Glutathione Facilitated Regeneration Of Reduced, Denatured Bovine Seminal Ribonuclease.

Glutathione Facilitated Regeneration of Reduced, Denatured Bovine Seminal Ribonuclease

by

Gary K. Smith

A Dissertation
Presented to the Graduate Committee
of Lehigh University
in Candidacy for the Degree of
Doctor of Philosophy
in
Chemistry

Lehigh University
1978

Approved and recommended for acceptance as a dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Date July 21, 1978

Stephen W. Schaffer
Professor in Charge

Accepted July 21 1978
Date

Special Committee directing
the doctoral work of
Gary K. Smith

Stephen W. Schaffer
Dr. S. W. Schaffer (Chairman)

Frederick M. Fowkes
Dr. F. M. Fowkes

Joseph R. Merkel
Dr. J. R. Merkel

Keith J. Schray
Dr. K. J. Schray

ACKNOWLEDGMENTS

The author wishes to thank his advisor, Dr. Stephen W. Schaffer, for knowledgeable assistance and direction. Further, thanks are expressed to the author's Ph.D. committee (Drs. F. M. Fowkes, J. R. Merkel, and K. J. Schray) individually and collectively for helpful discussions and criticism.

Sincere appreciation is expressed to the Newton Wayne Buch and Constance Nancie Buch Fellowship for two and one-half years of complete support.

The author wishes to thank Dr. D. B. Wetlaufer for the generous gifts of reagents, use of his circular dichroism instrument, and very helpful discussions without which much of this work would not have been possible.

Thanks are also expressed to Dr. G. D'Alessio for his gift of seminal ribonuclease to initiate the studies and for supplying Dr. S. W. Shaffer and the author with unpublished data.

The author expresses appreciation to Drs. W. Bank and L. F. Hass for the use of their amino acid analyzers and to Mr. A. Butz for the gift of seminal vesicles.

Mr. J. P. Chovan and E. C. Kulakowski are gratefully acknowledged for their aid in securing the seminal vesicles. Special thanks are extended to Ms. J. Loosbrock for typing this dissertation.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Totally reduced and denatured bovine seminal ribonuclease (seminal RNase) was regenerated from the reduced-denatured state with the glutathione redox system. The refolding kinetics were characterized according to redox state, temperature from 14 to 43°C, pH, and protein concentration. The maximal rate of regeneration occurred with 3mM reduced glutathione, 0.6mM oxidized glutathione, 24 to 30°C, and at pH 8.2.

The products of the refolding process were characterized by G-75 Sephadex gel filtration, SDS gel electrophoresis, enzymatic activity, circular dichroism, and amino acid analysis. The results indicate that the native dimeric form of the enzyme is not produced to any appreciable extent during refolding, rather the major product is monomeric. The purified monomer exhibits twice the activity of the native dimer toward yeast RNA. Its circular dichroism spectrum in the far UV is quite similar to that of RNaseA and different from the native enzyme. Amino acid analysis of the monomer showed that two molecules of glutathione are bound per chain indicating that cysteine 31 and 32 which normally form intermolecular disulfide bonds are blocked. The results are discussed in relation to other proteins and in particular RNaseA and IgG.

One major difference between RNaseA and seminal RNase folding with the glutathione system is the temperature dependence of the refolding kinetics. Although both processes yield monomeric species at either moderate or high temperature, the yield of seminal RNase activity is much lower at high temperature than is RNaseA. It was found that modification of cysteine 31 and 32 with a variety of sulfhydryl reagents decreased this large temperature dependence in seminal RNase. The results are compared with the rate of thermal denaturation of each enzyme and are discussed in relation to protein denaturation theory.

Since it was found that the regneration products of unmodified seminal RNase regeneration are not the native dimer, the effect of glutathione on the intermolecular disulfide bonds was studied. It was found that when native seminal RNase is incubated with 3mM reduced glutathione, 0.6mM oxidized glutathione, pH 8.2 at 30°C (conditions found optimal for regeneration), partial reduction of the intermolecular disulfide bonds resulted. G-75 gel filtration showed that approximately 30% of the enzyme dissociated to the monomer. The remaining dimer was shown by SDS gel electrophoresis to be covalent. The specific activity of the monomer is similar to that of the regenerated monomer. Further, amino acid composition and circular dichroism spectra of the monomeric and dimeric species from this reaction proved to be virtually identical

to the refolded monomer and native dimer, respectively. Thus, the monomeric species is a form of the enzyme which is accessible from both directions (regeneration or selective reduction).

In studying the products of refolding and selective reduction, a new affinity chromatography system for RNase was developed. The system is based upon the commercially available resin 5'UTP-Hexane-Agarose. Under the conditions used very little nonbiospecific binding was seen. Biospecificity of RNase binding was demonstrated by several techniques including competitive elution of enzyme with a soluble inhibitor. These results are discussed in relation to noncompetitive binding.

INTRODUCTION

The reversibility of protein denaturation is now a well accepted phenomenon; thus, primary structure determines tertiary structure in the protein's physiological milieu. However, the mechanism by which the primary structure dictates tertiary structure remains elusive. The process by which primary structure is translated into tertiary structure, protein folding, is the topic of the present dissertation.

Extensive reviews of the vast body of literature in support of protein folding have been published by several investigators in recent years (1-4); however, a discussion is also warranted here to put the present work in the proper perspective. This review will emphasize the recent literature but will also discuss the very important early work.

I. Protein Stability

In order to understand protein folding, one must understand the forces involved in protein stability and unfolding since the same forces which stabilize the protein should drive its folding. The problem of protein stability and denaturation was best stated by Tanford (5):

> (1) ...Can we account for the fact that the native state is thermodynamically stable under physiological conditions?
>
> (2) ...Can we further account quantitatively for the detailed course of transition from the native to the denatured state?

(3) Denaturants presumably do not act at long distances from the protein molecule. Can we identify the specific sites at which they act?

At that time he further stated that "the answers to these questions prove to be somewhat disappointing, and it is evident that the overall problem of protein denaturation is not solved." These statements were made in 1970. Since that time little advance has been made to further describe the factors of protein stability and to quantitate denaturation. However, it is presently (and indeed was in 1970) possible to qualitatively describe these forces.

Protein structure is presumably maintained by a complex balance of a number of forces including electrostatic, hydrogen bonding (both with the solvent and internally), and hydrophobic bonding (that is, the tendency of nonpolar groups to avoid water contact). It is generally accepted that the latter of these is most important in maintaining conformation (5); however, denaturation studies have indicated that ribonuclease A (RNaseA) and chymotrypsinogen are equally stabilized at their temperature of maximum stability by hydrogen bonding and hydrophobic bonding (6-8).

Generally, the importance of each stabilizing force is evaluated by determining the $\Delta G_{(transfer)}$ of small molecular weight model compounds between various solvents, but it is agreed that model compound studies can be misleading (9).

Briefly, it has been demonstrated that nonpolar groups have a favorable enthalpy of transfer from a hydrophobic medium to water; however, this is outweighed at moderate temperatures by a large unfavorable entropy of transfer (7,10). Presumably this is due to the entropically unfavored (but ΔH favored) formation of extensive water structure (H-bonding) around nonpolar groups exposed to water (often called clathrate formation) (11). This leads to a large stabilizing entropy contribution in the folded protein where virtually all hydrophobic groups are folded inward away from the solvent.

Model studies for peptide backbone hydrogen bonding suggest that this contribution to protein stability is small, though estimates disagree quantitatively. Studies comparing urea dimerization, which serves as a model for peptide hydrogen bonding (H-bonding), to urea H-bonding with water, which serves as a model for random backbone H-bonding with solvent in the denatured state, indicate that the free energy of stabilization would be small or positive (12,13). On the other hand, helix to random coil transitions in homopolyamino acids indicate that peptide H-bond is stronger than that with water (5). These conflicting data indicate that the point is not completely clear. However, it has been pointed out that perhaps the most important factor determining the strength of the hydrogen bond is the environment. Thus, internal

hydrogen bonding groups will certainly tend to be bonded in the nonpolar interior of the protein due to the large expenditure of free energy resulting from a lack of H-bonding (9). Indeed, it has been shown that 90% of internal polar groups are hydrogen bonded (14). Further, the cooperativity of α-helix hydrogen bonding also makes the small molecule a poor model (15).

Model compound studies of charged groups are rare, but protein ionic groups should behave like other charged moieties. Thus, water and salt interactions are favorable, and transfer of ionic groups to nonpolar environments is unfavorable (5). Further, virtually all charged protein groups are located at the surface of the folded molecule, so little change in environment or solvation will occur to these groups in the transition from denatured to native protein. Those internal ionic groups that do occur in proteins generally form salt bridges but are rare compared to the extensive internal hydrophobic and hydrogen bonding. Thus, they are likely to contribute little to the $\Delta G_{transfer}$.

Disulfide bonds are usually not discussed in protein stabilization models, but these structures are certainly a major stabilizing factor. The stability attributed to them results from a decrease in the entropy of the cross-linked denatured species compared to the species lacking crosslinks (16-19). As will be shown in the following

section, it is precisely this entropy which is the major factor driving denaturation. It has been demonstrated that introduction of a specific crosslink into native lysozyme results in an increase in the thermal denaturation temperature, which leads to a 5.2 kcal/mole increase in stabilization energy (19). This stabilization was shown to arise from a 20 E.U. decrease in the entropy of the denatured, crosslinked protein compared to that of the denatured unmodified protein. The crosslink caused virtually no change in ΔH. Similarly, protein stabilization from disulfide bonds is observed when comparing the denaturation of disulfide containing proteins in the presence and absence of reducing agents (to break the disulfides) (20,21).

In opposition to these stabilizing factors is the large conformational entropy of the denatured protein which accounts for the desire of each bond in the protein to randomize its conformation. This is the major destabilizing factor (5,6,9,11).

Based on data similar to the above, Tanford has calculated that RNaseA and β-lactoglobulin should not be stable (5)! This of course is impossible, but it illustrates the important point that protein tertiary structure is only marginally stable. Indeed, ΔG of stabilization for the several proteins studied is only approximately 10 kcal/mole (5).

In general, it is likely that protein stability is controlled by the large entropy of hydrophobic bonding. Internal hydrogen bonds endow rigidity with fixed distances and angles between groups, and the charged groups (as well as their catalytic roles) endow solubility (9).

II. Effect of Denaturants

A. Reversible vs. Irreversible Denaturation

As stated above, the reversibility of protein denaturation is now well accepted; however, a protein which undergoes a reversible transition under one set of conditions can undergo an irreversible transition under slightly different conditions. Irreversibility generally arises from aggregation of an otherwise reversibly denatured protein (20). In some cases there is direct evidence of intermolecular disulfide crosslinking (23-27). Irreversibility is most often observed in thermal denaturation near neutral pH where disulfide exchange can occur readily, but it does occur with other denaturants and during thermal denaturation at acid pH. It has been found that myoglobin, which does not possess disulfides or thiols, undergoes a reversible transition at 40°C, pH 4.4, but at higher temperatures the process becomes irreversible (28). Thus, clearly irreversible denaturation does not rely on intermolecular crosslinking. On the other hand, irreversible urea denaturation of ovalbumin and irreversible thermal denaturation of bovine

serum albumin have been directly linked to aggregation and the formation of intermolecular disulfides (23-25). Interestingly, Steinhardt, et al. (26) have shown that acid denaturation of horse ferrihemoglobin is reversible at short denaturation times and irreversible at longer times. Clearly, the irreversible character of denaturation is a real problem, which will invalidate thermodynamic analysis of denaturation. Thus, discussion of denaturation will be limited to the reversible processes unless otherwise noted.

B. The Two State Issue

Denaturation of proteins shows cooperativity in unfolding. That is, various probes of protein structure (for example: circular dichroism, fluorescence, ultraviolet absorbance, and enzyme activity) generally indicate that all parts of the molecule undergo the denaturation transitions simultaneously. This has caused several authors to conclude that denaturation is a two state process from the native to the denatured state with no stable intermediates. However, this has been shown to be an oversimplification for several proteins (RNaseA, carbonic anhydrase, nuclease), and although the overall process is highly cooperative, intermediates are detected by various techniques (29-32). Thus, the precise course of the transition can not yet be described in detail; however, the general mode of action of denaturants is

becoming clearer, and this may shed light on the pathway.

C. Effect of Denaturants

1. Alcohols and Dioxane

The effect of these denaturants is said to be on the hydrophobic groups. This conclusion has come from both model compound and intact protein studies. Ethanol and dioxane increase the solubility of various hydrocarbon amino acid side chains in water which results from a $-\Delta G$ transfer from water to solute:water (33). Similarly, long chain alcohols have been shown to be most effective while the glycols are the least effective denaturants for several proteins (chymotrypsinogen, myoglobin, cytochrome C, β-lactoglobulin, bovine serum albumin (BSA) and RNaseA) (34,35). Further, the thermal denaturation temperature for RNaseA also decreases as the chain length of the alcohol increases; that is, long chain alcohols are the most effective denaturants (35).

At the same time the peptide bond becomes less soluble with increasing alcohol concentration or carbon length (22); thus, secondary structure might be expected to be stabilized in these solvents. Indeed, there is considerable evidence which points to this exact effect (34,36,37). Jirgensons (36) has shown that proteins with little secondary structure in aqueous solvents are converted to structures with extensive secondary structure (resembling α-helix) in solvents containing high alcohol

concentrations.

The denaturation by these organic solutes appears to be at least a two step process. The proteins apparently first unfold (presumably by exposure of hydrophobic groups) and then reform conformations with high secondary structure as the concentration of solute is increased (34,38,39). Thus, there is a balance at any particular alcohol or dioxane concentration between increased hydrophobic solvation and desolvation of peptide bonds which together lead to exposure of apolar moieties and enhanced internal hydrogen bonding. This is actually an oversimplification, because, although high concentrations of ethanol do denature RNase A, low concentrations exhibit a rather large stabilizing force on the enzyme (8). Further, it has been pointed out that similar stabilizations can be seen at low alcohol concentrations in the denaturation of other proteins (22). Model studies of hydrophobic solubility in water have shown that solubility is decreased at low ethanol or dioxane concentration, while at higher concentrations the solubility is increased (40). Thus, this anomaly actually supports the proposed action of these solutes on hydrophobic interactions, but it is not known why low concentrations decrease the solubility of these groups (8).

2. Detergents

The effect of detergents is likely similar to that of the alcohol and dioxane. They disrupt hydrophobic bonding and normal structure but subsequently enhance helicity (41). Indeed, Jirgensons has demonstrated that proteins of low helix in the native state (RNaseA and bovine pancreatic trypsin inhibitor (BPTI)) are converted to species with a high helical content in the denatured state (42). However, the low concentrations necessary for the denaturation of proteins with detergents (<0.01M compared to several molar with alcohols) suggest very tight binding of the detergent to the protein (20,42). This has been verified by nuclear magnetic resonance (NMR) (43).

As stated above, it has been shown that alcohol effectiveness for denaturation increases with increasing chain length (34). Similarly, Jirgensons and colleagues have shown that alkyl sulfate detergent effectiveness increases logarithmically with chain length (44,45). These data also indicate similar mechanisms for alcohol and detergent mediated denaturation of proteins and that the hydrophobic moiety is important in both cases. However, it has been suggested that the ionic moiety may also be involved in detergent denaturation (46).

3. Urea and Guanidine Hydrochloride

Although the vast majority of protein denaturation studies make use of either urea or guanidine hydrochloride (GuHCl), the mechanism of denaturation by these presumably similar acting denaturants is in doubt. It is, however, well accepted that GuHCl is a better denaturant than urea (9,20,22).

Two basic mechanisms of denaturation by these solutes have been proposed. They are: 1) increased hydrophobic group solubility, and 2) decreased backbone hydrogen bonding. The salient features of each will be discussed; however, no attempt will be made to discuss all of the literature, much of which is conflicting (for example see reference 22).

Hydrogen bonding of urea and GuHCl to protein groups was proposed by Mirsky and Pauling (47) as the mechanism of denaturation. They pointed out that "the reagents which cause denaturation are all substances which affect hydrogen bond formation....they form hydrogen bonds with the protein side chains, which are thus prevented from combining with each other and holding the protein in its native configuration." However, due to the overwhelming number of peptide hydrogen bonds to side group hydrogen bonds, it is now thought that any effect on hydrogen bonding by denaturants will be at these peptide (backbone) bonds (5). Nonetheless, the general

effect of urea and GuHCl on hydrogen bonding still finds considerable support.

A negative ΔG is seen for the transfer of diglycine and triglycine (backbone models) from water to urea or GuHCl (5). Further, ΔG for transfer from water to GuHCl is larger than that for transfer to urea, which is in agreement with the result that GuHCl is a better denaturant (5).

Similarly, Robinson and Jencks (48) observed that urea and GuHCl increase the solubility of acetyl tetraglycine ethyl ester (another backbone model) in water solutions. They further pointed out that the increased solubility in the presence of these denaturants was not due to increased hydrophobic group accommodation since alkyl derivaties of urea and GuHCl have less effect on the solubility than the parent compounds themselves. Very recently, Bonner et al. (49) concluded from heat capacity studies that relative hydrogen bond strengths with the peptide carbonyl follow the sequence $-NH_2$ > OH_2 > NH. Thus, they concluded that the $-NH_2$ groups of urea and GuHCl could compete effectively with the peptide N-H and water for the peptide carbonyl hydrogen bonding.

These model studies are supported by protein denaturation data which indicate that destabilization of hydrophobic interactions can not be the sole contributor to urea and GuHCl denaturation. Jencks and collaborators

(48,50) have found that BSA is denatured more effectively by unsubstituted urea and GuHCl than by alkyl substituted analogs.

On the other hand, there is good evidence which indicates that these reagents act by stabilizing hydrophobic groups in aqueous solution. This could either be a direct effect on the nonpolar group itself or a long range effect on water hydrogen bonding (5,22). In a now classical paper Wetlaufer, et al. (10) found that the solubilities of several hydrocarbon gases in water solution increased in the presence of urea and especially GuHCl. The reason for this solubility increase was seen to be a positive $\Delta S_{transfer}$ for going from water to water plus denaturant solutions. Similarly, it has been shown that the leucyl, tryptophyl, phenylalanyl, methionyl, and histidyl side chains exhibit a negative $\Delta G_{transfer}$ from water to aqueous urea solution (51). Furthermore, urea destabilizes detergent micelles (52). Thus, urea and GuHCl stabilize the contact of apolar moieties with aqueous solutions, and effectively decrease the stability of the hydrophobic bond. This apparently arises from increased entropy of the solvated state in the presence of denaturant.

Brandts and Hunt (8) have noted that the temperature dependence of both these models (hydrogen or hydrophobic bond disruption) is not in agreement with the

temperature dependence for RNaseA denaturation by urea. They and several other authors have suggested that the true mechanism probably involves the destruction of both structures (5,22,49). The present author is inclined to agree with this point of view, not as a compromise but since it most readily accommodates all of the data which are convincing on both sides.

It was stated above that the denatured protein in "hydrophobic" denaturants (alcohols, dioxane, detergents) contains extensive secondary structure. In contrast, the completely denatured state in urea and GuHCl appears to be a random coil. Tanford and colleagues (53, 54) have investigated the denaturation of insulin, RNase-A, hemoglobin, myoglobin, β-lactoglobulin, chymotrypsinogen, glyceraldehyde-3-phosphate dehydrogenase, pepsinogen, aldolase, and BSA with 6M GuHCl in the presence of reducing agents. Based upon viscosity, sedimentation, and optical rotation data the proteins were characterized as random coils. Indeed, when disulfide bonds were left intact (reducing agents omitted), denaturation by 6M GuHCl led to a product which was characterized by the above criteria as a crosslinked random coil. In denatured RNaseA they also showed that the pK of all titrable groups became equal to that respective group exposed to the solvent (55). These data are for 6M GuHCl, and it is generally accepted that it is a better dena-

turant than urea; however, RNaseA, lysozyme, α-chymotrypsin, and β-lactoglobulin are also random coils in 8M urea (56-59).

Thermal denaturation can not occur by binding of the "denaturant" to the protein as has been implicated in solute denaturation and is thus different from the processes described above. However, the result is similar in that the transition is highly cooperative. Brandts and colleagues have shown that thermal denaturation of chymotrypsinogen and RNase A are consistent with an increase in destabilizing conformational entropy without a similar increase in the stabilizing hydrophobic bonding energy (6-8). In this model the conformational entropy term is shown to increase linearly with temperature, while the hydrophobic bonding term increases more slowly and levels off at high temperature. Thus, at sufficiently high temperature the destabilizing conformational entropy contributes more to the ΔG_{total} than the stabilizing forces and the protein undergoes a thermal transition. This is in qualitative agreement with the temperature behavior of hydrophobic model compounds (10). It is found that the solubilities of methane, ethane, propane, butane, and neopentane in pure water over the range 5 to 45°C decrease with increasing temperature, but level off at the higher temperatures. Thus, this corresponds to the increased stability of the hydrophobic bond at increased

temperatures, and the observation that the contribution levels off at the highest temperatures. Brandts and Hunt (8) have suggested that this stabilizing factor levels off at the high temperatures due to the "melting" of the clathrate structures which form around the apolar groups in water and drive hydrophobic bonding. Thus, the increase in conformational entropy and the destabilization of clathrates lead to better solvent accommodation of the hydrophobic groups, and denaturation.

Other evidence in support of this model can be found in the temperature of maximum stability for several proteins (6,8,60-62). Below (as well as above) this temperature, the protein is less stable; thus, denaturation occurs below this temperature more easily than at it. This "cold denaturation" results in a negative ΔS. This is virtually certain not to be due to increased ordering in the protein since the native protein is very likely characterized by "a nearly minimal degree of flexibility" (63). On the other hand, solubility of hydrocarbons increases with decreasing temperature (10); thus, it is likely that cold denaturation results from exposure of protein oily groups with only a small increase in conformational entropy. The denaturation temperature then occurs below the clathrate "melting point," and the increased solvent ordering on hydrophobic exposure results in an overall negative entropy change (63).

The thermal denatured protein does not appear to be a random coil in contrast to the urea and GuHCl denatured protein. Rather, considerable evidence indicates that structure remains even in the low pH, thermally denatured species. Evidence in support of this conclusion includes intrinsic viscosity (64), optical rotation (65), NMR (57,58,66) and side chain exposure (28). Very convincing support is seen in the GuHCl denaturation of heat denatured lysozyme (67). The thermally denatured enzyme shows an optical rotatory transition at around 2 to 3M GuHCl. Similarly, the proton NMR spectrum of heat denatured RNaseA is not that of a random coil (57, 58). However, the exact nature of this residual structure is not known.

The effect of denaturants (and denaturing conditions) appears to indicate that both hydrophobic and hydrogen bonding must be destroyed for complete denaturation. Thus, urea and GuHCl generate a random coil, while others (including neutral salts which appear to affect backbone hydrogen bonding (68,69)) lead to a less disordered chain.

III. Protein Folding

A. Thermodynamic Versus Kinetically Directed Folding

Although protein folding is a process of free energy minimization, the final structure assumed need not represent the global thermodynamic minimum free energy for the

specific amino acid sequence. Rather, the structure may become trapped in a local energy minimum with too large an activation energy to allow the protein to reach the global minimum under physiological conditions. This is referred to as the kinetically directed folding model. The original hypothesis of protein folding, put forth by Anfinsen and his colleagues (2), is the "thermodynamic hypothesis," that is, the native protein in its physiological environment is in its global free energy minimum conformation. The major evidence against the thermodynamic hypothesis is that protein synthesis and folding in vivo is very rapid. The time for RNaseA and lysozyme biosynthesis have been estimated to be about two minutes (70-73). Levinthal (74) pointed out that even in a small protein, a random search folding mechanism would be prohibitive in this short biological time. Indeed, it has been calculated that for a protein of 100 amino acids, a random search would take on the order of 10^{85} seconds (75). Thus, several authors have proposed that early kinetic events in the folding process, known as nucleations, must occur which restrict the possible conformations available to the protein later in the process (29, 74-76). Therefore, only the most rapidly forming conformations are reached during free energy minimization.

These two folding mechanisms also have experimentally testable predictive differences. The major

difference is in the number of intermediates formed during folding. The thermodynamic hypothesis predicts that all statistically possible intermediates will form during folding. On the other hand, the kinetic hypothesis predicts that relatively few of the possible intermediates will be formed since the folding pathway will be restricted. The second predictive difference is the result of nucleation sites, regions of the protein chain that gain specific structure first or independently of the rest of the molecule and direct subsequent folding. It has been pointed out that nucleating events and the thermodynamic hypothesis are mutually exclusive since formation of the former must limit the possible conformations forming later in folding (1).

