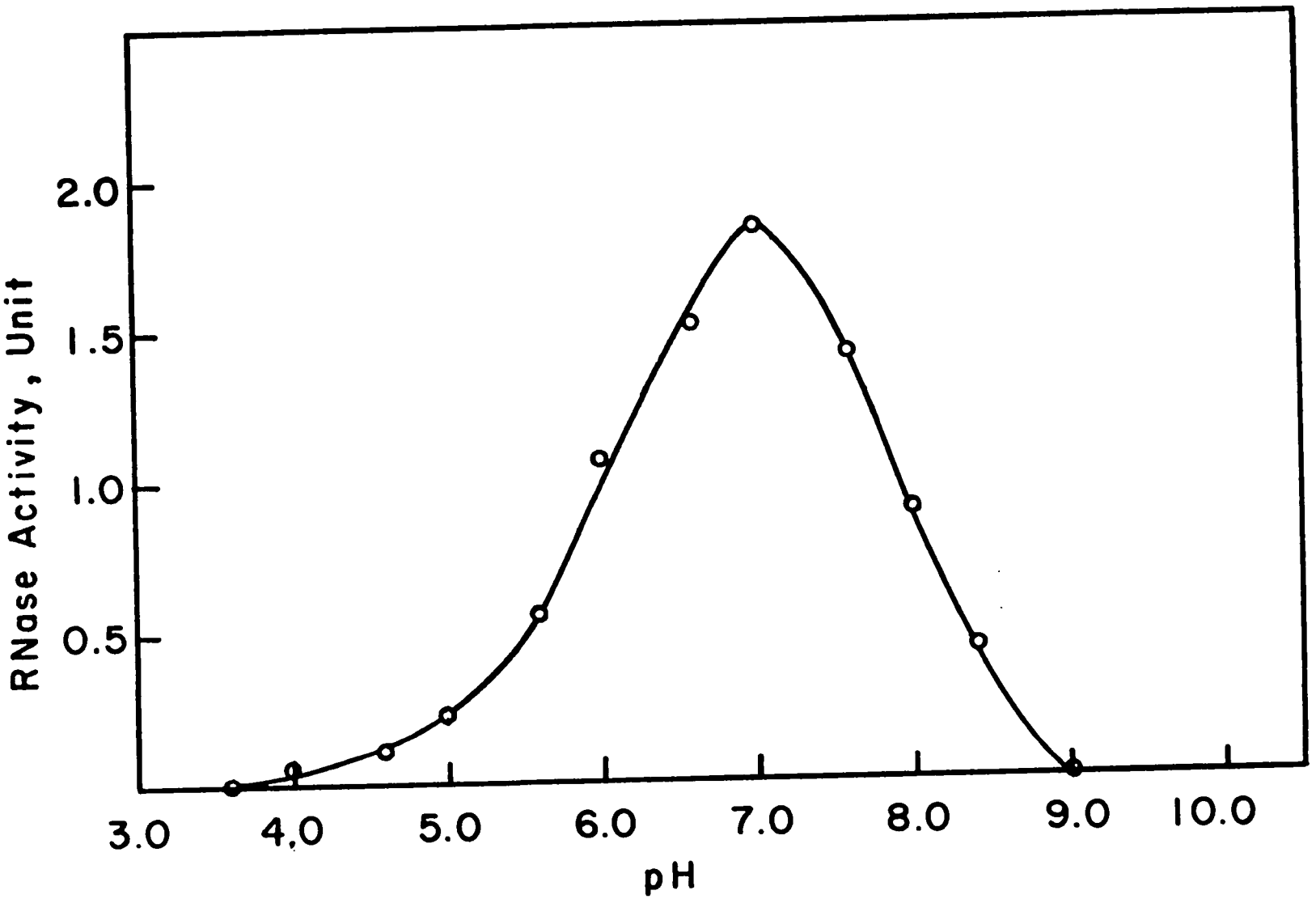
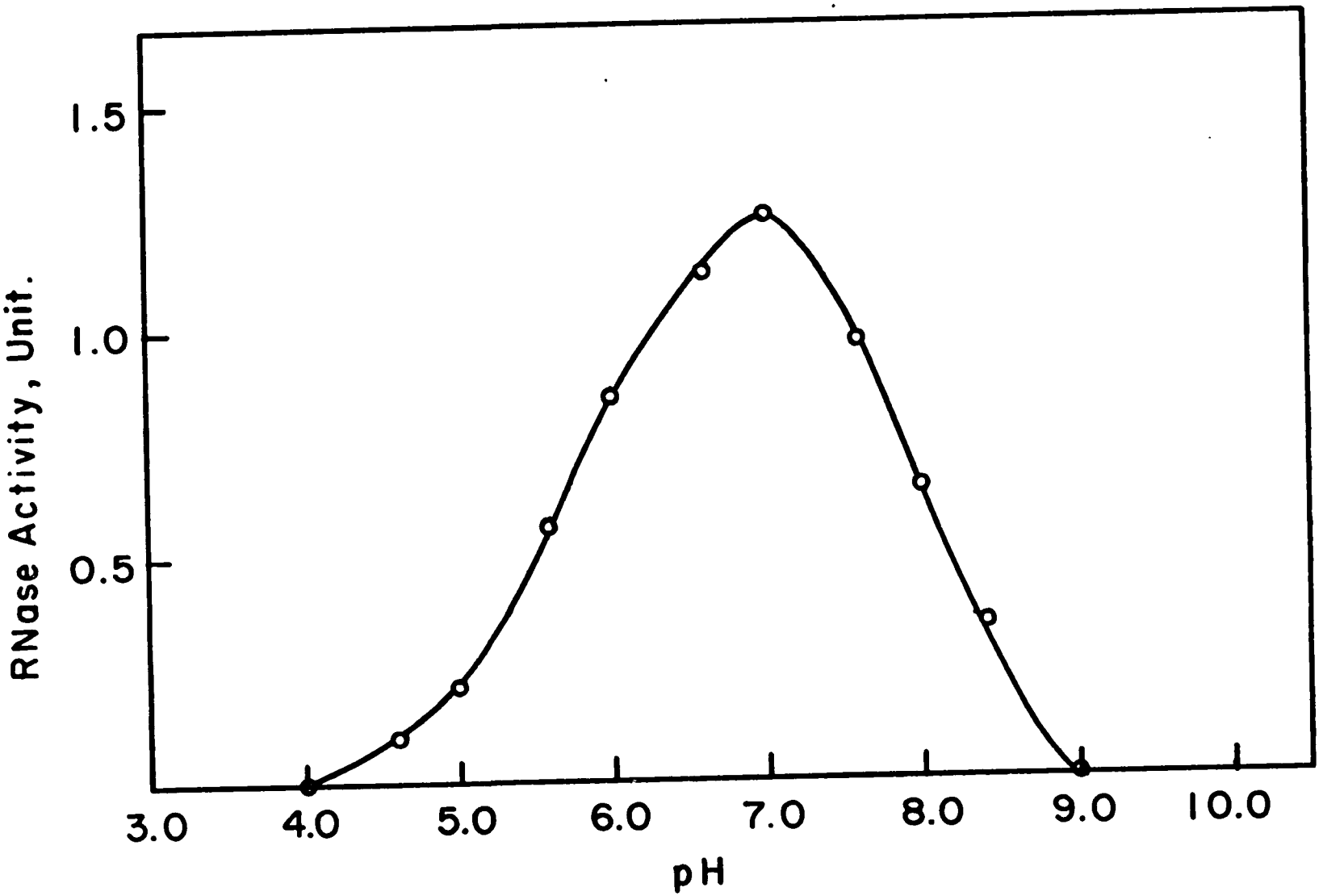

RNase Activity, Unit
2.0
1.5
1.0
0.5
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
pH

Figure 19

OPTIMUM pH FOR ENZYMATIC ACTIVITY OF RABBIT PANCREATIC RIBONUCLEASE.


RNase Activity, Unit.
1.5
1.0
0.5
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
pH

Source of RNase	Optimum pH	Reference
Bovine pancreas	7.7	75
Bovine aorta	7.0	247
Rabbit spleen	7.0	Present study
Rabbit pancreas	7.0	Present study
Rabbit reticulocyte	6.5	37
Human pancreas	7.5	22
Pig spleen	5.5	262
Cow spleen	5.5 and 7.8	263
Sheep pancreas	7.5	28
Rat liver	7.8	23
Rat pancreas	7.8	23
Rat serum	7.5	26, 264
Guinea pig serum	7.4	26
E. coli (alkaline RNase I)	8.1	265
Tetrahymena pyriformis	5.0	266
RNase T_1	7.5	267
RNase T_2	4.5	267

Table 6. COMPARISONS OF OPTIMUM pH FOR ENZYMATIC ACTIVITIES OF RIBONUCLEASES FROM DIVERSE SOURCES.

(B) INHIBITORS OF RABBIT RIBONUCLEASES

The effects of cations and other inhibitors on the enzymatic activities of rabbit spleen and pancreatic ribonucleases were studied by examination of the enzymatic activities in presence of different cations and some ribonuclease inhibitors such as heparin, sodium deoxycholate and sodium azide. The effects of chelating agents, EDTA and sodium citrate, on enzymatic activities of these rabbit ribonucleases were also studied.

Since relatively large amounts of metal ions (268, 269) are always found in commercial ribonucleic acid (RNA), it is necessary to remove them in order to obtain reliable results. These metal ions were removed by dialysis against EDTA according to the method of Roth (270). Yeast RNA (6%) solution was dialyzed against two changes of 10 volumes of 0.025 M EDTA at pH 7.0 for 24 hours, then against four changes of 10 volumes of 0.15 M NaCl for 24 hours and finally against five changes of 20 volumes of distilled water for another 24 hours at 4° C. This metal-free RNA was lyophilized.

A reaction mixture containing 0.01 M of cation was prepared by mixing 1.0 ml of the inorganic salt solution at a concentration of 0.016 mM per ml in 0.1 M Tris-HCl buffer, pH 7.3, with 0.2 ml of purified rabbit ribonuclease preparation in the same buffer, followed by addition of 0.4 ml of RNA solution at a concentration of 4 mg per ml of the same buffer. Immediately after the addition of RNA solution, the reaction mixture was incubated at 37° C for

30 minutes. At the end of the incubation period, 0.4 ml of the uranyl reagent, composed of 0.75% of uranyl acetate in 10% trichloroacetic acid, was added to stop the reaction and to precipitate the unreacted RNA molecules. After removing the precipitate by centrifugation at 1,000 x G for 10 minutes at 4° C, 0.2 ml of the supernatant was diluted to a total volume of 3.0 ml with distilled water. The optical density at 260 mμ was measured. In parallel, a blank was similarly prepared for each sample except that the uranyl reagent was added to inhibit the enzyme before the addition of substrate, RNA. For each tube, 3.7 μg of purified rabbit spleen ribonuclease was used. In the case of rabbit pancreatic ribonuclease, 23.5 μg was used in each tube. The results are expressed as % inhibition and shown in Table 7 and 8. The inhibitions of enzymatic activities of different ribonucleases by cations and inhibitors are compared and summarized in Table 9. The values listed for bovine pancreatic ribonuclease are based on the reports by Davis (271), Felling (272), Roth (273) and Houck (274) whereas the values for RNase T_1 have been reported by Egami et al. (267).

In the present study, the enzymatic activities of both rabbit spleen and pancreatic ribonucleases were not affected by monovalent cations except silver ion. Of all the divalent ions tested, copper and zinc inhibited the enzymatic activities of both rabbit ribonucleases. Heparin and sodium deoxycholate inhibited rabbit ribonucleases to an extent of 25-50%. Both rabbit ribonucleases were not affected by the metal chelating agents or p-chloromercuribenzoate.

Table 7

EFFECTS OF IONS AND INHIBITORS ON ENZYMATIC ACTIVITY OF RABBIT SPLEEN RIBONUCLEASE.

Effector	Concentration of effector	Enzymatic activity, unit	%Inhibition
None	--	1.80	0
NaCl	10^{-3} M	1.76	2.2
	10^{-2} M	1.75	2.8
KCl	10^{-3} M	1.75	2.8
	10^{-2} M	1.73	3.9
$AgNO_3$	10^{-3} M	0.58	67.8
	10^{-2} M	0.02	98.9
$CaCl_2$	10^{-3} M	1.80	0
	10^{-2} M	1.79	0.6
$CuSO_4$	10^{-3} M	1.07	40.6
	10^{-2} M	1.05	41.7
$MgCl_2$	10^{-3} M	1.78	1.2
	10^{-2} M	1.81	0
$ZnCl_2$	10^{-3} M	1.26	30.0
	10^{-2} M	0.91	49.5
Sodium azide	10^{-3} M	1.38	23.3
	10^{-2} M	1.17	35.0
Sodium citrate	10^{-3} M	1.78	1.1
	10^{-2} M	1.79	0.6
EDTA	10^{-3} M	1.80	0
p-chloromercuri-benzoate	10^{-3} M	1.81	0
Sodium deoxycholate	1%	1.10	38.9
Heparin	0.05 mg	1.17	35.0
	0.5 mg	0.91	49.5

Table 8

EFFECTS OF IONS AND INHIBITORS ON ENZYMATIC ACTIVITY OF RABBIT PANCREATIC RIBONUCLEASE.

Effector	Concentration of effector	Enzymatic activity, unit	% Inhibition
None	--	1.86	0
NaCl	10^{-3} M	1.85	0.5
	10^{-2} M	1.84	1.1
KCl	10^{-3} M	1.84	1.1
	10^{-2} M	1.86	0
$AgNO_3$	10^{-3} M	0.17	90.9
	10^{-2} M	0	100
$CaCl_2$	10^{-3} M	1.87	0
	10^{-2} M	1.88	0
$CuSO_4$	10^{-3} M	0.97	47.8
	10^{-2} M	0.96	48.4
$MgCl_2$	10^{-3} M	1.90	0
	10^{-2} M	1.85	0.5
$ZnCl_2$	10^{-3} M	1.45	22.0
	10^{-2} M	1.22	34.4
Sodium azide	10^{-3} M	1.70	8.6
	10^{-2} M	1.47	21.0
EDTA	10^{-3} M	1.86	0
Sodium citrate	10^{-3} M	1.85	0.5
	10^{-2} M	1.85	0.5
p-chloromercuri-benzoate	10^{-3} M	1.86	0
Sodium deoxycholate	1%	0.98	47.3
Heparin	0.05 mg	1.41	24.2
	0.5 mg	1.20	35.5

Inhibitor	Rabbit RNase		Bovine RNase		RNase T1	
	Conc.	%Inhibit.	Conc.	%Inhibit.	Conc.	%Inhibit.
Na^{+}	$10^{-2}M$	0	$10^{-2}M$	0	$10^{-2}M$	0
Ag^{+}	$10^{-3}M$	100	$10^{-4}M$	100	$10^{-3}M$	100
Ca^{++}	$10^{-2}M$	0	$2x10^{-2}M$	0	$10^{-2}M$	25-30
Cu^{++}	$10^{-3}M$	40-48	$2x10^{-4}M$ $-2x10^{-3}M$	70-100	$10^{-3}M$	50-80
Mg^{++}	$10^{-2}M$	0	$2x10^{-2}M$	0	$10^{-1}M$	40
Zn^{++}	$10^{-3}M$ $-10^{-2}M$	30-50	$2x10^{-3}M$ $-2x10^{-4}M$	45-95	$10^{-3}M$	100
Heparin	0.05-0.5 mg	25-50	0.064%	38-59	0.01-0.05%	0
Sodium azide	$10^{-3}M$ $-10^{-2}M$	10-35	--	--	$10^{-2}M$	0-5
EDTA	$10^{-3}M$	0	$10^{-2}M$	0	$10^{-2}M$	Activation 25-50
p-chloro-mercuri-benzoate	$10^{-3}M$	0	$10^{-2}M$	0	$10^{-3}M$	0

Table 9

COMPARISONS OF INHIBITION OF RIBONUCLEASES FROM DIFFERENT SOURCES.

(C) ISOELECTRIC POINTS OF RABBIT RIBONUCLEASES

Electrophoresis was carried out on cellulose acetate membrane in a Beckman Microzone electrophoresis apparatus, Model R-101, with an applied voltage of 250 volts for one hour. Tris-HCl buffer solutions were prepared at pH 8.0 and 8.8 of the same ionic strength, μ =0.1. Glycine-NaOH buffers at pH 10.0 and 10.8, ionic strength 0.1, were also employed. The samples of purified rabbit spleen and pancreatic ribonucleases, as well as of bovine pancreatic ribonuclease A, were applied to the same cellulose acetate membrane for each experiment carried out at a given pH value; the bovine pancreatic ribonuclease served thus as reference. After electrophoresis, the membrane was fixed and stained with a dye solution composed of 0.2% Ponceau-S stain, 3.0% trichloroacetic acid and 3.0% of sulfosalicylic acid in distilled water and the migration distance of each sample was measured (Table 10). The isoelectric points were obtained from Figure 20 as the pH values at which the enzymes did not migrate in the electric field. The isoelectric points for rabbit spleen ribonuclease, pancreatic ribonuclease and bovine pancreatic ribonuclease were thus estimated to be 10.84, 9.93 and 10.12 respectively.

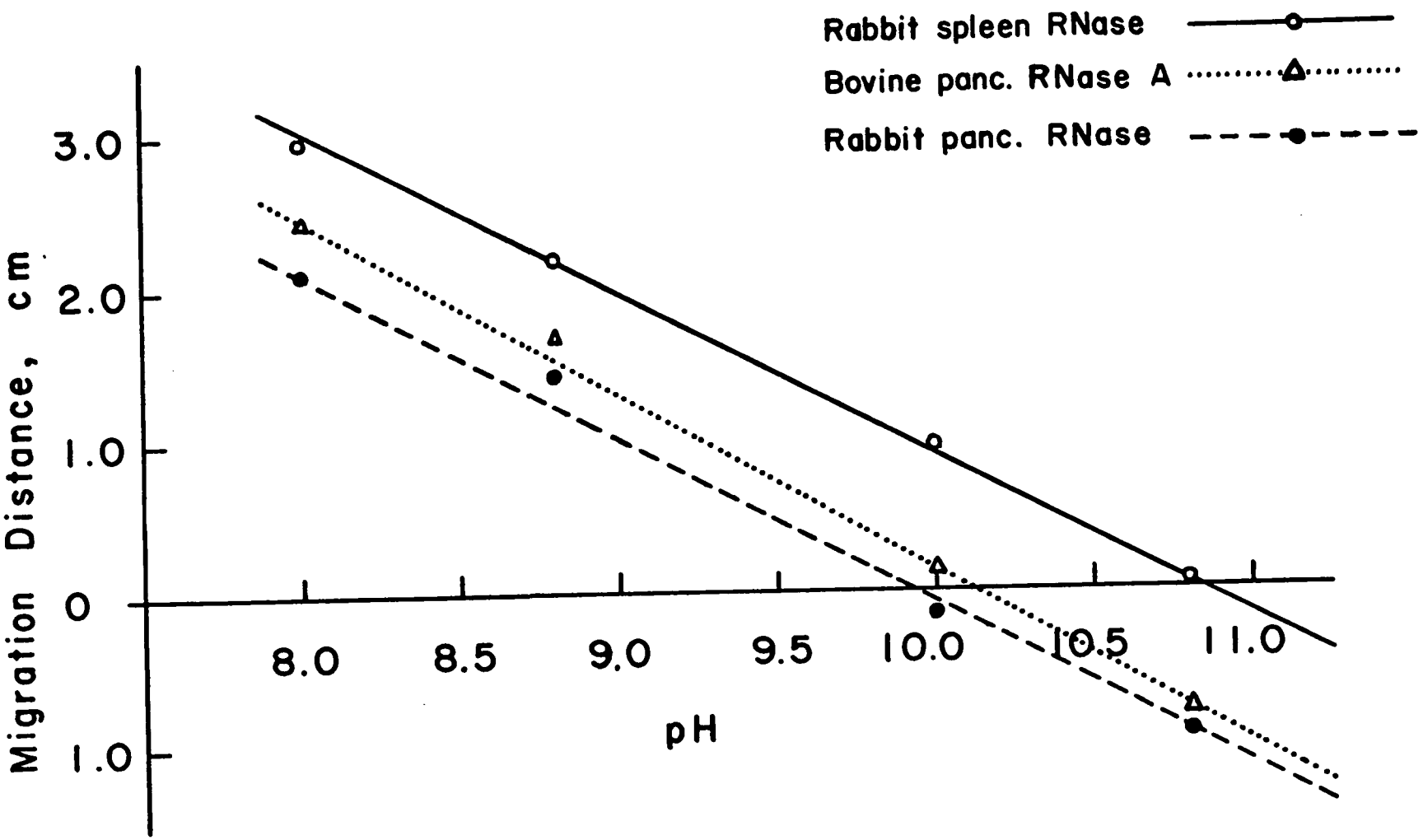
pH	Distance migrated, cm		
	Rabbit spleen RNase	Rabbit panc. RNase	Bovine panc. RNase
8.0	+ 2.96	+ 2.10	+ 2.45
8.8	+ 2.19	+ 1.42	+ 1.69
10.0	+ 0.95	- 0.16	+ 0.14
10.8	+ 0.06	- 0.95	-0.80

Table 10

ELECTROPHORETIC MOBILITIES OF RIBONUCLEASES.

Figure 20

ISOELECTRIC POINTS OF RIBONUCLEASES.


Rabbit spleen RNase
Bovine panc. RNase A
Rabbit panc. RNase
Migration Distance, cm
3.0
2.0
1.0
0
1.0
8.0
8.5
9.0
9.5
10.0
10.5
11.0
pH

(D) EFFECTS OF SUBSTRATE CONCENTRATION ON ENZYMATIC ACTIVITIES OF RABBIT RIBONUCLEASES

The Michaelis-Menten constant, which is the substrate concentration at half maximum velocity of an enzyme-catalyzed reaction, can be determined by studying the effect of variation of substrate concentration on the rate of enzyme reaction.

The purified rabbit spleen and pancreatic ribonucleases were dissolved respectively in 0.15 M phosphate buffer, pH 7.3, to give a concentration of 19 μg of spleen RNase per ml, and 130 μg of pancreatic RNase per ml. A two-fold serial dilution of the substrate, yeast RNA, was prepared with the same phosphate buffer. The enzyme solution (0.2 ml) was added to the substrate solution (consisting of 24 mg of RNA in 1.0 ml) at 37 ^{o} C and incubated for 30 minutes. After the incubation period, 0.4 ml of the uranyl reagent (composed of 0.75% uranyl acetate in 10% trichloroacetic acid) was added to stop the reaction. After centrifugation at 1,000 x G for 10 minutes at 4 ^{o} C, 0.2 ml of the supernatant was diluted to a final volume of 3.0 ml with distilled water. The optical density at 260 mμ was measured. A blank of the same composition was similarly prepared for each tube except that the uranyl reagent was added to the mixture before the addition of enzymes.

The results are shown in Table 11, in which S represents the substrate concentration in mg of RNA per ml and V represents the velocity of reaction as the increase in optical density at

260 mμ caused by the acid soluble nucleotides liberated in 30 minutes at 37° C. The Michaelis-Menten constants were determined graphically by the double reciprocal plot method (26, 275). Straight lines were obtained when the reciprocal values of substrate concentration (S) were plotted against the reciprocal values of reaction velocity (V). The straight lines intersected the vertical axis at a point which gave 1/Vm and intersected the base line at a point giving - 1/Km, where Vm represented the maximum velocity. The straight line had a slope of Km/Vm. The Michaelis-Menten constants determined from Fig. 21 for rabbit spleen and pancreatic ribonucleases were found to be 0.52 and 1.47 respectively.

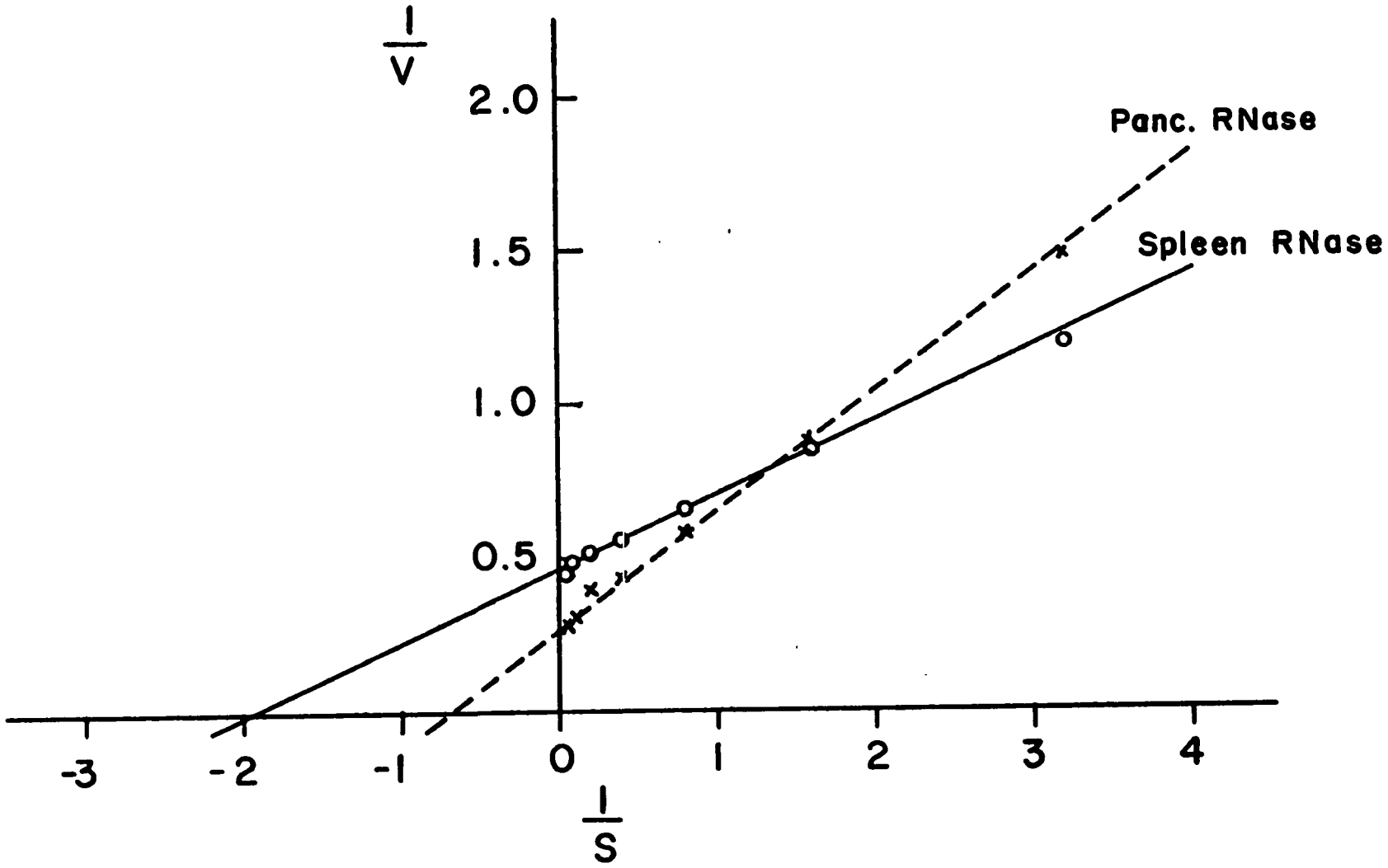
S mg/ml	1/S	Spleen RNase		Pancreatic RNase	
		V, unit	1/V	V, unit	1/V
20.0	0.05	2.21	0.452	3.56	0.281
10.0	0.10	2.07	0.483	3.23	0.309
5.0	0.20	1.92	0.521	2.52	0.397
2.5	0.40	1.76	0.568	2.30	0.435
1.25	0.80	1.53	0.654	1.72	0.582
0.625	1.60	1.18	0.849	1.14	0.877
0.312	3.20	0.84	1.190	0.68	1.470
0.156	6.40	0.52	1.923	0.41	2.439

Table 11

EFFECTS OF SUBSTRATE CONCENTRATION ON ENZYMATIC ACTIVITIES OF RABBIT RIBONUCLEASES.

Figure 21

EFFECTS OF SUBSTRATE CONCENTRATION ON ENZYMATIC ACTIVITIES OF RABBIT RIBONUCLEASES.


1/V
2.0
1.5
1.0
0.5
Panc. RNase
Spleen RNase
-3
-2
-1
0
1
2
3
4
1/S

(E) MOLECULAR WEIGHT DETERMINATIONS BY GEL FILTRATION

The method for estimation of molecular weights of proteins by Sephadex gel filtration, reported by Andrews (276) was followed with slight modification.

A column of 2.5 x 160 cm was maintained at 4° C by circulating the 4° C water through the glass jacket of the column. Sephadex G-75 (100-125 mesh) gel was allowed to swell in excess of distilled water for two days. The fine particles were removed by decantation and the gel was recovered on a Buchner funnel. The gel was then resuspended in 0.15 M phosphate buffer, pH 7.3, for 24 hours with several changes of the buffer in a cold room at 4° C. After the gel suspension was deaerated under reduced pressure, it was poured carefully into the chromatographic column which was half filled with the same buffer. When a 10 cm layer of the gel had settled out, the column outlet was opened and the column was continuously filled with the gel suspension. After prolonged settling and passage of the buffer through the column, a stable level of gel was achieved. The gel was packed to a height of 150 cm. A buffer reservoir was connected to the top of the column and the gel was washed for two days at a flow rate of 50 ml per hour until a constant level of gel bed was obtained. The height of the bed was adjusted to exactly 150 cm.

Protein samples of 5 to 10 mg were dissolved in 2 ml of the phosphate buffer and clarified by centrifugation at 15,000 x G for 20 minutes. The sample was applied to the column cautiously

so that the gel was not disturbed. After the sample had sunk into the gel, two 1-ml portions of the buffer were used to wash in any solution adhering to the column, the first being allowed to sink into the gel before the second portion was applied. The buffer reservoir was connected to the top of the column and the flow rate was adjusted to 30 ml per hour. The collection of effluent was started as soon as the sample had sunk into the gel. An LKB drop-counting fraction collector was employed. The elution volume, Ve, was measured as the effluent volume corresponding to maximum concentration of protein within the emerging fraction, which was detected by extrapolating both sides of the protein peak to the apex. The proteins of known molecular weights listed in Table 12 were used as reference standards for calibration.

The void volume, Vo, of the column can be determined by measuring the elution volume of a substance of high molecular weight which is well above the exclusion limit of the dextran gel used. Blue dextran of molecular weight 2,000,000 which is well above the exclusion limit of Sephadex G-75, served for this purpose. The void volume was measured on alternate runs and found to be fairly constant during the whole operation. It was in the range of 269.5 to 271.1 for six determinations, average 270.1 S.D. $\pm$ 0.6 ml.

The ratio of the elution volume to the void volume (Ve/Vo, Table 13) was plotted against the logarithm of molecular weights of the proteins as shown in Fig. 22. A linear relationship was

Protein	Literature value of mol. wt.	Method of determination *	f/f_o	Reference
Cytochrome C	13,400	S.D.	1.00	277
Bovine panc. RNase A	13,700	A.A.S.	1.04	5
Myoglobin	16,900	S.D.	1.11	278
α -Chymotrypsin	22,500	S.D.	1.12	279
Trypsin	23,800	S.D.	1.20	280
Pepsin	35,500	S.D.	1.08	281

* A.A.S. = amino acid sequence.
S.D. = sedimentation velocity-diffusion.

Table 12

MOLECULAR WEIGHTS OF STANDARD PROTEINS.

Protein	Elution Vol. (V_e), ml	Ve/Vo	Mol. wt.	Log mol. wt.
Cytochrome C	597.5	2.213	13,400	4.1271
Bovine panc. RNase	588.0	2.178	13,700	4.1367
Myoglobin	532.0	1.970	16,900	4.2279
α -Chymotrypsin	448.2	1.659	22,500	4.3522
Trypsin	440.4	1.630	23,800	4.3766
Pepsin	323.1	1.196	35,500	4.5502
Rabbit spleen RNase	576.8	2.136	(14,420)*	(4.159)*
Rabbit panc. RNase	579.7	2.146	(14,190)*	(4.152)*

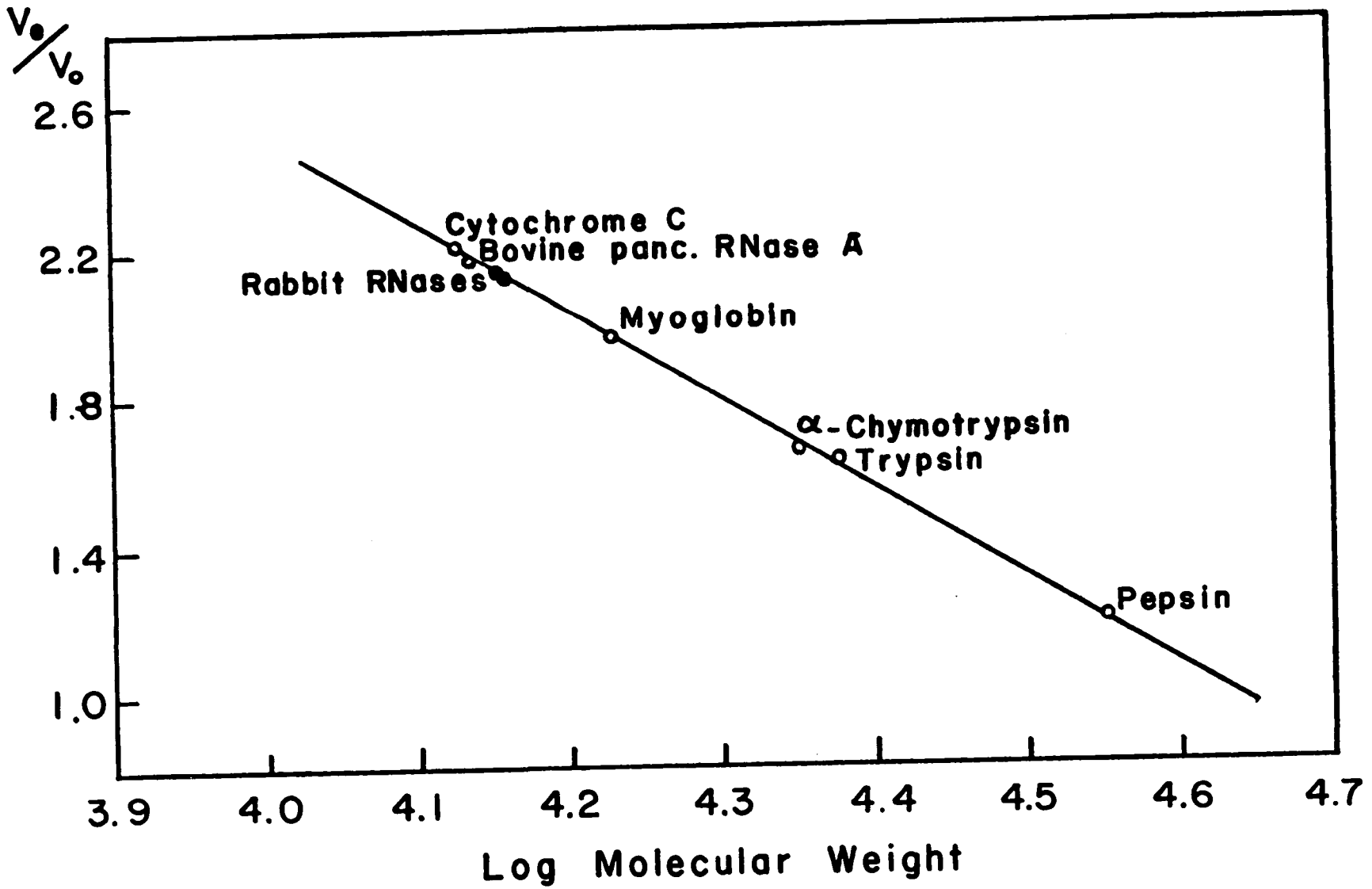
* Values determined from Fig. 22.

Table 13

ELUTION VOLUMES OF PROTEINS ON SEPHADEX G-75 COLUMN (2.5x150 cm).

Figure 22

DETERMINATION OF MOLECULAR WEIGHTS BY GEL FILTRATION ON SEPHADEX G-75 COLUMN (2.5x150 cm).


Ve/Vo
2.6
2.2
1.8
1.4
1.0
Cytochrome C
Bovine panc. RNase A
Rabbit RNases
Myoglobin
α-Chymotrypsin
Trypsin
Pepsin
3.9
4.0
4.1
4.2
4.3
4.4
4.5
4.6
4.7
Log Molecular Weight

observed. The rabbit spleen and pancreatic ribonucleases were found to have a molecular weight of 14,420 and 14,190 respectively as determined from Fig. 22.

(F) AMINO ACID COMPOSITIONS OF RABBIT RIBONUCLEASES.

The amino acid compositions of rabbit spleen and pancreatic ribonucleases were determined in a Beckman automatic amino acid analyzer, Model 120B. The pure rabbit spleen and pancreatic ribonucleases (4 to 7 mg) which were considered homogeneous in terms of the results obtained by electrophoresis, column chromatography and optical ultracentrifugation (as mentioned in Chapter III), were hydrolyzed in 6 N hydrochloric acid at 105 ^{o}C for 24 hours in sealed tubes. After removal of the hydrochloric acid by lyophilization, the amino acids were dissolved in 4 to 6 ml of distilled water. Aliquots (0.5 ml) of these sample were applied to the columns of the analyzer. Two chromatographic column were used, i.e. a short column (0.9 x 15 cm) operated at pH 5.3 to separate the basic amino acids, ammonia and tryptophan, and the long column (0.9 x 150 cm) operated at pH 3.25 and 4.25 for the separation of the remaining common amino acids. The amount of each amino acid was expressed as micromoles of amino acid per gram of protein sample as shown in Table 14 and Table 15. The ratio of micromoles of each amino acid to the amino acid in smallest corresponding value, which was methionine, was taken as the mole ratio of these amino acids. Taking the nearest integral number of each amino acid, the total number of amino acid residues was calculated as 129 for rabbit spleen ribonuclease, and 127 for rabbit pancreatic ribonuclease. Based on the amino acid compositions of these rabbit ribonucleases, the exact

Amino acid	μ mole/gm protein	Ratio	Nearest integral number
Aspartic acid	669.93	15.76	16
Threonine	288.01	7.87	8
Serine	334.63	7.87	8
Glutamic acid	379.60	8.93	9
Proline	294.83	6.94	7
Glycine	429.97	10.12	10
Alanine	558.76	13.14	13
Cysteine	241.99	5.69	6
Valine	283.33	6.67	7
Methionine	42.51	1.00	1
Isoleucine	249.32	5.87	6
Leucine	335.49	7.89	8
Tyrosine	229.82	5.40	5
Phenylalanine	51.50	1.21	1
Lysine	590.30	13.89	14
Histidine	87.45	2.06	2
Arginine	367.72	8.65	9
Amide NH_2	701.31	16.40	16

Table 14

AMINO ACID COMPOSITION OF RABBIT SPLEEN RIBONUCLEASE.

Amino acid	μ mole/gm protein	Ratio	Nearest integral number
Aspartic acid	681.82	11.78	12
Threonine	342.40	5.92	6
Serine	353.35	6.10	6
Glutamic acid	1016.41	17.56	18
Proline	541.87	9.36	9
Glycine	616.50	10.65	11
Alanine	697.28	12.05	12
Cysteine	248.39	4.29	4
Valine	466.57	8.06	8
Methionine	57.88	1.00	1
Isoleucine	267.53	4.62	5
Leucine	512.79	8.86	9
Tyrosine	137.62	2.38	2
Phenylalanine	141.84	2.45	2
Lysine	768.08	13.27	13
Histidine	188.17	3.25	3
Arginine	345.89	5.98	6
Amide NH_2	789.78	13.65	14

Table 15

AMINO ACID COMPOSITION OF RABBIT PANCREATIC RIBONUCLEASE.

molecular weights can be computed as 14,305 for the spleen ribonuclease and 13,971 for the pancreatic ribonuclease. A comparison of the amino acid compositions of rabbit ribonucleases with those of ribonucleases from various sources is listed in Table 16.

Amino acid	Rabbit spleen RNase	Rabbit panc. RNase	Bovine panc. RNase	Rat panc. RNase	B. subtilis RNase	RNase T_1
Aspartic	16	12	15	15	17	15
Threonine	7	6	10	10	9	6
Serine	8	6	15	15	9	17
Glutamic acid	9	18	12	12	8	10
Proline	7	9	4	7	5	4
Glycine	10	11	3	8	11	12
Alanine	13	12	12	4	8	7
Cysteine	6	4	8	8	0	4
Valine	7	8	9	7	4	7
Methionine	1	1	4	4	1	0
Isoleucine	6	5	3	5	7	2
Leucine	8	9	2	3	7	3
Tyrosine	5	2	6	4	8	8
Phenylalanine	1	2	3	3	6	4
Lysine	14	13	10	11	9	1
Histidine	2	3	4	5	3	3
Arginine	9	6	4	6	6	1
Tryptophane	0	0	0	0	4	1
Amide NH_2	16	14	17	15	15	11
Total	129	127	124	127	122	105

Table 16

COMPARISONS OF AMINO ACID COMPOSITIONS OF DIFFERENT RIBONUCLEASES.

DISCUSSION

(A) OPTIMUM pH FOR ENZYMATIC ACTIVITIES OF RABBIT RIBONUCLEASES

The pH optimum for enzymatic activity has been used traditionally in classifying nucleolytic enzymes. Ribonucleases are generally divided into two groups, acid ribonucleases and alkaline ribonucleases, according to the pH optimum. Since the pH optimum varies considerable with the conditions for assay, such as substrate concentration, ionic strength, and types of buffer, it is not an absolute value and is defined as a function of these variables.

It is generally found that ribonucleases are active in the pH range of 5-9 with a pH optimum around 7.0 (75, 282, 283, 284). The pH optimum varies with the source of RNA. A pH optimum of about 7.6 was reported for bovine pancreatic ribonuclease using yeast RNA as substrate (75). On the other hand, an optimum pH of 6.5 was reported by Maver et al. (283) using rat liver RNA as substrate. The enzymatic activity of ribonuclease is also strongly affected by variations in ionic strength (271, 285). Kalnitsky (286) has also shown that the effects of ionic strength on the hydrolysis of RNA by ribonuclease are pH dependent.

As seen in Table 6, most of the mammalian ribonucleases show optimum activity at pH around 7.0. The spleens of pig and cow possess acid ribonucleases which have an optimum pH at 5.5 in addition to the alkaline ribonucleases. The ribonucleases from micro-organisms show a wide range of optimum pH. In

Taka-diastase both acid (T_2) and alkaline (T_1) ribonucleases are present. In present study, both rabbit spleen and pancreatic ribonucleases show an optimum pH at 7.0 like other mammalian ribonucleases. These rabbit ribonucleases are free of any acid ribonuclease as seen in Figures 18 and 19.

(B) INHIBITORS OF RABBIT RIBONUCLEASES

It has been known for a long time that ribonucleic acid is capable of binding significant quantities of metal ions (268, 269, 270). Wacker et al. (269, 270) demonstrated that relatively large amounts of metal ions were firmly bound to nucleic acid from diverse sources. These metal ions may be important in stabilizing the conformation of the nucleic acid (269, 287, 288) and in maintaining its biological activity (289). However, these bound metal ions in RNA may influence the accuracy of the results in studies of the inhibition of ribonuclease activities. Consequently they must be removed by dialysis against EDTA in the first place. Wojnar (270) reported that bovine pancreatic ribonuclease was inhibited by copper and zinc ions at very low concentrations so that the ions present in RNA can affect the results of inhibition of ribonuclease activity by metal ions. In order to obtain reliable results, the preparation of metal-free RNA was essential. In addition, all glassware was cleaned with a mixture of H_2SO_4-HNO_3 (1:1, v/v).