Wetlaufer (75) envisions nucleations as short amino acid sequences 8 to 18 residues in length since a chain 8 residues in length can form a significant amount of structure and 18 residues can fold in biological time. However, it is conceivable that they could also be hydrophobic areas which rapidly coalesce to avoid solvent (S. W. Schaffer, personal communication), though it seems that the latter mechanism would lead to intermolecular aggregation, especially at high protein concentration. Nonetheless, evidence for such initial structure formation would support the kinetic hypothesis. The predictions of each mechanism will be discussed further in relation

to experimental evidence favoring each model.

B. Folding Simulations

It is well accepted that primary structure determines tertiary structure, and prediction of protein structure from the amino acid sequence is certainly the long term goal of all protein folding experiments. Thus, the derivation of schemes to simulate and predict the precise three dimensional structure of a protein is important. However, to say that the schemes have been "successful" is somewhat optimistic. As pointed out by Wetlaufer and Ristow (1): "A result of 80% correct prediction...is surely gratifying from one point of view, but to predict the three dimensional structure of a protein or polypeptide from its amino acid sequence, one has to be 99+% correct."

A review of this topic has appeared very recently (77), and the reader is referred to it and references included therein. However, a few comments are warranted here. The popular schemes used presently employ simplified representations of the protein to be folded (78-83). These simplifications vary, but they include representation of each residue as a sphere of specified size and properties (polar, apolar, ionic, etc.), and allowing rotation about ψ and ϕ angles alone (assuming a planar, trans peptide bond). Success of the folding scheme is then often measured as root mean square (rms) deviation

from the crystal structure. Values of 4 to 8 Å rms have been obtained, globularity of the "folded" protein as well as correct bends have also been observed (79,80). Thus, the similarity to the early x-ray diffraction studies is apparent. However, similar results have also been obtained by representing the entire protein (BPTI, a popular model) with alanine residues interspersed with glycines at positions normally occupied by glycine, aspartate, or asparagine (known structure breakers) (83). Starting with the extended chain, as is normal in these studies, they obtained a globular folded product with rms deviation of 6.2 Å and folds similar to the native protein. Thus, a sequence entirely different from the correct sequence gave results well within the error range found in simulations where each amino acid is represented uniquely. However, they pointed out that on close inspection, their resulting structure, and indeed those of the more extensive schemes, exhibit differences between the predicted and actual structures that are more significant than the similarities.

Kuntz, et al., (80) are optimistic "that there is much to learn from low resolution models, but, unlike [low resolution] x-ray studies, there is no assurance that the model calculations can ever be refined to high accuracy." The present author is in agreement and would point out that more experimental evidence is needed on

the precise mechanism of protein folding. For example, should the folding simulations involve a global energy minimization (79-83), or should nucleating structures be "forced" to form first and the remaining protein be allowed to fold around them (84-86)? Clearly, the answer lies in the thermodynamic versus kinetic hypotheses, and more experimental evidence is required to decide this issue.

C. Regeneration Studies

1. Early Work

In an excellent series of papers before 1940 Anson, Mirsky, and others clearly showed that under the proper conditions, usually acid pH, the denaturations of hemoglobin, trypsin, egg albumin, chymotrypsin, serum albumin, pepsin, carboxypeptidase, trypsinogen, chymotrypsinogen, and pepsinogen are either completely or partly reversible (87-93). Much of this work is reviewed by Anson (93).

Specifically, acid or heat denatured hemoglobin was found to renature to 67% of initial when allowed to refold at room temperature and near neutral pH (87). The heat denaturation of trypsin in acid solution was completely reversible, and the presence of 10% "alcohol" decreased the denaturation temperature but had no effect on reversibility (89). Most interestingly, they also found that the denaturation of hemoglobin, trypsin, and

egg albumin followed by several criteria (solubility, absorbance spectrum, proteolytic digestibility, enzyme activity, or exposure of titratable thiol groups) showed the same transition point (91,92). Further, analysis showed that molecules in the transition zone were either completely native or completely denatured. They concluded: "Protein denaturation is a definite chemical reaction: different quantitative methods agree in estimates of the extent of denaturation,....A protein molecule is either native or denatured. The denaturation of some proteins can be reversed" (91). Aside from the implication that denaturation is a two state phenomenon, these conclusions have proven true.

2. Recent Studies

a. Regeneration Systems

The modern study of protein folding was initiated with the discovery that RNase A with its 4 disulfides reduced by thioglycolic acid in 8M urea could regain activity (94). (The earlier denaturations had been carried out with disulfide bonds intact, and it could thus have been argued that the protein was not truly in a random state.) In this first report the yield of activity was low, 12 to 19% after 68 hours. Later it was found that an impurity in the thioglycolate reacted with protein amine groups and was responsible for the poor activity regain (95). Thus, when the protein was reduced

with purified thioglycolate or 2-mercapto-ethanol (2-ME) in 8M urea, yields of up to 80% activity could be obtained in 20 hours at room temperature, pH 8.0, by bubbling air through the solution to reoxidize the disulfides (95,96). The structure of the purified air oxidized enzyme proved to be indistinguishable from the native enzyme by ion exchange chromatography, peptide mapping, optical rotatory dispersion, viscosity, ultraviolet absorbance spectrum, x-ray crystallography, and quantitative immunoassay (97). Thus, the reoxidation of reduced and denatured RNaseA led to native enzyme and not some other species, or combination of species, with biological activity. This clearly demonstrated that (for RNaseA at least) the amino acid sequence does determine the tertiary structure.

However, the rate of regeneration under the above conditions is slow and variable (98,99). Further, the reaction only proceeds to 30-35% activity at 37°C, physiological temperature (98,99). Enzymatic regeneration of RNaseA, on the other hand, does proceed well at 37°C, pH 7.4, and exhibits a half time for activity regain of 5 minutes under optimal conditions (100-102). This system relies on an "enzyme" which has been found in microsomal fractions of rat and bovine liver; chicken, pigeon, and porcine pancreas; bovine lymphatic gland, testis, liver, ovary, thyroid, lung, parotid gland, kidney, brain,

heart, and spleen, as well as plant and microbial sources (100-104), though it has not been located in bovine pancreas, the source of RNaseA. The enzyme has been purified from bovine liver microsomes, has a molecular weight of 42,000 daltons (105), and, like those from the other sources, requires small amounts of low molecular weight thiols to keep the active site cysteine in its reduced and active form (105,106). Rather than acting to oxidize thiols to disulfides, its mode of catalysis is thought to be disulfide exchange; that is, the enzyme shuffles wrongly paired disulfides (106). This was clearly demonstrated by its ability to reactivate RNaseA with randomly paired disulfides (produced by oxidizing reduced RNaseA in urea) (106).

There has been a recent report that two interchange enzymes are found in the microsomes (107). It was shown that one is located on the cytoplasmic side and the other on the luminal side of the endoplasmic reticulum. These authors suggested that one system could be responsible for folding extracellular proteins after they pass through the endoplasmic reticulum membrane, and the other is responsible for intracellular protein folding.

There is, however, some doubt about the physiological significance of the system. The enzyme will accelerate the reactivation of lysozyme but only in the absence of the low molecular weight factor which is important for

RNaseA reactivation (108). If the factor is present, reactivation of lysozyme is severely inhibited. Further, reactivation of reduced denatured rabbit IgG with 46 half cystines is not accelerated by the enzyme (102), while the reactivation of Bacillus subtilis α-amylase, which contains no disulfide bonds, is enhanced (109).

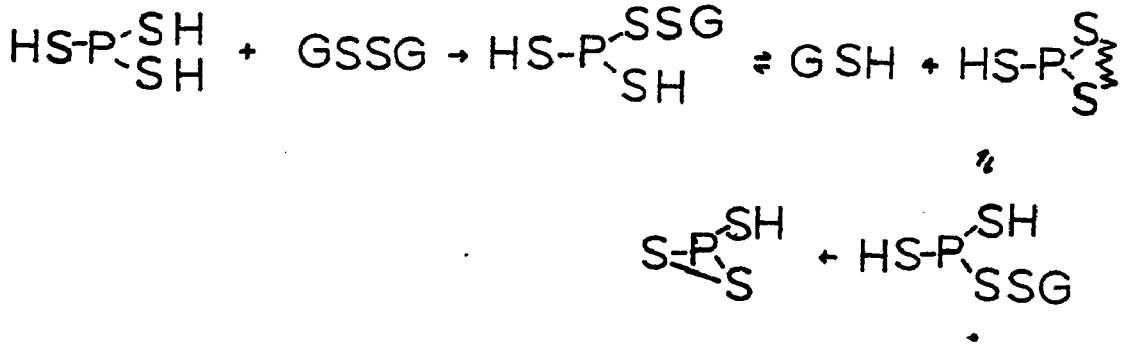
Recently other apparently unrelated proteins have been shown to accelerate reactivation of proteins. The disulfide interchange enzyme from porcine liver has been shown to be inactive in the presence of 3 mM reduced glutathione, 0.25 mM cystamine, 0.25 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and glutathione reductase (physiological concentrations); however, when a microsomal mixed function oxidase, which catalyzes the oxidation of low molecular weight thiols, was added, regeneration was accelerated (110,111). Further, this oxidase also accelerates the reactivation in the absence of disulfide interchange enzyme. They suggested that such a system would be preferred over the disulfide interchange enzyme in vivo which is inoperative in the presence of glutathione reductase.

Similarly Anderson and Tomasi (112) have noted that air oxidation of disulfide bonds is metal catalyzed (113) and investigated the effect of metal containing proteins on lysozyme reactivation. They showed that transferrin and lactoferrin accelerated the regeneration,

but the rate was not as rapid as with the disulfide interchange enzyme.

Nonetheless, enzyme or protein facilitated folding appears rather complex, and some of the acceleration may be simply due to physical interaction with the refolding protein (as in the action of the interchange enzyme on α-amylase). More investigation is required to clarify the issue.

A simpler system of reoxidation and disulfide exchange of regenerating proteins has been devised by Saxena and Wetlaufer (113). This system accomplishes oxidation of reduced protein and reduction of incorrect disulfide bonds by a mixture of reduced and oxidized glutathione (GSH and GSSG, respectively). The suggested mechanism is shown below (113).



where straight lines represent correct disulfide bonds and zigzag lines incorrect disulfide bonds.

Levels found optimal for GSH and GSSG, 3 mM GSH/

0.03 to 0.6mM GSSG (99,113), are the concentrations found *in vivo* (114,115); thus, the system has physiological potential. The glutathione redox system was originally used for lysozyme where the half time for regain of activity at pH 8, 37°C was 5 minutes (113), comparable to that found with the disulfide interchange enzyme (108). The major fraction after ion exchange chromatography, which accounted for 67% of the total protein, was indistinguishable from native lysozyme. However, another fraction, accounting for 13% of the total protein, had 51% activity and eluted from the ion exchange column earlier than the native enzyme; presumably this (and an inactive fraction, also found) had wrongly paired disulfide bonds.

Since the original demonstration of this system, several other proteins have been reoxidized with it including soybean protease inhibitor (116), human IgG (117), BPTI (118), BSA (119), trypsinogen (120), and RNaseA (99,121). The most significant of these to the present study is the regeneration of reduced, denatured RNaseA (99,122). The optimal regeneration was again found to occur under reducing conditions, and, unlike air oxidation, it proceeds well at 37°C (98,99). The half time for this enzyme in tris-acetate buffer (the buffer most often used in regeneration studies) is 75 minutes, far below the *in vivo* rate. However, with 0.5 to 1M phosphate present, the half time is less than

5 min at a protein concentration of 0.02 mg/ml (122). It is interesting that at high protein concentration the rate of return and yield of active enzyme decreases in phosphate (122), while no effect of protein concentration is seen in tris-acetate alone (99). This phenomenon has been attributed to aggregation in the presence of phosphate (S. W. Schaffer, personal communication), a good salting out electrolyte (22). One wonders if the acceleration due to phosphate is related to the increased intermolecular interactions in this solution and mechanistically similar to disulfide interchange enzyme catalyzed reactivation. The latter is strongly inhibited at high disulfide interchange enzyme levels (102,108,123).

The presence of disulfide bonds complicates the folding process. Thus, several investigators have chosen to study protein folding on proteins lacking disulfide bonds. Indeed, the rate of reactivation of enzymes without disulfides, or without prior reduction of disulfides, is faster than those containing disulfide bonds (2,29,30, 124). It has been suggested that either disulfide exchange or the conformational changes accompanying it is rate limiting for enzymes containing disulfides (2,113, 120).

b. The Role of Metals, Cofactors and Metabolites

Several proteins have been found to fold most rapidly

and with the highest yield in the presence of some specific ion or metabolite. Takagi and Isemura (125) found that Aspergillus oryzae taka-amylase A requires Ca^{++} for its regeneration from the reduced, denatured state. The ion is tightly bound to the native enzyme and is required for structural stability. In the presence and absence of Ca^{++}, protein thiols were found to be oxidized at the same rate during air oxidation regeneration; however, in the absence of Ca^{++} the oxidation led to less than 5% activity after 20 hours of regeneration, while in the presence of equimolar Ca^{++} concentration, 70% reactivation was obtained in 200 minutes. The inactive material produced in the absence of Ca^{++} had an optical rotatory dispersion spectrum considerably different from the native molecule but could be reactivated to native protein upon addition of Ca^{++} and 2-ME (to shuffle incorrect disulfides) (125, 126).

In a very interesting study, Bornmann, et al. (127) have found the renaturation of Saccharomyces carlsbergensis pyrvate kinase to be completely dependent upon L-valine and to a lesser extent Mn^{++} or Mg^{++}. The valine is tightly bound to the enzyme in the native form and dissociates upon denaturation (128). In the absence of valine or Mn^{++} (or Mg^{++}) regeneration does not occur. In the presence of 0.1 mM L-valine alone, the reactivation plateaus at 60% in less than 10 min, and with both L-

valine and Mn^{++} (or Mg^{++}) present the reactivation proceeds to 75%. Further, the action of L-valine is completely stereospecific; D-valine is ineffective.

Reactivation of GuHCl, dithiothreitol (DTT) denatured rabbit muscle phosphofructokinase was shown to be accelerated by the presence of adenosine triphosphate, but not fructose-6-phosphate, in the medium (129). In this case the half time for reactivation in the absence of ATP was found to be approximately 80 minutes and in the presence of 5 mM ATP was about 15 minutes.

Teipel and Koshland (124,130) have shown that cofactors affect the refolding kinetics and yield of several enzymes from the reduced, GuHCl denatured state. Porcine heart fumarase was shown to be rapidly reactivated in the presence of L-malate, but not in its absence. Similarly, Mg^{++} and nicotinamide adenine dinucleotide (NAD) have a large effect on the reactivation of rabbit muscle enolase and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), respectively. To a lesser extent, rabbit muscle aldolase, porcine heart lactate dehydrogenase (LDH), and malate dehydrogenase (MDH) reactivation can be accelerated by fructose-1,6-bis phosphate (FBP), NAD, and oxalacetate plus reduced nicotinamide adenine dinucleotide (NADH), respectively. These studies further indicate the presence of a rapidly forming intermediate in the refolding of G3PDH. NAD added to the reaction mixture at zero time has a large

acceleration effect on the reactivation, while NAD added less than one minute after regeneration has started has little or no effect although virtually no activity reforms at this early time (124).

The effect of Zn^{++} on the renaturation of bovine carbonic anhydrase shows similar properties (131). This enzyme refolds in 1M GuHCl in the presence of Zn^{++} provided the ion is added at t=0; however, if it is added later to a Zn^{++} free reactivation, continued regneneration occurs at an extremely low rate (even after several weeks it is not complete). These data suggest that Zn^{++} in carbonic anhydrase and NAD in G3PDH bind to the protein very early in reactivation and direct subsequent folding, while in the absence of these factors there is a rapid formation of some intermediate which can not bind Zn^{++} or NAD and can not be readily converted to the native species. This is supported by the report that Zn^{++} is coordinated to the otherwise random coil form of bovine carbonic anhydrase in 6M GuHCl (132,133).

An interesting effect of substrate on reactivation has been demonstrated with acid denatured rabbit muscle aldolase (134). The addition of FBP at t=0 causes refolding to abort and the enzyme to aggregate. However, if it is added at a time after the refolded monomer is formed (as determined by density gradient ultracentrifugation) the subunits associate to the native tetramer and become

completely active. These studies were done at pH 5.5, conditions where little subunit association occurs from the refolded monomer in the absence of FBP; however, at pH 7.0 rapid association and activation occurs. It is interesting that Tiepel and Koshland (124) found that the presence of FBP at zero time accelerates the reactivation of aldolase. This apparent discrepancy in results, however, may be explainable, because Teipel and Koshland examined the renaturation of GuHCl denatured enzyme while Vimard, et al., investigated the reactivation of acid denatured enzyme. These two denatured states are likely different and the mechanism of inactivation from each may also be different.

Perhaps a similar situation to acid denatured aldolase exists in the reactivation of GuHCl denatured spinach leaf glyoxylic acid reductase where addition of substrate at t=0 leads to severe inhibition of renaturation (135). Addition at other times was not reported. Interestingly, however, NADH accelerates the reaction when added at t=0.

The effect of cofactors, etc., clearly demonstrates the importance of environmental conditions on regeneration and indicates the presence of intermediates in several cases. Further, it is apparent that in some cases the factor plays the role of initiation analogous to a nucleation site. It should be pointed out, however, that not all enzymes containing cofactors require them for rena-

turation. A notable example is staphylococcal nuclease, which requires Ca^{++} for activity. The refolding of this enzyme is little affected by the presence of Ca^{++} (136).

c. Multisubunit Proteins

In the previous section much was said about multisubunit enzymes, since many of the proteins requiring cofactors during regeneration are multisubunit. However, these proteins constitute a special case since folding must lead to proper intersubunit interaction as well as intrachain interactions. The present dissertation deals with a multisubunit enzyme, and some insight into this problem may be gained by examining other oligomeric proteins.

The regenerations of oligomeric proteins appear to exhibit one characteristic common to all. Intrachain folding precedes subunit association. This is a nontrivial conclusion, especially in the case of proteins with different subunits. The necessity of subunit interactions during chain folding would render the folding process more complex by increasing the number of conformations and interactions to be searched. An excellent example of chain folding preceding subunit association is the regneration of IgG which has both intrachain and interchain disulfide bonds. The regeneration of completely reduced and denatured rabbit IgG was first demonstrated by Freedman and Sela (137). However, due to the extreme insolubility of

the reduced, denatured protein in aqueous solution, it was necessary to attach polyalanine chains to many of the protein amino groups to increase the solubility. The undenatured, modified protein retained most of it antigenic and antibody properties (138). Optimal regeneration was found to occur when heavy (H) and light (L) chains were first allowed to reoxidize separately and then incubated together to form interchain disulfide bonds. This suggested to the authors that each chain normally folds before association and intermolecular disulfide formation.

The order of intermolecular disulfide formation was demonstrated by Petersen and Dorrington (117) using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) to observe the disulfide intermediates. The results suggest that the preferred pathway of covalent association is as follows:

$$L + H \rightarrow L - H \overset{H}{\rightarrow} L - H_2 \overset{L}{\rightarrow} L_2H_2$$

The evidence in support of this is that the major intermediates are L-H and LH_2, and LH_2 forms as LH disappears. These results have recently been supported and extended by Beychok and colleagues (139,140). They also found that LH and LH_2 were major intermediates; however, they pointed out that the probability of LH formation is only twice that of H_2 formation. The data indicate a non-random cooperative mechanism, but do not fit any obligatory scheme (140).

Subunit folding also appears to precede association of skeletal and heart muscle LDH. Using an ingenious quenching and electrophoretic technique to trap and separate intermediates, Tenenbaum-Bayer and Levitzki (141) showed that from the acid denatured state, the major intermediates in both enzymes were folded monomer, dimer and native tetramer. Very little trimer was found in either case. Kinetic data also support a similar mechanism (142).

Similarly, the regenerations of Saccharomyces carlsbergensis pyruvate kinase, rabbit muscle aldolase, and Bacillus megaterium glucose dehydrogenase indicate that subunit folding is followed by association (127,134,143).

d. Detection of Intermediates

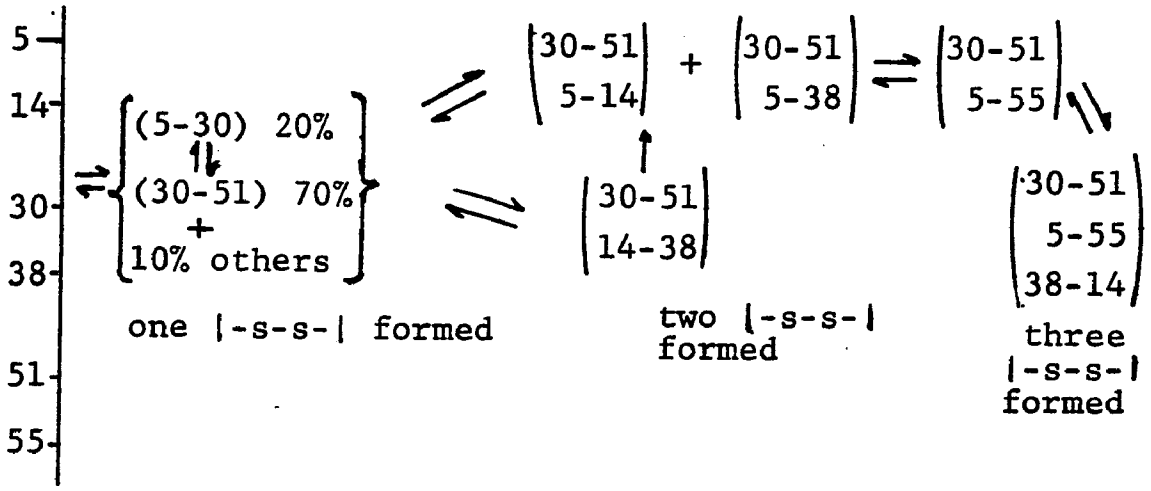
As stated above, the effects of cofactors on the regeneration of several proteins have inferred intermediates in the refolding process; thus, the strict two state behavior of protein folding is discounted. Similarly, Treipel and Koshland (130) observed the regeneration of a number of oligomeric enzymes by increases in intrinsic fluorescence upon dilution from GuHCl. They noted that the initial rate of fluorescence intensity increase was very rapid, and the half time of less than 30 seconds for this process was much faster than activity return. It was suggested that this indicated rapid gross structure formation followed by a slower adjustment to yield active

enzyme. A similar burst of structure formation, followed by a slower reactivation, has been observed for lysozyme (113), RNaseA (29), carbonic anhydrase (30), and staphylococcal nuclease (31).

A transient intermediate apparently observed in the high temperature refolding of lysozyme is the native enzyme (144). Regeneration of this enzyme at 60 to 90°C with the glutathione redox system shows a maximum of activity followed by subsequent decline. This inactivation is reversible when the temperature is returned to 37°C. The native enzyme under the same conditions is not thermodynamically stable; thus, the authors pointed out that this was in support of kinetic control of lysozyme folding at high temperature.

Disulfide intermediate trapping has also proven a very useful tool for observing the pathway of folding. Creighton (118,145-148) has thoroughly mapped the pathway of folding for BPTI, a single chain protein with 3 disulfide bonds making it a good model system. Covalent intermediates were trapped with iodoacetate or iodoacetamide, the chain was cleaved, and peptides were isolated and characterized to determine their disulfide pairing throughout the course of refolding. The results (shown below) clearly indicate a limited search mechanism with relatively few of the possible disulfide intermediates forming along the route.

Scheme 1:



The numbers in parentheses represent paired disulfides in one molecule; thus, $\binom{30\text{-}51}{14\text{-}38}$ means that there is an intermediate with 2 disulfides formed between residues 30 and 51, and 14 and 38. Interestingly, modification of residues 38 and 14 with iodoacetate blocks the formation of $\binom{30\text{-}51}{5\text{-}55}$ during refolding, and modification of either residue 38 or 14 individually allows $\binom{30\text{-}51}{5\text{-}55}$ to form but at one tenth the normal rate. Thus, it was concluded that a "wrong" two disulfide intermediate [$\binom{30\text{-}51}{5\text{-}14}$ and/or $\binom{30\text{-}51}{5\text{-}38}$] must form before the correct two disulfide intermediate can form (as shown in the scheme). Clearly, the pathway cannot be random.

Lysozyme contains 8 half cystines compared to 6 in BPTI. Thus, the disulfide pattern in the former is more complex. However, trapping experiments have led to a characterization of the early disulfide intermediates

along the pathway (149,150). The results show that a limited search mechanism is used, and that the early disulfide bonds are formed in one section of the chain, the linear sequence 62 through 96. Though it is possible that this represents a nucleating segment, it is certainly not proof thereof. The authors pointed out that nucleation could occur in a segment with no disulfide bonds (150). Nonetheless, it does indicate that this section of the protein obtains structure before the entire molecule.