Although the inhibition of ribonuclease activity by different metal ions varied to some extent depending on the methods used by different workers, it is generally agreed that zinc and copper ions are inhibitory (49, 246, 271, 274, 277 290). These two ions are among the most inhibitory cations which affect the enzymatic activity of ribonucleases from diverse sources (48). Findlay (291) suggested that the inhibition by zinc ions was due to the formation of a ternary complex consisting of RNase, Zn^{++}, and cytidine-3-phosphate. Some years later, this view was

supported by the work of Breslow (292) who demonstrated that in the absence of 3'-cytidylic acid, each cupric ion was distributed among a set of approximately four spectrally similar sites; each site appeared to consist mainly of a single imidazole side chain. In the presence of 3'-cytidylic acid, binding of both cupric and zinc ions was strengthened and the cupric-binding sites were altered. Therefore the formation of a ternary complex consisting of a cupric or zinc ion, 3'-cytidylic acid and ribonuclease was suggested. Saundry (293) reported that cupric ions were bound to the imidazole groups of histidine residues at the active site.

Heparin was found to be inhibitory of the enzymatic activities of most mammalian ribonucleases, including bovine pancreatic (273), rat kidney and liver (273, 293), rat- and guinea pig-serum ribonucleases (26). In the present study rabbit spleen and pancreatic ribonucleases were inhibited by heparin just like other mammalian ribonucleases. The enzymatic activity of RNase T_1 from Taka-diastase was not inhibited by heparin at all. The inhibition of bovine pancreatic ribonuclease which is a basic protein has been explained in terms of its electrostatic combination with heparin which is anionic polymer. Similar finding was reported by Roth (273) that Treburon which is a synthetic, heparin-like, sulfated polygalacturonic acid, also inhibited the bovine pancreatic ribonuclease as well as rat kidney and liver ribonucleases. On the other hand, RNase T_1 being an acidic protein with an isoelectric point of pH 2.9

does not combine with heparin, and consequently is not inhibited by heparin. Being basic proteins, rabbit spleen and pancreatic ribonucleases are therefore inhibited by heparin (Table 9). The inhibition of bovine ribonuclease by heparin was found to be competitive (272) and pH dependent (273). Dickman (285) reported that the enzymatic activity of bovine pancreatic ribonuclease was inhibited by a group of anionic detergents including sodium lauryl sulfate and alkyl aryl sulfonate but not affected by four non-ionic detergents.

As seen in Table 9, rabbit ribonucleases are not affected by metal chelating agent, EDTA and sodium citrate. This implies that no metal ions are required for the enzymatic activities of rabbit ribonucleases. Since p-chloromercuribenzoate has no effect on the enzymatic activities of rabbit spleen and pancreatic ribonucleases, it appears that the rabbit ribonucleases are not sulfhydryl enzymes. Adachi (38) reported that sulfhydryl reagents, p-chloromercuribenzoate and cysteine did not affect the enzymatic activity of rabbit reticulocyte ribonuclease.

On the basis of these overall properties, it may be concluded that rabbit spleen and pancreatic ribonucleases behave similarly as other mammalian ribonucleases in response to different effectors, rather than the ribonucleases from yeast (34) or other micro-organisms (266, 267). The ribonucleases from micro-organisms are always inhibited or stimulated by EDTA.

(C) ISOELECTRIC POINTS OF RABBIT RIBONUCLEASES

One of the intrinsic characteristics of a protein is the isoelectric point which is the pH value at which the protein does not migrate in an electric field. Bovine pancreatic ribonuclease has been known to be a basic protein with an isoelectric point of 9.45 (294) as determined in glycine-NaOH buffer of ionic strength =0.01. Tanford (295) reported that the isoelectric points of bovine pancreatic ribonuclease were pH 9.604 and 9.658 in 0.001 M and 1.0 M KCl respectively. The basic property of bovine pancreatic ribonuclease is attributed to the high content of basic amino acids (lysine, arginine and histidine).

In the present study, rabbit spleen and pancreatic ribonucleases were also found to be basic proteins. The basic behaviour of these ribonucleases revealed on electrophoresis is in accord with their amino acid compositions, as is evident from Table 16 in Section (F) these rabbit ribonucleases have high contents of the basic amino acids lysine, arginine and histidine, i.e. 19.4 and 17.3% respectively for the spleen and pancreatic ribonucleases. Since the isoelectric point of a protein varies with the ionic strength of the buffers used, the electrophoretic mobilities of the rabbit ribonucleases isolated in this study were compared with that of bovine pancreatic ribonuclease under identical conditions of pH, ionic strength and applied voltage. It was thus found that rabbit spleen RNase was more basic than bovine RNase, whereas the rabbit pancreatic RNase was less basic than the later (Fig. 20).

The bovine pancreatic ribonuclease had an apparent isoelectric point of 10.13 as determined by zone electrophoresis under the conditions used in the present study. This value is slightly higher than the value of 9.45 determined by Alberty (294) by moving boundary electrophoresis in a Tiselius apparatus, with glycine-NaOH buffers of ionic strength 0.01, i.e. at a salt concentration ten times lower than that used in the present study.

(D) EFFECTS OF SUBSTRATE CONCENTRATION ON ENZYMATIC ACTIVITIES OF RABBIT RIBONUCLEASES

In most cases, the rate of an enzyme-catalyzed reaction increases with increasing substrate concentration except where the product is inhibitory to the enzyme. Ordinarily the rate increases until a maximum is reached. In 1913 Michaelis and Menten (296) sought to extend Henri's work (297) and suggested that the enzyme first formed a complex with its substrate and it subsequently broke down to give the free enzyme and the products of the reaction.

The Michaelis-Menten constant (K_m) refers to the substrate concentration at half-maximum reaction rate. That is, $K_m = S$ when $V_m = 2\ v$. The constant, K_m, can be expressed as follows:

$$K_m = \frac{(V_m - v)\ S}{v} \qquad \text{.....(Eq. 1)}$$

where V_m is the maximum velocity,
v is the velocity of reaction,
S is the concentration of substrate.

The Michaelis-Menten equation can be written in a linear form in the following three ways (298, 299, 300, 301, 302):

(i) $$\frac{1}{v} = \frac{K_m}{V_m} \cdot \frac{1}{S} + \frac{1}{V_m} \qquad \text{.....(Eq. 2)}$$

(ii) $$\frac{S}{v} = \frac{1}{V_m} \cdot S + \frac{K_m}{V_m} \qquad \text{.....(Eq. 3)}$$

(iii) $$V_m = v + \frac{v}{S} \cdot K_m \qquad \text{.....(Eq. 4)}$$

These linear equations have many advantages in the graphical evalution of the Michaelis-Menten constant. The double reciprocal plot obtained from Eq. 2 is most widely used (303).

The results of the present study indicate that rabbit spleen ribonuclease has a lower value of K_m than the pancreatic ribonuclease. Since a low K_m value indicates a high enzyme-substrate affinity and vice versa, rabbit spleen ribonuclease must therefore have a higher affinity towards the RNA substrate than the pancreatic ribonuclease. Recalling the results in Chapter III which indicate that the purified rabbit spleen ribonuclease has a higher specific activity than the pancreatic ribonuclease, these results are therefore in agreement. It is evident that having a low value of K_m, rabbit spleen ribonuclease acts on RNA molecules faster than the pancreatic ribonuclease, hence a higher specific activity is observed.

Rabinovitch (26) studied the effects of substrate (RNA) concentration on enzymatic activities of guinea pig serum ribonuclease and bovine pancreatic ribonuclease and reported the values of Michaelis-Menten constant for guinea pig serum ribonuclease and bovine pancreatic ribonuclease to be 2.24 and 1.48 respectively. Edelhoch (304) reported a value of Michaelis-Menten constant for the hydrolysis of RNA by bovine pancreatic ribonuclease to be 1.25 mg per ml. These values are comparable to those for rabbit ribonucleases determined in the present study.

(E) MOLECULAR WEIGHT DETERMINATIONS BY GEL FILTRATION

Although the molecular weight of a protein can be determined by various physicochemical methods such as sedimentation equilibrium, sedimentation velocity-diffusion, and osmotic pressure, gel filtration has proved to be useful because of the extreme simplicity of this procedure (276). Of a variety of gel media used for separation of proteins, Sephadex which are cross-linked dextrans (305), and polyacrylamide (306) gave better separation than agar gels (307). Andrews (276) obtained a correlation between molecular weights of proteins and their corresponding elution volumes, V_e, on gel filtration through Sephadex G-75 and G-100 ranging from 3,500 to 150,000. Whitaker (308) obtained an excellent linear correlation between the logarithm of the molecular weight of a protein and the ratio of its elution volume to the void volume, V_o. By using Sephadex G-75, he covered the molecular weight range of 13,000-42,200, and with Sephadex G-100 for the range of 13,000-76,000. Leach (309) reported that proteins of molecular weight up to 225,000 could be determined on a column of Sephadex G-200.

In the present study an excellent linear relationship (Fig. 22) was obtained for proteins of molecular weight range 13,000-36,000 on a column of Sephadex G-75. The rabbit spleen and pancreatic ribonucleases have almost the same molecular weights which are slightly greater than that of the bovine pancreatic ribonuclease A. Since Leach (309) found that a 2 cm diameter column gave higher resolution than a 0.9 cm diameter

column, a 2.5 cm diameter column was used in the present study. The column employed has a void volume, V_o, of 270.1 ml. As suggested by Whitaker (308) one should use a column with void volume greater than 100 ml, in order to obtain a molecular weight which is significant to three figures without interpolation to the nearst 0.1 ml. The main sources of error introduced in the determination of molecular weight by gel filtration method are the changes in protein conformation, dissociation or association of protein molecules, and adsorption of proteins on the dextran gel. The reference proteins used in the present study are nearly spherical (Table 12, f/f_o = approximately 1.0), so that the effect of molecular shape on the elution volume is expected to be negligible.

(F) AMINO ACID COMPOSITION OF RABBIT RIBONUCLEASES

One of the valuable characteristics of a protein is the amino acid composition which provides useful information for the elucidation of complete amino acid sequence of the protein. The comparisons of the amino acid compositions of rabbit spleen and pancreatic ribonucleases with some mammalian ribonucleases such as bovine (310) and rat (311), and also ribonucleases from micro-organisms like B. subtilis (112) and RNase T_1 (267) as seen in Table 12, reveal several interesting points. The most obvious feature is the high content of basic amino acids in the rabbit and other mammalian ribonucleases, but not in RNase T_1. The percentage of basic amino acids in rabbit spleen RNase, rabbit pancreatic RNase, bovine pancreatic RNase, rat pancreatic RNase, B. subtilis RNase and RNase T_1 are found to be: 19.4%, 17.3%, 14.5%, 17.3%, 14.8% and 4.8% respectively. Having high percentage of basic amino acids, mammalian ribonucleases always have an isoelectric point around 9 to 10. In this connection, it is interesting to note that RNase T_1 with exceptionally low content of basic amino acids, has an isoelectric point of pH 2.9 (267).

Another feature which is noteworthy is the even number of cysteine residues in these ribonucleases. They form intramolecular disulfide bridges in these ribonucleases without any free sulfhydryl group. Ribonuclease from B. subtilis has no disulfide bond so that the molecule is more flexible. From the amino acid composition determined in this study it may be inferred that rabbit spleen

ribonuclease has three intramolecular disulfide bridges, whereas rabbit pancreatic ribonuclease has only two, just as the RNase T_1. The total number of amino acid residues for these ribonucleases are rather similar with a molecular weight of approximately 13,000 to 14,000.

Taking an overall view of the results presented, it may be concluded that the amino acid compositions of rabbit spleen and pancreatic ribonucleases are significantly different from those of bovine pancreatic ribonuclease, although some similarities are observed. As will be shown in Chapter V, sheep anti-rabbit spleen ribonuclease antibodies (Fig. 24) did not cross-react with bovine pancreatic or rat pancreatic ribonucleases which would suggest that due to the differences in amino acid compositions of these ribonucleases, the antigenic determinants might be different. The actual molecular weights for rabbit spleen and pancreatic ribonucleases as calculated from the number of amino acid residues are found to be 14,305 and 13,971 respectively. These values are in accord with the molecular weights determined by gel filtration method as mentioned previously in Section (E) of this Chapter.

CHAPTER V

PURIFICATION OF RABBIT RIBONUCLEASE BY THE USE OF IMMUNOSORBENTS

INTRODUCTION

Since the first report by Campbell et al. (312), many types of immunosorbents have been synthesized and used for the isolation of antibodies and antigens (43, 222, 223, 313, 314). Because of the exquisite specificity of antigen-antibody reactions, the use of immunosorbents leads in general to the isolation of antibodies and antigens in a state of purity not readily achieved by physicochemical methods. Highly monospecific antibodies against different determinants on a protein antigen have been isolated by the use of immunosorbents (228, 229). Givas et al. (228), in this laboratory, employed a synthetic heptapeptide corresponding to the C-terminal of myoglobin to elute monospecific antibodies from an appropriate immunosorbent consisting of myoglobin coupled to ethylene maleic anhydride copolymer. Monospecific antibodies against C-terminal portion of staphylococcal nuclease were isolated by means of immunosorbent (229). Furthermore, monospecific anti-hapten antibodies have also been isolated by the utilization of immunosorbents (231, 233).

Immunosorbents are commonly prepared by polymerizing the antigens or antibodies by use of bi-functional or multi-functional molecules like ethyl chloroformate (41), glutaraldehyde

(225), N-acetyl homocysteine thiolactone (226) and ethylene maleic anhydride copolymer (42, 43), or attaching the antigens or antibodies to insoluble supporting media such as carboxymethyl cellulose (222), bromoacetyl cellulose (223, 224) and p-amino-phenylbutyryl-aminoethyl-cellulose (314).

In Chapter III, chromatographic procedures for the purification of rabbit spleen and pancreatic ribonucleases in a pure form were described. However, the methods used were time-consuming and laborious. Consequently it was decided to explore the possibility of obtaining pure rabbit ribonuclease in a one-step procedure by use of reverse immunosorbents synthesized by attachment of 'monospecific' antibodies to spleen ribonuclease to an insoluble matrix. For this purpose, sheep antibodies against rabbit spleen ribonuclease were insolubilized by two methods, i.e. with ethyl chloroformate by the method of Avrameas (41), and with ethylene maleic anhydride by the method developed by Centeno and Sehon in this laboratory (42, 43). The resulting reverse immunosorbents were successfully employed to isolate pure rabbit spleen ribonuclease in high yields.

MATERIALS AND METHODS

RABBIT RIBONUCLEASES: Rabbit spleen and pancreatic ribonucleases were isolated and purified as described in Chapter III.

REAGENTS: All commercial chemicals were reagent grade; bentonite, agarose and ethyl chloroformate were purchased from Fisher Scientific Company. Ethylene maleic anhydride copolymer (EMA-31) was generously provided by Monsanto Company, La Salle, Quebec, Canada. Freund's complete adjuvant was purchased from Difco Laboratories, Michigan, U.S.A. Millipore filters were purchased from Millipore Filter Corporation, Bedford, Massachusetts, U.S.A.

PRODUCTION OF SHEEP ANTISERUM AGAINST RABBIT SPLEEN RIBONUCLEASE:

Ten mg of the pure rabbit spleen ribonuclease (Preparation V as mentioned in Chapter III) dissolved in 1.0 ml of physiological saline was emulsified with 1.3 ml of complete Freund's adjuvant with a MulsiChurn (made by MulsiJet Inc., Elmhurst, Illinois). A female sheep weighing about 200 lbs was injected intramuscularly with this emulsion. Subsequent injections were made at two weeks' intervals. The sheep was bled one week after the fourth injection. The blood obtained was allowed to clot at 4° C overnight and the antiserum was separated by centrifugation at 5,000 x G for 20 minutes. After passing through a Millipore filter (pore size 0.45 μ), the antiserum was stored in sterile bottles at 4° C. Immunization of the sheep was continued for production of additional amounts of antiserum.

IMMUNODIFFUSION: Immunodiffusion was carried out in agar gel on microscope slides (2.5 x 7.5 cm). Agar (1% w/v) in physiological saline was heated in boiling water until all the agar dissolved and then poured over onto some microscope slides in a plastic tray to make a uniform layer of gel (about 1.5 mm thick) on the slides. After the gel had solidified, some wells were made with a gel punch by LKB-PRODUKTER AB, Sweden. The enzymes and antiserum were placed into corresponding wells and allowed to stand at room temperature in a humid chamber for 24 to 48 hours. To remove unreacted proteins, the slides were washed with saline for 24 hours, covered with filter paper, dried at room temperature, and then stained in 1% Amido Black 10B in 5% acetic acid. The precipitin bands could be seen after washing the slides with 5% acetic acid, when the background was clear.

IMMUNOELECTROPHORESIS: For immunoelectrophoresis in agarose gel the LKB 6800A apparatus made by LKB-PRODUKTER AB, Sweden, was used with veronal buffer of ionic strength 0.10 at pH 8.6 containing 0.01 M diethylbarbituric acid, 0.05 M sodium diethyl-barbiturate and 0.05 M sodium acetate. The agarose gel (1%) was also prepared in this buffer. Troughs and wells were punched in the gel layer. After applying about 5 µl of sample in the wells, direct current at 250 volts was applied for one hour. The gel in the troughs was removed and then filled with the immune serum. It was allowed to stand in a humid chamber at room temperature for 20 hours. After washing off the excess proteins with 1% sodium chloride solution, the gel was covered with wet filter

paper and dried in air. Finally the gel was stained with acid fuchsin solution, consisting of 0.5 gm of acid fuchsin in a solution of 125 ml absolute methanol, 100 ml distilled water and 25 ml of glacial acetic acid. The excess dye was removed by rinsing the slides in 5% acetic acid.

COLUMN CHROMATOGRAPHY: A column (2.5 x 100 cm) was packed with Sephadex G-75 gel equilibrated in 0.1 M phosphate buffer at pH 7.3. Protein samples dissolved in 10 ml of the buffer were applied to the column and then eluted with the same buffer. Six-ml fractions were collected at a flow rate of 1 ml per minute.

ANALYTICAL ULTRACENTRIFUGATION: It was performed at 59,780 rpm in a Beckman model E optical ultracentrifuge, the rotor temperature being maintained at 20° C. The protein sample was dissolved in 0.15 M phosphate buffer at pH 7.3 to give a 1% solution.

ELECTROPHORESIS: Cellulose acetate electrophoresis was performed in barbital buffer of ionic strength 0.075 at pH 8.6 in a Beckman Microzone electrophoresis cell, Model R-101, as described in Chapter III.

RESULTS

The presence of antibodies in the antiserum were readily demonstrated by the ring test as well as by immunodiffusion and immunoelectrophoresis. From the immunodiffusion patterns shown in Figures 23 and 24, it is evident that the antibodies gave only one precipitin band with the purified or crude rabbit spleen ribonuclease preparations as well as with the pure or crude rabbit pancreatic ribonuclease preparations. The precipitin bands of the antibodies with both rabbit spleen and pancreatic ribonucleases showed a reaction of identity. The antibodies did not react with ribonucleases isolated from other species, such as bovine pancreas, rat liver, rat spleen or pancreas. These results were corroborated on immunoelectrophoresis as demonstrated in Figures 25 and 26. Both the pure and crude preparations of rabbit spleen ribonuclease gave only a single precipitin band. All these results prove that the resulting antibodies are 'monospecific' to the rabbit ribonuclease.

PREPARATION OF REVERSE IMMUNOSORBENTS:

(A) ETHYL CHLOROFORMATE METHOD (41)

The method of Avrameas (41) was followed. Ethyl chloroformate (1.2 ml) was added dropwise to a mixture of 20 ml of antiserum and 1.0 ml of 2 M acetate buffer at pH 5.0 with constant stirring. The pH of the reaction mixture was maintained at 4.5 to 5.0 by dropwise addition of 1 N sodium hydroxide. A precipitate was formed as soon as the ethyl chloroformate was added. After completion of

Figure 23

IMMUNODIFFUSION PLATE OF RABBIT RIBONUCLEASES.

Well (1): Sheep antibodies to rabbit spleen RNase.

Well (2): Crude extract of rabbit pancreas.

Well (3): Purified rabbit pancreatic RNase.

Well (4): Purified rabbit spleen RNase.

Well (5): Crude extract of rabbit spleen.

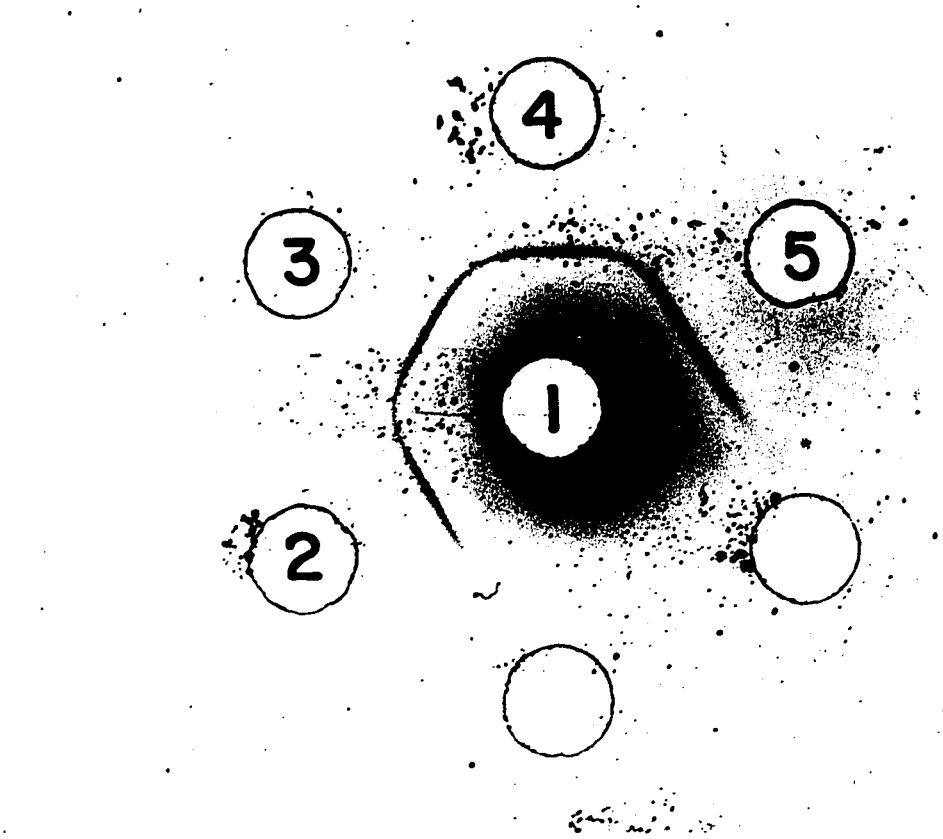

4
3
5
1
2

Figure 24

IMMUNODIFFUSION PLATE OF RIBONUCLEASES FROM VARIOUS SOURCES.