Trapping experiments with RNaseA have indicated that the early stage of disulfide bond formation is random (121). Peptide mapping studies yielded no disulfide peptides after 5 minutes of regeneration when an average of 3 protein disulfides were formed. This result is expected for random disulfide pairing since all statistically possible disulfide peptides will be present at low concentrations; thus, none will be detectable. After 17 minutes it was found that the major disulfide peptides present were those of the native protein. Although the early steps clearly appear random, it is not clear if the later disulfide bonds, which are largely native, form randomly or are directed by kinetic steps. A conformational change detected by tyrosine fluorescence was observed to occur during refolding at a time intermediate between the observed random disulfide pairing and native disulfide pairing. One wonders if this conformational

change directs subsequent folding and disulfide pairing. Further characterization of this phenomenon would be of interest.

3. Folding within Domains

The ability of separate sections, or domains, of a protein molecule to fold independently of the remaining molecule would decrease structure searching time by shortening the effective chain length in any one domain. Several proteins have been shown to fold in this way. BSA appears to be composed of three domains (151,152), and Teale and Benjamin (119,153,154) have clearly demonstrated that each domain folds separately. When native structure was judged by recovery of antigenic determinants present in the native protein and not present in the unfolded protein, the results show that each domain obtains native structure at a different rate. Further, each domain folds independently of the others as demonstrated by cleaving the molecule between domains. Moreover, each fragment was shown to regain its antigenicity at rates faster than in the intact molecule (154). Thus, the domains do fold independently, but some interdomain interactions would appear to occur in the native molecule which decrease the rate of folding. Interestingly, the carboxy terminal 1/3 of each domain regains structure faster than the amino terminal 2/3 (154).

The β-chain of Escherichia coli tryptophan synthetase appears to fold as 2 separate domains (155). The enzyme can be cleaved between these domains and the fragments separated in 6M urea. When denaturant is removed each fragment appears to assume the conformation it had in the "nicked" protein; thus, the sum of the circular dichroism (CD) spectra of the separated, refolded fragments is virtually the same as the spectrum of the "nicked" native protein.

Chavez and Scheraga (156) have shown that the carboxy terminal 1/3 of RNaseA obtains native antigenic determinants before the amino terminal 2/3. These authors suggested that the rapidly folding region may be an independently forming nucleation region. The validity of this hypothesis remains to be proven. However, it is interesting that removal of a 20 amino acid peptide from the amino terminal end of RNaseA allows the return of a small amount of enzyme activity from the reduced denatured fragment (157), but reoxidation of fragment 1-120 (minus a 4 amino acid peptide from the carboxy terminal end of the intact enzyme) causes the chain to assume a random structure (158). Further, electron spin resonance (ESR) spin label studies indicate that the amino terminal end of the enzyme has a lower thermal unfolding temperature than does the carboxy terminal end (159).

Similarly lysozyme would appear to possess an independently folding region. A fragment of this enzyme, 13-105, with a 12 amino acid peptide removed from the amino terminal end and a 24 amino acid peptide removed from the carboxy terminal end has the ability to attain native structure as judged by inhibitor binding, antigenic determinant return, and correct disulfide pairing (160). Further, this fragment possesses two of the four disulfides of the native protein, and these are the same residues involved in the early disulfide pairing of the intact molecule (150). Thus, a fragment of lysozyme which folds independent of the rest of the chain, possesses a segment which obtains structure early in the folding scheme of the intact molecule.

Clearly, protein folding studies have progressed considerably over the past twenty years since Anfinsen's group first demonstrated the ability of the protein amino acid sequence to determine the three dimensional structure. Presently the emphasis in this field is on how this structure is determined from the sequence. The above discussion has introduced several techniques that have been adopted in this monumental problem. In the present study, a slightly different approach has been taken. We have recognized some of the structural similarities and differences between two RNases, RNaseA and seminal RNase, and hope to gain some insight into their folding mechan-

isms by comparing and contrasting their folding kinetics and products.

IV. Choice of Proteins

RNaseA is synthesized in the pancreas (where it is isolated) and secreted into the intestines as a digestive enzyme. This protein possesses one polypeptide chain of 124 amino acids with well characterized sequence and crystal structure (161). As discussed above, much is known about its refolding process.

Bovine seminal RNase is synthesized in the seminal vesicles and secreted into the semen where it is isolated (162,163). However, the physiological role of this protein remains elusive (163). This protein is composed of two identical polypeptide chains of 124 amino acids each and with an amino acid sequence entirely homologous with RNaseA (80% of the amino acids are conserved) (164,165). The individual subunits are held together by two intermolecular disulfide bonds. The residues responsible for these interchain bonds are 31 and 32, which are lysine and serine, respectively, in RNaseA (161,164). The remaining eight half cystines of the seminal RNase subunits are in the same positions and form the same intrachain disulfide bonds as those in RNaseA (161,165). Thus, seminal RNase has ten half cystines per chain compared to eight in RNaseA, which leads to more potential random disulfides in seminal RNase per chain than RNase A, 945 compared to

105. If folding is completely random, and a process dependent upon disulfide exchange is rate limiting, as has been suggested (2,113), then the half time for seminal RNase regeneration should be 9 times as slow as RNaseA!

Other differences between the seminal RNase and RNaseA chains are 14 lysine residues in the former and 10 in the latter, 5 prolines in the former and 4 in the latter, and 4 tyrosines in the former with 5 in the latter (161,165). Interestingly, hydrophobicity is virtually identical between the two proteins, and the largest changes are seen in the hydrogen bonding groups (161,165).

The mechanism of catalysis of each protein is similar and all active site residues are conserved (161,163,165). However, seminal RNase is a more basic protein than RNaseA, having an isoelectric pH of 10.3 compared to 9.5 for RNaseA (163). This presumably plays an important role in its ability to degrade double stranded RNA and RNA:DNA hybrids while RNaseA can not (166-169).

Thus, the many similarities between the two proteins and the obvious differences make the comparison of the regenerations of these two proteins quite instructive.

This enzyme was mainly attractive for the above reasons; however, this is also the first enzymatic protein with intermolecular disulfide bonds to be shown to refold. The only other protein with intermolecular disulfide bonds shown to refold was IgG (137). As dis-

cussed above, IgG required as many as 800 alanine residues attached to the protein before regeneration was accomplished, and this is clearly not a physiological condition.

As mentioned earlier, there are three systems available for the regeneration of disulfide containing proteins: air oxidation, enzymatic regeneration, and the glutathione redox system (98,106,113). We have chosen the latter of these, the glutathione system, for our regeneration studies for several reasons. First, it is the simplest system that gives rapid and reliable kinetics. Second, it uses glutathione concentrations normally found in cells. Third, Dr. S. W. Schaffer has thoroughly characterized RNaseA folding with this system (9,122, 170); thus, a direct comparison can be made.

The immediate goal of this work was fourfold: (1) to determine optimal conditions for glutathione regeneration of seminal RNase; (2) to determine if the monomer folds before subunit association takes place; (3) to characterize the products of regeneration under optimal conditions; and (4) to compare and contrast this regeneration with RNaseA. These goals have all been realized.

Materials:

Bovine seminal RNase was prepared by the method of D'Alessio, et al. (163). Bovine pancreatic RNaseA (5X crystallized) type 1-A (Lot No. 115C-008) was purchased from Sigma Chemical Co., St. Louis, Missouri. Crystalline catalase was a product of Mann Research Laboratories, New York, N.Y. (Lot No. A6715). Hemoglobin substrate powder was purchased from Worthington Biochemical Corp., Freehold, N.J. Yeast RNA was purchased from Boehringer Mannheim (control No. 7305317), thoroughly dialized against 0.1M $NH_3 \cdot H_2O$ and lyophilized.

5'-Uridinetriphosphate-hexane-agarose (5'-UTP-agarose) was purchased from P. L. Biochemicals, Milwaukee, Wisconsin (Lot. No. 455131), and stored at -20°C in 50% glycerol until used. In this form it was stable for months. However, stability was less than one month at 4°C, pH 5.3. The suppliers have informed us that alkaline pH causes hydrolysis of the 5'-UTP-hexane linkage, and the present results tend to agree with this.

2-Mercaptoethanol, iodoacetic acid, iodacetamide, N-ethylmaleimide, dithiothreitol, reduced and oxidized glutathione were purchased from Sigma Chemical Co., St. Louis, Missouri. Electrophoresis reagents were products of Bio Rad Laboratories, Rockville Centre, N.Y. Ethyleneimine was a product of Columbia Organic Chemicals, Inc.,

Columbia, S.C. Piperazine was purchased from Aldrich Chemical Co., Metuchen, N.J. Ultra pure tris(hydroxymethyl)aminomethane and urea were gifts of Chemzymes, Inc.

All Sephadex gels were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Cellex-P ion exchange resin was purchased from Bio Rad Laboratories, Rockville Centre, N.Y.

Maleimides M-1, M-2, and M-3 in Figure 4 were generous gifts of Dr. D. B. Wetlaufer.

Methods

I. Purification of Bovine Seminal Ribonuclease

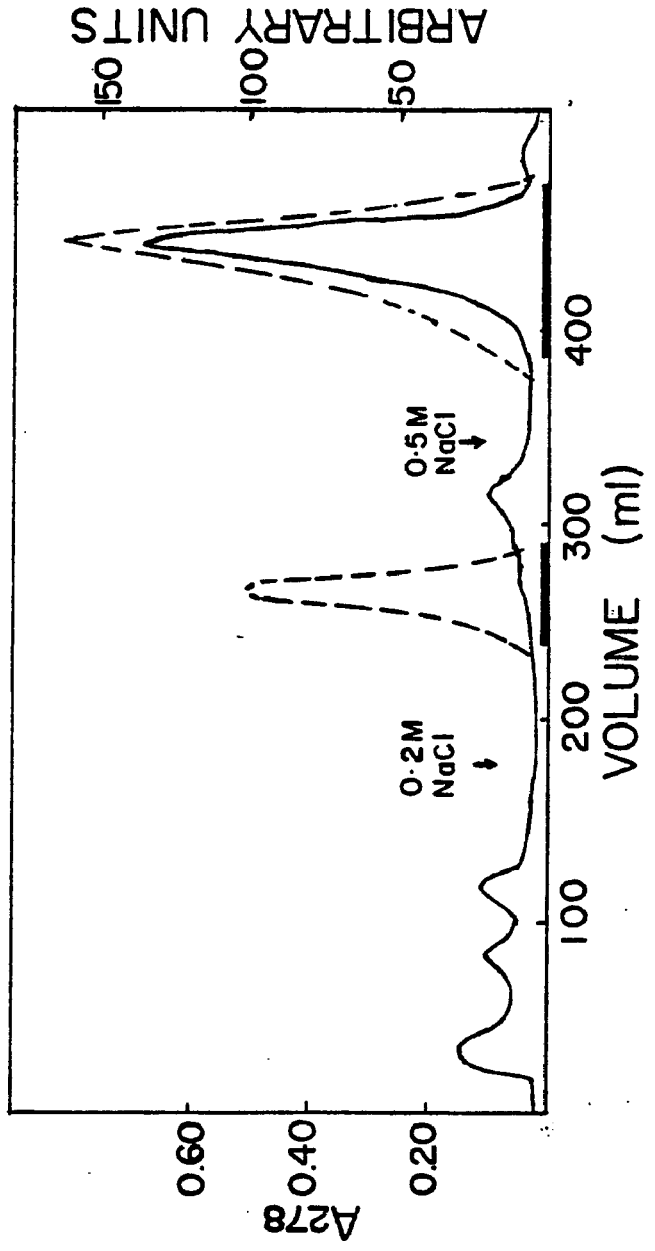
The seminal RNase purification procedure, with minor modifications, was according to D'Alessio, et al. (163). A typical procedure went as follows: frozen bull semen was thawed and the cells removed by centrifugation at 11,000 RPM for 20 minutes. The seminal plasma was then freeze dried and subsequently dissolved to 50 mg/ml by weight in water. The pH was adjusted to 3.5 with 1M H_2SO_4, and a precipitate formed. The solution was stored at -2°C over night, after which the precipitate was removed by centrifugation at 11,000 RPM for 20 minutes. All subsequent centrifugations used the same conditions.

The next step in the purification procedure was heat treatment. The solution was separated into two equal parts and swirled in Erlenmeyer flasks (3:1 ratio of Erlenmeyer size to solution volume) at 60°C for 2 minutes. The solutions were then moved to a 100°C water bath where they were swirled for 5 minutes. A precipitate was formed during these two heating steps and increased when the pH was adjusted to 5.0 with 1M NaOH. The precipitate was discarded following centrifugation.

Ammonium sulfate fractionation was the third step. Solid ammonium sulfate (390 g/l) was added to the supernatant from above and stirred at 4°C for 1.5 hours. A precipitate which formed was centrifuged out. The super-

Figure 1. SP Sephadex chromatography of seminal RNase. Crude seminal RNase (80 mg) was added to the (60 x 1.5 cm) column in 20 ml of 5mM tris-Cl, 50mM NaCl, pH 8.0 and eluted with the same buffer at 4°C. Where indicated by arrows, the buffer was changed to 5mM tris-Cl containing 0.2M NaCl or 0.5M NaCl, respectively. The active fractions eluting after addition of 0.2M NaCl and 0.5M NaCl were labeled RNase BS-2 and seminal RNase, respectively, and pooled as shown by black bars. Flow rate was 1 drop/ 12 seconds, and 3 ml fractions were collected. Elution was followed by A_{278} (————) and RNase activity (-------).

FIGURE 1

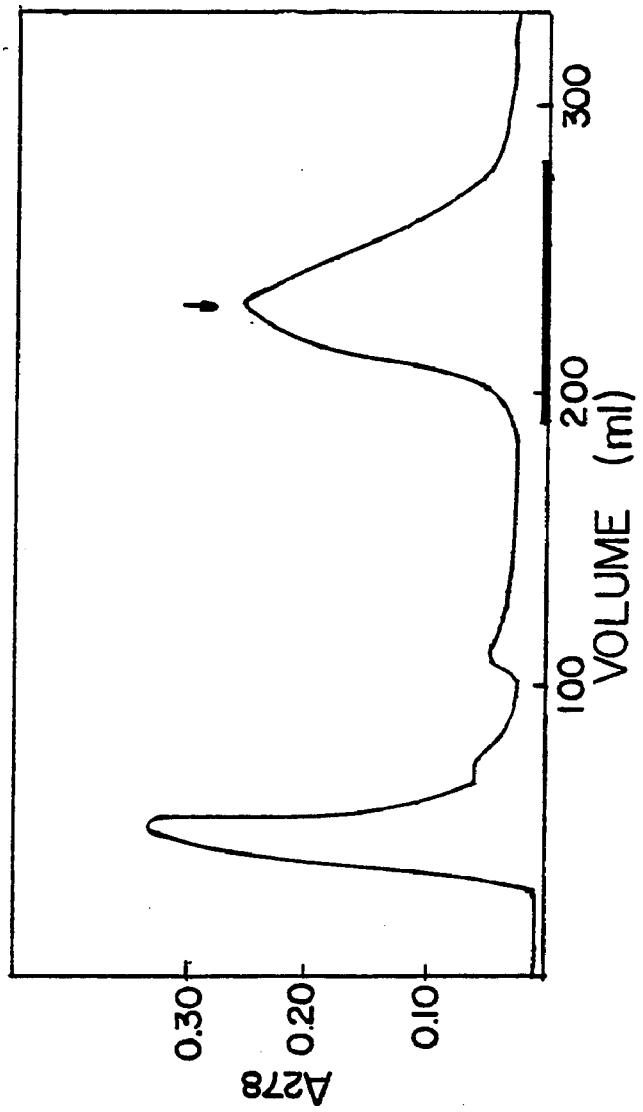

ARBITRARY UNITS
150
100
50
0·5M NaCl
0·2M NaCl
100
200
300
400
VOLUME (ml)
A278
0.60
0.40
0.20

natant was saved and the precipitate was redissolved to the original volume in water. Solid Ammonium sulfate (390 g/l) was added to the redissolved precipitate and stirred for 1.5 hours. The precipitate was centrifuged out and discarded. The two ammonium sulfate supernatants were combined, 230 g/l of ammonium sulfate added, and stirred overnight. The resulting precipitate was recovered by centrifugation, and the supernatant was discarded. The precipitate was dissolved in a small volume of water, dialized against several changes of distilled water, and freeze dried.

The initial column chromatography step employed discontinuous ion exchange chromatography on SP-Sephadex. Crude enzyme was dissolved in a small volume of 5mM tris, 0.05 M NaCl, pH 8.0 and added to a column (1.5 x 60 cm) of SP-Sephadex C-50 equilibrated with the same buffer. Approximately 60 fractions of 3 ml each were collected at a rate of 13 sec/drop. The elution buffer was switched to 5mM tris, 0.2M NaCl, pH 8.0, and another 60 fractions were collected. Finally, the elution buffer was switched to 5mM tris, 0.5M NaCl, pH 8.0, and about 100 fractions were collected. Figure 1 shows the Sephadex elution pattern. The major peak of RNase activity was pooled as shown and dialyzed against several changes of distilled water. The solution was then freeze dried.

Figure 2. Cellex-P chromatography of seminal RNase. Partially purified seminal RNase was added to the (20 x 1.3 cm) Cellex-P column in 18 ml of 0.3M ammonium formate, pH 6.5. The column elution was begun with a linear gradient of 0.3 to 1M ammonium formate, pH 6.5 at 4°C. At the arrow, 1M ammonium formate was added to finish elution. Enzyme was pooled as shown by black bar. Flow rate was 1 drop/7 seconds, and 2 ml fractions were collected.

FIGURE 2



The final purification step employed Cellex-P chromatography. The enzyme was dissolved in water, and dialyzed against 0.3M ammonium formate, pH 6.4. The resulting solution was then added to a Cellex-P column in the sodium form (20 x 1.5 cm), equilibrated with the same buffer, and eluted with a linear gradient of 0.3 to 1M ammonium formate, pH 6.4 (100 ml of each solution). 2 ml fractions were collected. When the gradient had finished an additional 100 ml of 1.0M ammonium formate was eluted through the column. Elution pattern is shown in Figure 2. The active fractions were pooled as shown, dialyzed against several changes of water, and freeze dried. The yield was typically 0.5 to 0.75 mg seminal RNase per ml of semen.

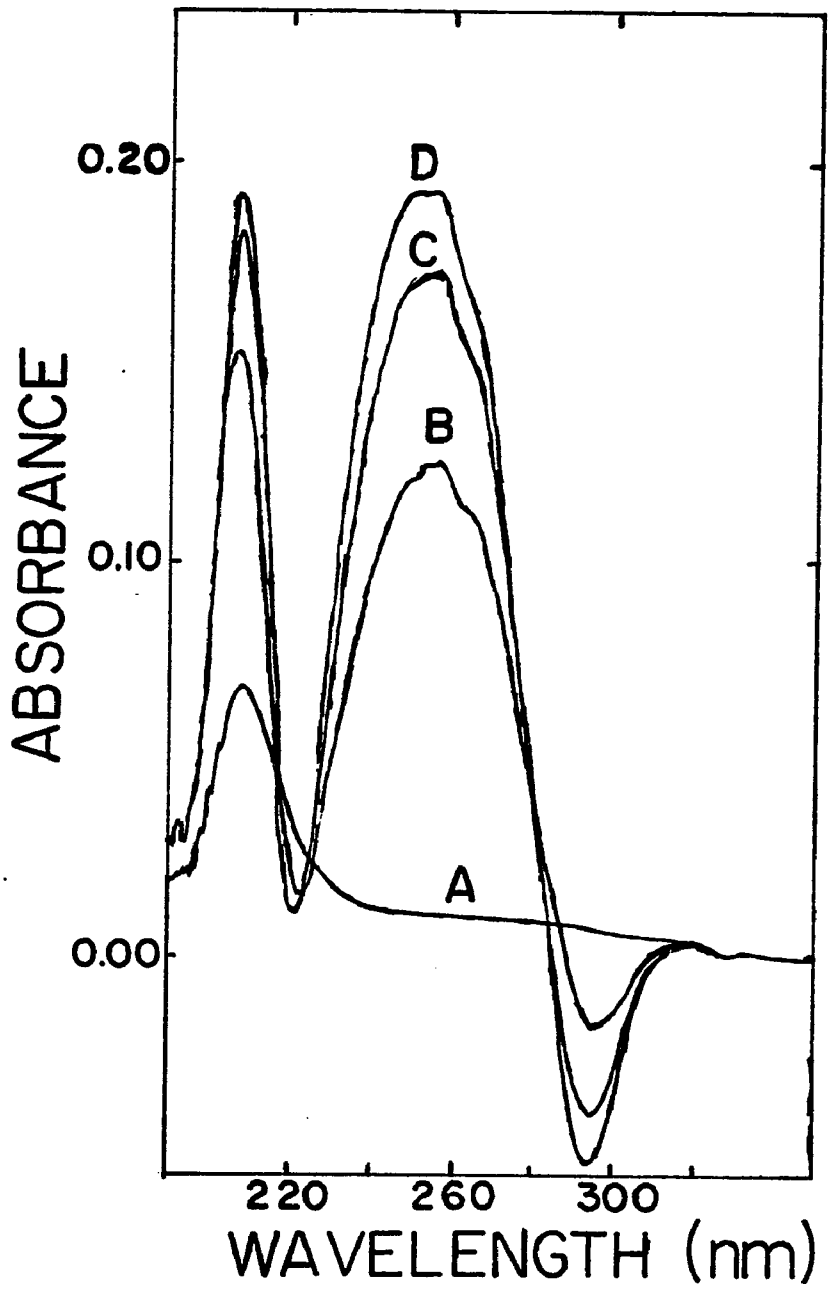
The enzyme was used without further handling unless otherwise stated.

II. Assays

A. Ribonuclease Assay. RNase activity was determined using a modification of the continuously recording method of Fletcher and Hash (171). It was carried out at 30°C in a thermostatted Perkin-Elmer Hitachi 200 UV-Vis spectrophotometer. The assay was typically run by adding 0.020 ml of RNase solution to 3 ml of 0.075 mg/ml RNA in 0.1M sodium acetate buffer, pH 5.0, in a quartz spectrophotometer cell. The change in absorbance at 260 nm as a function of time was recorded

Figure 3. Difference spectra for yeast RNA and hydrolysis products. RNA (0.075 mg/ml) in 0.1M sodium acetate was placed in reference and samples cells of Perkin-Elmer Hitachi 200 UV-Vis Spectrometer at 30°C; the resultant UV spectrum served as the baseline, A. RNAseA (0.020 ml of 0.1 mg/ml) was added to the sample cell, and spectra were observed at 10 minutes, B; 1 hour, C; and 3.5 hours, D.

FIGURE 3



with full scale at 0.05 and chart speed at 20 sec./cm; a difference spectrum for RNA and products of hydrolysis is shown in Figure 3. 260 nm was chosen as a compromise between a large increase in absorbance on hydrolysis and a small background absorbance. The reaction rate employed was the initial velocity. Arbitrary units were calculated as

$$1 \text{ unit} = 5 \times 10^{-4} \Delta A/20 \text{ sec.}$$

This assay was chosen for its relative simplicity, sensitivity, and the small amount of enzyme required as compared to the precipitation assay of Kalnitsky, et al. (172). The useful range for RNaseA and seminal RNase were found to be 0.005 to 0.065 and 0.015 to 0.29 mg/ml, respectively.

Protein concentration was determined sprectrophotometrically at 278 nm, $E^{1\%}_{1cm} = 4.65$ (163).

B. Sulfhydryl Content. Free sulfhydryls were assayed according to the method of Ellman (173) using 5,5'-dithiobis-(2-nitrobenzoic acid) (NBs_2), $E_{412} = 1.36 \times 10^4 \ M^{-1}Cm^{-1}$. NBs_2 was dissolved in 0.05M sodium acetate buffer, pH 5.0, at 1 mg/ml; 0.2 ml of this solution was added to 3.8 ml 0.2M tris-Cl, 10M urea, pH 8.6. To this solution 1 ml of enzyme solution was added, and after 10 minutes at room temperature, A_{412} was read.

C. Catalase Assay. This activity was determined spectrophotometrically by following the disappearance of H_2O_2 at 240 nm, pH 7.0, 30°C (174).

III. Regeneration Studies

A. RNase Reduction. Reduction of seminal RNase and RNaseA were carried out by a modification of the method of Anfinsen, et al. (96), in which 2 to 3 mg of native enzyme were incubated in 2.0 ml of 8.0M urea, 0.01M tris-Cl, 0.01M EDTA, and 500 fold excess of 2-mercaptoethanol (2ME), pH 8.5. The reduction was allowed to proceed for 3.5 hrs. and was stopped by decreasing the pH to approximately 3.5 with 0.1M acetic acid. The reduced, denatured protein was desalted on a G-25 Sephadex column (40 to 60 x 1.5 cm) which was preequilibrated with 0.1M acetic acid. Following elution, RNaseA was freeze dried and stored at -20°C for up to 6 months with no loss of thiols. Seminal RNase, on the other hand, was used immediately or freeze dried and used the next day; longer periods of storage at -20°C resulted in loss of thiol content and led to altered regeneration kinetics.

B. RNase Regeneration. The regeneration was a modification of the method of Ahmed, et al. (99). Approximately 0.5 to 3 mg reduced RNase were dissolved in 3.0 to 10.0 ml of distilled water and the concentration determined spectrophotometrically. The regeneration

began by pipetting 1.5 ml of the reduced protein into 3.5 ml of 0.1M tris-acetate, 0.001M EDTA (except in the air oxidation experiments where the EDTA was omitted), pH 8.2 buffer containing the desired concentration of reduced and oxidized glutathione and equilibrated at the desired temperature. Glutathione was always added just prior to the addition of the protein to the reaction mixture. The regeneration kinetics were followed by removing 0.020 ml aliquots at the desired times for assay as described above.

C. Preparative Reduction-Regeneration. A typical preparation went as follows: 5 to 15 mg of native RNase were reduced-denatured in 2 to 5 ml of 0.1M tris-Cl, 8M urea, 1 mM EDTA and a 500 fold excess of 2-Me, pH 8.5 as described above. The reduced protein was desalted on G-25 Sephadex, 0.1M acetic acid. For regeneration tris and EDTA were added to the desalted protein to give a final concentration of 0.1M and 1mM, respectively, and the pH was adjusted to 8.2 with 1M NaOH. (In the air regenerations, EDTA was omitted.) Regeneration began with addition of solid glutathione. The protein concentration varied from 0.1 to 0.3 mg/ml. The reaction was allowed to proceed overnight (15 to 20 hrs.).

Preparative air regeneration was performed in a 10 x 1.4 cm test tube at 25°C for 24 to 30 hrs.

IV. Affinity Chromatography on 5'-UTP-agarose

A. General Methods. Column sizes varied from 1 x 0.5 to 3 x 0.5 cm. A typical experiment, modified from the method of Stewart and Stevenson (175), is described as follows: a few mg of RNaseA and/or catalase in 3 to 10 ml of 0.025M piperazine-HCl, pH 5.3, were applied to the column equilibrated with the same buffer at 4°C. Following application of the sample, 10 to 20 ml of the piperazine buffer solution were passed through to remove any unbound material. Finally, RNase was eluted from the column with 0.25M sodium phosphate buffer at pH 3.0 or 5.45. 1 ml fractions were collected, and elution was followed by protein absorbance and enzymatic activity.

B. Competitive Elution Experiments on 5'-UTP-Agarose. In these experiments, 0.7 mg of RNaseA in 0.050 ml of 0.025M piperazine-HCl, pH 5.3, were added to a 4.9 x 0.5 cm 5'-UTP-agarose column equilibrated with an identical buffer containing varying concentrations of 2'(3')uridine monophosphate (2'(3')-UMP). The enzyme was eluted with the piperazine-HCl buffer containing UMP at 4°C and collected in 0.45 ml (10 drops) fractions at a rate of 13 drops per min.

V. Reformation of Intermolecular Disulfide Bonds from Regenerated and Selectively Reduced Monomers

Either affinity chromatography pure regenerated or selectively reduced monomer was incubated for 24 hours in 0.1M tris-acetate, 1mM EDTA, pH 8.2, containing various concentrations of reduced and oxidized glutathione. At specified times, the reaction was stopped with a ten fold excess of either iodoacetamide or N-ethylmaleimide (NEM). The products were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the absence of 2-ME.

VI. Selective Reduction of Native Seminal RNAse with Glutathione

Native seminal RNase, at a concentration of 0.1 mg/ml, was incubated for 24 hours in 0.1M tris-acetate, 1mM EDTA, pH 8.2 buffer containing the desired glutathione concentrations at 30°C. The modified enzyme was concentrated in 0.1M acetic acid or 0.05M tris-Cl, 1mM EDTA, pH 7.2 on a YM-10 Amicon diaflow membrane, and applied to a Sephadex G-75 column (85 to 120 x 1.5 cm), which was equilibrated with the same buffer. Protein elution was followed by absorbance at 225 nm and enzymatic activity. The protein peaks were pooled and reconcentrated on the YM-10 diaflow membrane.

VII. Product Characterization.

A. Gel Filtration. The reaction mixtures were concentrated on a UM-10 or YM-10 diaflow membrane and passed through a Sephadex G-75 column (85 to 120 x 1.5 cm). Elution from the column was followed by absorbance at 225 nm and enzymatic activity; void volume was established with blue dextran. Two ml fractions were collected. The first protein peak was pooled and stored in either the freeze dried state or at 4°C. The second peak, following concentration by ultrafiltration, was routinely stored at 4°C to avoid formation of protein aggregates during the freeze drying step.

B. SDS Polyacrylamide Electrophoresis. SDS-PAGE was performed by a modification of the method of Weber and Osborn (176). Free thiols were blocked with a 10 fold excess of NEM (over total thiol concentration) at 25°C for 15 minutes in the pH range 7.0 to 8.2. If glutathione was previously removed, blocking was omitted. The protein was denatured by mixing 0.10 ml of 0.05 to 1 mg/ml protein with 0.10 ml of SDS dialysis buffer, which contained 0.01M sodium phosphate, 0.1% SDS at pH 7.0. This solution was incubated for approximately 2 hours at 37°C. Fifty to one hundred microliters of the solution were then added to 0.005 ml tracking dye (0.05% bromphenol blue in water), 1 drop of glycerol, and 0.050 ml of dialysis buffer. 2-ME was omitted from

the denaturation medium to prevent reduction of the intermolecular disulfide bonds.

Ten percent SDS-polyacrylamide gel solution was prepared by mixing 13.5 ml of acrylamide solution (22.2% acrylamide and 0.6% methylene bis acrylamide in water), 15 ml gel buffer (7.8 gm $NaH_2PO_4 \cdot H_2O$, 20.5 gm Na_2HPO_4, and 2 gm SDS per liter), 1.5 ml ammonium persulfate solution (15 mg/ml prepared fresh), and 0.050 ml TEMED (N,N,N',N'-tetramethyl ethylenediamine). This solution was added to each of 12 gel tubes, which were closed off at the bottom with rubber stoppers, to within 1 cm of the top, and covered with a thin layer of water. Polymerization at room temperature required about 1 hour, after which the rubber stoppers were removed and the water was pipetted off.

The upper and lower electrophoresis chambers were filled with 1:2 dilution of the gel buffer. Ten to one hundred microliters of the SDS denatured protein were layered on top of the gels through the top chamber buffer. Electrodes were connected to each chamber, anode in the lower, and a current of 8 ma/gel was applied. Electrophoresis was carried out until bromphenol blue band was approximately 1 cm from the gel bottom, usually requiring 3 to 4 hours.

After removal from the tubes, the gels were stained in 10 ml of coomassie blue stain (1.25 gm coomassie blue,

454 ml of 50% methanol, and 46 ml of glacial acetic acid) for 2 hours. Destaining was carried out in a solution containing 7.5% acetic acid, 5.0% methanol, and 87.5% water.

Densitometer scans of the destained gels were carried out on an ISCO densitometer. Integration of the peaks was performed by cutting out the peaks and weighing them.

C. Reisfeld Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis of basic proteins was performed by a modification of the method of Reisfeld et al. (177). To 0.1 ml of protein solution (0.1 mg/ml) was added 0.005 ml tracking dye (0.4% solution of methylene blue in water) and one drop of glycerol.

Gel tubes were filled with 7.5% acrylamide gel solution containing 2.5 ml of solution A (48 ml 1M KOH, 17.2 ml glacial acetic acid, 4.0 ml TEMED, adjusted to 100 ml with water) 2.5 ml solution B (60 gm acrylamide, 0.4 gm methylene bisacrylamide, adjusted to 100 ml with water), 5 ml water, 10 ml ammonium persulfate solution (0.28% in water). The gels polymerized in approximately 1 hour.

Electrophoresis buffer (31.2 gm β-alanine, 8.0 ml glacial acetic acid, and water to 1000 ml) was added to upper and lower chambers. The protein solution (0.01 to 0.1 ml) prepared above was layered onto the gels

through the upper chamber buffer. Electrodes were connected to each chamber, cathode in lower, and a 2 ma/gel current was applied. After 5 minutes this was increased to 6 ma/gel. Electrophoresis was carried out until the methylene blue band was within 1 cm of the gel bottom, usually 1-1.5 hours.

Staining and destaining were as described under SDS-PAGE.

D. Amino Acid Analysis. Hydrolysis was carried out on affinity chromatography pure protein by the method of Moore and Stein (178). 0.6 to 1.6 mg of protein were either desalted on G-25 Sephadex equilibrated with 0.1M acetic acid or by several ultrafiltration washings with water, and freeze dried in a 25 ml drying ampule. One ml of 6 N HCl was added to the freeze drying ampule to dissolve the protein. The solution was frozen with liquid N_2, evacuated to 50 microns, thawed under vacuum slowly to allow O_2 to bubble out, and then reevacuated to 50 microns. The tube was sealed with a propane torch and placed in a $110^{\circ}C \pm 1^{\circ}C$ oven for 24 hours. The tube was broken open and the HCl pulled off at $40^{\circ}C$ with a water aspirator. Hydrolysis products were dissolved in 0.5 ml water, and 0.5 ml of 0.2M sodium phosphate, pH 6.5 was added to oxidize cysteine to cystine. The solution was allowed to stand for 4 hours at room temperature. Thirty microliters of 1 N HCl were

added to decrease the pH, and finally, enough pH 2.2, 0.1 N citric acid-HCl, 0.5% thiodiglycol was added to bring the final volume to 3 ml. Alternatively, the hydrolysis products were dissolved directly in the analyzer buffer. No difference in results was noted between the two procedures.

Amino acid analysis was performed according to Spackman (179) on a Beckman model 120 amino acid analyzer. Only the long column amino acids were examined. Integration of peaks was accomplished by the following technique.

1. Baseline of the peak was determined.

2. The height of the peak was determined at its center.

3. The baseline was subtracted from the peak height (yielding the net height).

4. The net height was divided by two and the result was added back to the baseline to give the half height.

5. All the black dots above the half height, except the first, were counted, and the result was multiplied by 4.

6. The red dots between the half height and first and last black dots were counted, and the distance between the half height and the first and last red dots was estimated as tenths. The result, added to the result

of #5, was taken as the peak width.

7. The peak area was determined by multiplying net height times width.

8. When two peaks overlapped, only the half that reached the baseline was used to count the dots, and the results was multiplied by two.

9. The expected number of amino acids per chain was divided by the area. The average of all "amino acids per chain divided by area," excluding those that were clearly out of the average, was determined. The average times the area, including those not used in the average, was then taken as the number of each amino acid found per chain.

E. Circular Dichroism. The regeneration and selective reduction products were fractionated by G-75 Sephadex as described above. The separate protein peaks were then purified of inactive material by 5' UTP agarose affinity chromatography. The purified RNase was then washed several times on a UM or YM-10 diaflow membrane with water and once with 0.05M $KClO_4$, pH 3.9. The native enzyme was also subjected to affinity chromatography prior to analysis to ensure homogeneity.

Circular dichroism (CD) measurements were performed on a Durrum-Jasco J-10 circular dichrometer which was calibrated according to Cassim and Yang (180). The measurements were routinely taken at an instrument

sensitivity of 2×10^{-4} $\Delta A/cm$ and a path length of 0.05 or 0.1 cm. Molar elipticities were calculated according to the equation (42):

$$[\theta] = 2.303\ (4500/\pi)\Delta\varepsilon = 3300\ \Delta\varepsilon$$

where $\Delta\varepsilon$ = the difference in the molar absorptivities of the left and right circularly polarized light. If θ is determined on the basis of mean residue weight, which in this case is 118, and the instrument constants are included, the working equation becomes:

$$[\theta]_\lambda = \frac{3300(118\ gm/mole)(2 \times 10^{-4} \Delta A/cm)(X)(94/99)}{(Protein\ conc.,\ mg/ml)(path\ length, cm)\ 3.3}$$

where "X" is the instrument deflection (in cm) at each wavelength, and 94/99 and 3.3 are instrument constants.

VIII. Modification of Cysteines 31 and 32

A. Selective Reduction with Dithiothreitol. Seminal RNase was selectively reduced with dithiothreitol (DTT) at cysteine 31 and 32 according to the method of D'Alessio, et al. (181). Seven mg of seminal RNase were dissolved in 1 ml of 0.1M tris-Cl, 2.5mM DTT, pH 8.5 and allowed to react for 15 minutes at room temperature.

B. Modification with Maleimides, Iodacetate, or Iodacetamide. After selective reduction with DTT, the pH of the reaction was adjusted to 7.0 with 1M HCl.

Modification of the free cysteine 31 and 32 with the maleimides was performed with a 10 fold excess of reagent over total thiol. However, the iodoacetamide and iodo-

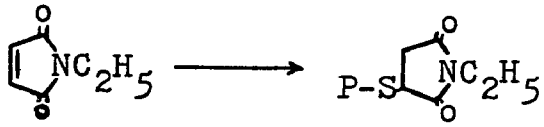
Figure 4. Thiol modifying agents and products of reaction. A, iodoacetate; B, iodoacetamide; C, N-ethylmaleimide (NEM); D, M-1; E, M-2; F, M-3; G, ethyleneimine.

FIGURE 4

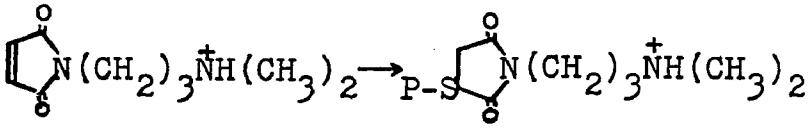
A.

B.

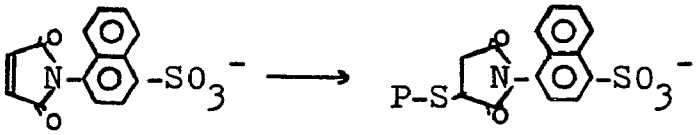
C.



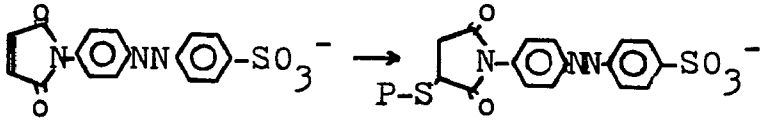
D.



E.



F.



G.

acetic acid modifications were carried out in the presence of 5mM 2'(3') UMP to protect the active site histidines and with a 5 fold excess of modifying reagent. In all cases the reaction was allowed to proceed for 5 to 10 minutes. Typically, the products were then desalted on G-25 Sephadex (40 x 1.5 cm) in 0.1M acetic acid and freeze dried. Subsequently, the typical reduction-denaturation and regeneration procedures were followed. However, in the case of reagents of M-2 and M-3 (see Figure 4), the protein precipitated upon addition of the reagent. Thus, 0.005 ml of 2-ME were added and allowed to react with the excess modification reagent for 5 minutes, then solid urea was added to achieve a final concentration of 8M to solubilize the protein. Two ml of the typical reduction-denaturation medium were then added. The modified protein was reduced and denatured as described above, and was then used for regeneration studies.

C. Modification with Ethyleneimine. The modification method used was according to Parente, et al. (182). Native enzyme (1.5 ml of 0.25mM) was selectively reduced with a 10 fold molar excess of DTT as described above; however, the buffer for the reduction was changed to 2.5M tris-Cl, pH 8.6. At the end of 15 minutes, 0.005 ml of ethyleneimine was added and allowed to react for 10 minutes. The ethyleneimine was added and incubated three

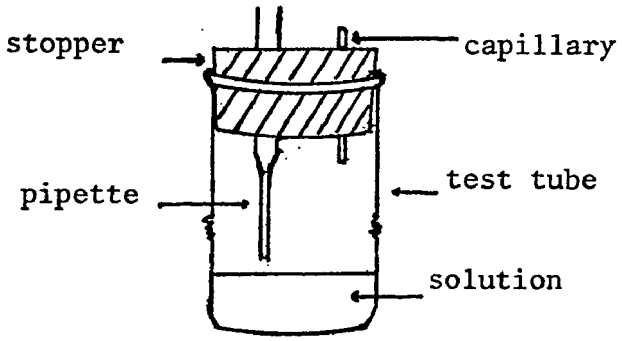
more times, and the products were desalted on a G-25 Sephadex column (40 x 1.5 cm) equilibrated with 0.1M tris-acetate, pH 8.2. (This high pH is necessary to prevent methionine modification (183,184)). If the enzyme was to be used for degeneration studies, it was incubated in 3M urea for 2 hours to ensure dissociation (182), and the urea was then dialyzed out against 0.1M sodium-acetate, pH 5.0. If it was used for regeneration studies, the tris-acetate (G-25 Sephadex) buffer was dialyzed out against water, and the enzyme was freeze dried. Denaturation with 8M urea and 2 ME was as described above.

When using the modified RNase, protein concentration was determined by the method of Lowry (185) since several of the modifying reagents had a significant absorbance at 278 nm. None of them were found to interfere with the Lowry assay.

IX. Degeneration Studies

Degeneration of native and ethyleneimine modified seminal RNase were performed at approximately 0.1 mg/ml, and the degeneration of RNaseA was performed at approximately 0.05 mg/ml. The enzyme was dissolved in 0.1M tris-acetate, 1mM EDTA. (In the case of modified seminal RNase solid tris and EDTA were added to the solution of 0.1M sodium acetate after dialysis and the volume was adjusted with 0.1M tris-acetate, 1mM EDTA.) GSH and

GSSG were added to give final concentrations of 3mM and 0.3mM, respectively. Final pH was always adjusted to 8.2. Ten ml of solution was typically used and the reaction was performed in a 20 x 2.5 cm test tube. N_2 was bubbled through the solution at room temperature for 15 to 30 minutes through a Pasteur pipette attached to the N_2 tank. Then the pipette was taken out of the solution (to avoid bubble denaturation), secured above the latter, and N_2 passed over it at 42°C. The apparatus is shown below.



N_2 was allowed to escape through a (2mm ID) capillary tube; thus, the reaction could be carried out at 1 atmosphere. (If the escape tube was blocked off, a 1 psi pressure increase changed the results toward more denatured.) Under the above condition only about 10 to 15% of initial thiols were oxidized; thus, the GSSG concentration did not rise above 0.6mM, well within the optimal regeneration range (see results, page 104).

At desired times the stopper was removed and 0.020 ml of reaction mixture was assayed as usual.

X. Determination if Dimer is Purification Artifact

Ten ml of seminal plasma was adjusted to pH 6.5 and solid NEM was added to a final concentration of 0.1M. The solution was allowed to react at room temperature for 15 minutes and at 4°C for 15 minutes. The solution was desalted at 4°C on G-25 Sephadex (70 x 1.5 cm) equilibrated with 0.05M sodium phosphate, 0.85% NaCl, 1mM EDTA, pH 7.4. The pH of the eluting protein fraction was adjusted to 3.5 with 1M H_2SO_4, and subsequently seminal RNase was purified as described above except for one minor change. All solutions prior to the SP-Sephadex chromatography step contained 1mM EDTA.

XI. Search for Dimerization Factor in Vivo

A. In Seminal Plasma. Sperm cells were removed from 2 ml of semen by centrifugation. The seminal plasma was mixed with 2 ml of 1 mg/ml refolded monomer and incubated at room temperature for 1 hour. The solution was then chromatographed on a G-75 Sephadex column (85 x 1.5 cm), which was equilibrated with 0.05M tris-Cl at 4°C.

B. In Crude Seminal Vesicle Microsomes. Four seminal vesicles were removed from two steers (not bulls) within 1/2 hour after killing. It was not completely certain that seminal vesicles had been found. The vesicles and ducts were attached directly to the urinary

bladder under membranous tissue. The vesicles were excised and kept for approximately 2 hours on ice in 0.01M tris-Cl, 0.25M sucrose, pH 8.0. Preparation of the microsomes was according to the liver preparation of Goldberger, et al. (100). Fat and connective tissue were removed and the free vesicles (which looked like convoluted tubules) were minced with scissors. The minced vesicles were then homogenized for 3 to 5 minutes (in 30 second periods since there was heating during the homogenization) in tris-Cl sucrose, 150 ml total volume with a Waring blender. The resulting homogenate was centrifuged 20 minutes at 1,500 RPM and the precipitate discarded. The supernatant was then centrifuged at 17,000 X G for 20 minutes, and the precipitate discarded. The supernatant was used in the experiment without further handling.

Two ml of the seminal vesicle extract was mixed with 2 ml of 1 mg/ml refolded monomer and incubated at room temperature for 60 min. The resulting product was then passed through a G-75 Sephadex column (85 x 1.5 cm) as described previously for the seminal plasma preparation.

Results:

I. Enzyme Purification

Purification of seminal RNase was carried out using a slight modification of the procedure of D'Alessio, et al. (163). Figures 1 and 2 show the purification profile of seminal RNase on SP-Sephadex and Cellex-p, respectively. The major active peak in Figure 1 was labeled seminal RNase. As can be seen in Figure 2, all of the activity did not elute from Cellex-p with the linear gradient; thus, it was necessary to flush the column with 1M ammonium formate. Further, it was necessary to dialyze the pooled enzyme after Cellex-p against water before freeze drying could be accomplished. Other than these two modifications, the literature procedure was followed. The preparation gave an enzyme which was homogeneous on SDS gel electrophoresis and had an activity approximately 25% of that of RNaseA (Sigma 5 X crystallized). The activity varied approximately 10% over several preparations including those given to us by G. D'Alessio, University of Naples, Italy.

II. Affinity Chromatography

A. Biospecificity. The characterization of refolding and selective reduction products (described later), necessitated the removal of any inactive enzyme components. For this purpose a new affinity chromatography system for RNase was devised using the commercially

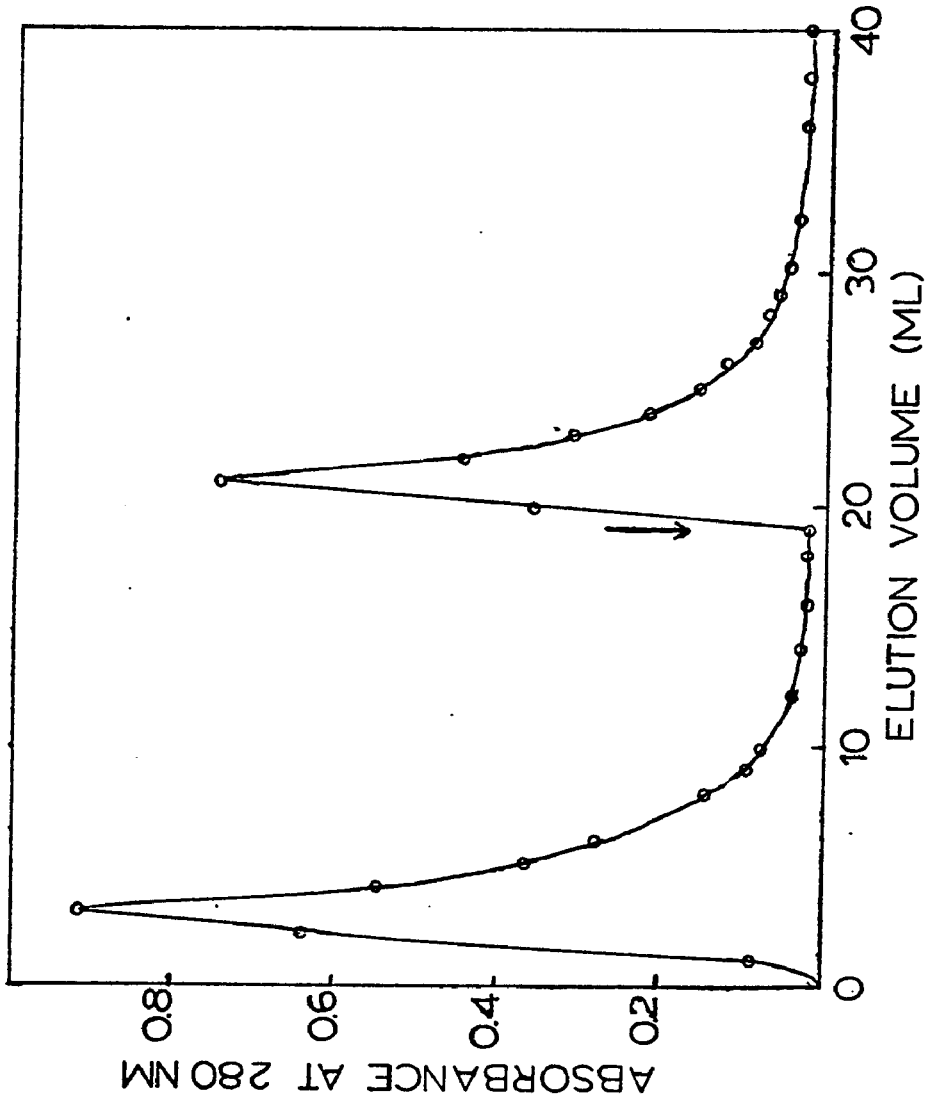
available resin material 5'-UTP-agarose. Due to the limited availability of seminal RNase and its relative expense compared to RNaseA, the latter was chosen to characterize the affinity system. This substitution is valid since the two proteins are entirely homologous and possess identical active sites (161,165).