Well (1): Sheep antibodies to rabbit spleen RNase.

Well (2): Rabbit spleen RNase.

Well (3): Rabbit pancreatic RNase.

Well (4): Bovine pancreatic RNase.

Well (5): Rat spleen RNase.

Well (6): Rat pancreatic RNase.

Well (7): Rat liver RNase.

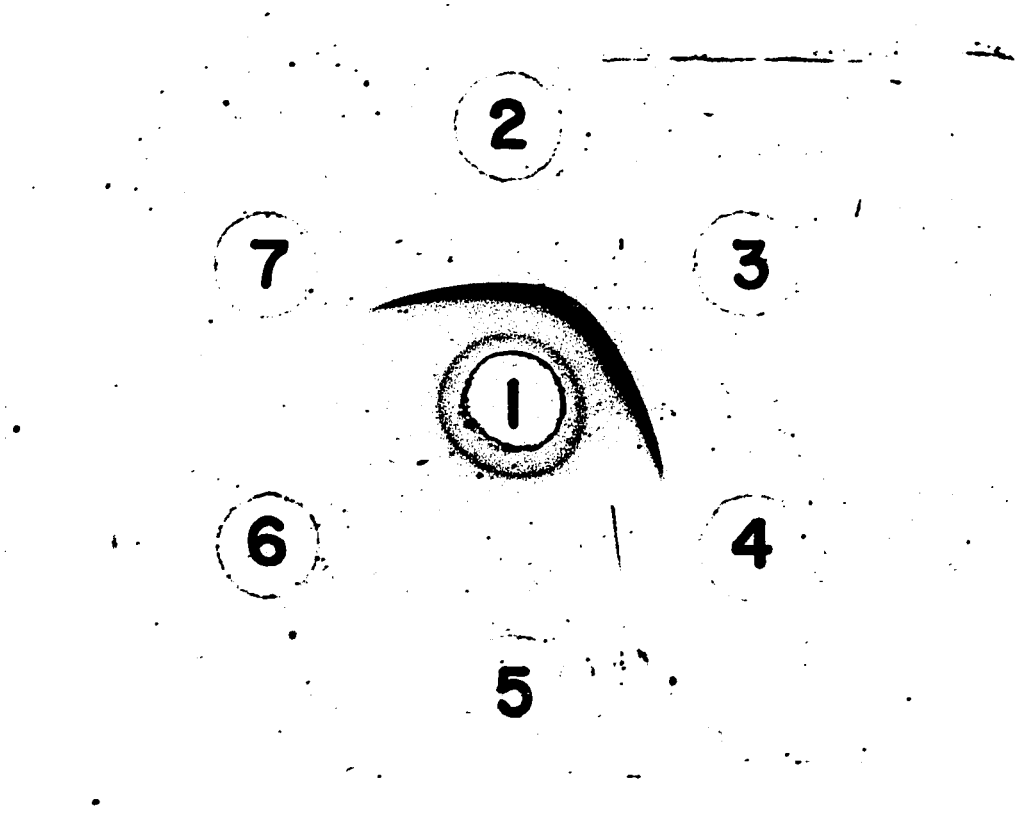

2
7
3
1
6
4
5

Figure 25

IMMUNOELECTROPHORESIS OF RABBIT SPLEEN RIBONUCLEASE.

Well (1): Purified rabbit spleen RNase.

Well (2): Crude extract of rabbit spleen.

Trough (Ab): Sheep antibodies to rabbit spleen RNase.

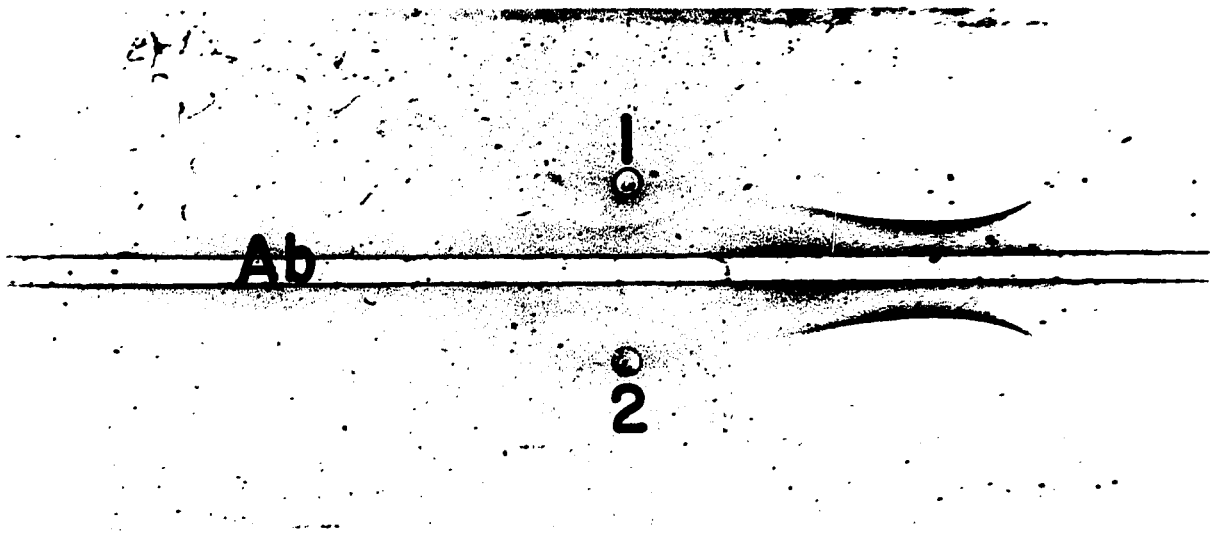

1
Ab
2

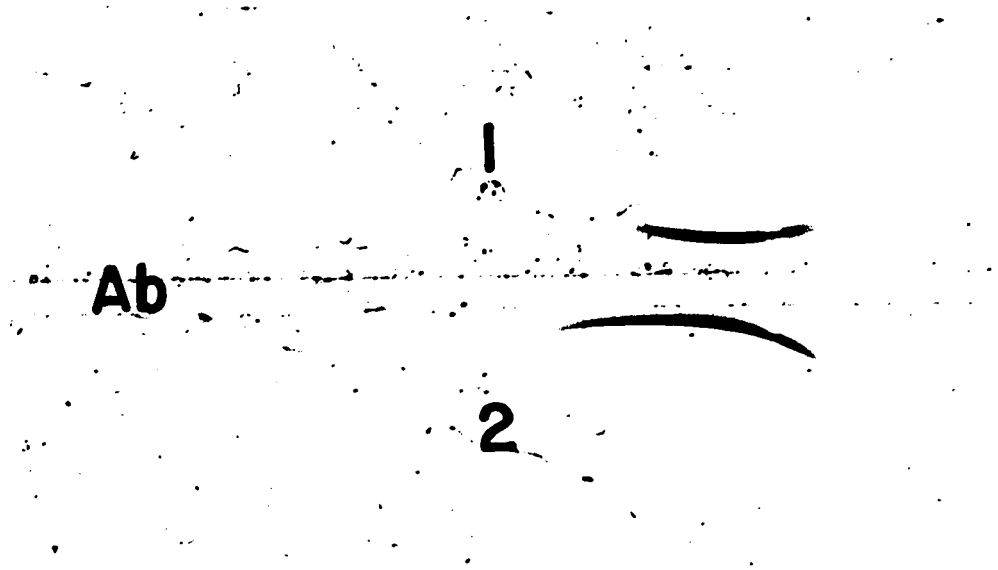
Figure 26

IMMUNOELECTROPHORESIS OF RABBIT SPLEEN AND PANCREATIC RIBONUCLEASES.

Well (1): Rabbit spleen RNase.

Well (2): Rabbit pancreatic RNase.

Trough (Ab): Sheep antibodies to rabbit spleen RNase.


1
Ab
2

the polymerization which took place within 30 minutes, as judged by the measurement of the absorbance of supernatant at 280 mμ, the mixture was stirred into 20 ml of 2 M acetate buffer, pH 5.0 and allowed to stand at room temperature for one hour. After the addition of 200 ml of phosphate-buffered saline (PBS, consisting of 0.85% NaCl in 0.02 M phosphate buffer, pH 7.2), the polymer was homogenized for one minute with a Virtis homogenizer. The reverse immunosorbent was collected by centrifugation at 15,000 x G for 15 minutes. The immunosorbent was washed successively with PBS (approximately 3 liters), 0.1% sodium carbonate (500 ml), PBS (500 ml) and glycine-HCl buffer (pH 2.0, 0.4 M) until the optical density of the eluates was zero at 280 mμ and subsequently with PBS (2 liters) until the pH of the eluate was 7.2.

For the adsorption of rabbit ribonuclease, 80 ml of the crude extract of rabbit spleen ribonuclease with enzymatic activity of 6,560 units, was treated with the reverse immunosorbent for two hours at room temperature. After centrifugation at 15,000 x G for 20 minutes the supernatant which contained negligible enzymatic activity was removed. The immunosorbent was washed repeatedly by successive suspension in PBS and sedimentation by centrifugation at 15,000 x G for 20 minutes until the optical density of the supernatant at 280 mμ was zero (approximately 10 times). The antigen was subsequently eluted with three 100-ml portions of 0.4 M glycine-HCl buffer at pH 2.0. The eluates which were collected by centrifugation at 15,000 x G for 20 minutes were neutralized immediately with 1 N sodium hydroxide and dialyzed against distilled

water. The reverse immunosorbent was further washed with PBS (roughly 2 liters) until the pH of the eluate was neutral. The reverse immunosorbent was then ready for use again.

The results of the batchwise elution of antigen from the reverse immunosorbent are shown in Table 17. The specific activity of the ribonuclease eluted from the reverse immunosorbent increased very significantly. A 49-fold purification was achieved with a recovery of 82.6%. With the immunosorbent prepared with 10 ml of antiserum, at least 6.3 mg of ribonuclease could be isolated.

(B) EMA METHOD (42, 43):

A gamma globulin preparation, which was precipitated from 10 ml of the antiserum at 2.5 M concentration of ammonium sulfate, was dissolved in 75 ml of phosphate-buffered saline and cooled to 4° C. A 1% (w/v) solution of EMA-31 (45 ml) in acetone was added dropwise with constant stirring. The amount of EMA required for polymerization was established previously by adding various volumes of the EMA solution into 0.4 ml of antiserum which was diluted to 3.0 ml with PBS. The unreacted proteins remaining in the supernatant were determined by Lowry's method as described in Chapter III. The results in Table 18 show that 1.8 ml of EMA solution was needed to polymerize 0.4 ml of the antiserum.

After stirring the mixture for one hour at 4° C, the reverse immunosorbent was collected by centrifugation at 5,000 x G for 10 minutes. The washing of reverse immunosorbent was carried out by the standard procedure described below. The precipitate was washed

Sample	Volume, ml	Enz. activity, unit/ml	Proteins, mg/ml	Specific activity
Crude RNase extract	80	82.00	9.213	8.9
Proteins not adsorbed	80	0	7.710	0
Dissociation step I *	100	52.75	0.121	436.1
Dissociation step II *	100	1.45	0.004	362.3
Dissociation step III *	100	0	0	0

* With 0.4 M glycine-HCl buffer, pH 2.0.

Table 17

BATCHWISE ELUTION OF SPLEEN RIBONUCLEASE FROM REVERSE IMMUNOSORBENT MADE WITH ETHYL CHLOROFORMATE.

Vol. (ml) of 1% EMA solution added to 0.4 ml of antiserum diluted to 3.0 ml.	Proteins remaining in supernatant, mg/ml
0.1	4.05
0.2	3.45
0.6	1.42
1.0	0.99
1.5	0.22
1.8	0
2.0	0
4.0	0

Table 18

AMOUNT OF EMA REQUIRED TO POLYMERIZE THE ANTIBODIES.

many times with PBS until the optical density of the supernatant was lower than 0.01 at 280 mμ. It was suspended in a mixture of 100 ml of PBS and 10 ml of 1 M disodium phosphate and stirred overnight in a cold room at 4° C. The immunosorbent was washed again with PBS for several times. The immunosorbent was resuspended in a mixture of 100 ml of PBS and 10 ml of monosodium phosphate at 4° C and stirred overnight. Hydrochloric acid (1 N) was added dropwise until the pH decreased to 3.0 to elute any material which may be released from the immunosorbent at low pH, since the subsequent dissociation of the enzyme from the immunosorbent involved treatment of the column with acid. All the washing was done by centrifugation at 5,000 x G for 15 minutes. After washing the immunosorbent which was prepared with 30 ml of the antiserum three more times with cold PBS, it was dispersed homogeneously in Sephadex G-25 (coarse) which was equilibrated with PBS. The mixture was packed into a chromatographic column (4 x 70 cm) which contained a layer of Sephadex G-25 of one centimeter height. After washing the column with PBS overnight, the column washed with 0.4 M glycine-HCl buffer at pH 2.0 until the effluent was free from any proteins. Finally the column was washed with PBS until the pH of the effluent was neutral. The column at this stage was ready for use. Absorption of antigen was achieved by applying 70 ml of the concentrated crude spleen extract which contained 7,510 units of ribonuclease activity to the immunosorbent column. The flow rate was adjusted to 10 ml per hour. After the column was washed with PBS overnight at this slow flow rate, the eluate was subsequently allowed to flow fast at a rate of 10 ml per

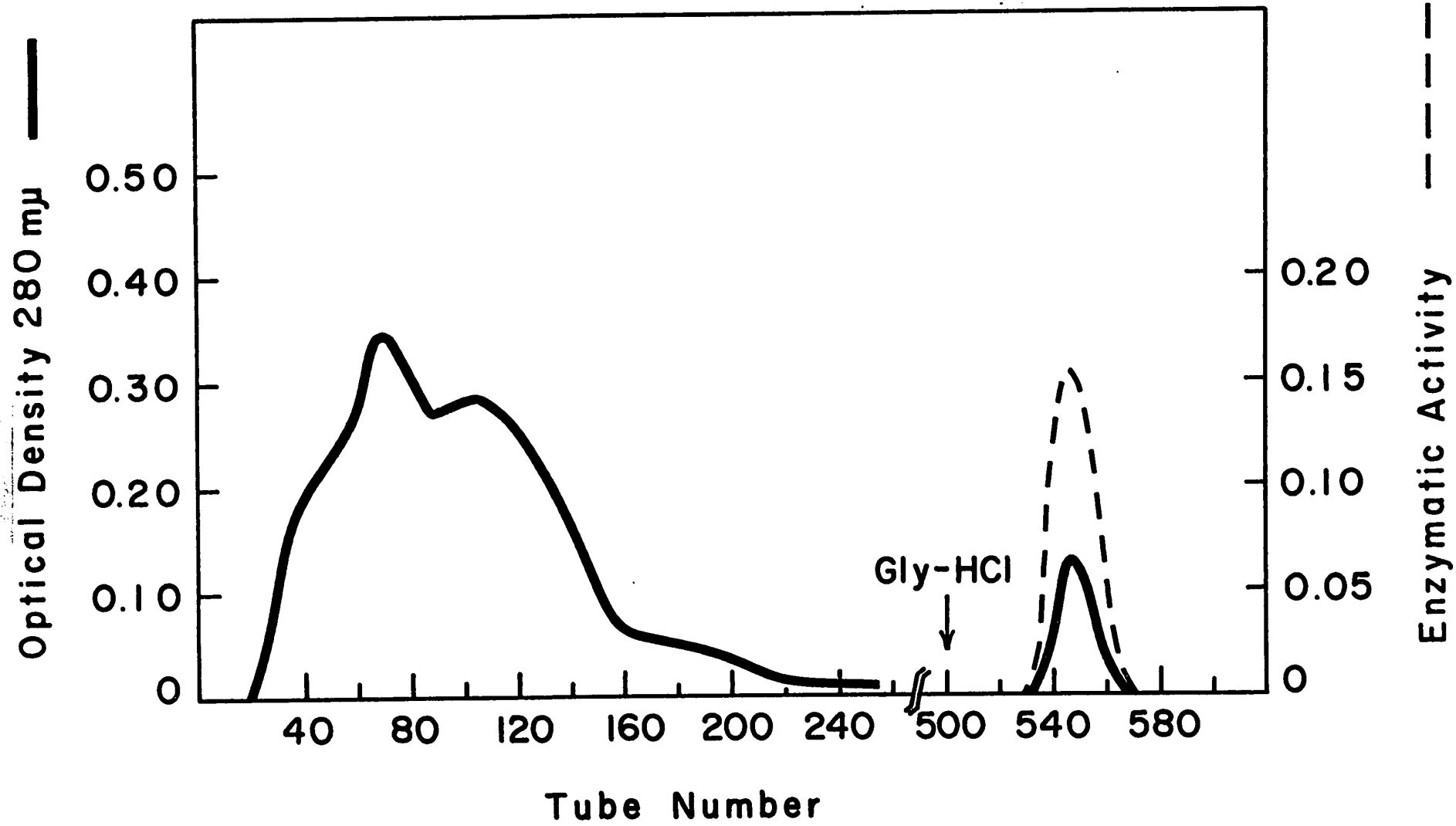
minute. The column was washed with large amount of PBS (5 to 6 liters) until the optical density of the effluent at 280 mμ was zero. Glycine-HCl buffer (0.4 M, pH 2.0) was then applied to elute the absorbed ribonuclease. As seen in Fig. 27, the RNase activity corresponding to a total of 6,480 units was associated with theprotein located in a narrow volume range. The fractions containing the enzyme were pooled and dialyzed immediately against distilled water. After a few changes of water, the enzyme was lyophilized. As shown in Table 19, the enzyme was purified 48-fold with a recovery of 86.2%. The immunosorbent column was further washed with a large volume of PBS until the pH of the effluent was neutral; then the column was ready for use again.

On examination of the ribonuclease, which was eluted from the immunosorbent, by optical ultracentrifugation in a Beckman Model E analytical ultracentrifuge (Fig. 28) a symmetrical peak with a sedimentation coefficient of 1.6S was observed.

The eluted ribonuclease was further subjected to chromatography on Sephadex G-75 (column of 2.5 x 100 cm) which had been equilibrated with 0.1 M phosphate buffer at pH 7.3. From the elution profile (Fig. 29) it is evident that the bulk of the ribonuclease activity was associated with the major protein; in addition to a small amount of inactive protein emerged from the column right after the major peak. This finding was further confirmed by the result of electrophoresis on cellulose acetate. As seen on the electrophorogram (Fig. 30) small amounts of impurities were present in the ribonuclease eluted from the immunosorbent. These impurities were removed after

Figure 27

ELUTION PROFILE OF SPLEEN RIBONUCLEASE FROM REVERSE IMMUNOSORBENT MADE WITH EMA.


Optical Density 280 mμ
0.50
0.40
0.30
0.20
0.10
0
Gly-HCl
0.20
0.15
0.10
0.05
0
Enzymatic Activity
40
80
120
160
200
240
500
540
580
Tube Number

Sample	Volume, ml	Enz. act. unit/ml	Proteins, mg/ml	Specific act. unit/mg
Crude extract concentrated	70	107.29	11.176	9.6
Proteins not absorbed	2,635	0	0.286	0
RNase eluted	492	13.17	0.028	470.3

Table 19

ELUTION OF SPLEEN RIBONUCLEASE FROM REVERSE IMMUNOSORBENT MADE WITH EMA.

Figure 28

ULTRACENTRIFUGAL PATTERN OF RABBIT SPLEEN RIBONUCLEASE ELUTED FROM REVERSE IMMUNOSORBENT.

Pictures were taken at intervals of 4 minutes.

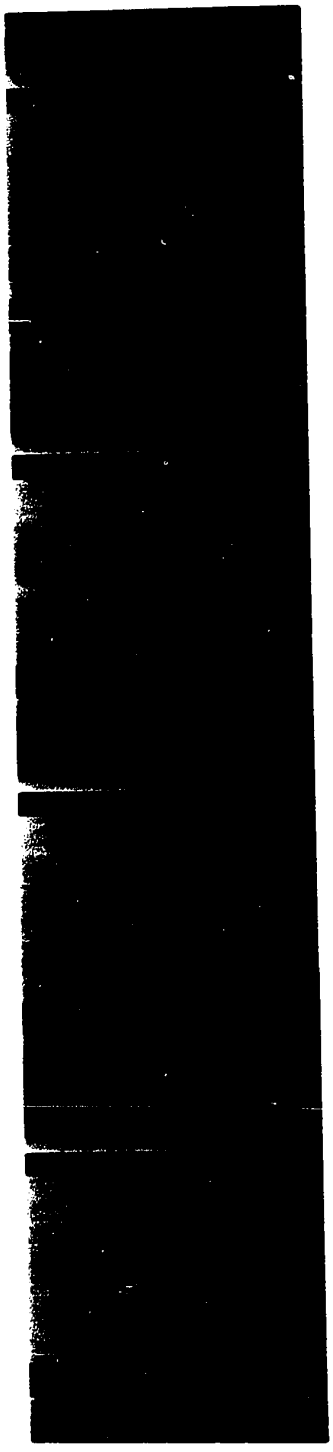


Figure 29

CHROMATOGRAPHY OF RABBIT SPLEEN RIBONUCLEASE ELUTED FROM REVERSE IMMUNOSORBENT ON SEPHADEX G-75.