Elimination of nonspecific binding in affinity chromatography is critical for good purification; thus, it was important to optimize our system in this respect. It has been shown that the buffer used to bind and elute the specific enzyme is important to ensure biospecificity of adsorption and complete elution (186). Thus, several buffers, most of which were suggested in earlier affinity chromatography articles, were tested to maximize biospecificity. These included 0.025M sodium acetate, 0.2M ammonium acetate, and 0.025M piperazine-HCl, pH 5.3 to bind the enzyme and 0.2M acetic acid or 0.25M sodium phosphate at pH 3 or 5.45 to elute the enzyme (175,187, 188). Of the buffers tested, 0.025M piperazine-HCl, pH 5.3, binding buffer and 0.25M sodium phosphate, pH 5.45, eluting buffer were found to be optimal for binding specificity and complete elution.

The 0.025M sodium acetate buffer originally used by Wilchek and Gorecki (187) showed significant nonspecific adsorption of cationic proteins, such as catalase and hemoglobin; however, the nonspecific binding

Figure 5. Reduction of non-biospecific protein binding to 5' UTP-agarose with 0.025M, pH 5.3 piperazine-HCl buffer: 3 ml of a 2 mg/ml solution of hemoglobin in 0.025M, pH 5.3 sodium acetate buffer were added to the column and eluted initially with acetate buffer followed at the arrow by 0.025M, pH 5.3 piperazine-HCl buffer.

FIGURE 5



could be largely eliminated with 0.025M piperazine-HCl. This is shown for hemoglobin in Figure 5; clearly a significant fraction of the nonspecific protein, still bound to the column after washing with sodium acetate, could be removed with the piperazine buffer. Chaiken and Taylor (188) have shown that the higher ionic strength buffer, 0.2M ammonium acetate, decreased nonspecific binding of RNaseA on another RNase affinity column. However, it was found in the present study that this buffer decreased RNaseA binding to the 5'-UTP-agarose column by approximately 20%; thus, the piperazine-HCl system was chosen.

Acetic acid, often used to elute the specific enzyme in affinity chromatography, was found to incompletely elute RNaseA in agreement with earlier studies in which other resins were employed (175,186,187). However, this problem did not occur with 0.25M sodium phosphate, pH 3.0 or 5.45, where typically 95 to 99% of the enzyme was recovered.

Stewart and Stevenson (175) suggested that the low pH and high ionic strength of pH 3.0 sodium phosphate buffer, as well as acetic acid, induced distortions in RNase which aided in the elution of the enzyme from Sepharose-5'(4-aminophenylphosphoryl)uridine 2'(3') phosphate (Sepharose-aPhpUp), another RNaseA affinity column. However, in the case of 5'-UTP-agarose, pH 5.45 sodium phosphate buffer eluted the enzyme slightly

Figure 6. Effect of pH on RNaseA elution from 5' UTP-agarose: 3 ml of a 0.32 mg/ml RNase solution in 0.025M, pH 5.3 piperazine-HCl buffer were applied to the column and eluted at the arrow with either pH 5.45 (■) or pH 3.0 (▲), 0.25M sodium phosphate buffer.

FIGURE 6

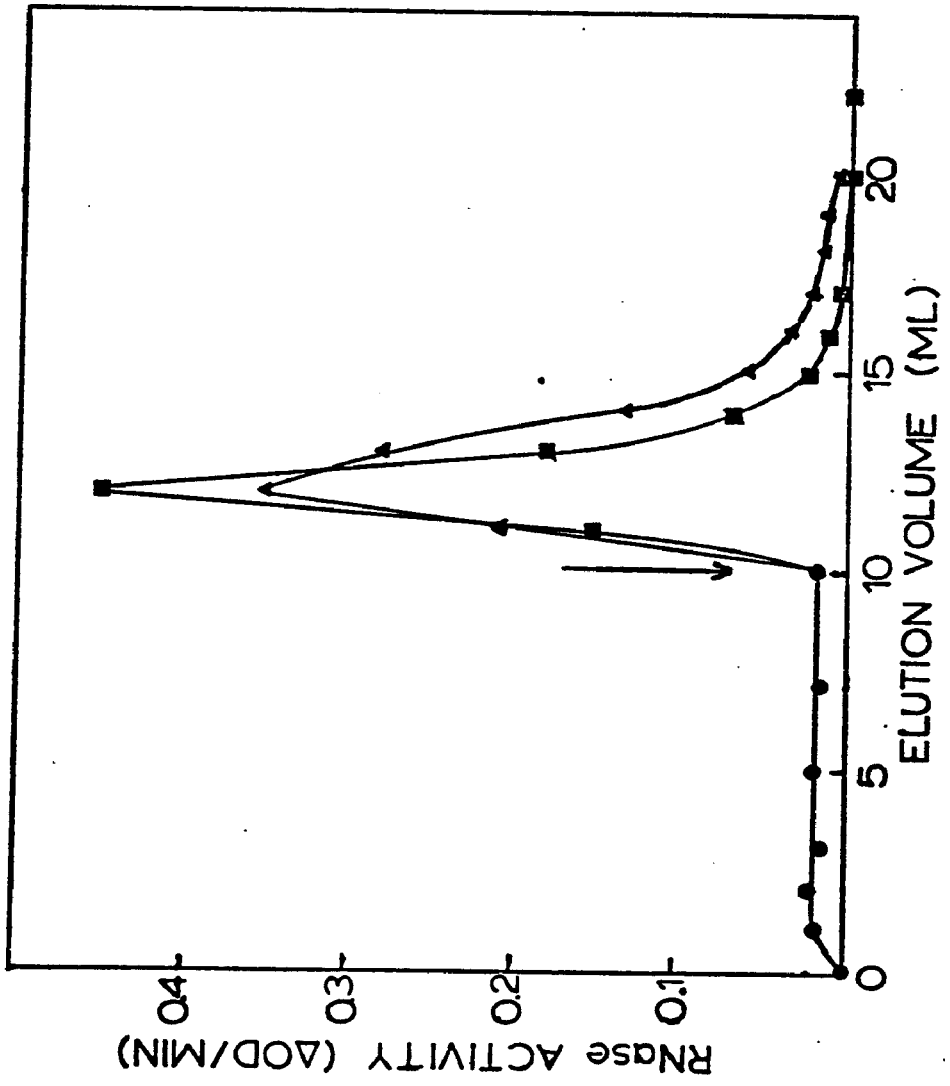

RNase ACTIVITY (ΔOD/MIN)
0.4
0.3
0.2
0.1
0
5
10
15
20
ELUTION VOLUME (ML)

Figure 7. Purification of Sigma 5X crystallized RNaseA by affinity chromatography on 5' UTP-agarose: (--O--O--), $A_{277.5}$, (~~XXX~~), RNase activity. The enzyme was added to the column in 3 ml of 0.025M, pH 5.3 piperazine-HCl buffer and eluted as indicated by the arrow with 0.25M, pH 3.0 phosphate buffer.

FIGURE 7

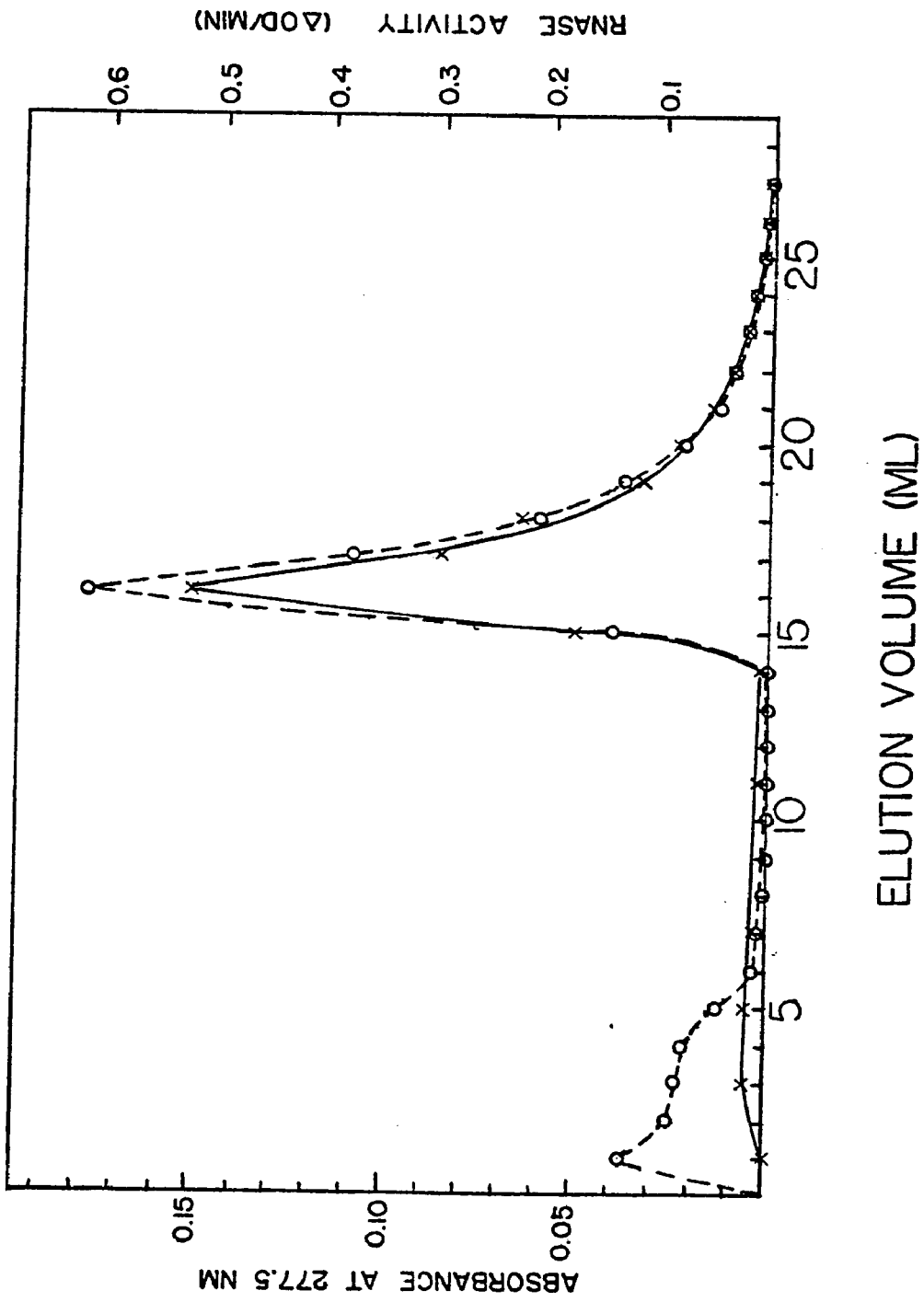
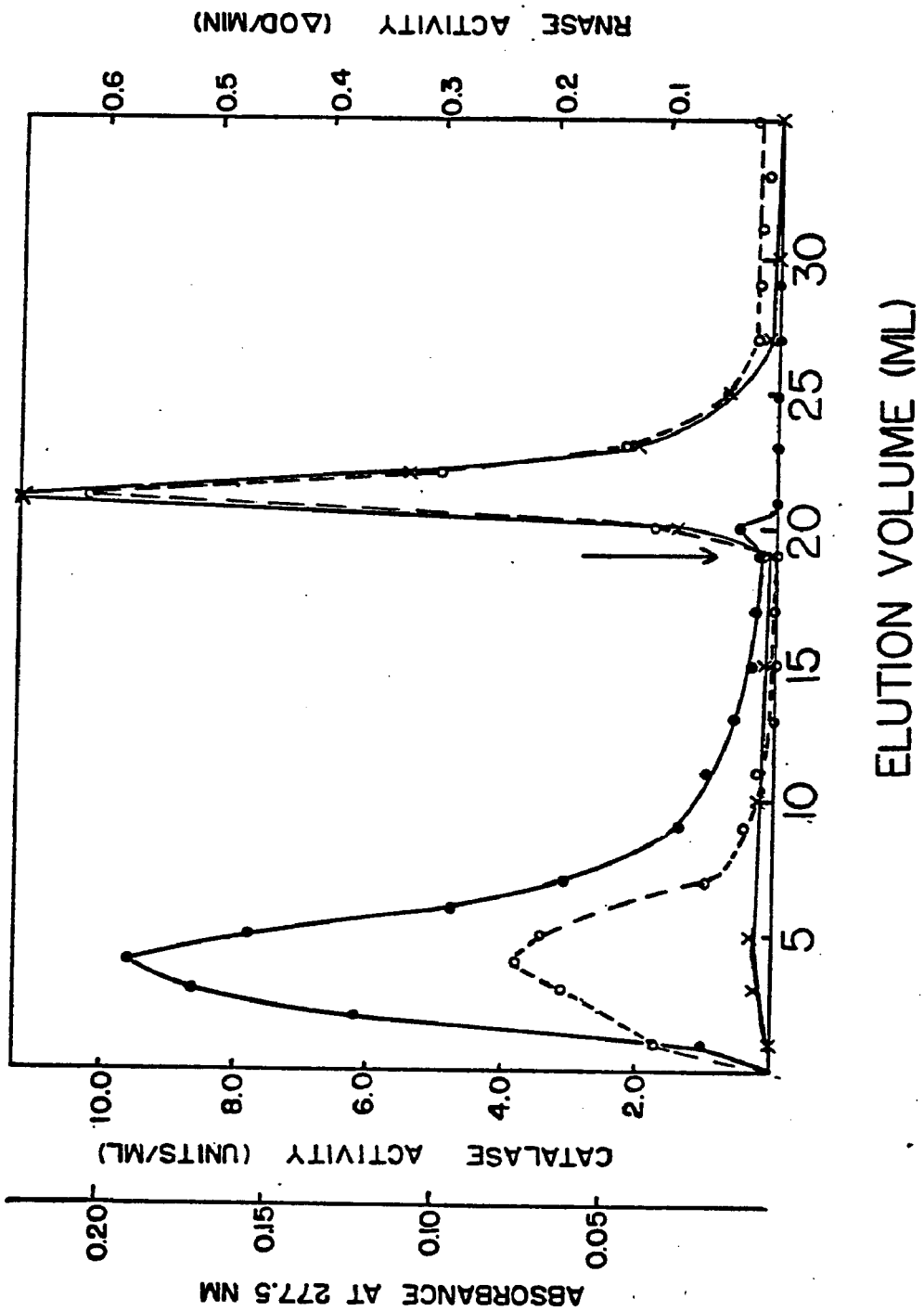

RNASE ACTIVITY (ΔOD/MIN)
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE AT 277.5 NM
0.15
0.10
0.05
5
10
15
20
25
ELUTION VOLUME (ML)

Figure 8. Separation of catalase contamination from RNaseA on 5' UTP-agarose: (--O--O--), $A_{277.5}$; (———), catalase activity; (~~XXX~~), RNase activity. 5.2 ml of enzyme solution were added to and eluted from the column as in Figure 7.

FIGURE 8


RNASE ACTIVITY (ΔOD/MIN)
0.6
0.5
0.4
0.3
0.2
0.1
ELUTION VOLUME (ML)
5
10
15
20
25
30
CATALASE ACTIVITY (UNITS/ML)
10.0
8.0
6.0
4.0
2.0
ABSORBANCE AT 277.5 NM
0.20
0.15
0.10
0.05

more efficiently than the low pH phosphate buffer (Figure 6). This phenomenon is likely due to the stronger competitive binding of phosphate at pH 5.5 where it binds tightest (K_d = 4.26mM) (189) and supports the view that the 5'-UTP-agarose column functions principally as an affinity system.

Further evidence for the biospecific nature of RNase binding to this column when using the piperazine/phosphate buffers can be seen in Figures 7 and 8. Figure 7 shows the purification of Sigma 5 X crystallized RNaseA on 5'-UTP-agarose. Although nearly all the enzymatic activity was retained by the column, an inactive component eluted with the piperazine wash. In Figure 8 it is seen that essentially all of the RNase bound to the column, although catalase did not.

Recently it has been shown that the best proof of biospecific adsorption is the use of competitive elution experiments (188,190). In these studies, the enzyme is eluted from the column with varying concentrations of a competitive inhibitor. For biospecific adsorption, the elution volume will increase with decreasing inhibitor concentration according to equation 1 (190):

$$\frac{1}{V - V_o} = {}^{K}_{IM/V_o[IM]} + {}^{[I]K}_{IM/V_o[IM]K_i} \qquad (1)$$

where V is the volume required to elute half the biospecifically retained protein, V_o the volume to elute

Figure 9. Competitive elution of RNaseA from 5' UTP-agarose with 2'(3') UMP: 0.7 mg of RNaseA was added to the column and eluted with 5.0 x 10^{-4}M, ▲; 8.3 x 10^{-5}M, ■; 3.5 x 10^{-5}M, ○ 2'(3') UMP in 0.025M, pH 5.3 piperazine-HCl as described in the Methods. Bed volume was 0.96 ml, and 0.45 ml fractions were collected.

FIGURE 9

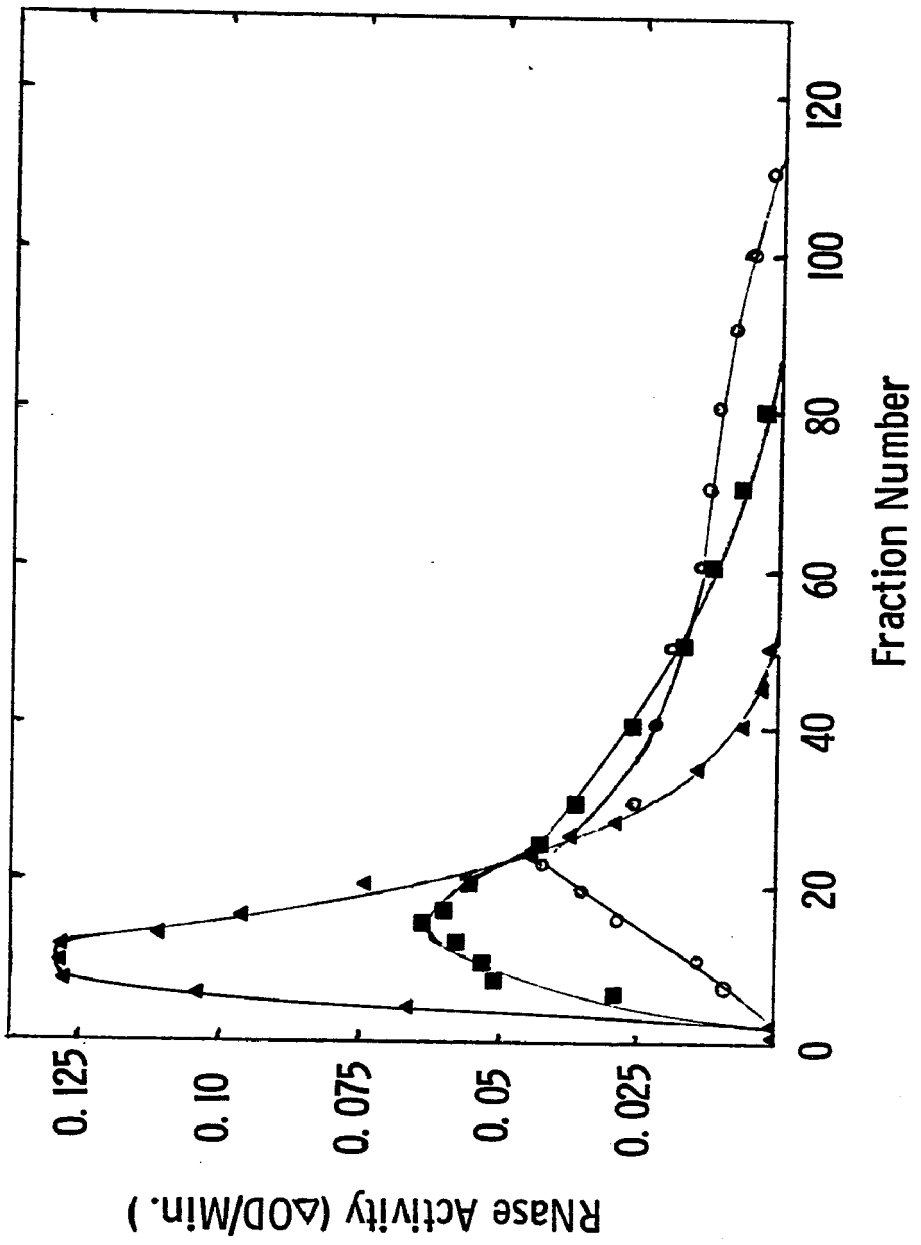
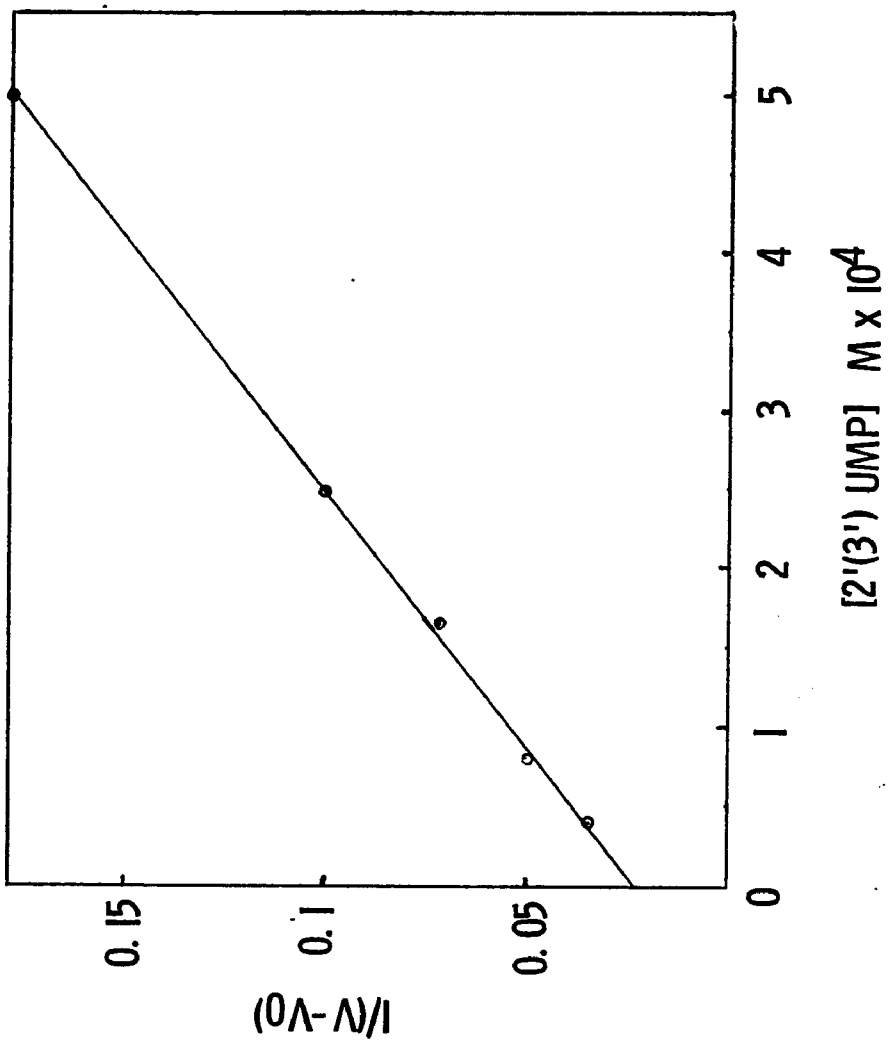

RNase Activity (ΔOD/Min.)
0.125
0.10
0.075
0.05
0.025
0
20
40
60
80
100
120
Fraction Number

Figure 10. Plot of $1/(V-V_o)$ vs. competitive inhibitor concentration for the determination of K_I and $K_{\overline{IM}}$: $1/(V-V_o)$ was calculated from Figure 9 (profiles for 1.66 and 2.49 x 10^{-4} M 2'(3') UMP were not shown). From the intercept and slope of this line K_I and $K_{\overline{IM}}$ can then be calculated according to equation 1.

FIGURE 10



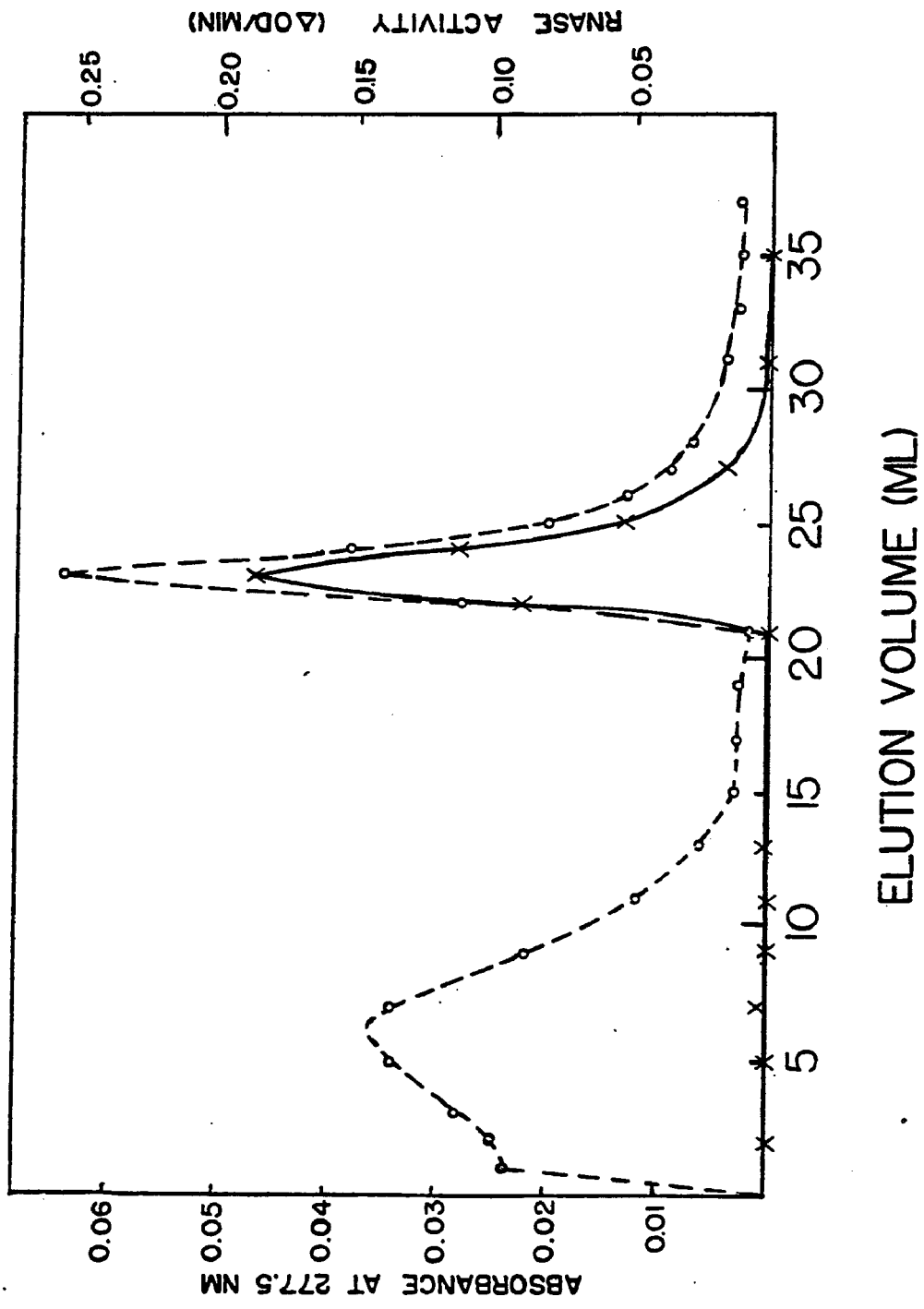
half the unretained protein, K_{IM} the dissociation constant for the immobilized ligand, [IM] the effective concentration of immobilized ligand sites, K_I the dissociation constant for the soluble ligand, and [I] the soluble ligand concentration. [IM] was found to be 0.70mM by determining the maximal amount of RNaseA that would bind to the column.

These experiments were performed in 0.025M piperazine-HCl, pH 5.3, and elution was effected with the competitive inhibitor 2'(3')UMP in the same buffer. The elution of RNaseA from 5'-UTP-agarose with varying concentrations of 2'(3')UMP is shown in Figure 9. It is apparent that the volume to elute half the enzyme activity increased with decreasing inhibitor concentration. Equation 1 predicts a linear plot for $1/(V - V_o)$ versus soluble inhibitor concentration for biospecific adsorption and elution. The data from Figure 9, when plotted in this manner, were linear (Figure 10). The K_I and K_{IM} at pH 5.3 were calculated to be 75 and 130 μM, respectively. The value for K_I calculated here is in excellent agreement with the reported K_I for 2'(3')UMP obtained by difference spectra (189).

Since the column was intended for characterization of refolded RNase, it was necessary to determine if inactive RNase would bind under the conditions used. Thus, the binding characteristics of air oxidized, refolded

Figure 11. Removal of inactive RNase from air oxidized, reduced, denatured RNaseA on 5' UTP-agarose: (--O--O--), $A_{277.5}$; (~~XXX~~), RNase activity. 7.4 ml of enzyme solution were added to and eluted from column as in Figure 7.

FIGURE 11


RNASE ACTIVITY (ΔOD/MIN)
0.25
0.20
0.15
0.10
0.05
ABSORBANCE AT 277.5 NM
0.06
0.05
0.04
0.03
0.02
0.01
5
10
15
20
25
30
35
ELUTION VOLUME (ML)

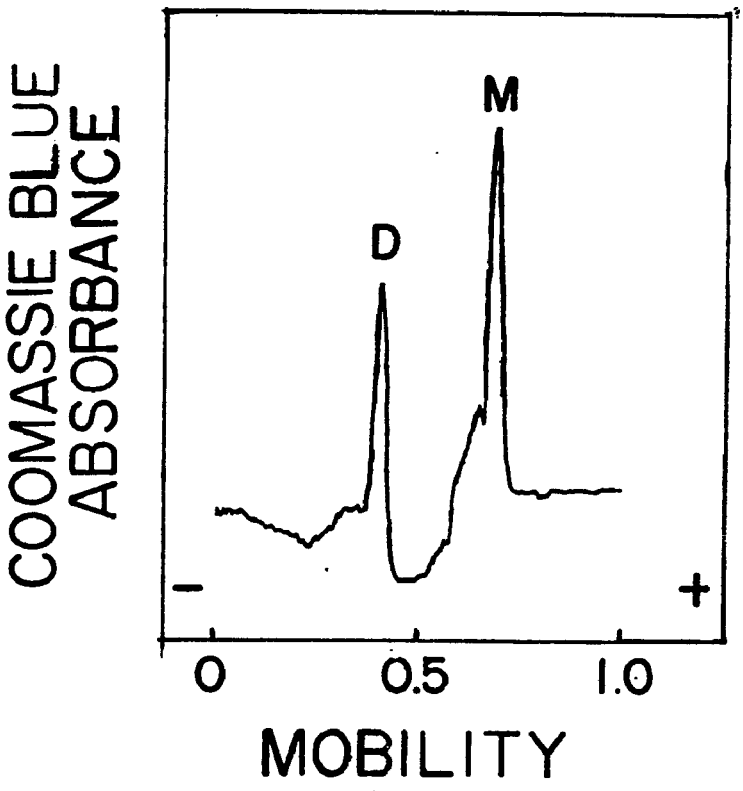
RNaseA were investigated. Completely reduced-denatured RNaseA was regenerated for 29 hours at 25°C in 0.1M tris-acetate at pH 8.2 and a protein concentration of 0.1 mg/ml. Final activity of the regenerated protein was approximately 50% of the native enzyme. Following ultrafiltration and washing with piperazine-HCl, the enzyme was applied to the 5'-UTP-agarose column. Figure 11 shows quite clearly that the inactive enzyme was not retained by the column while essentially all of the active RNase was. The RNase which eluted with the phosphate had a specific activity equal to the native enzyme indicating that the fraction was pure.

B. Binding of Seminal RNase Monomer and Dimer. Both native seminal RNase and the seminal RNase monomer (whose production will be discussed later) bind tightly to the column and elute with phosphate. Interestingly, twice as much dimer (by weight) will bind as will monomer. This indicates that only one site on the dimer is used to bind the affinity column.

C. BS-2 RNase Purification. This column was also used to effect a further purification of the minor ribonuclease fraction from SP-Sephadex (Figure 1). The peak was pooled, dialyzed against water, freeze dried, and dissolved in piperazine-HCl. This solution was then bound to and eluted from 5'-UTP-agarose. Very little protein was recovered since the pooled peak from SP-

Figure 12. SDS-PAGE of affinity chromatography purified RNase BS-2. D, mobility of seminal RNase dimer; M, mobility of seminal RNase monomer. Electrophoresis was performed as described in Methods in the absence of 2-ME.

FIGURE 12



Sephadex was small. However, sufficient quantity was available to perform SDS-PAGE in the absence of 2-ME. The results are shown in Figure 12. Two bands were evident; one had a mobility of the native seminal RNase dimer and the other migrated like the monomer. Thus, the results suggest that the minor ribonuclease peak from SP-Sephadex contains a mixture of molecules. No further characterization was possible.

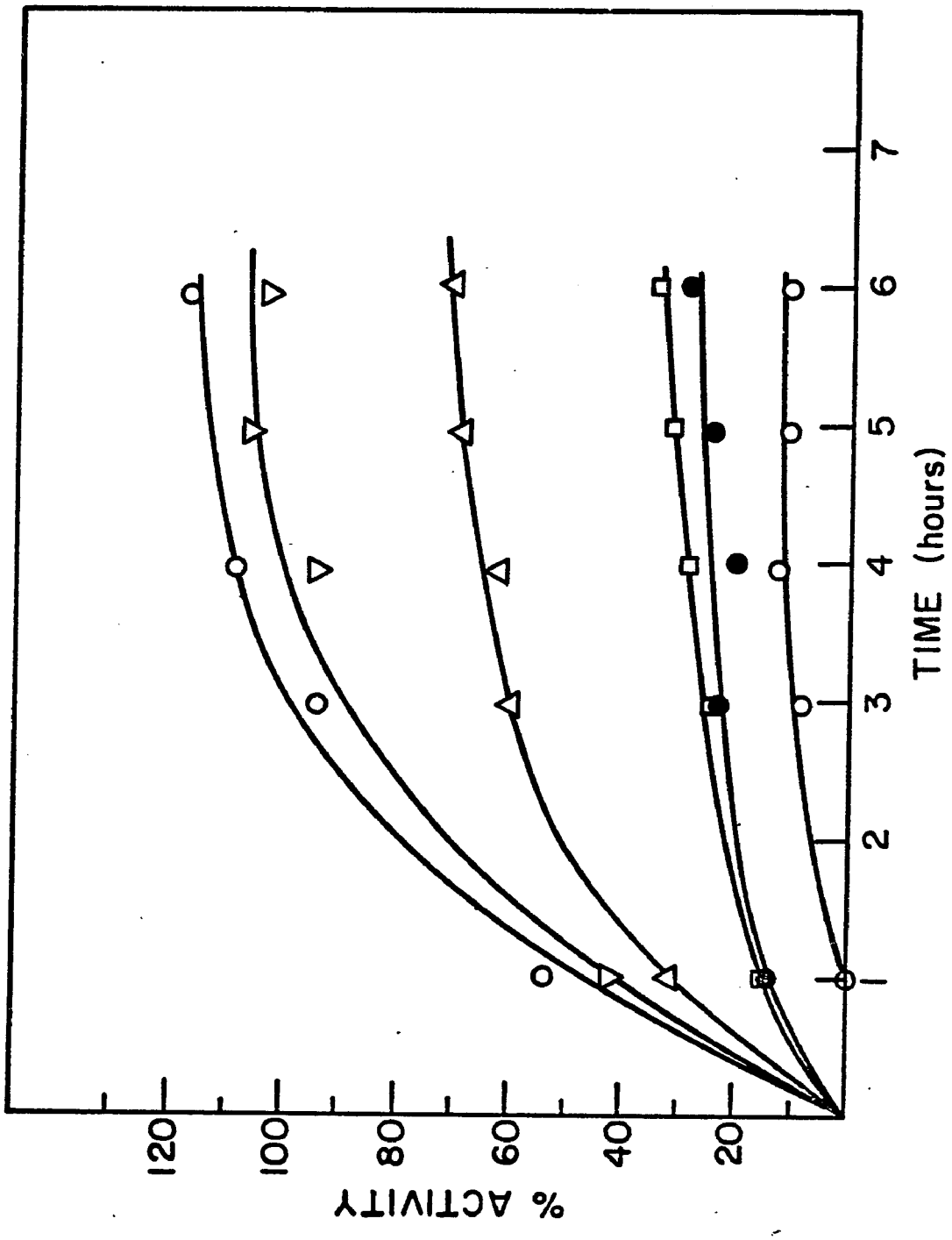
III. Regeneration Studies

A. Characteristics of the Reduced, Denatured Protein. Seminal RNase was reduced and denatured at room temperature, desalted on G-25 Sephadex (equilibrated with 0.1M acetic acid), and freeze dried. The reduced protein was very labile, and, unlike RNaseA, which can be stored for months, reduced seminal RNase can only be stored in a desiccator at -20°C for 1 or 2 days. Longer storage leads to insolubility, irreproducible and incomplete regenerations. It is assumed that cysteines 31 and 32 are responsible for the lability since they are the only thiol differences between the two proteins. The number of thiols per reduced dimer quickly drops from 20 to 15 during storage. Thus, the protein was always used within a day.

B. Kinetics. The regeneration of seminal RNase was followed by activity measurements. 0.020 ml aliquots were removed and immediately assayed for activity

Figure 13. Effect of glutathione concentration and redox state on regeneration of seminal RNase. Seminal RNase (0.051 mg/ml) was regenerated in 0.07M tris-acetate, 0.7mM EDTA, pH 8.2 buffer containing: o, 3.0mM GSH, 0.6mM GSSG; ∇, 3.0mM GSH, 0.3mM GSSG; Δ, 3.0mM GSH, 3.0mM GSSG; □, 0.3mM GSH, 3.0mM GSSG; ●, 3.0mM GSH, 10.0mM GSSG; ○, 30.0mM GSH, 3.0mM GSSG at 30°C.

FIGURE 13



at pH 5.0. Previous studies have shown that disulfide interchange is slow at this pH (113). By employing these conditions, the regeneration could be essentially stopped and activity assayed at the same time.

It was found that over the times used in these regeneration studies bubbling N_2 through the solution before starting the regeneration to prevent air oxidation was unnecessary. The presence of 1mM EDTA was sufficient.

Regeneration was maximized according to its rate and final activity as a function of several factors: glutathione concentration and redox state, protein concentration, temperature, and pH. The kinetics and final activity were found to be dependent upon several of these factors. The optimal regeneration in tris-acetate buffer was found to occur at 25 to 30°C, pH 8.2, in 3mM GSH/0.6 mM GSSG. Variation from these conditions caused reduced rates of regeneration and recovery of enzymatic activity.

The effect of glutathione upon regeneration is shown in Figure 13. The optimal regeneration was found to occur under reducing conditions over a narrow range of GSH and GSSG concentration. It can be seen that more reducing or oxidizing conditions greatly alter the regeneration kinetics. These results are in agreement with the effect of glutathione on the regeneration of RNaseA and other proteins (99,113). It can also be seen in Figure 13 that the regeneration under optimal conditions

Figure 14. Effect of protein concentration on regeneration of seminal RNase under optimal and nonoptimal glutathione conditions. Regenerations were performed in tris-acetate, pH 8.2, 30°C buffer medium containing either 3.0mM GSH/0.6 GSSG (upper curve) or 3.0mM GSH/3.0mM GSSG (lower curve) and a reduced seminal RNase concentration as follows: ◇, 0.293; ●, 0.140; ○, 0.137; ▽, 0.070; ■, 0.060; o, 0.035, △, 0.030; ▼, 0.029; □, 0.018 mg/ml.

FIGURE 14

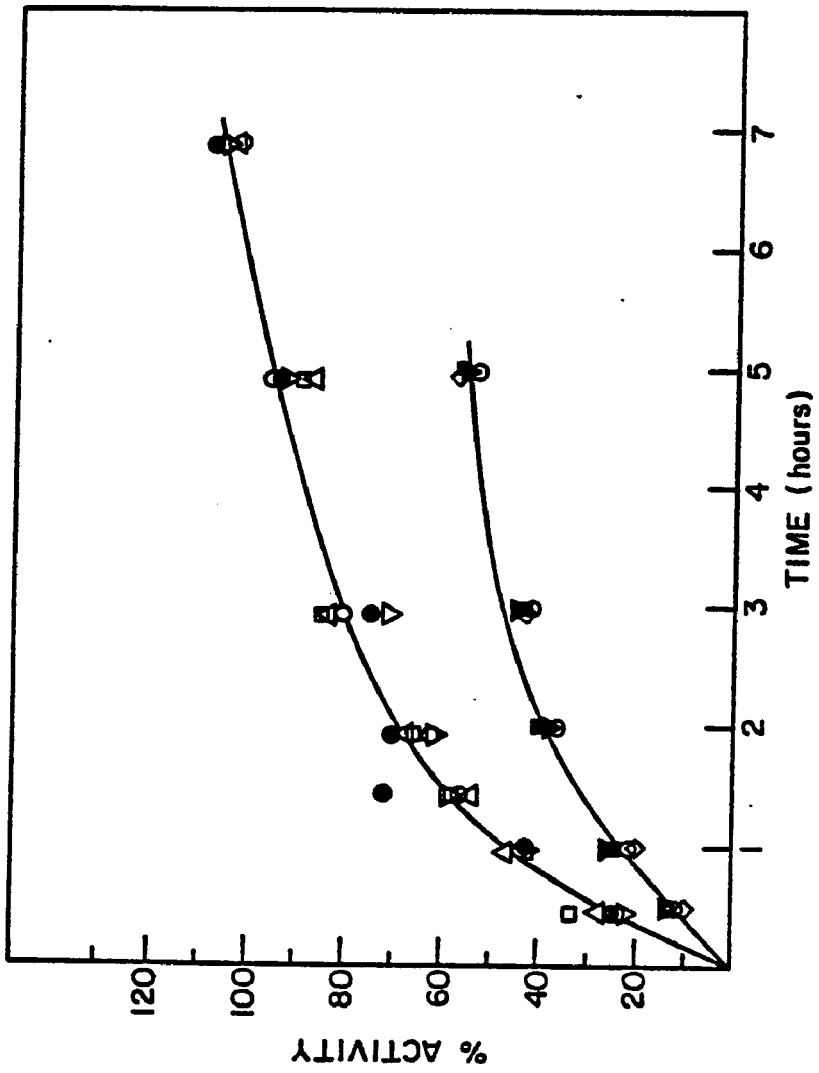
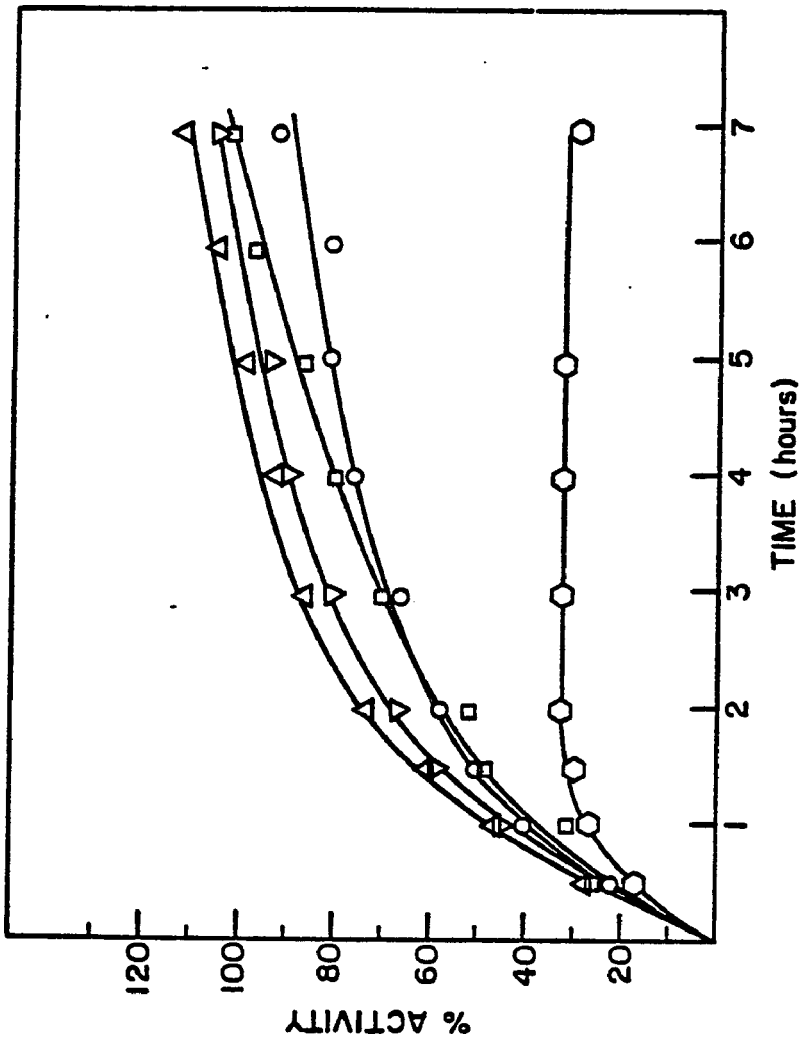


Figure 15. Effect of temperature on seminal RNase regeneration. Regenerations were carried out in a medium containing 0.091 mg/ml reduced seminal RNase and 3.0mM GSH and 0.6 mg GSSG and at a temperature of Δ, 24°C; ▽, 30°C; □, 14°C; o, 36°C; and ○, 42.7°C.

FIGURE 15



proceeded to over 100% activity; this phenomenon was observed in all experiments when optimal conditions were used and will be discussed later.

The regeneration exhibited no dependence on reduced seminal RNase concentration over a ten fold range using the optimal and more oxidizing conditions. Higher protein concentrations, up to 0.31 mg/ml, were used in some of the optimal regeneration product characterizations (see page 117), and no dependence could be detected even at these higher concentrations. The effect of protein concentration on the regeneration is shown in Figure 14. A similar lack of protein concentration dependence was noted in the glutathione regeneration of RNaseA (99).

The effect of temperature on seminal RNase regeneration is shown in Figure 15. The optimal temperature for regeneration occurred in the range 25 to 30°C. At lower temperatures the kinetics were slower, though the final products had activity similar to the optimal temperature regeneration. However, above 30°C, and most obviously above 36°C, regenerations were characterized by decreases in rate and extent of enzymatic activity recovery. When the regenerations were allowed to proceed for 24 hours (completion), the products obtained from experiments performed at 14 to 30, 36 and 43°C exhibited 110 to 125, 90, and 33% activity, respectively. This sharp temperature dependence above physiological

Figure 16. Effect of pH on regeneration: Regenerations were performed in tris-acetate, 30°C buffer containing 3mM GSH/0.6mM GSSG. Protein concentration was either 0.12 or 0.17 mg/ml. Time points were determined at (●), 60 min; (□), 120 min; and (Δ), 180 min.