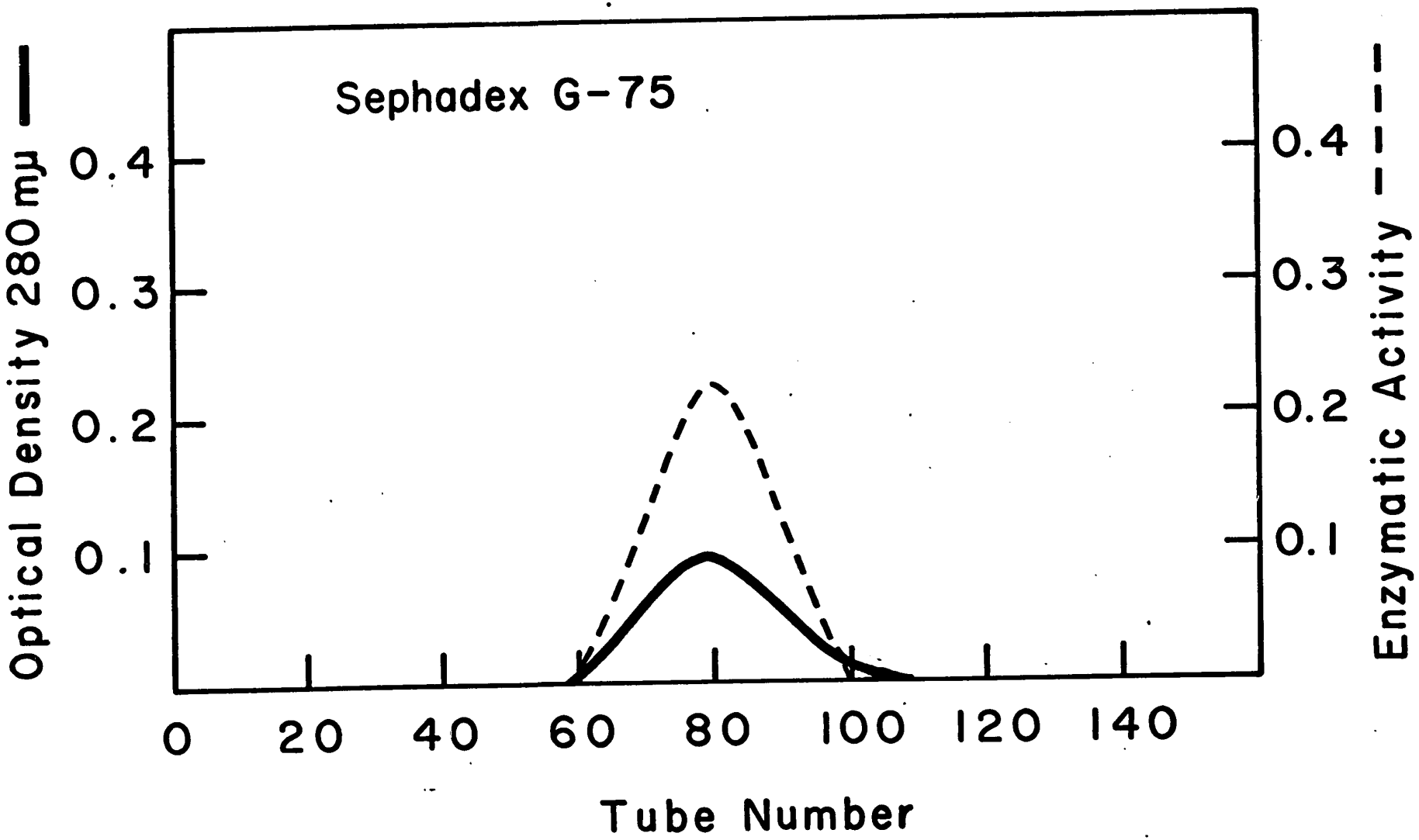

Sephadex G-75
Optical Density 280mμ
Enzymatic Activity
0.4
0.3
0.2
0.1
0
20
40
60
80
100
120
140
Tube Number

Figure 30

ELECTROPHOROGRAM OF RABBIT SPLEEN RIBONUCLEASE ELUTED FROM REVERSE IMMUNOSORBENT.

A: Crude extract of rabbit spleen.

B: RNase eluted from immunosorbent.

C: RNase from Sephadex G-75 column after eluted from immunosorbent.


C
B
A

passing through the Sephadex G-75 column. With the reverse immunosorbent prepared with 10 ml of antiserum, 6 to 7 mg of purified ribonuclease was recovered.

DISCUSSION

Rabbit spleen ribonuclease, like other proteins, induced the production of antibodies in the animal in which it was injected. The resulting antibodies were highly species specific as shown in Fig. 24. The formation of a single precipitin band on immunodiffusion (Figures 23 & 24) and immunoelectrophoresis (Figures 25 & 26) was considered as evidence for the purity of the enzyme isolated in this study and used for eliciting the antibody response in sheep. However, this does not imply that a homogeneous population of antibodies was present, since in all probability antibodies directed against different determinants of the ribonuclease molecule would have been produced.

In the present study, reverse immunosorbents were prepared by two different methods. The first method of Avrameas (41) involves the cross-linking of antibodies by the small bi-functional organic compound, ethyl chloroformate, whereas in the procedure developed in this laboratory EMA-31 plays the dual role in attaching covalently the antigen or antibodies to the polymeric backbone of ethylene maleic anhydride, and in providing a lattice for the immunosorbent.

The antigens absorbed on immunosorbents may be eluted by many reagents such as buffers at pH 2-3 (209, 210, 212, 315, 316), 5 M potassium iodide (41) or 0.1 N hydrochloric acid (210, 317). The elution of rabbit spleen ribonuclease from the reverse immunosorbent

by 0.4 M glycine-HCl buffer at pH 2.0 was found advantageous in the present study since ribonucleases were very stable at low pH. In fact, ribonucleases were extracted from tissues with 0.25 N sulfuric acid and shown to retain their enzymatic activities at pH 2.0 for at least three days.

Both reverse immunosorbents proved to be efficient as regards the high recovery of ribonuclease, i.e. 81 to 88% of the active ribonuclease was recovered in both cases. It was necessary to wash the immunosorbent prepared with ethyl chloroformate for a longer time, since some proteins appeared to be trapped in the particles of this immunosorbent. These trapped proteins could only be removed by repeated washing with large volume of PBS. On the other hand, the particles of the immunosorbent prepared with EMA appeared to be looser, and the proteins which had been nonspecifically absorbed could be removed more readily.

To ascertain that ribonuclease eluted from the reverse immunosorbent was not denatured, the enzyme was examined by analytical ultracentrifugation and column chromatography on Sephadex G-75. On analytical ultracentrifugation, the eluted RNase was found to have a sedimentation coefficient of 1.6S which was the same as the rabbit ribonuclease isolated by physicochemical methods. Moreover, the recovered enzyme emerged from a Sephadex G-75 column at tube no. 80, which was the same as the fraction in which ribonuclease had been previously isolated. Furthermore, the fact that the eluted ribonuclease had a high specific activity approaching that of the enzyme isolated by physicochemical

methods demonstrated that the eluted ribonuclease was in fairly pure state.

The purity of the rabbit ribonuclease eluted from the immunosorbent was further revealed by electrophoresis on cellulose acetate membrane. Besides the major ribonuclease component, minor impurities were present, which might represent some proteins nonspecifically absorbed within the immunosorbent lattice, which had not been released on washing with PBS, but which could be eluted with glycine-HCl buffer. These impurities could be removed by column chromatography on Sephadex G-75. Since the ribonuclease eluted from immunosorbents had the same electrophoretic mobility as that of the enzyme isolated by physicochemical methods, the eluted ribonuclease was considered to have the same net electric charge as the one isolated by physicochemical methods. All these results demonstrate strongly that the eluted ribonuclease is identical to the ribonuclease isolated by physicochemical methods in all respects so far studied.

The capacity of the reverse immunosorbent for absorption of ribonuclease could not be readily determined because, in addition to ribonuclease, small amounts of other nucleases capable of degrading RNA were also present in the crude spleen extract, thus masking the effect of ribonuclease alone. In practice, it was shown in this study that 6 to 7 mg of purified ribonuclease could be recovered from the reverse immunosorbent prepared with 10 ml of antiserum. After repeated use of this immunosorbent, smaller amounts of ribonuclease were recovered.

The gradual decrease of the 'capacity' of the immunosorbent was probably due to irreversible absorption of some proteins which masked the combining sites of the insolubilized antibodies, or denaturation of these antibodies due to repeated exposure to low pH.

Considering the fact that at least five to six purification steps involved in the conventional physicochemical methods for the purification of rabbit ribonucleases, and the fact that by the use of reverse immunosorbents one purification step would produce fairly homogeneous rabbit ribonuclease, the method by immunosorbent could be regarded as a simple and rapid method for purification of rabbit ribonuclease. Furthermore, only 2 to 5 fold increase in ribonuclease activity was achieved in each step of purification by using conventional column chromatographic methods whereas 48 fold purification was achieved in one step by using immunosorbent.

CHAPTER VI

EFFECT OF SHEEP ANTI-RABBIT RIBONUCLEASE ANTIBODIES ON ENZYMATIC ACTIVITY OF RABBIT RIBONUCLEASE

INTRODUCTION

A vast amount of works has been done on the inhibition of enzymatic activity by antibodies to enzymes such as papain (318), tyrosinase (319, 320), catalase (321, 322), alkaline phosphatase (323, 324), amylase (325, 326, 327), lysozyme (328, 329), ribonucleases (79, 185, 186, 194, 330, 331), deoxyribonuclease (332, 333) etc. Almost all of the antibodies inhibited the enzymatic activities of the enzymes but to a different extent, the inhibitory capacity depending also on the course of immunization (183, 334). Moreover, in a few cases the production of antibodies capable of enhancing the enzymatic activity has also been reported (183, 334). Thus, Cinader (183) reported that in a series of six rabbits which were immunized with a polyalanine-polytyrosine derivative of bovine pancreatic ribonuclease, five of them produced inhibitory antibodies, but one rabbit produced consistently activating antibodies which increased the activities of the chemically modified ribonuclease as well as of the native ribonuclease. Pollock (181) detected antibodies to penicillinase which possessed the capacity of increasing the penicillinase activity by a factor of ten.

The extent of inhibition by antibodies varies from enzyme to enzyme; some enzymes were completely (328, 329, 330, 331), some partially (185, 322, 326) and some were not at all inhibited (323, 324, 335). The inhibitory effect of antibodies depends not only on the molecular weight of the substrate, but also on the molecular weight of the products (183).

Bovine pancreatic ribonuclease has been reported to be inhibited by 20 to 98 percent (79, 183, 185) by the homologous antibodies. The inhibitory capacity of antibodies to ribonuclease increased during the progress of immunization (183). Gordon (244) reported that rabbit antibodies to rat liver ribonuclease inhibited the rat liver and kidney ribonucleases to the extent of 52-70% and 83% respectively. He suggested that this residual ribonuclease activity which remained in the supernatant was probably due to formation of soluble ribonuclease-antibody complexes. However, no evidence was provided to prove the presence of the soluble complexes.

In the present study, the inhibitory effects of the soluble and insolubilized sheep antibodies to rabbit spleen ribonuclease were examined, and unequivocal evidence for the presence of soluble complexes consisting of ribonuclease and its homologous antibodies was provided.

MATERIALS AND METHODS

REAGENTS: All chemicals were reagent grade. Sephadex G-75, Sephadex G-100 and SE-Sephadex C-50 were obtained from Pharmacia, Uppsala, Sweden. Bentonite was purchased from Fisher Scientific Company. Rabbit anti-sheep gamma globulins were purchased from Pentex Co., Illinois.

RABBIT SPLEEN RIBONUCLEASE: Rabbit spleen ribonuclease was isolated and purified by conventional column chromatographic methods as described in Chapter III.

CHROMATOGRAPHY ON SEPHADEX G-100: The chromatography was carried out at 4 °C with a column of 2.5 x 160 cm equilibrated with 0.15 M phosphate buffer at pH 7.3. Serum (10 to 20 ml) was applied to the column and eluted with the same buffer. Fractions (7 ml) of the effluent were collected at a flow rate of 1.0 ml per minute.

CHROMATOGRAPHY ON SEPHADEX G-75: The Sephadex G-75 gel was equilibrated in 0.15 M phosphate buffer at pH 7.3 and packed into a column of 2.5 x 100 cm. The sample to be fractionated (in 1 to 3 ml volume) was applied to the column and eluted with the same phosphate buffer at a flow rate of 1.2 ml per minute, the effluent being collected in 6-ml fractions.

ANALYTICAL ULTRACENTRIFUGATION: All sedimentation velocity measurements were performed in a Beckman model E optical ultracentrifuge. The ultracentrifugation was performed at 59,780 rpm and the rotor temperature was maintained at 20° C.

RESULTS

(A) REMOVAL OF ENDOGENOUS RIBONUCLEASES PRESENT IN THE ANTISERUM:

The endogenous ribonucleases present in the antiserum were removed by a combination of precipitation with ammonium sulfate, treatment (adsorption) with bentonite and by column chromatography on Sephadex G-100.

Saturated ammonium sulfate solution (20 ml) was added dropwise from a separatory funnel to a portion of the antiserum (30 ml) in a beaker at 4°C until 40% saturation saturation had been attained. The mixture was stirred constant and kept at 4°C for 30 minutes. The precipitate formed was collected by centrifugation at 18,000 x G for 20 minutes. It was dissolved in 20 ml of 0.01 M phosphate buffer at pH 6.0 and dialyzed against the same buffer overnight at 4°C.

The antiserum was further treated with bentonite. Commercial bentonite was purified before use according to the method described by Fraenkel-Conrat (102). A suspension of bentonite in water (15 gm/300 ml) was centrifuged at 1,000 x G for 15 minutes to remove the fine particles. The supernatant was centrifuged again at 8,000 x G for 20 minutes. The precipitate was collected and suspended in 300 ml of 0.1 M EDTA at pH 7.0 for two days at room temperature. After centrifugation at 8,000 x G for 20 minutes, the precipitate was suspended in 300 ml of 0.01 M phosphate buffer at pH 6.0 for 24 hours. Finally the precipitate was centrifuged again and resuspended in 300 ml of the phosphate buffer. The

bentonite suspension thus obtained was ready for use. The amount of bentonite needed for adsorption of endogenous ribonucleases in the antiserum was determined in a preliminary experiment. Various amounts of bentonite were added to 0.25 ml of antiserum diluted to 1.0 ml. After stirring for 30 minutes at 4° C, the bentonite was removed by centrifugation at 15,000 x G for 20 minutes. A portion (0.2 ml) of the supernatant was assayed for ribonuclease activity by using yeast RNA as substrate (286). Another aliquot (0.2 ml) of the supernatant was assayed for protein content by Folin reagent (255). The results in Table 20 show that 10 ml of the bentonite suspension was needed for 30 ml of the antiserum.

A portion of the bentonite suspension (10 ml which contained 500 mg of bentonite) was added to 30 ml of the antiserum which had been diluted with equal volume of 0.01 M phosphate buffer at pH 6.0 and stirred for one hour at room temperature. The bentonite was removed by centrifugation at 15,000 x G for 20 minutes. The supernatant containing antibodies was dialyzed against distilled water and lyophilized.

The lyophilized sample was dissolved in 20 ml of 0.15 M phosphate buffer at pH 7.3, and then applied to a column (2.5 x 160 cm) of Sephadex G-100. It is evident from the elution profile shown in Fig. 31 that the main peak, which contained the antibodies, was separated from the fractions which showed ribonuclease activity. The fractions containing antibodies were pooled and dialyzed overnight against distilled water. After lyophilization the proteins were dissolved in 30 ml of phosphate-buffered saline.

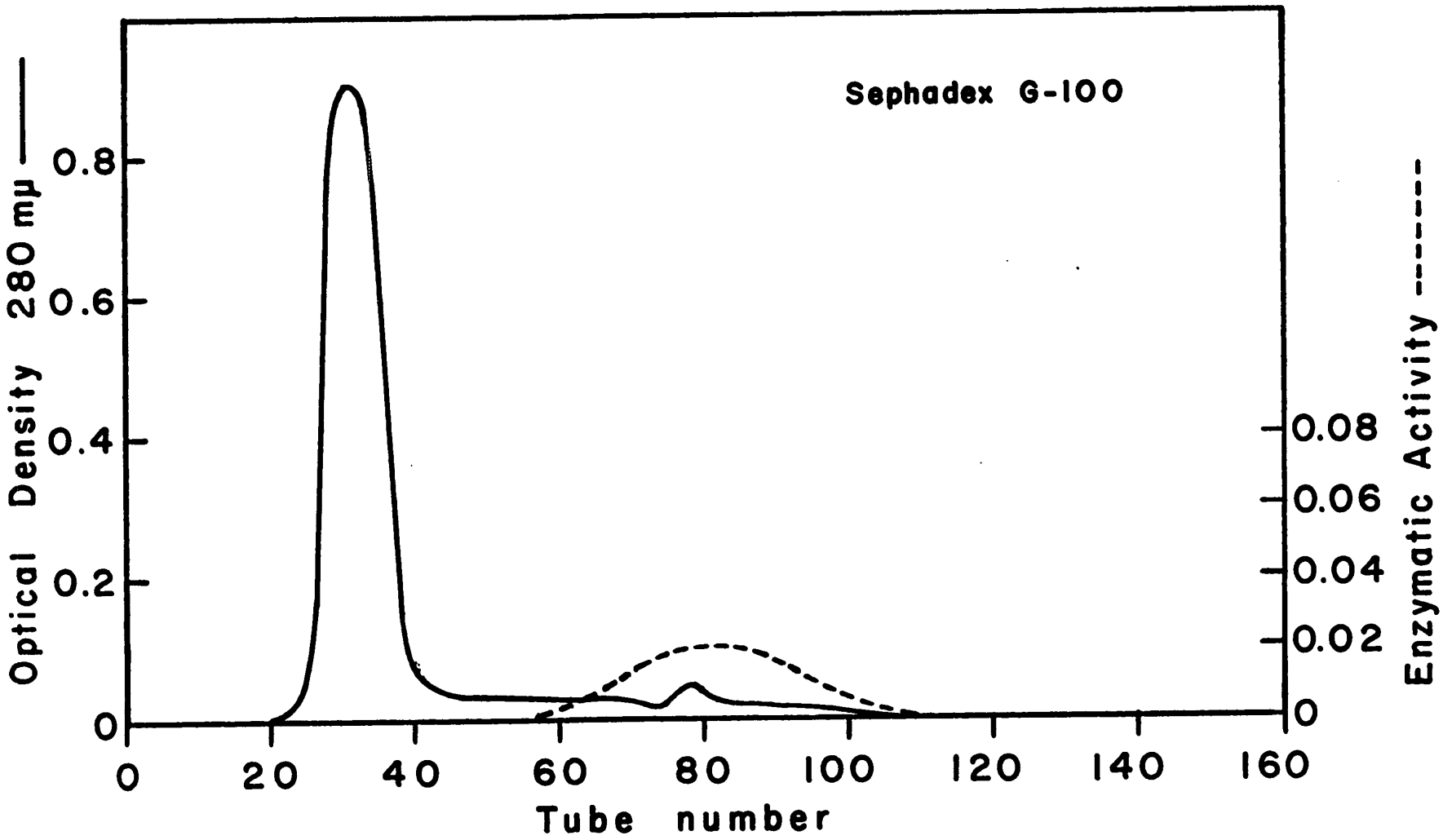
Bentonite added, mg	Residual RNase activity in supernatant, unit.	Residual proteins in supernatant, mg.
0	11.8	9.90
0.6	4.6	8.40
1.2	2.1	7.80
2.5	0.7	7.72
5.0	0	7.52
10.0	0	7.14
20.0	0	6.88
30.0	0	6.20

Table 20

ADSORPTION OF ENDOGENOUS RIBONUCLEASES FROM ANTISERUM WITH BENTONITE.

Figure 31

CHROMATOGRAPHY OF ANTISERUM ON SEPHADEX G-100 COLUMN (2.5 x 160 cm).


Sephadex G-100
Optical Density 280 mμ
0.8
0.6
0.4
0.2
0
0.08
0.06
0.04
0.02
0
Enzymatic Activity
0
20
40
60
80
100
120
140
160
Tube number

The antibodies at this stage were found to be devoid of any ribonuclease activity, and were then used to study their effect on the enzymatic activity of rabbit ribonuclease.

Since the adsorption of ribonuclease by bentonite is non-specific, some other proteins were also adsorbed which led to some losses of antibodies. Further experiments as seen in Table 21, showed that batchwise treatment of this antiserum with SE-Sephadex C-50 was as effective as treatment with bentonite for the removal of endogenous ribonucleases. Moreover, only ribonucleases, but not the antibodies, were adsorbed by SE-Sephadex which was equilibrated with 0.4 M sodium chloride in 0.1 M acetate buffer at pH 4.5,

Antiserum (30 ml) was added to SE-Sephadex C-50 equilibrated with the acetate buffer (90 ml) and stirred for 20 minutes at 4° C. The supernatant which was collected by means of a Buchner funnel. The gel was washed with 50 ml of the same buffer. These filtrates were combined and dialyzed against distilled water overnight. The antibodies were recovered in higher yield, leaving the ribonucleases adsorbed on the gel. Consequently loss of antibodies could be reduced appreciably.