FIGURE 16

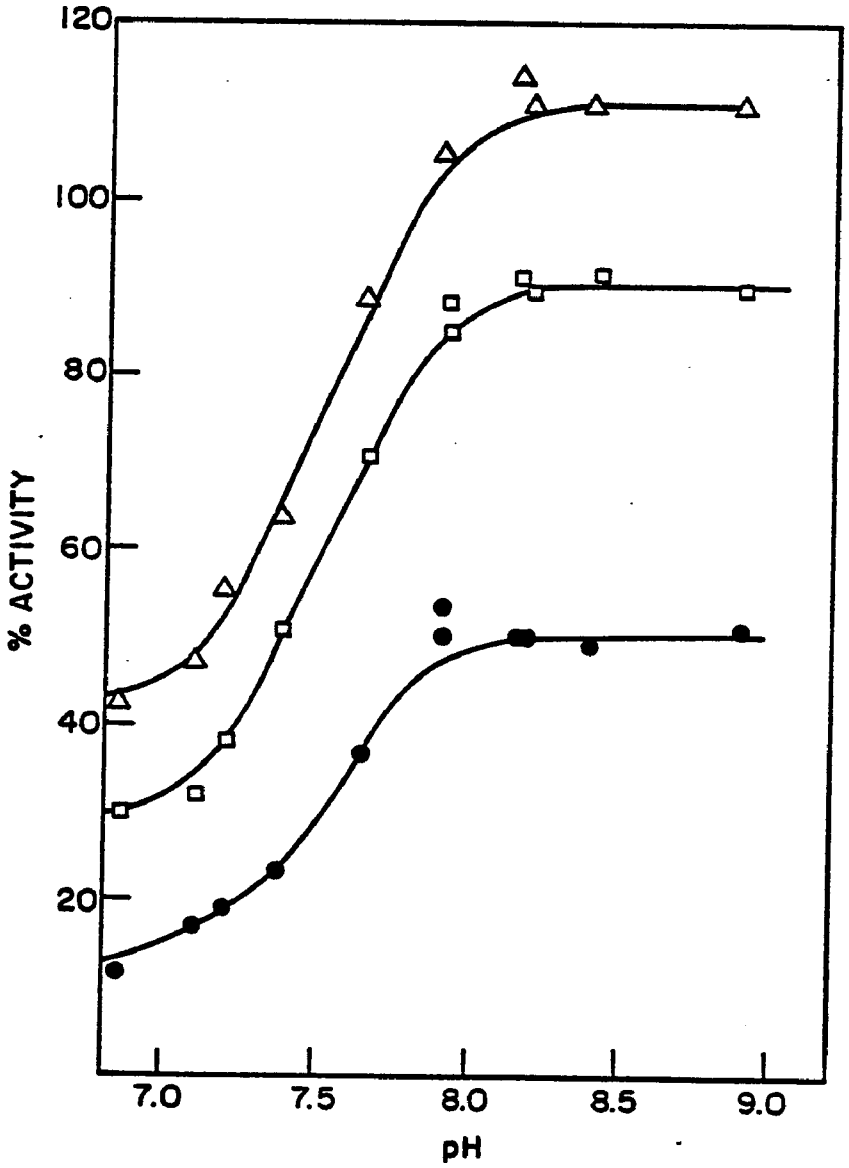
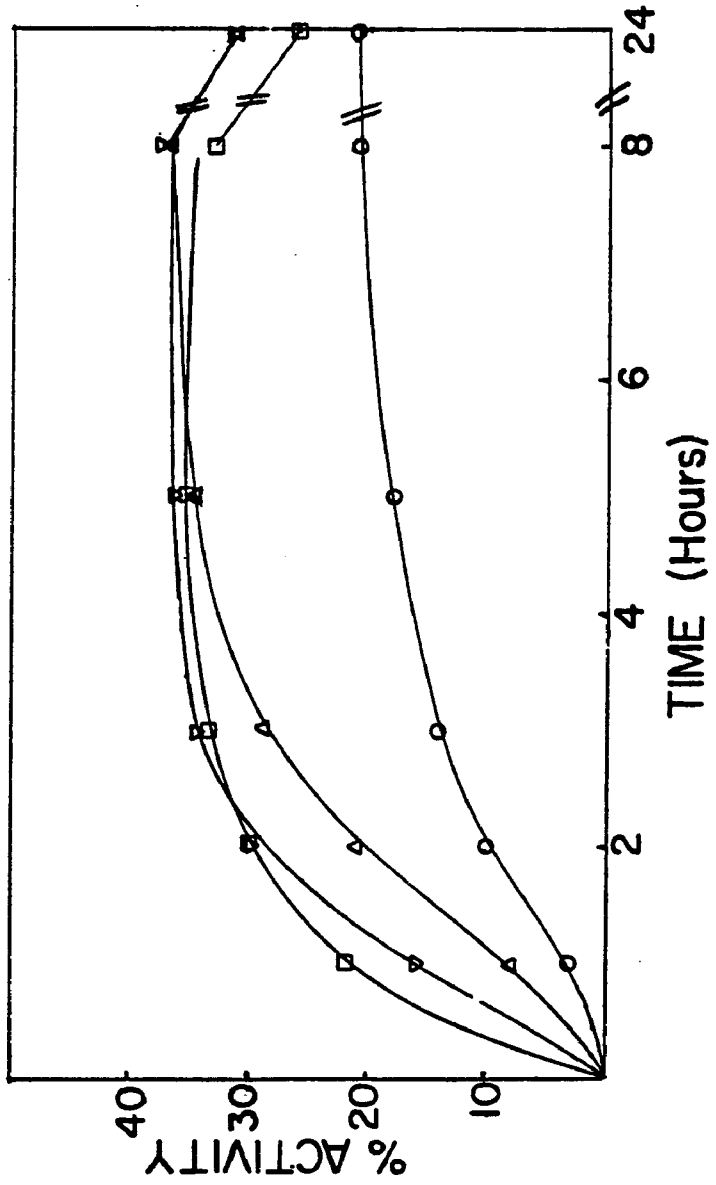


Figure 17. Effect of pH on regeneration at 42°C. Regenerations were performed in tris-acetate buffer, 42.3°C containing 3mM GSH/0.6mM GSSG. Protein concentration was 0.1 mg/ml. Solution pH's were as follows: (□), 8.2; (▽), 7.5; (Δ), 7.1; (o), 6.5.

FIGURE 17



temperatures was not observed in the glutathione regeneration of RNaseA (99) and will be examined later. Interestingly, this temperature dependence at 43°C was largely reversed (activity returned) when the temperature of the regeneration solution was dropped to 25°C after 24 hours of 43°C incubation and allowed to stand for 96 hours.

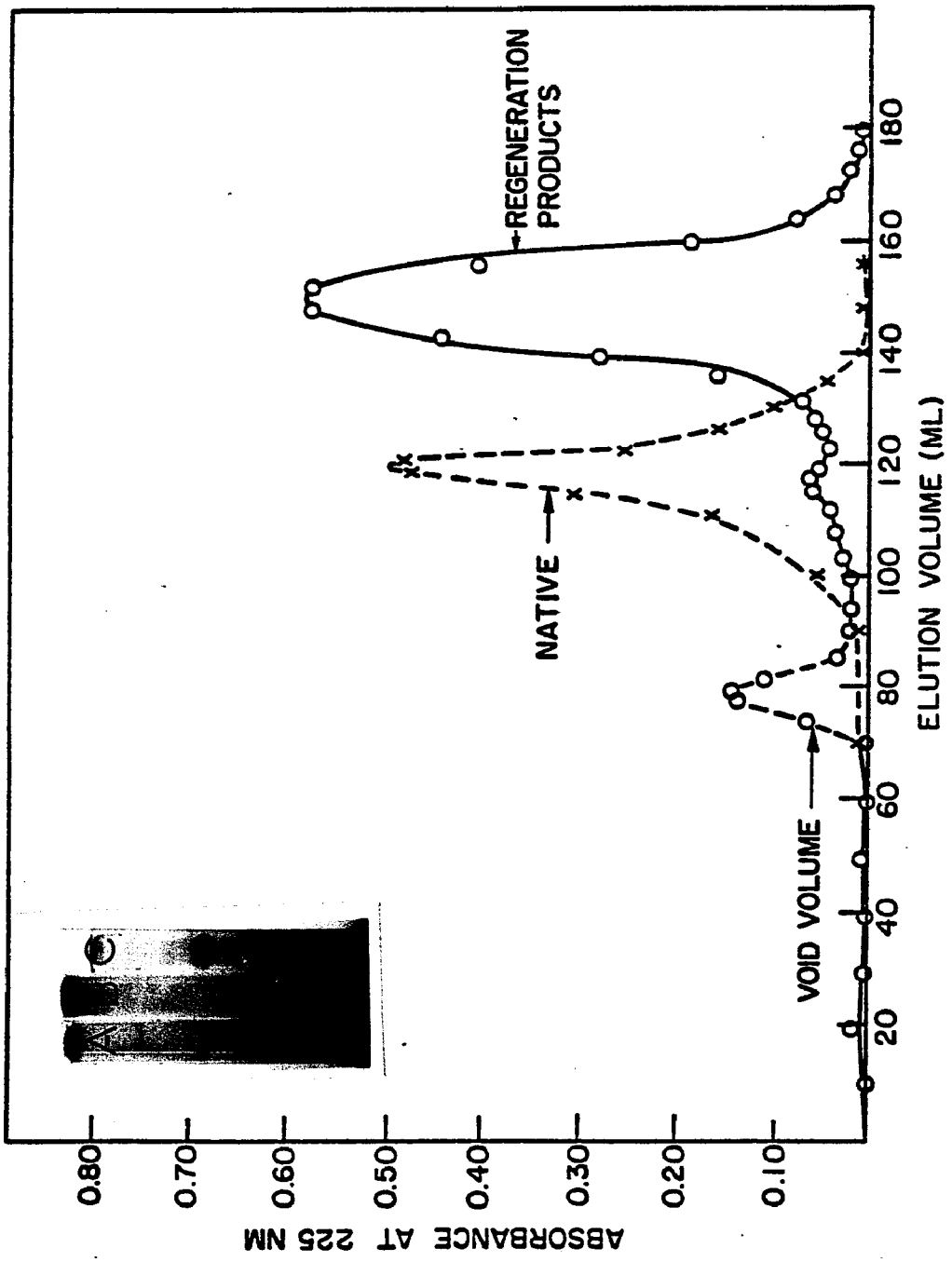
The rate of regeneration at 30°C was found to be highly dependent upon pH over the range pH 6.8 to 8.0 and independent of pH above pH 8.0. This is shown in Figure 16. The results suggest that a group with a pK of approximately 7.5 controls the rate of regeneration over this range; however, this type of experiment is inadequate for the identification of the group. Similar pH dependence was observed in the glutathione and air regenerations of RNaseA but not in the regeneration with the disulfide interchange enzyme (98,99,106). Activity of the final products was not affected by pH since the regeneration at pH 6.8 reached 120% after 24 hours. Thus, unlike high temperature, low pH does not halt the regeneration, it merely slows it.

A similar dependence of regeneration rate upon pH was also noted at 43°C (Figure 17). However, the final products contained approximately 35% activity at all pH conditions. Thus, the high temperature effect could not be reversed by low pH, and although rate of regeneration

Figure 18. Sephadex G-75 elution pattern of both native and optimally regenerated seminal RNase. The regeneration was performed in 0.1M tris-acetate, 1.0mM EDTA, pH 8.2 buffer containing 3.0mM GSH/0.6mM GSSG and 0.15 mg/ml of reduced seminal RNase. Following the 24 hour incubation at 30°C the protein was concentrated and applied to an 89 x 1.5 cm Sephadex G-75 column and eluted with 0.1M acetic acid at room temperature. Flow rate was 1 drop/3 seconds, and 2 ml fractions were collected.

Inset: SDS-PAGE of native seminal RNase and regeneration products. Electrophoresis of the native enzyme (A), G-75 Sephadex monomer peak (B), and G-75 Sephadex dimer (C) was carried out in the absence of β-mercaptoethanol.

FIGURE 18


REGENERATION PRODUCTS
NATIVE
VOID VOLUME
ELUTION VOLUME (ML)
ABSORBANCE AT 225 NM
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
20
40
60
80
100
120
140
160
180

became dependent upon pH, the final products were solely dependent upon temperature. If the temperature was dropped to 25°C after 24 hours at 43°C, the percent activity rose to 100% within 96 hours.

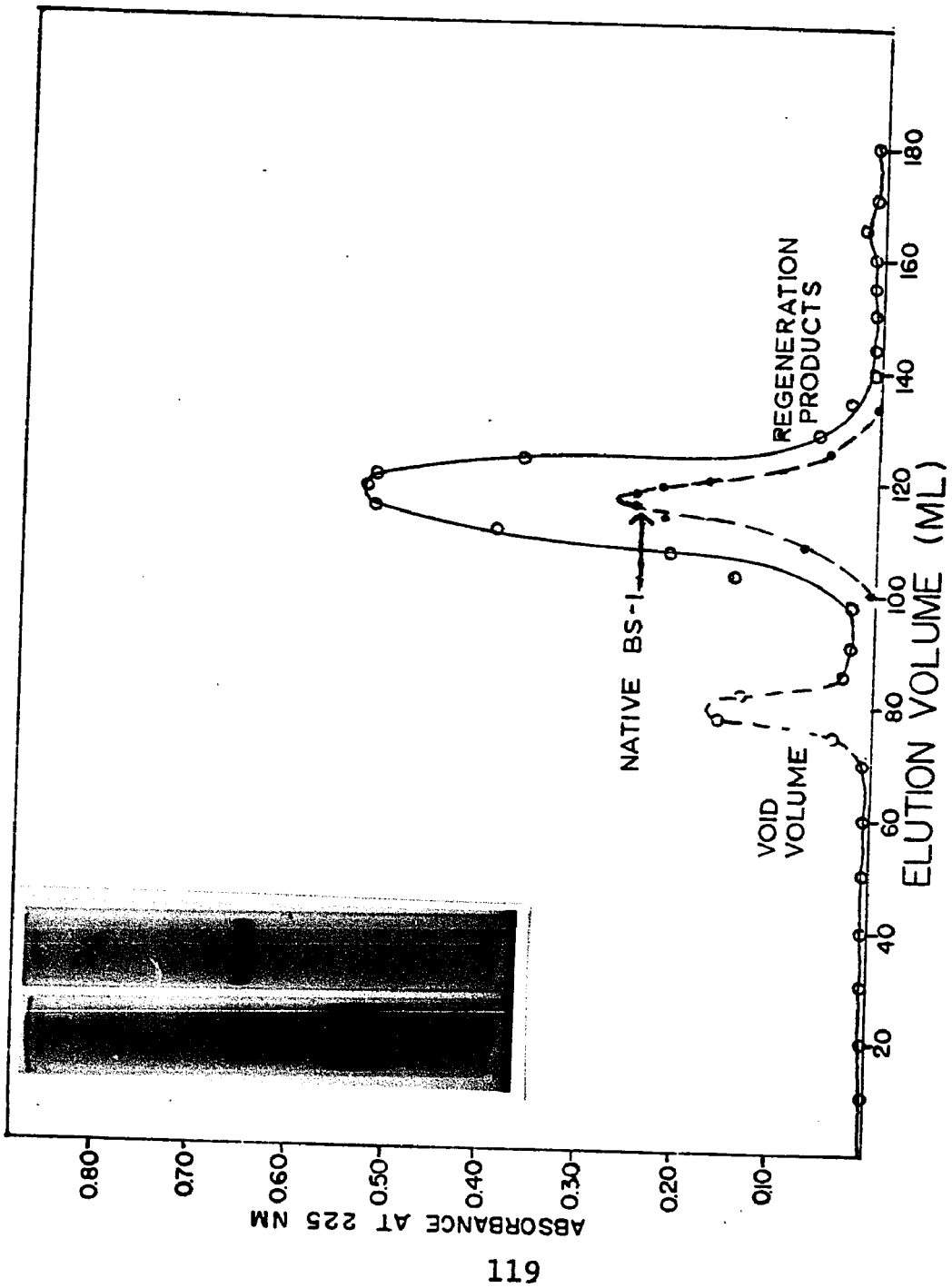
C. Characterization of Regeneration Products. Figure 18 shows the Sephadex G-75 elution pattern of regnerated seminal RNase, prepared by refolding 0.1 to 0.3 mg/ml reduced protein under optimal conditions (pH 8.2, 3mM GSH, 0.6mM GSSG, 1mM EDTA, 0.1M tris-acetate, 30°C), and that of the native enzyme. It can be seen that the major product of the reaction does not elute with the native enzyme. Rather, the major peak corresponds to the seminal RNase monomer. The pooled dimer peak contains primarily noncovalent dimer since most of it dissociates to the monomer on SDS-PAGE carried out in the absence of 2-ME (Figure 18, inset). The small percent of covalent dimer present in the G-75 peak fractionates in the SDS gel into two species with slightly different mobilities. However, due to their low concentration in the regeneration mixture, it was not possible to establish if one of them corresponded to the native enzyme.

Attempts were made to increase the yield of native covalent dimer by increasing the concentration of GSSG in the regeneration medium. A two fold increase in GSSG concentration to 1.2mM, while maintaining GSH concentration at 3mM, had no effect upon the final percent activity

Figure 19. Sephadex G-75 elution pattern of native and nonoptimally regenerated seminal RNase. Regeneration was performed at 30°C in 0.1M tris-acetate, 1mM EDTA, pH 8.2 containing 3mM GSH/3mM GSSG, and 0.14 mg/ml reduced enzyme. After 17 hours the protein was concentrated and applied to G-75 Sephadex as in Figure 18.

Inset: SDS-PAGE of native seminal RNase and regenerated dimer. Electrophoresis of native (A), and regenerated (B) enzyme was carried out in the absence of 2-ME.

FIGURE 19


ABSORBANCE AT 225 NM
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
ELUTION VOLUME (ML)
20
40
60
80
100
120
140
160
180
VOID VOLUME
NATIVE BS-1
REGENERATION PRODUCTS

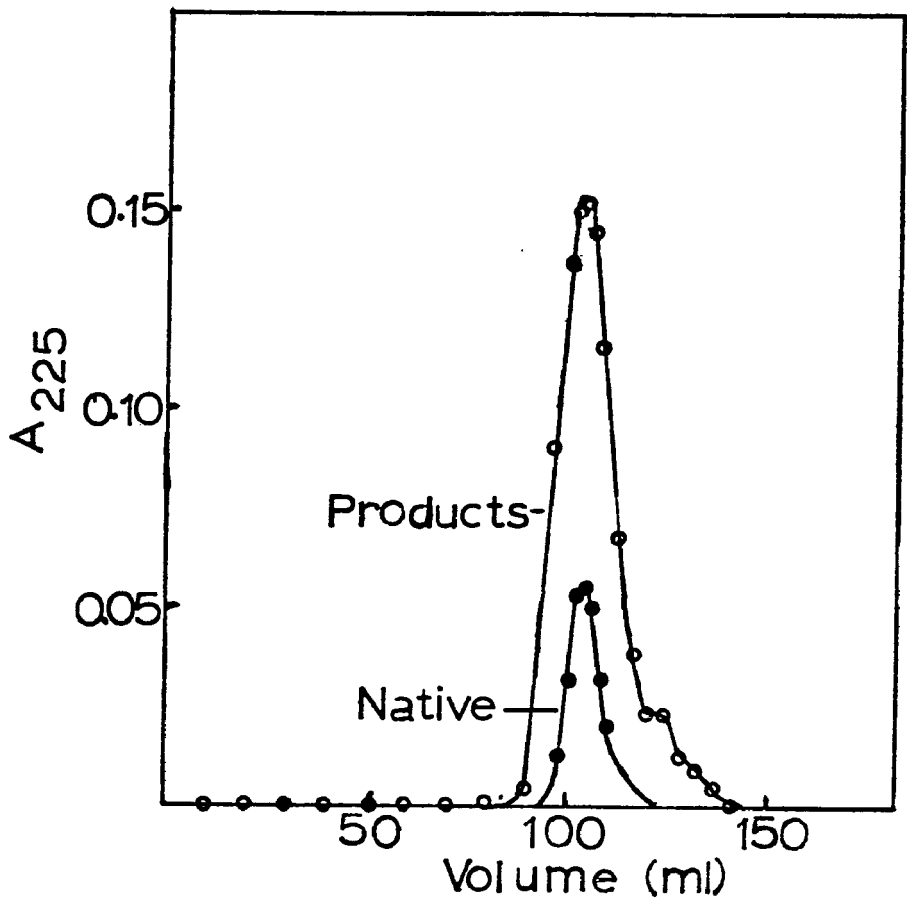
or the distribution of products. However, a further increase of GSSG concentration to 3mM drastically reduced the amount of regenerated monomer. Figure 19 shows the Sephadex G-75 chromatographic pattern of the products from a 24 hour regeneration in the presence of 3mM GSH and 3mM GSSG. It can be seen that the products are dimeric. In other experiments some monomer was observed, but the dimer was always dominant. Although these results suggest that a considerable amount of covalent dimer was formed in the regeneration, this interpretation was not substantiated by SDS-PAGE. Figure 19 (inset) shows that the dimer from G-75, which exhibits approximately 50% activity, is largely noncovalent.

The free regenerated monomer contains no free thiols and is a mixed disulfide with 2 molecules of glutathione, as will be shown later. Thus, the thiols which normally form the intermolecular disulfide bonds are blocked. A preparative regeneration was run under more reducing conditions in an attempt to reduce the blocking disulfides and allow formation of the intermolecular disulfide bonds. However, the regeneration carried out with 3mM GSH and 0.06mM GSSG produced little or no dimer and essentially all monomer.

Air Regeneration of Seminal RNase. It has been reported that completely reduced, denatured seminal RNase regenerates to a covalent dimeric species under

Figure 20. Sephadex G-75 elution pattern of native and air oxidation regenerated seminal RNase. Regeneration was performed at room temperature in 0.1M tris-acetate, pH 8.2 containing 0.31 mg/ml reduced enzyme. After 29 hours the protein was added directly to G-75 Sephadex and eluted as in Figure 18.

FIGURE 20



air oxidation conditions (D'Alessio personal communication). Considering the glutathione regeneration results, in which only active monomer was formed under optimal conditions, it seemed important to verify the air oxidation results. Thus, a regeneration of seminal RNase in air was undertaken. The buffer used was 0.1M tris-acetate, pH 8.2 at 25°C, in which EDTA and glutathione were omitted. Regeneration was performed at a protein concentration of 0.3 mg/ml and allowed to proceed for 29 hours. Final activity yield was 65%. Sephadex G-75 elution pattern of the products is shown in Figure 20. Essentially no monomer is apparent and the major product elutes with the native enzyme. Furthermore, SDS-PAGE showed very little monomer and greater than 90% covalent dimer.

D. Characterization of Refolded Monomer. The regenerated monomer eluting from G-75 Sephadex had a specific activity approximately 1.5 times that of the native enzyme. Purification of this monomer by affinity chromatography on 5'-UTP-agarose gave a preparation with 199 ± 7% activity, twice that of the native enzyme. The monomer appeared homogeneous upon gel electrophoresis carried out in the presence and absence of SDS (176,177).

Thiol assay of the monomer with NBs_2 showed that the species contains 0.005 to .018 free thiols per 14,500 daltons which indicates that cysteines 31 and 32 are not

TABLE I

Partial amino acid composition of native seminal ribonuclease and the refolded monomer.[a]

Amino Acid	Native Protein	Refolded Monomer
Alanine	8	8.17
Aspartate	11	11.0
Glutamate	11	13.1
Glycine	6	7.95
Isoleucine	3	2.92
Leucine	2	2.12
Phenylalanine	3	2.96

[a]Compositions are expressed as moles of residues per moles of subunits. Only stable amino acids from the long column are included.

Figure 21. SDS-PAGE of attempted dimerization of regenerated monomer. Regenerated monomer (0.1 mg/ml) was incubated for 19 hours at 30°C in pH 8.2 tris-acetate buffer containing: A, 3mM GSSG; B, 3mM GSH/0.6mM GSSG; C, 0.3mM GSH/3mM GSSG; D, no additions (air oxidation); E, SDS-PAGE of native and monomeric seminal RNase is also included. Electrophoresis was performed in the absence of 2-ME.