Molarity of NaCl in 0.1 M acetate buffer, pH 4.5 *	Proteins eluted, mg	RNase activity in eluate, unit	Precipitin test in agar gel
0.2	187.8	0	+
0.4	105.6	0	+
0.6	42.2	56.2	−
0.8	5.8	127.9	−
1.0	2.7	287.5	−

* Antiserum (8 ml) in SE-Sephadex C-50 was eluted with 100-ml portions of the buffer at different NaCl concentrations.

Table 21

REMOVAL OF ENDOGENOUS RIBONUCLEASES FROM ANTISERUM WITH SE-SEPHADEX C-50.

(B) EFFECT OF ANTIBODIES TO RABBIT RIBONUCLEASE ON ENZYMATIC ACTIVITY OF RABBIT RIBONUCLEASE:

The capacity of the anti-RNase antibody preparation to inhibit the enzymatic activity was established by adding increasing amounts of antibodies to a series of test tubes containing a constant amount of ribonuclease.

The purified rabbit spleen ribonuclease, obtained by the procedures described in Chapter III, was dissolved in 0.15 M phosphate buffer at pH 7.3 to give a concentration of 36 μg per ml. A volume of 0.4 ml of this RNase solution was added to each tube which contained 1.0 ml of the RNase-free antibody preparation, which had been diluted to different extent with 0.15 M phosphate buffer to give the final concentrations of antibodies listed in Table 22. The tubes were incubated at 37 ^{o}C for one hour and allowed to stand at room temperature for another hour. The tubes were centrifuged at 5,000 x G for 20 minutes to remove any precipitate. A portion (0.2 ml) of the supernatant was removed and assayed for ribonuclease activity by using yeast RNA as substrate. The results are summarized in Table 22 and shown diagrammatically in Fig. 32. It is obvious that complete inhibition of enzymatic activity could not be achieved over a wide range of antibody concentration and that only 40 to 60% of the activity could be inhibited with antibodies from different bleeding.

Normal sheep serum was purified as described previously in Section (A) to remove endogenous ribonuclease activity. The above

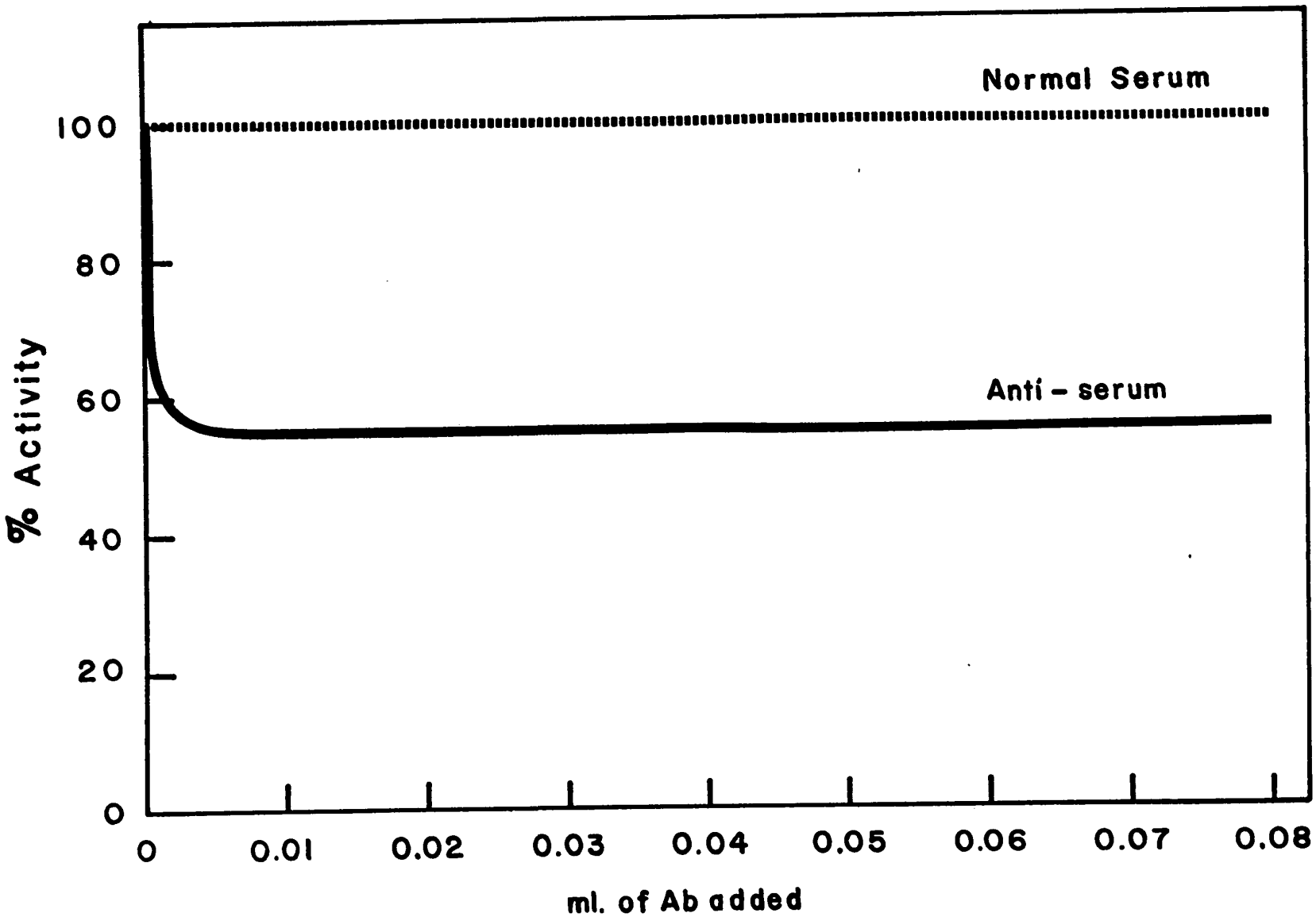
Vol. of antibodies added, ml	Residual RNase activity in supernatant, unit	% Activity
0	7.10	100.0
0.0002	5.65	79.6
0.0006	4.80	67.2
0.001	3.70	52.1
0.002	3.30	45.8
0.004	2.75	38.8
0.006	2.55	36.0
0.008	2.45	34.6
0.01	2.50	35.2
0.05	2.60	36.6
0.1	2.65	37.4
0.2	2.45	34.6

Table 22

INHIBITION OF RABBIT RNase ACTIVITY BY ANTIBODIES.

Figure 32

INHIBITION OF RABBIT RIBONUCLEASE ACTIVITY BY ANTIBODIES.


Normal Serum
Anti – serum
100
80
60
40
20
0
% Activity
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
ml. of Ab added

inhibition experiments were repeated but normal sheep gamma-globulins were used in place of the antibodies. The results (Fig. 32) show that normal sheep gamma-globulins had no inhibitory effect on the enzymatic activity of the rabbit ribonuclease. This strongly suggested that the inhibition of RNase activity was due to specific antigen-antibody reaction. Since some residual enzymatic activity was found in the supernatant even in the presence of large excess of antibodies, it was suspected that some soluble antigen-antibody complexes remained in the supernatant and that they were responsible for the residual ribonuclease activity. A comparison of the inhibitory capacities of antibodies against various ribonucleases is shown in Table 23.

Antibody	Antigen	% Inhibition	Reference
Rabbit anti-rat pancreatic RNase	Rat pancreatic or spleen RNase	100	Gordon (244)
Rabbit anti-rat liver RNase	Rat liver RNase	52-70	Gordon (244)
Rabbit anti-rat liver RNase	Rat kidney	83	Gordon (244)
Rabbit anti-bovine pancreatic RNase	Bovine pancreatic RNase	20-98	Brown (79) Branster (185)
Sheep anti-rabbit spleen RNase	Rabbit spleen RNase	40-60	Present study

Table 23

COMPARISON OF THE INHIBITORY CAPACITIES OF VARIOUS ANTIBODIES AGAINST DIFFERENT RIBONUCLEASES.

(C) EFFECT OF INSOLUBILIZED ANTIBODIES ON ENZYMATIC ACTIVITY OF RABBIT RIBONUCLEASE:

Sheep antibodies to rabbit spleen ribonuclease were freed of endogenous ribonuclease by treatment with ammonium sulfate precipitation, adsorption with bentonite and column chromatography on Sephadex G-100 as described in Section (A). Reverse immunosorbent was prepared by polymerizing these antibodies with ethylene maleic anhydride copolymer (EMA-31) as described in Chapter V. After polymerization the reverse immunosorbent was washed successively with phosphate-buffered saline (PBS), a solution of monosodium phosphate, PBS, a solution of disodium phosphate, PBS, 0.4 M glycine-HCl buffer at pH 2.0 and PBS until it was free of any soluble proteins. Finally the reverse immunosorbent was suspended in 0.15 M phosphate buffer, pH 7.3, at a concentration corresponding to the original concentration of the antiserum, i.e. the immunosorbent prepared from 10 ml of antiserum was suspended in 10 ml of the phosphate buffer.

A portion (0.4 ml) of the purified rabbit spleen ribonuclease at a concentration of 36 μg per ml was added to each of a series of test tubes. Different volumes of the suspension containing the immunosorbent were added to each tube and made up to 1.0 ml with phosphate buffer. The mixture was incubated at 37° C for one hour followed by standing at room temperature for another hour. The immunosorbent was removed by centrifugation at 5,000 x G for 20 minutes. A portion of the supernatant (0.2 ml) was transferred

to another tube and assayed for ribonuclease activity using yeast RNA as substrate. The results are summarized in Table 24 and shown diagrammatically in Fig. 33. The complete inhibition of ribonuclease activity indicated that all the ribonuclease molecules could be removed from the supernatant by the insolubilized antibodies.

When the insolubilized gamma-globulins from the normal sheep serum were added to the ribonuclease as described above, no inhibition of the ribonuclease activity was observed.

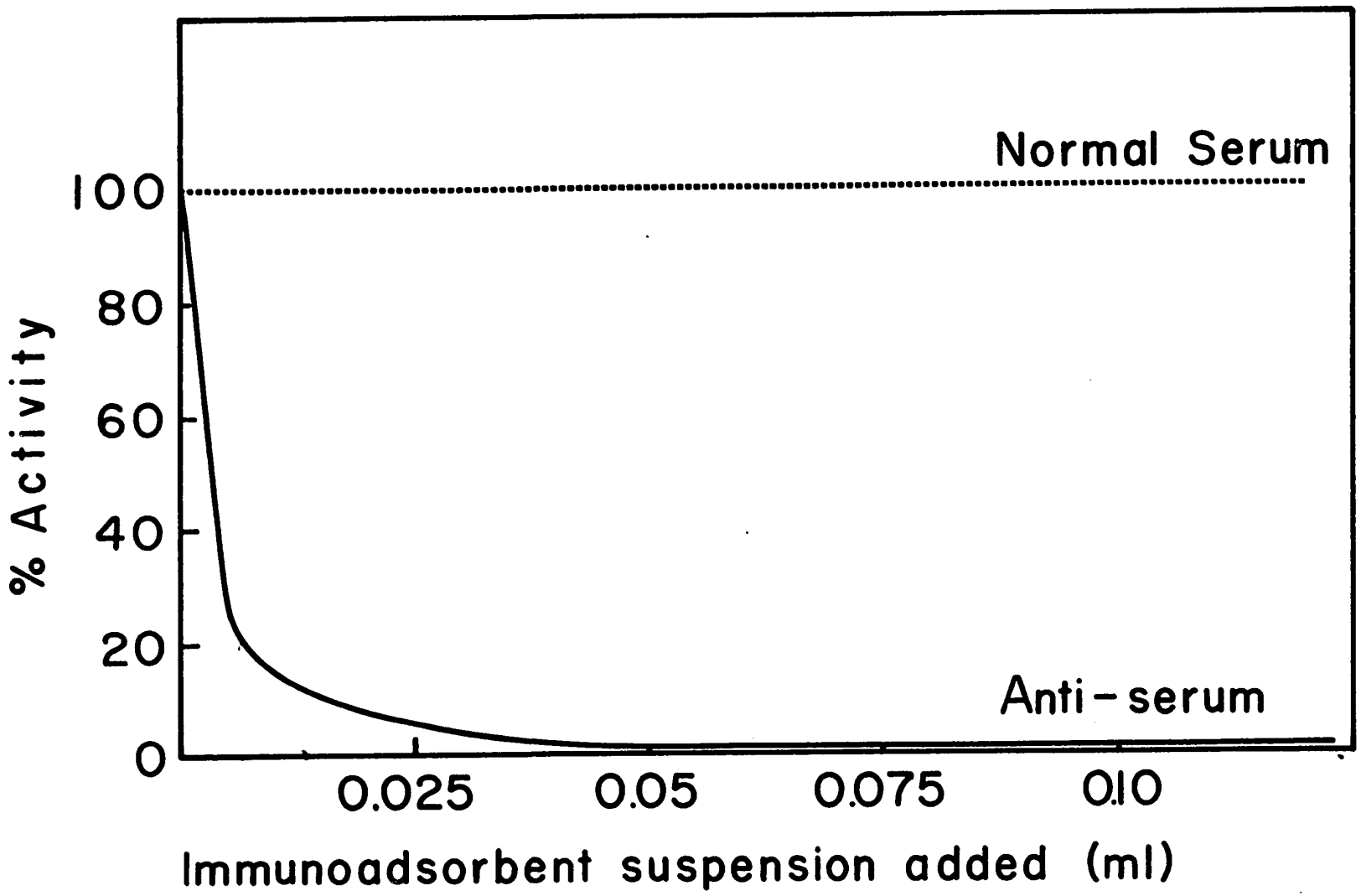
Volume of reverse immunosorbent suspension added, ml	Residual enzymatic activity in supernatant, unit	% Activity
0	7.35	100.0
0.00078	7.20	95.2
0.00156	6.10	83.0
0.00312	4.00	54.4
0.00625	1.50	20.4
0.0125	1.00	13.6
0.025	0.60	8.2
0.05	0.10	1.4
0.10	0.05	0.7
0.20	0.10	1.4

Table 24

INHIBITION OF RABBIT RIBONUCLEASE ACTIVITY BY INSOLUBILIZED ANTIBODIES.

Figure 33

INHIBITION OF RABBIT RIBONUCLEASE ACTIVITY BY INSOLUBILIZED ANTIBODIES.


Normal Serum
Anti-serum
100
80
60
40
20
0
% Activity
0.025
0.05
0.075
0.10
Immunoadsorbent suspension added (ml)

(D) DEMONSTRATION OF THE PRESENCE OF SOLUBLE RIBONUCLEASE-ANTIBODY COMPLEXES:

(a) By the use of anti-antibodies:

Rabbit antibodies to sheep gamma-globulins were employed as the anti-antibodies. The endogenous ribonuclease activity was eliminated as described above in Section (A). Sheep antibodies to rabbit spleen ribonuclease (1.0 ml), devoid of ribonuclease activity, were added in excess to purified rabbit spleen ribonuclease (1.2 mg in 0.2 ml of 0.15 M phosphate buffer, pH 7.3). The mixture was incubated at 37° C for one hour followed by standing at room temperature for one hour. The precipitate formed was removed by centrifugation at 5,000 x G for 20 minutes. A portion (0.1 ml) of the supernatant was diluted 5 times and assayed for ribonuclease activity. To another portion (0.5 ml) of this supernatant which contained ribonuclease activity was added 0.5 ml of rabbit antibodies to sheep gamma-globulins, which had been freed of endogenous ribonuclease activity. The mixture was incubated at 37° C for one hour and then at room temperature for one hour. After removing the precipitate by centrifugation at 5,000 x G for 20 minutes, a portion (0.2 ml) of the supernatant was assayed for ribonuclease activity using yeast RNA as substrate. No RNase activity was found in this supernatant as indicated in Table 25. These results demonstrated that the soluble RNase-antibody complexes were precipitated by the anti-antibodies, so that no ribonuclease activity remained in the supernatant.

Sample	Residual enz. act. in supernatant, unit	% Activity
Purified RNase alone	48.56	100.0
Supernatant from RNase + Sheep anti-RNase	27.24	56.2
Supernatant from RNase + Sheep anti-RNase + Rabbit anti-sheep gamma-globulins	0	0

Table 25

DEMONSTRATION OF THE SOLUBLE RIBONUCLEASE-ANTIBODY COMPLEXES BY ANTI-ANTIBODIES.

(b) By means of gel filtration:

Sheep antibodies to rabbit spleen ribonuclease (1.0 ml) being free of endogenous ribonuclease activity were added to 1.5 mg of purified rabbit spleen ribonuclease in 0.5 ml of 0.15 M phosphate buffer at pH 7.3. The mixture was incubated at 37° C for one hour and at room temperature for one hour. The supernatant which contained ribonuclease activity was collected by centrifugation at 25,000 x G for 15 minutes. The supernatant was subsequently applied to a column (2.5 x 100 cm) of Sephadex G-75 equilibrated with 0.15 M phosphate buffer at pH 7.3, and eluted with the same buffer. From the elution profile shown in Fig. 34, it is obvious that the proteins, which were excluded from the gel, were associated with ribonuclease activity. This finding demonstrates that ribonuclease emerged from the gel in the form of ribonuclease-antibody complexes.

In a control experiment, normal sheep gamma-globulins which were free of endogenous ribonuclease activity were used in place of antibodies. The supernatant from the mixture of normal sheep gamma-globulins and rabbit spleen ribonuclease was applied to the same column of Sephadex G-75 under the same conditions. The elution profile in Fig. 35 shows that the ribonuclease did not bind to the normal gamma-globulins so that it emerged from the column much later at tube number 80.

Figure 34

CHROMATOGRAPHY OF THE SUPERNATANT FROM A MIXTURE OF SPLEEN RIBONUCLEASE AND ITS HOMOLOGOUS ANTIBODIES ON SEPHADEX G-75 COLUMN (2.5 x 100 cm).

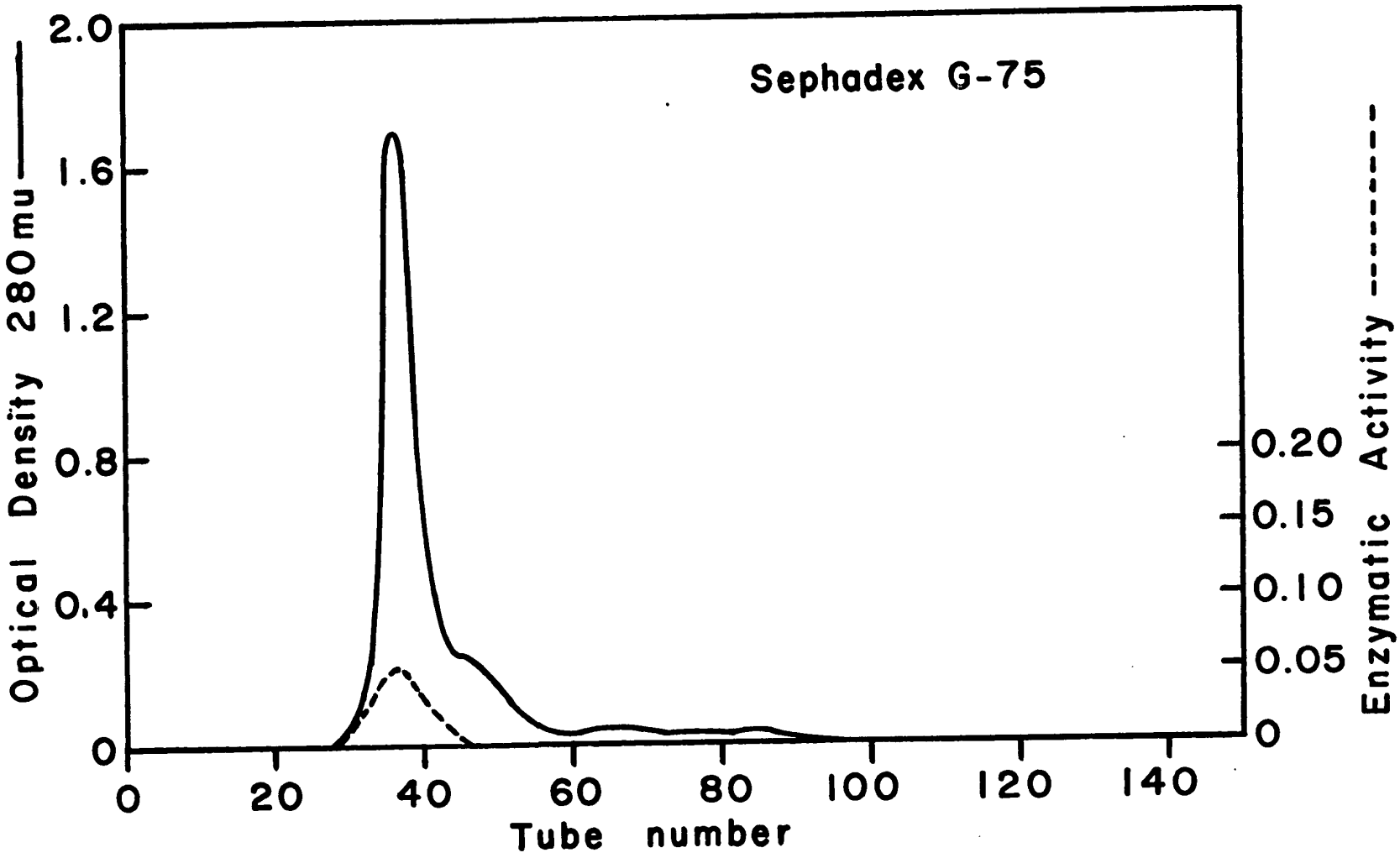
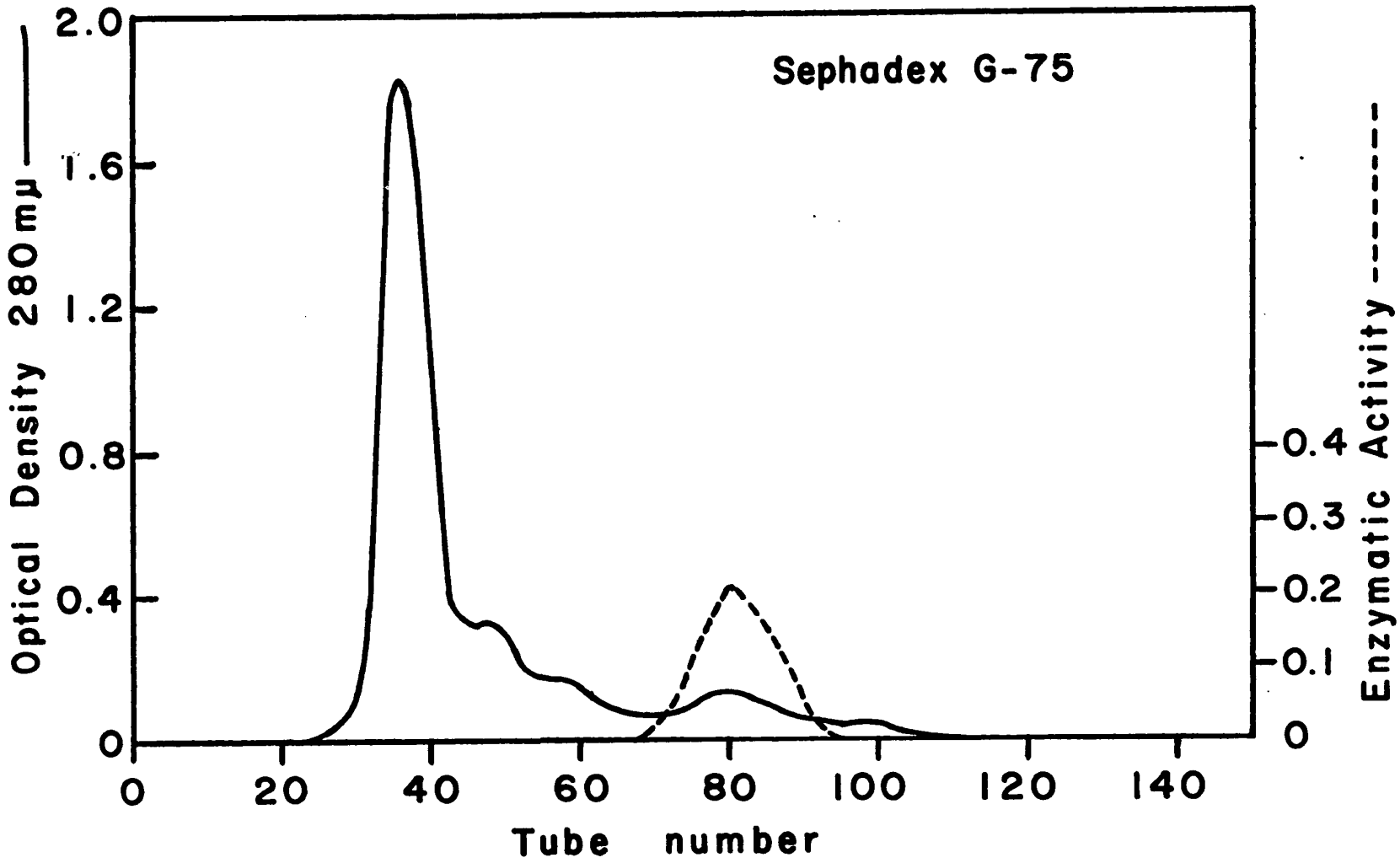

Sephadex G-75
Optical Density 280mu
Enzymatic Activity
2.0
1.6
1.2
0.8
0.4
0
0.20
0.15
0.10
0.05
0
0
20
40
60
80
100
120
140
Tube number

Figure 35

CHROMATOGRAPHY OF A MIXTURE OF SPLEEN RIBONUCLEASE AND NORMAL SHEEP SERUM ON SEPHADEX G-75 COLUMN (2.5 x 100 cm).


Sephadex G-75
Optical Density 280mμ
Enzymatic Activity
2.0
1.6
1.2
0.8
0.4
0
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
Tube number

(E) DEMONSTRATION OF THE EFFECTIVENESS OF INHIBITION OF RABBIT RIBONUCLEASE ACTIVITY BY INSOLUBILIZED ANTIBODIES:

The substrate, yeast RNA, was precipitated from a mixture of acetic acid and ethanol to remove low molecular weight RNA according to the method of Klee (15) as described in Chapter III. This RNA was dissolved in 0.15 M phosphate buffer at pH 7.3 to give a concentration of 2 mg per ml and analyzed in a Beckman model E optical ultracentrifuge at 59,780 rpm in a synthetic boundary cell. A single peak of 3.7S was observed as shown in Fig. 36 (a).

In the second experiment a solution of purified rabbit spleen ribonuclease (20 µg in 1.0 ml of 0.15 M phosphate buffer at pH 7.3) was added to 1.0 ml of the substrate solution containing 4 mg of yeast RNA. The mixture was incubated at 37 ^{o} C for 30 minutes and then analyzed in the optical ultracentrifugation. The result in Fig. 36 (b) shows that the substrate was degraded into small oligonucleotides as indicated by the absence of the RNA peak.

In a parallel experiment, a suspension (2.0 ml) of the reverse immunosorbent made with EMA was centrifuged at 5,000 x G for 20 minutes. The supernatant was removed and discarded. A solution of 20 µg of purified rabbit spleen ribonuclease in 1.0 ml of the phosphate buffer was added to the reverse immunosorbent. The mixture was stirred for 30 minutes at room temperature. After centrifugation at 5,000 x G for 20 minutes, a portion (0.5 ml) of the supernatant was transferred and added to 0.5 ml of the

Figure 36

DEMONSTRATION OF EFFECTIVENESS OF INHIBITION OF RIBONUCLEASE ACTIVITY BY INSOLUBILIZED ANTIBODIES ON OPTICAL ULTRACENTRIFUGE.

(a)
RNA

(b)
RNA
+ RNase

(c)
RNA + Supernatant
from RNase
+ Immunosorbent

substrate solution containing 4 mg of yeast RNA. This mixture was incubated at 37° C for 30 minutes. At the end of incubation period, the mixture was analyzed immediately in the optical ultracentrifuge at 59,780 rpm. A single peak having a sedimentation coefficient of 3.7S, which was identical to the substrate RNA peak, was detected (Fig.36 C) indicating that the ribonuclease was removed completely by the reverse immunosorbent so that the substrate remained intact.

DISCUSSION

Before one can proceed to study the effect of antiserum on the enzymatic activity of ribonuclease, the endogenous ribonucleases present in the antiserum must be removed, since ribonucleases had been found in mammalian serum (26, 264, 336). In the present, sheep antibodies to rabbit spleen ribonuclease were also found to contain considerable amounts of endogenous ribonucleases.

Gordon (244) reported that endogenous ribonucleases in the rabbit antiserum could be removed by ammonium sulfate precipitation at 40% saturation followed by gel filtration on a column of Sephadex G-75. However, in this study residual ribonuclease activity was found after these two treatments. Further treatment with bentonite or preferably by chromatography on SE-Sephadex C-50 was found necessary to remove the last trace of RNase activity.

The inhibition of ribonuclease activity with specific antibodies had been demonstrated by many workers (79, 185, 186, 194, 244, 330, 331) for several ribonucleases from different sources. The ribonucleases were inhibited to various extent by the antibodies (Table 23). The results of Gordon (244) showed that rat liver and kidney ribonucleases were not completely inhibited by antibodies. He suggested that the incomplete inhibition was due to the formation of soluble complexes, but no evidence was provided for this interpretation. Cinader (183) reported that bovine pancreatic ribonuclease could be removed completely from the supernatant by appropriate antibody preparations.

In the present study, only partial inhibition of rabbit spleen ribonuclease could be achieved with homologous antibodies. Since the residual ribonuclease activity in the supernatant was always in the order of 40 - 60%, two possibilities could be postulated to explain this finding, i.e. (i) the formation of soluble RNase-antibody complexes possessing enzymatic activity, or (ii) some RNase molecules remained free in the supernatant because of a low binding affinity between RNase and the corresponding antibodies. However, the findings that rabbit ribonuclease could be completely removed from the supernatant by insolubilized antibodies (Fig. 33) or on addition of the second antibody (rabbit anti-sheep gamma-globulins, Table 25), strongly support the first alternative. The results of the experiments of gel filtration on Sephadex G-75 add further support to this interpretation, since if the small ribonuclease molecules had not been bound to antibodies, they should have emerged from the gel after the corresponding antibodies (Figures 34 and 35) and not in the same elution volume with the latter.

Since the insolubilized antibodies were found to be capable of removing the purified rabbit ribonuclease completely from the supernatant as shown in Fig. 33, it was expected that this reverse immunosorbent could be used as an effective ribonuclease inhibitor. The results in Fig. 36 demonstrate that this reverse immunosorbent was indeed capable of removing ribonuclease, since the substrate used (i.e. RNA) was unaffected.

The damage of ribonucleic acid caused by ribonuclease has been a serious problem in the field of nucleic acid research particularly as regards the isolation of intact polysomes. Although some nuclease inhibitors like bentonite (102, 103), Macaloid (105), heparin (104), copolymer of tyrosine and glutamate (337), polyvinyl-sulphonic acid (337) had been used, they were not found completely satisfactory probably because of two reasons. First, these inhibitors may not be capable of inhibiting totally the nuclease activity. Thus, Sela (104) reported that the extent of inhibition of bovine pancreatic ribonuclease activity depended on the pH value of the buffer as well as the concentration of the inhibitors. The RNase was inhibited completely by polyvinylsulphonic acid at pH 5.0 but not at pH 7.4. Second, many types of nucleases exist in the tissues and it is conceivable that no single inhibitor will inhibit all the nucleases. Obviously, a similar limitation can be visualized for the inhibitory capacity of anti-RNase antibodies.

As shown earlier, the purified rabbit ribonuclease could be removed completely by the reverse immunosorbent. However, when the crude extract of rabbit spleen was mixed with the reverse immunosorbent, some residual nuclease activity (approximately 10 to 20%) was found in the supernatant and this may be attributed to other nucleases which did not react with the insolubilized antibodies.

In spite of this low residual activity, reverse immunosorbent is considered more efficient for removing ribonuclease activity as compared to heparin and bentonite. As mentioned earlier in

Chapter IV (Table 9), heparin inhibits rabbit spleen and pancreatic ribonucleases only to an extent of 25 - 50%, whereas 38 - 59% inhibition was reported for bovine pancreatic ribonuclease. On the other hand, the adsorption of ribonuclease by bentonite has the disadvantage of nonspecific adsorption. It adsorbs not only ribonuclease but also some other proteins. It was, therefore, expected that the reverse immunosorbent would be more efficient than heparin or bentonite for the removal of ribonuclease activity. The utilization of this reverse immunosorbent for removal of ribonuclease activity during the isolation of polysomes has been studied and described in detail in Chapter VII.

CHAPTER VII

REMOVAL OF RIBONUCLEASE ACTIVITY BY REVERSE IMMUNOSORBENT IN THE ISOLATION OF POLYSOMES

INTRODUCTION

Polyribosomes have been shown to be the active units of protein synthesis in gamma globulin-producing cells from different species of animals, including rabbit lymph nodes (96, 97), mouse plasma cell tumors (98, 104, 106, 338) and hepatomas (74, 339). Uhr (96, 97) demonstrated that nascent protein synthesis was associated with polyribosomes of approximately 200S in the extracts of lymph node cells removed from hyperimmunized rabbits. Askonas et al. (106) reported that heavy chains of immunoglobulins were synthesized on the large polyribosomes in the 300S region. The main difficulty of isolating intact polyribosomes has been attributed to the release of ribonucleases during cellular disruption. Numerous attempts have been made to minimize or eliminate the harmful effect of RNase on the polysomes. Heparin and sodium deoxycholate have been commonly used to inhibit the RNase activity and release the membrane-bound polysomes (98, 100, 101, 104). The use of Macaloid, i.e. sodium magnesium lithofluorosilicate, as nuclease inhibitor in the isolation of polysomes has also been reported (106). More recently, rat liver cellular ribonuclease inhibitor (107) has been utilized to prevent the degradation of polysomes and higher yield of polysomes was obtained.

Recently, more rapid procedures for the isolation of polysomes have been developed (104, 106). Williamson et al. (106) introduced a method for the fractionation of polysomes by sucrose density gradient centrifugation without prior separation from other cell components. In this laboratory, Davis et al. (104) introduced a lytic zone, which contained detergents (sodium deoxycholate plus TX-100), on top of the sucrose density gradient. The cell suspension was placed on top of the lytic zone, so that cell lysis occurred during the precipitation of the cells through this zone on centrifugation. The time that polysomes were exposed to the crude cell lysate was thus decreased to a few minutes and any nucleases released were expected to remain in the lighter zones while the polysomes were migrating to lower zones; indeed higher yields of undegraded polysomes were obtained with this procedure from mouse myeloma tissue.

As stated in the first chapter, the main reason for the choice of rabbit tissues for this study was that this species has been commonly used for immunological experiments. It was anticipated that antibodies to rabbit RNase might be employed as inhibitors of ribonuclease in the study of biosynthesis of rabbit antibodies, in particular as regards the isolation from lymphoid cells of intact m-RNAs from polysomes coding for the amino acid sequence of the heavy and light chains of the immunoglobulins of this species.

In this phase of the study, in an attempt to remove the ribonuclease activity in the isolation of polysomes from rabbit spleen cells, the reverse immunosorbent which was prepared by

polymerizing the sheep antibodies to rabbit spleen RNase with ethylene maleic anhydride copolymer has been employed. As has been shown in the preceding chapter, the reverse immunosorbent was capable of removing RNase activity and, therefore, the possibility of using it for the isolation of intact polysomes from rabbit spleen cells was investigated.

MATERIALS AND METHODS

BUFFERS AND REAGENTS: TMK-1 buffer contains 10 mM Tris, pH 7.3, 20 mM $MgCl_2$ and 100 mM KCl. TMK-2 buffer is composed of 10 mM Tris, pH 7.3, 3 mM $MgCl_2$ and 15 mM KCl. Phosphate-buffered saline (PBS) is 0.14 M sodium chloride in 0.02 M phosphate buffer at pH 7.3. Heparin was purchased from Riker Pharmaceutical Co. Ltd., Cooksville, Ontario. Sodium deoxycholate (DOC) was obtained from British Drug House Ltd. Bovine serum albumin is a product of Pentex Co. Merck's sucrose was used in the sucrose density gradient.

PREPARATION OF REVERSE IMMUNOSORBENT: The sheep antibodies to rabbit spleen ribonuclease, which were freed of endogenous RNase activity, was polymerized with ethylene maleic anhydride (EMA-31) as described in Chapter V.

SUCROSE DENSITY GRADIENTS: Linear 30-ml sucrose gradients, 10 to 40 percent (w/v) sucrose in TMK-2 buffer were prepared. After applying 2.0 ml of sample to the top of gradients, the tubes were spun at 25,000 rpm for 165 minutes, using a Spinco SW.27 swinging bucket rotor. At the end of centrifugation the gradients were fractionated by an LKB drop-count fraction collector. The optical density of each fraction was measured at 260 mμ after diluted with TMK-2 buffer.

ISOLATION OF POLYSOME FRACTIONS: Freshly excised rabbit spleen was rinsed in phosphate-buffered saline and cut into small pieces

followed by teasing through a 200 mesh stainless steel grid into 5 ml of TMK-1 buffer containing 0.01 percent heparin and 1 percent bovine serum albumin. The contaminating erythrocytes were lysed by hypotonic treatment. Dilute NaCl solution (4 ml of 0.36% NaCl) was added to the cell pellet and stirred for forty seconds followed by addition of 0.57 ml of 5% NaCl to restore isotonicity. The cells were collected by centrifugation at 1,000 x G for 15 minutes, washed twice in TMK-2 buffer, and finally suspended in the buffer at a concentration of 1×10^8 cells per ml. All operations were conducted at 2° - 4° C.

A portion of the cell suspension (2.0 ml) containing 2×10^8 cells was added to reverse immunosorbent (1.0 ml of the sediment) and put into a teflon-glass homogenizer (made by Tri-R Instruments, Jamaica, N.Y.) which was cooled to 2° C before used. The breaking of cells was accomplished by application of four strokes at 500 revolutions per minute with an electric motor made by Tri-R Instruments, according to the method of Bloemendal et al. (339). The cell debris and the reverse immunosorbent were removed by centrifugation at 10,000 x G for ten minutes. The supernatant containing the ribosomes was applied to the top of a linear sucrose density gradient (10-40%, w/v). Another portion of cell suspension (2.0 ml containing 2×10^8 cells) was added to a mixture of 0.4 ml of sodium deoxycholate (5%) and 0.1 ml of 1% heparin and homogenized as mentioned above. After centrifugation at 10,000 x G for ten minutes, the supernatant was applied to another sucrose density gradient. In a control experiment 2.0 ml of the cell suspension

containing 2 x 10^8 cells was homogenized in the same manner but without any ribonuclease inhibitor.

The preparations were spun at 25,000 rpm for 165 minutes, using a Spinco SW.27 rotor. After centrifugation, the gradients were fractionated through a hole punctured in the bottom of the cellulose nitrate tubes. The effluent was collected in twenty drop fractions (approximately one ml) by an LKB automatic fraction collector. Each fraction was diluted with appropriate volume of TMK-2 buffer (usually 5 to 10 volumes) and the optical density at 260 mμ was measured in a Zeiss spectrophotometer.

RESULTS

The optical density profiles of the polysomes isolated under different experimental conditions, i.e. with and without inhibitors including the use of reverse immunosorbent, are shown in Fig. 37. From these results it can be concluded that approximately the same amounts of polysomes were obtained in the presence of heparin plus detergent as on addition of reverse immunosorbent, and that the addition of these nonspecific and specific RNase inhibitors resulted in a substantially higher recovery of heavier polysomes, i.e. in fractions 1-17.

To further improve the yield of polysomes, an attempt was made to combine the 'lytic zone method' (104) with the use of reverse immunosorbent. In this experiment, reverse immunosorbent was incorporated in the lytic zone which was on top of the linear sucrose density gradient (10-40%, w/v). The fractionation procedure was essentially the same as described above except that the cell suspension, without homogenization, was applied to the top of the lytic zone and the tubes were centrifuged in a brief initial run at a low speed (5 minutes at 800 rpm) before increasing the speed to 25,000 rpm. However the yield of polysomes could not be improved by this method. In an attempt to gain further insight into this problem, some experiments were performed to examine the effects of sucrose and detergent on the binding of ribonuclease with reverse immunosorbent. The results showed that the presence of sucrose or detergent inhibited the binding of ribonuclease with

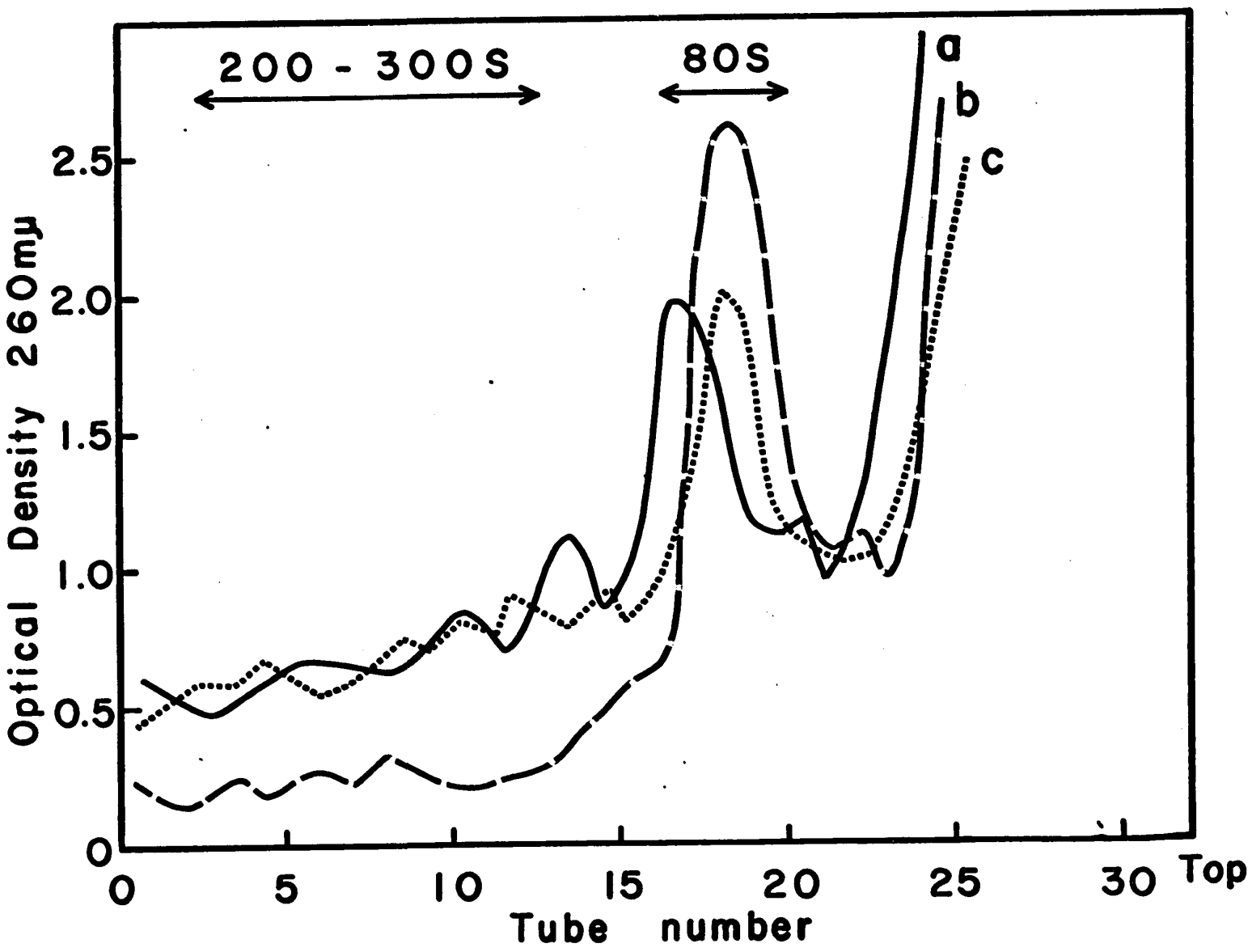
Figure 37

POLYSOME PROFILES OF RABBIT SPLEEN CELL HOMOGENATE.

(a) With reverse immunosorbent as RNase inhibitor.

(b) Without any RNase inhibitor.

(c) With heparin plus DOC as RNase inhibitors.


200 - 300S
80S
a
b
c
Optical Density 260mμ
2.5
2.0
1.5
1.0
0.5
0
0
5
10
15
20
25
30
Top
Tube number

reverse immunosorbent. Thus, in presence of 20% sucrose, the supernatant from a mixture of ribonuclease and reverse immunosorbent contained 63% of the ribonuclease activity; i.e. the higher the sucrose concentration, the higher was the residual ribonuclease activity in the supernatant. The result of another experiment demonstrated that the presence of sodium deoxycholate also interferred with the binding of ribonuclease by the reverse immunosorbent, decreasing it by about 15%.

DISCUSSION

It has been shown in the preceding chapter that the reverse immunosorbent was capable of removing ribonuclease effectively. It was therefore expected that the use of reverse immunosorbent would improve the yield of polysomes. However, the results in Fig. 37 showed clearly that it was only as effective an inhibitor as heparin plus sodium deoxycholate. Recalling the finding that the reverse immunosorbent could remove only approximately 85% of the ribonuclease activity in the crude extract of rabbit spleen, as mentioned in Chapter VI, the residual activity might be attributed to the presence of nucleases other than ribonuclease in the crude extract. Therefore, it is likely that these nucleases, which were not inhibited by this monospecific reverse immunosorbent, are responsible for the degradation of some polysomes. For example, Spahr et al. (340) reported that a phosphodiesterase from E. coli could degrade m-RNA.

The use of the procedure combining the lytic zone method with reverse immunosorbent was limited by the effects of sucrose and sodium deoxycholate on the binding of ribonuclease with reverse immunosorbent. As would be expected, some of the ribonuclease, which had combined with reverse immunosorbent during the rupture of cells, would be released when passing through the sucrose gradient. In addition, the reverse immunosorbent was heavier than the cell debris and therefore would separate out from the latter on sedimentation. Although the rate of immunochemical reactions is very fast, their half life time being in the order of milli-second (341, 342), it is conceivable that some reverse immunosorbent would

have migrated out of the lytic zone before the cells were lysed. Moreover, the presence of other nucleases in the cell lysate would have also contributed to lowering the yield of polysomes.

In order to prevent the effects of detergent and sucrose on the binding of ribonuclease with reverse immunosorbent, conventional method for cell lysis by homogenization was subsequently employed. The cells were homogenized in the presence of reverse immunosorbent, so that any ribonuclease released from the cell would be bound by the immunosorbent. The reason for the removal of cell debris and reverse immunosorbent before applying the polysomes to the sucrose density gradient is to prevent the release of ribonuclease from the reverse immunosorbent on passing through the sucrose gradient. The results in Fig. 37 shows that the yield of polysomes is indeed increased in presence of the reverse immunosorbent with respect to the control experiment without ribonuclease inhibitor. Furthermore the use of heparin plus detergent as nuclease inhibitors resulted in a similar yield of polysomes as isolated with reverse immunosorbent (Fig. 37). It can be concluded that the reverse immunosorbent is only as effective as heparin plus detergent for the removal of ribonuclease activity in the isolation of polysomes.

However, one may suggest that this procedure could be improved by the use of antibodies against <u>all</u> the nucleases in the crude tissue extract. These antibodies would be expected to inhibit the activities of all nucleases, including ribonuclease. In principle, it should be possible to isolate by gel filtration the nucleases, which have relatively low molecular weights, from

polysomes or m-RNAs, or other biopolymers present in the crude extract. Antibodies directed against this crude nuclease preparation would be more efficient, as implied from the results of the present study, for the inhibition of nuclease activity in the isolation of polysomes.

CHAPTER VIII

GENERAL DISCUSSION

Prior to the characterization of a particular enzyme or protein, the most important requirement is to obtain the enzyme or protein in a highly purified state. The most commonly used techniques for the isolation of a single enzyme from a complex mixture of proteins, such as from a crude extract of a given tissue of the animal, are based on their physicochemical properties. Among these techniques, salting-out, gel filtration (by which separation is achieved according to the molecular sizes of proteins) and ion-exchange chromatography (which is based on the charge differences of the protein molecules) have been widely employed.

Although ribonucleases have been isolated and purified from different organs of animals, plants and micro-organisms, rabbit ribonucleases had received little attention prior to this study probably because of the low content of ribonuclease in this species (36). In the present study the use of conventional physicochemical methods, including ammonium sulfate precipitation, gel filtration and ion exchange column chromatography, were found useful for the isolation of highly purified forms of rabbit spleen and pancreatic ribonucleases, but the procedures employed were lengthy and laborious. In search for a more rapid method for the purification of rabbit ribonuclease, the use of reverse immunosorbent, which absorbed ribonuclease specifically from the crude tissue extract, proved successful. The absorbed ribonuclease could be eluted from

the reverse immunosorbent by glycine-HCl buffer at pH 2.0 without denaturation of the ribonuclease, as judged by optical ultracentrifugation, electrophoresis, column chromatography and by its enzymatic activity.

The physicochemical characterization of rabbit spleen and pancreatic ribonucleases revealed that these ribonucleases possessed the characteristic properties of mammalian ribonucleases. The optimum pH for enzymatic activity, the effect of some cations and inhibitors on enzymatic activity, and isoelectric points of these rabbit ribonucleases were similar to other mammalian ribonucleases. The molecular weights of these rabbit ribonucleases, as determined by gel filtration and from their amino acid compositions, were in excellent agreement with those of other mammalian ribonucleases.

The purity of the rabbit spleen ribonuclease isolated by physicochemical method was further demonstrated by the evidence that antibodies elicited in sheep by immunization with this ribonuclease preparation gave rise to only a single precipitin band on immunodiffusion as well as on immunoelectrophoresis (Figures 24 & 26). The resulting antibodies did not react with ribonucleases from other species, such as bovine and rat (Fig. 24) indicating that the antigenic determinants in these ribonucleases were different. This view is further supported by the fact that their amino acid compositions are significantly different as seen in Table 16. Furthermore, rabbit spleen and pancreatic ribonucleases did not react with rabbit anti-rat liver ribonuclease or anti-rat pancreatic ribonuclease antibodies which were kindly provided by

Dr. J. Gordon (244) indicating that rabbit and rat ribonucleases were not antigenically related.

The specificity of the antibodies against rabbit spleen ribonuclease was further demonstrated by the fact that these antibodies reacted only with ribonucleases from the spleen and pancreas of rabbit, but not with ribonucleases present in other organs or serum of rabbit. This observation is a direct demonstration that even within a given species, ribonucleases from various organs might be immunologically different. It is interesting to note that similar findings were reported on rat ribonucleases by Gordon (244), i.e. that rabbit antibodies against rat pancreatic ribonuclease reacted identically on immunodiffusion with rat pancreatic and spleen ribonucleases, but not with ribonucleases from other rat organs, such as liver and kidney.

Extensive studies on the inhibition of enzymatic activity of bovine pancreatic ribonuclease by its antibodies have been reported by Cinader (183, 185, 186, 331), Brown (79), Richards (194) and other workers (330, 341). The extent of inhibition varied widely from 20 to 100% depending on the methods for assay of ribonuclease activity and the course of immunization. The inhibitory capacity increased with the course of immunization (183, 334). Higher degree of inhibition was found when macromolecular RNA was used as substrate rather than the small cyclic cytidylic acid molecules (185, 186).

The effect of inhibition of enzymatic activity by antibodies can be studied in three ways: (i) assay of the enzymatic activity of the whole mixture of the enzyme and antibodies. (ii) assay of

enzymatic activity of the supernatant after the precipitate has been removed, (iii) assay of enzymatic activity of the precipitate separated from the supernatant. The observation that homologous antibodies inhibited the enzymatic activity of bovine pancreatic ribonuclease, as reported by many workers, was mainly based on the first approach which obviously does not distinguish between the activity of ribonuclease complexed in the precipitate and that remaining in the supernatant. Therefore, the second or third approach would provide a direct answer as regards the activity in the supernatant or in the precipitate, or in both. Cinader (183) reported that the enzymatic activity (30%) found in the mixture of bovine pancreatic ribonuclease and excess of antibodies was almost entirely associated with precipitate which could be separated from the supernatant. Smolens et al. (341) studied the enzymatic activity of the ribonuclease-antibody precipitate which was separated from a mixture of ribonuclease and its homologous antibodies, and found 20-30% inhibition of the ribonuclease activity. Gordon (244) examined the inhibition of rat ribonucleases by rabbit anti-rat pancreatic ribonuclease and anti-rat liver ribonuclease antibodies, by assay of enzymatic activity in the supernatant from mixture of ribonuclease with excess of antibodies. He reported that rat pancreatic or spleen ribonucleases could be removed completely from the supernatant by the anti-rat pancreatic RNase antibodies, whereas only 52 to 70% of the rat liver ribonuclease could be removed from the supernatant by its homologous antibodies. The presence of soluble ribonuclease-antibody complexes in the supernatant

was suspected but no evidence had been provided prior to this study.

In the present investigation, the enzymatic activity of the supernatant was assayed in most cases, since one of the main original purposes of this study was to develop a simple procedure for the removal of ribonuclease activity so that its harmful effect on the integrity of polysomes might be eliminated or minimized. In order to get further insight into the effect of inhibition of rabbit spleen ribonuclease activity by its homologous antibodies, the precipitate was separated from a mixture of ribonuclease and its antibodies. After washing the precipitate twice with phosphate-buffered saline, it was assayed for enzymatic activity by suspending it in 1.2 ml of 0.15 M phosphate buffer at pH 7.3; the precipitate possessed 18% of the enzymatic activity and the supernatant 40%. It may be worth reiterating that the whole suspension of the precipitate formed on addition of antibodies had 60% of the initial enzymatic activity, which represents almost quantitatively the additive effect of the enzymatic activities of the precipitate and of the supernatant.

The residual ribonuclease activity in the supernatant was proved to be attributable to the presence of soluble ribonuclease-antibodies complexes as demonstrated by the following facts: (a) This residual activity could be removed completely by the addition of anti-sheep gamma globulins, (b) the ribonuclease activity could be entirely removed with the insolubilized antibodies, and (c) the protein components with ribonuclease activity in the supernatant were excluded from a column of Sephadex G-75 in the void volume,

indicating that ribonuclease had been bound with antibodies to form molecular complexes larger than ribonuclease itself. These results demonstrate unequivocally the presence of soluble ribonuclease-antibody complexes in the mixture of rabbit ribonuclease and its homologous antibodies.

In the present investigation, the reverse immunosorbent, prepared by insolubilizing the antibodies against rabbit spleen ribonuclease, was capable of removing ribonuclease activity completely from a solution of pure rabbit ribonuclease. On the other hand, this reverse immunosorbent removed only about 85% of the enzymatic activity from a crude extract of rabbit spleen. These results may be construed as indicating that the reverse immunosorbent removed only ribonuclease activity specifically, without affecting other nonspecific nucleases or phosphodiesterases which are also capable of degrading ribonucleic acids at a lower rate as compared to ribonuclease itself. The residual activity remaining in the crude extract was, therefore, considered to be attributable to the presence of nucleases other than ribonucleases.

The isolation of intact polysomes is of great interest in molecular biology in general, because polysomes have been shown to be the active units of protein synthesis which is governed by the specific mRNA linking the ribosomes into the active unit. The main difficulty of isolating intact polysomes is due to the release RNA-degrading enzymes during cellular disruption. Attempts have been made to eliminate the harmful effect of ribonucleases by the addition of nonspecific inhibitory agents such as bentonite (102, 103), Macaloid (105, 106), heparin (100, 104) and detergents (98,

99, 100). However, no ideal nuclease inhibitor has as yet been discovered.

Since the reverse immunosorbent which was prepared by insolubilization of antibodies to rabbit spleen ribonuclease was capable of removing ribonuclease activity effectively, the possibility of using it for the isolation of intact polysomes from rabbit spleen cells was investigated. Although it had been shown in Chapter IV (Table 7) and Chapter VI (Table 24) that the reverse immunosorbent could be successfully used for removal of ribonuclease and thus for the inhibition of rabbit ribonuclease activity to a greater extent than a mixture of heparin and sodium deoxycholate, the use of this immunosorbent did not lead to a higher yield of polysomes. Since the reverse immunosorbent is highly specific for ribonuclease only and does not combine with other nucleases present in the cell lysate, it does not prove advantageous for the isolation of polysomes. However, one may suggest that this procedure could be improved by the use of antibodies against <u>all</u> the nucleases in the crude tissue extract. These antibodies would thus be expected to inhibit the activities of all nucleases.

A further extension of the present study for the establishment of the complete amino acid sequences of rabbit ribonucleases would be of great interest. From a comparison of the primary structure of rabbit ribonucleases with those of other mammalian ribonucleases, one would be able to derive information regarding the nature of the immunodominant loci of these proteins and of the mutational events which have affected them on the evolutionary scale. From the protein

sequence data, it is possible to derive a phylogenetic tree which shows in detail the nature of the ancestral relationships of present-day species. Thus, by analogy, the study of comparisons of amino acid sequences of cytochrome C from different species has led to the derivation of a phylogenetic tree for these species. It is conceivable that catalytic site of ribonuclease has undergone no, or only a negligible, change in the course of evolution, since otherwise the catalytic properties of these enzymes would not have been maintained. Highlight on this problem will be shed once the primary structures of ribonucleases from diverse species are established.

CHAPTER IX

CONCLUSIONS

Rabbit spleen and pancreatic ribonucleases have been purified 50- and 23-fold, respectively, by conventional physicochemical methods of sequential column chromatography on Sephadex G-75, DEAE-Sephadex A-50, SE-Sephadex C-50 and CM-Sephadex C-50. From the results of analytical ultracentrifugation, electrophoresis and column chromatography on Sephadex G-75, the purified ribonucleases were judged homogeneous. These preparations were devoid of DNase, acid and alkaline phosphatase and phosphodiesterase activities.

The physicochemical characteristics of rabbit spleen and pancreatic ribonucleases showed some similarities, but not identity, to the bovine pancreatic ribonuclease. The optimun activity of both rabbit ribonucleases was found at pH 7.0. Of all the cations tested, Cu^{++}, Zn^{++} and Ag^{+} were inhibitory to the enzymatic activities of rabbit ribonucleases, whereas Na^{+}, K^{+}, Ca^{++} and Mg^{++} had practically no effect. The enzymatic activity was not affected by metal ion chelating agents, such as sodium citrate and EDTA, indicating that metal ions were not required for enzymatic activities of rabbit ribonucleases. The insensitivity of rabbit ribonucleases to p-chloromercuri-benzoate suggests that these ribonucleases do not belong to the group of sulfhydryl enzymes. The rabbit ribonucleases are basic proteins with isoelectric points at pH 10.84 and 9.93, respectively. The results of effect of substrate (RNA) concentration on enzymatic activity indicate that the spleen ribonuclease has

higher affinity towards the substrate than the pancreatic RNase. The Michaelis-Menten constants for rabbit spleen and pancreatic ribonucleases were found to be 0.52 and 1.47 respectively. By means of gel filtration on Sephadex G-75, the molecular weights for these ribonucleases were estimated to be approximately 14,000. The exact molecular weights for rabbit spleen and pancreatic ribonucleases were calculated from the amino acid compositions and found to be 14,305 and 13,971, respectively. From their amino acid compositions these rabbit ribonucleases were found to have a high content of basic amino acids as is the case for bovine pancreatic ribonuclease. Therefore, one may make a general statement to the effect that both rabbit spleen and pancreatic ribonucleases have similar physicochemical properties to those of bovine pancreatic ribonuclease and other mammalian ribonucleases.

The purified rabbit spleen ribonuclease was used for immunization of a sheep which produced 'monospecific' antibodies as judged by the formation of single precipitin band on immunodiffusion as well as on immunoelectrophoresis. Insolubilization of the resulting antibodies by either cross-linking with ethyl chloroformate or polymerization with ethylene maleic anhydride (EMA-31) co-polymer, produced reverse immunosorbents which were capable of removing rabbit ribonuclease activity effectively. Incorporation of the reverse immunosorbent into a column of Sephadex G-25 gel yielded an effective means for the isolation and purification of rabbit ribonuclease in a rapid and simple manner with high yield (86%) and high purity. When a crude extract of rabbit spleen was passed

through the reverse immunosorbent column, the ribonuclease was absorbed specifically. After elimination of the unabsorbed protein contaminants by washing with phosphate-buffered saline, the ribonuclease was eluted from the column by 0.4 M glycine-HCl buffer at pH 2.0, without any denaturation of the enzyme.

The study of effect of antibodies on the enzymatic activity of rabbit ribonuclease showed that the supernatant from a mixture of ribonuclease and excess of antibodies contained some residual ribonuclease activity. Nevertheless, residual ribonuclease activity was not found in the supernatant when the enzyme was mixed with insolubilized antibodies. This observation indicated the formation of soluble ribonuclease-antibody complexes in the supernatant. Indeed, the presence of soluble complexes in the supernatant was proved unequivocally by complete removal of the residual ribonuclease activity on (i) double immuno-precipitation and (ii) on chromatography through a column of Sephadex G-75, the enzymatic activity appearing in the first protein component excluded from this gel. An attempt has been made to use the reverse immunosorbent as an ribonuclease inhibitor in the isolation of intact polysomes. Unfortunately, the use of this reverse immunosorbent was only as effective as the use of a mixture of heparin and sodium deoxycholate. The limitation of this procedure for the isolation of polysomes in higher yields was attributed to the presence of nucleases other than ribonuclease in the cell lysate, which were not inhibited by the specific reverse immunosorbent.

CLAIMS TO ORIGINALITY

1. By the application of physicochemical procedures, i.e. gel filtration and ion-exchange chromatography, it was possible to isolate pure rabbit spleen ribonuclease.

2. Rabbit pancreatic ribonuclease was obtained in a pure form by similar physicochemical procedures, i.e. salting-out, gel filtration and ion-exchange chromatography.

3. For effective desalting of rabbit ribonucleases a column (4 x 80 cm) of Sephadex G-25 was used with distilled water as eluant.

4. The physicochemical properties such as pH optima, Michaelis-Menten constants, isoelectric points, effect of ions and inhibitors on enzymatic activity, molecular weights and amino acid compositions, of rabbit spleen and pancreatic ribonucleases showed the characteristics of mammalian ribonucleases.

5. An excellent linear relationship between molecular weights of proteins and their elution volumes from a Sephadex G-75 column (2.5 x 150 cm) was obtained. The molecular weights of rabbit spleen and pancreatic ribonucleases determined by this method were in agreement with the values calculated from their amino acid compositions.

6. Sheep antibodies against rabbit spleen ribonuclease were produced and found to be 'monospecific' to rabbit ribonucleases as judged by the formation of a single precipitin band on immunodiffusion and immunoelectrophoresis.

7. On immunodiffusion rabbit spleen ribonuclease cross-reacted with pancreatic ribonuclease and showed a reaction of identity.

8. The sheep antibodies against rabbit spleen ribonuclease were highly species specific, since they gave no reaction with bovine or rat ribonucleases.

9. A reverse immunosorbent was prepared by insolubilizing sheep antibodies against rabbit spleen ribonuclease with ethyl chloroformate. This reverse immunosorbent was capable of absorbing ribonuclease specifically from the crude extract of rabbit spleen. The absorbed ribonuclease could be subsequently eluted with glycine-HCl buffer, pH 2.0, in a state of high purity.

10. Cross-linking of the sheep antibodies against rabbit spleen ribonuclease with ethylene maleic anhydride copolymer produced a reverse immunosorbent which could be dispersed in a Sephadex G-25 column and used for the isolation of rabbit ribonuclease in a simple and rapid manner.

11. The incomplete inhibition of rabbit ribonuclease activity by homologous antibodies was found to be attributable to the formation of soluble ribonuclease-antibody complexes.

12. The presence of soluble ribonuclease-antibody complexes was proved unequivocally from the studies of inhibition of enzymatic activity by insolubilized antibodies, double immunoprecipitation with anti-antibodies, and gel filtration.

13. A combination of precipitation with ammonium sulfate, treatment (adsorption) with bentonite and column chromatography on Sephadex G-100 resulted in the removal of endogenous ribonuclease activity present in the sheep antiserum.

14. On exposure of a solution of high molecular weight yeast RNA to ribonuclease the RNA was degraded as detected by analytical ultracentrifuge, the fast sedimenting RNA (3.7S) was degraded on treatment with ribonuclease to small oligonucleotides. This analytical method was found useful in providing information of the effectiveness of inhibitors in protecting RNA from degradation. Thus the reverse immunosorbent was demonstrated to be highly effective in inhibiting the enzymatic activity of ribonuclease.

15. Up to the present study, only nonspecific nuclease inhibitors were used in the isolation of intact polysomes. In this study, an attempt was made to use the reverse immunosorbent as a specific ribonuclease inhibitor in the isolation of polysomes from rabbit spleens. However, its inhibitory capacity did not exceed that of a mixture of heparin and sodium deoxycholate. This limitation was attributed to the presence of other nucleases in the cell lysate. One can speculate that this procedure could

be further improved by the use of reverse immunosorbent prepared by insolubilization of antibodies against all the nucleases in the crude tissue extract.

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