FIGURE 21

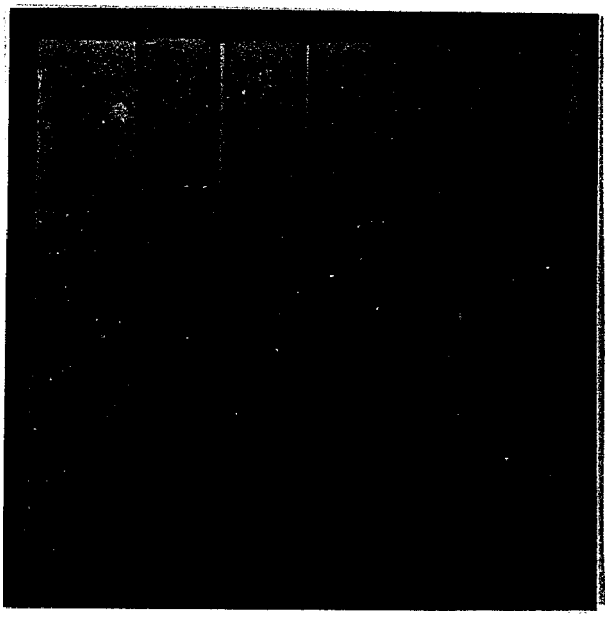
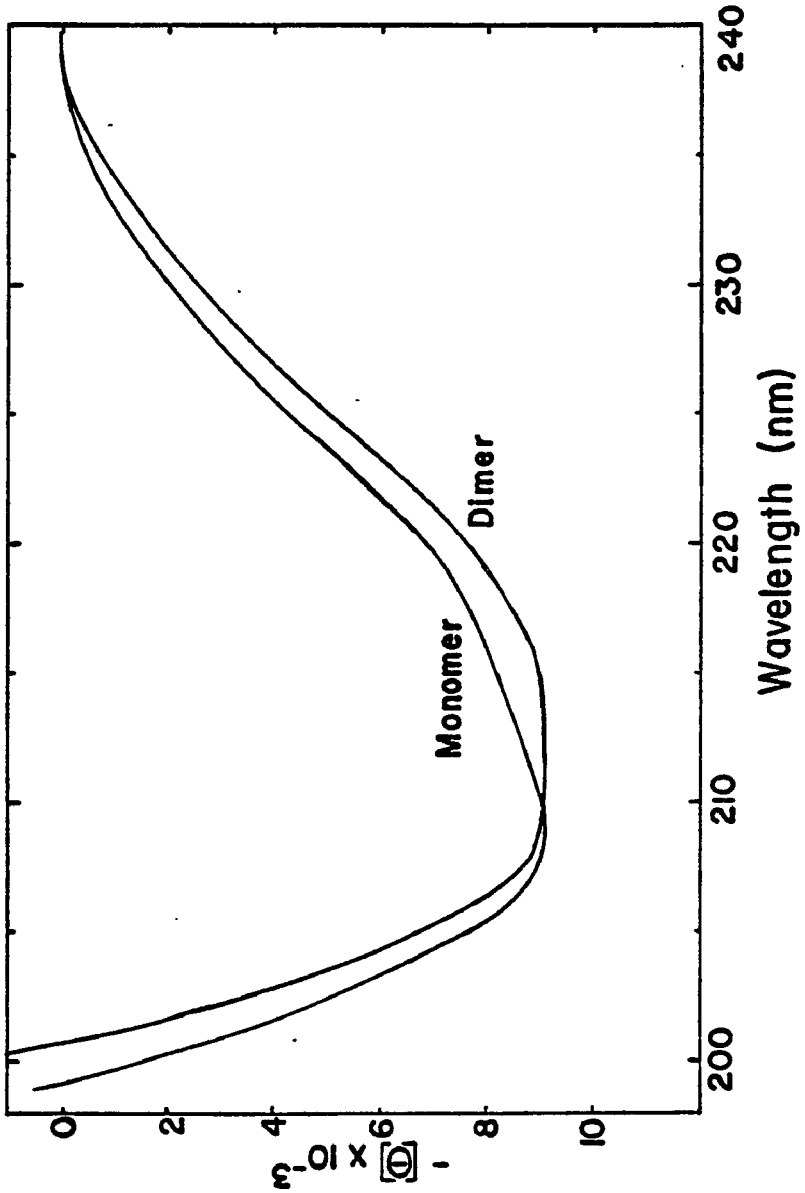


Figure 22. Circular dichroism of native seminal RNase and regenerated monomer. Circular dichroism spectra were carried out at pH 3.9 and 0.05M $KClO_4$ on a Durrum-Jasco J-10 circular dichrometer. The concentration of the monomer and the dimer were 0.774 mg/ml and 0.643 mg/ml, respectively. [θ] represents the mean residue molar ellipticity.

FIGURE 22


Dimer
Monomer
Wavelength (nm)
200
210
220
230
240
0
2
4
6
8
10
- [θ] x 10^-3

free. Furthermore, partial amino acid analysis of the regenerated monomer (Table 1) showed that two moles of glutathione were incorporated into the monomer, which suggests that two mixed disulfides were formed with glutathione.

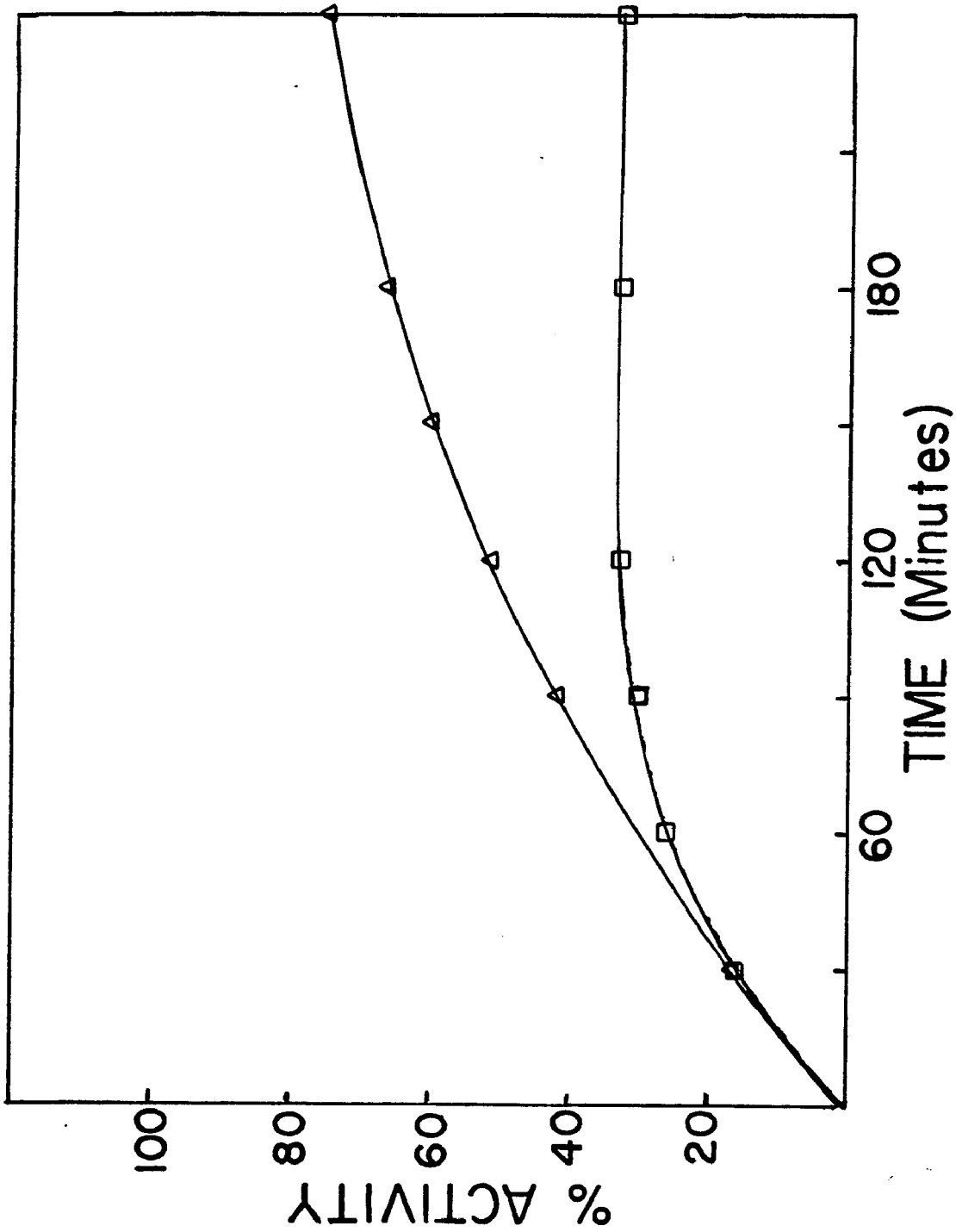
The 200% active glutathione refolded monomer proved to be very resistant to dimerization. Attempts to cause significant covalent dimerization all failed. To test its stability the monomer was incubated with each of the following: 3mM GSH/0.6mM GSSG; 0.3 to 10mM GSSG, 3mM GSSG/0.3mM GSH; or 0.0mM glutathione (air oxidation) for 1 hour or overnight. The reaction was stopped with a ten fold excess of iodoacetamide (over total thiol), and SDS-PAGE was performed on the products. The results are shown in Figure 21. It can be seen that dimerization only occurred in the presence of 3mM GSH/0.6mM GSSG and under air oxidation conditions. In both cases only very small amounts of dimerization did occur.

The presence of substrate (0.5 mg/ml RNA) or a salting out agent (0.5M phosphate) did not alter the extent of dimerization.

Circular dichroism spectra of native seminal RNase and regenerated monomer, both purified by affinity chromatography, are shown in Figure 22. It is apparent that the spectra are different indicating structural differences between the two forms. The major differences

Figure 23. Regeneration of RNaseA (Δ) and seminal RNase (□) at 42°C. Both enzymes were regenerated in 0.1M tris-acetate. RNaseA regeneration contained 3mM GSH/0.3mM GSSG and 0.02 mg/ml reduced RNaseA; seminal RNase regeneration contained 3mM GSH/0.6mM GSSG and 0.1 mg/ml reduced seminal RNase.

FIGURE 23



are a blue shifted trough ($[\theta]_{max}$) and blue shifted crossover ($[\theta]=0$) for the monomer compared to the native enzyme. Particularly noteworthy are the similarities between the CD spectra of the regenerated seminal RNase monomer and RNaseA (native or regenerated) (170). The data suggest that the tertiary structure of the native molecule is affected by its quarternary structure. When the subunits refold the monomer assumes a conformation similar to RNaseA.

E. Mechanistic Studies of the High Temperature Regeneration. It is apparent from the results presented above that the regeneration of seminal RNase is very similar to that of RNaseA under similar conditions, and the products have similar properties. However, as mentioned earlier, one major difference between them is the temperature dependence of the two regenerations, as shown in Figure 23. This difference indicates different molecular mechanisms for the two regenerations. It would be of importance to the present studies to discern the chemical basis for the difference; thus, studies were undertaken for this purpose.

One major structural difference between seminal RNase and RNaseA is the presence of two extra half cystines per chain in the former, which form intermolecular disulfide bonds in the native enzyme. It was reasoned that the sharp temperature dependence could be due

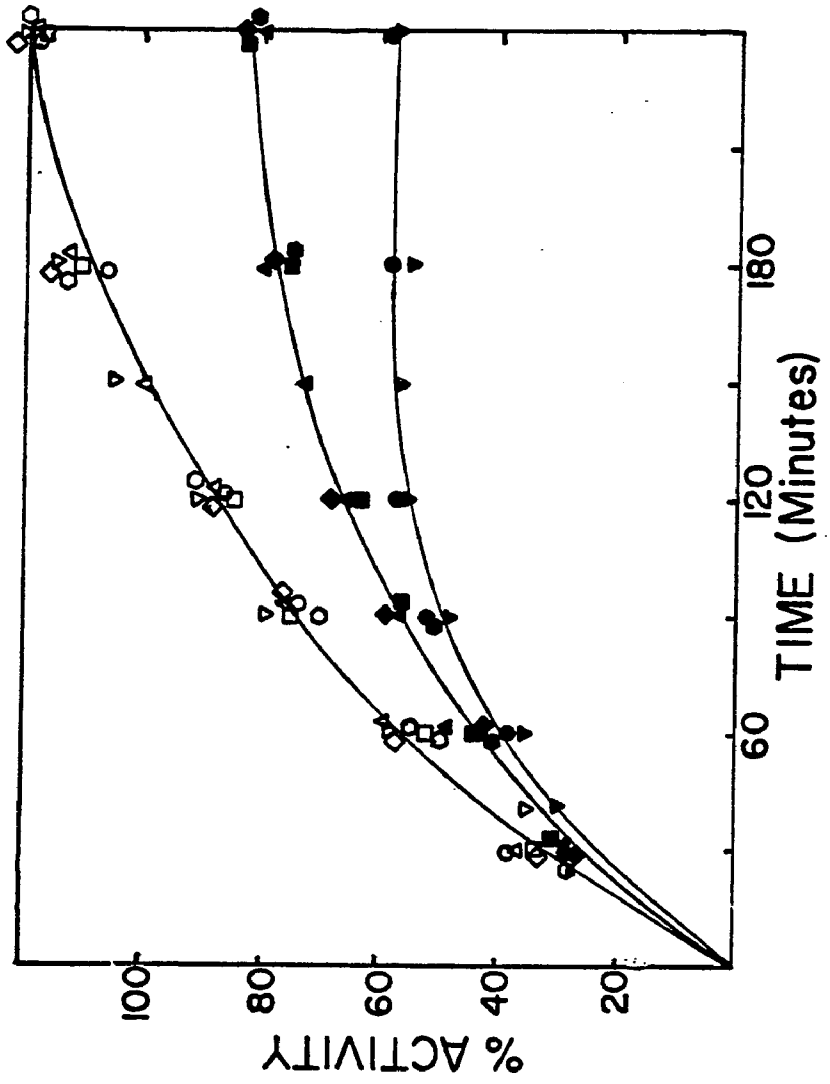
to the formation of incorrectly paired intermolecular disulfide bonds at higher temperatures where more collisions would be possible. To test this, SDS-PAGE was performed on the products of the 43°C regeneration after stopping the reaction with a 10 fold excess of NEM. The results were quite similar to those of Figure 18 (inset) where little covalent dimer was found. Thus, formation of irreversible intermolecular disulfide bonds could not be responsible for the differences. However, this does not eliminate cysteines 31 and 32 from the high temperature mechanism since they could also be involved in wrongly paired intramolecular disulfide bonds.

The intermolecular disulfide bonds of native seminal RNase can be selectively reduced and modified while the remaining disulfide bonds stay intact. The resulting product is a free monomer and has an activity greater than that of the native protein (181,182). Thus, it became of interest to determine the effect of blocking these two thiols upon the regeneration of seminal RNase at moderate and high temperatures.

Native seminal RNase at a concentration of 0.25mM was selectively reduced with 2.5mM DTT (10 fold molar excess), blocked with either a maleimide, iodoacetate, iodoacetamide, or ethyleneimine, and then totally reduced with a 500 fold excess of 2-ME in 8M urea. Effect of modifying group charge on the regeneration was deter-

Figure 24. Regeneration of modified seminal RNase. Filled symbols represent experiments performed at 42°C and open symbols represent experiments carried out at 25 to 30°C. ▽, iodoacetate; o, M-2; □, iodoacetamide; ◇, M-1; Δ, NEM; and ○, ethyleneimine modified seminal RNase. Regenerations were performed in 0.1M tris-acetate buffer containing 1mM EDTA, 3mM GSH/ 0.6mM GSSG, and 0.05-0.1 mg/ml reduced modified seminal RNase (see Figure 4 for modification reagent structures).

FIGURE 24



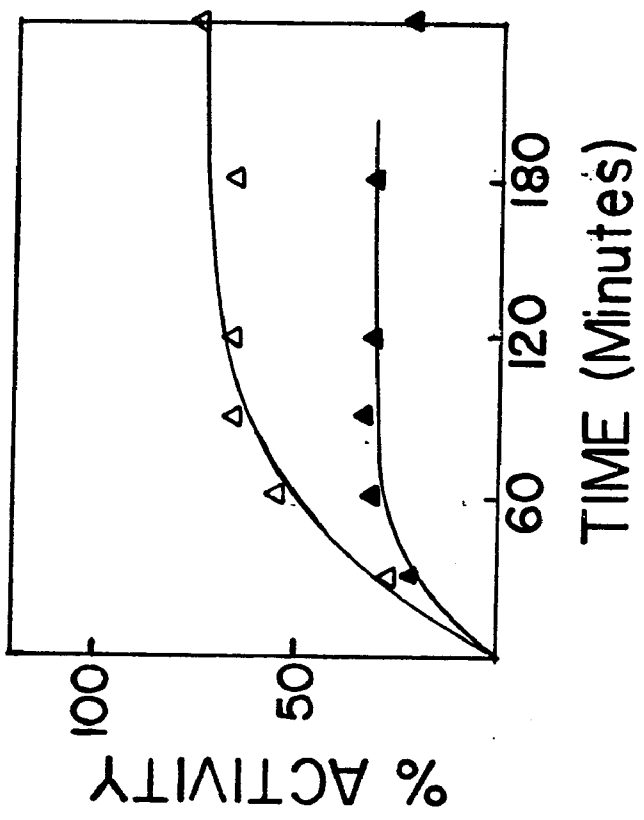
mined by testing 2 neutral, 3 negative, and 2 positive charged blocking reagents.

The modification reagents used and expected product upon reaction with cysteine are shown in Figure 4. It can be seen that NEM and iodoacetamide lead to neutral, iodoacetate, M-2, and M-3 lead to negatively charged, and M-1 and ethyleneimine result in positively charged products. Previous work with ethyleneimine, iodoacetate, and iodoacetamide has shown that the procedure yields complete modification of cysteine 31 and 32 and no modification of other amino acid residues (181,182).

As a control, each regeneration was run at 25 to 30°C as well as 43°C to be certain that the modification did not affect optimal refolding. The regenerations were performed under the optimal glutathione conditions in pH 8.2, 0.1M tris-acetate buffer containing 1mM EDTA. Figure 24 shows the effect of the various modification reagents (excluding M-3) on the regeneration at 43 and 25 to 30°C. The modifications had no large effect upon the regenerations at the moderate temperatures; however, in every case the sharp temperature dependence at 43°C in the regeneration of the native enzyme is at least partially reversed. Moreover, the neutral and positively charged modifications mimic the high temperature regeneration of RNaseA more closely than that of the negatively charged modifications. This result is in

Figure 25. Regeneration of M-3 modified seminal RNase. Filled symbols represent experiment performed at 42°C, and open symbols represent experiment performed at 25°C. Regenerations were performed as in Figure 24 at a protein concentration of 0.1 mg/ml.

FIGURE 25



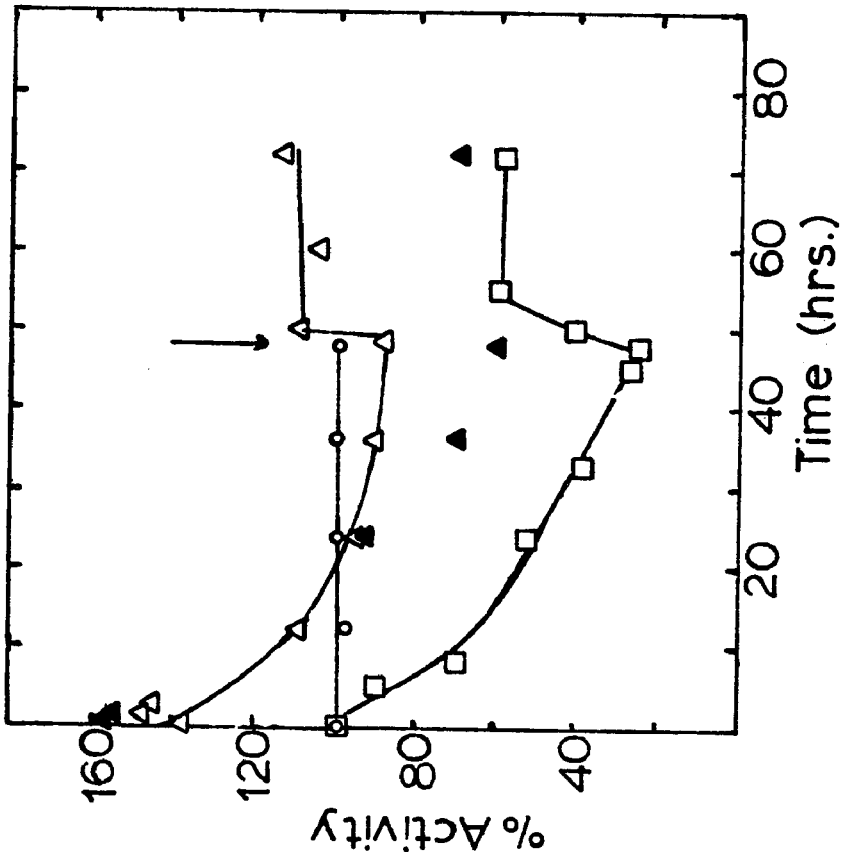
agreement with the fact that RNaseA contains a lysine (positive) and a serine (neutral) at residues 31 and 32, respectively, while seminal RNase has two negatively charged cysteines at these positions (161,165). Thus, the temperature dependence in the regeneration of seminal RNase is largely due to cysteines 31 and 32.

One modification, that of M-3, did not give good regenerations and was not included in the interpretation. This is shown in Figure 25. It can be seen that even the moderate temperature regeneration was poor; thus, this modification altered the basic regeneration mechanism and cannot be used in a comparison with the native state. This modification reagent was the least soluble in aqueous solution of all the reagents used. Thus, it is assumed that the reagent endowed the modified cysteines with hydrophobic character and caused them to form internal hydrophobic bonds or induced protein aggregation.

If the high temperature regenerations were allowed to proceed for longer periods of time, the percent regeneration steadily decreased to approximately 25, 50 or 65% after 48 hours with NEM, M-1, or iodoacetamide, respectively. However, little change was noted for the iodoacetate or ethyleneimine blocked experiments up to 48 hours.

Figure 26. Degeneration of RNaseA (o), seminal RNase (□), and ethyleneimine modified seminal RNase (Δ,▲). Native enzyme was incubated at 42°C in pH 8.2 tris-acetate containing 3mM GSH/0.3mM GSSG under a nitrogen barrier. Protein concentrations were 0.04 mg/ml for RNaseA and 0.085 mg/ml for native and modified seminal RNase. At the arrow, the temperature was decreased to 25°C.

FIGURE 26

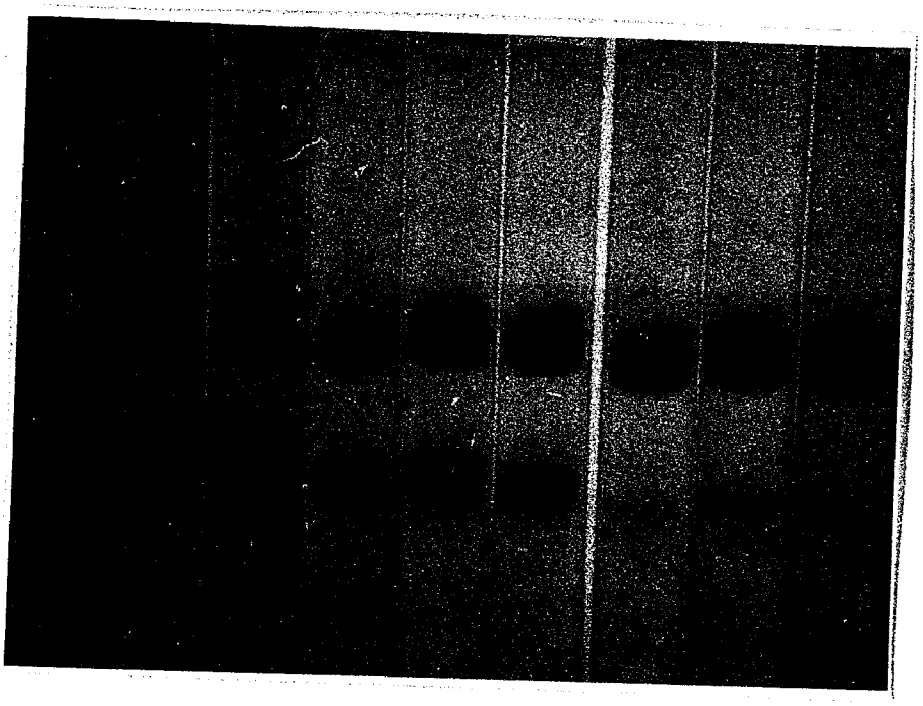


F. High Temperature Degeneration Studies. The effect of these modifications on the regeneration of seminal RNase apparently arises from increased thermal stability of the modified protein. The effect of incubating native RNaseA, native seminal RNase, or ethyleneimine modified seminal RNase in 3mM GSH/0.3mM GSSG, 0.1M tris-acetate, 1mM EDTA, pH 8.2 at 42°C is shown in Figure 26. Clearly RNaseA maintains 100% of its initial activity while native seminal RNase loses its activity. After two days the temperature was decreased to 25°C, and some seminal RNase activity returned; however, since the return of activity leveled at 60% rather than over 100% the process is partly irreversible. If the 42°C incubation exceeded two days, activity continued to decrease, and the process became more irreversible. On the other hand, it can be seen that ethyleneimine modified seminal RNase loses less activity under the same conditions. The activity loss was variable and the extent of irreversible denaturation increased with increased loss, but it was consistently found that the modified protein retained more activity than the unmodified protein.

The irreversible nature of the reaction does not allow the use of thermodynamics; thus, it is not clear where the stabilization arises. However, it is apparent that the modification does impart the enzyme with

Figure 27. SDS-PAGE of selective reduction with GSH. Native enzyme (0.1 mg/ml) was incubated for 14 hours at 30°C in 0.1M tris-acetate, 1mM EDTA, pH 8.2 containing: A, 60mM GSH; B, 60mM GSH/3mM GSSG; C, 60mM GSH/12mM GSSG; D, 3mM GSH; E, 3mM GSH/3mM GSSG; F, 3mM GSH/12mM GSSG; G, 0.3mM GSH; H, 0.3mM GSH/3mM GSSG; I, 0.3mM GSH/12mM GSSG. Electrophoresis was performed on the trapped products in the absence of 2-ME.

FIGURE 27



increased thermal stability.

IV. Reductive Cleavage of Intermolecular Disulfide Bonds

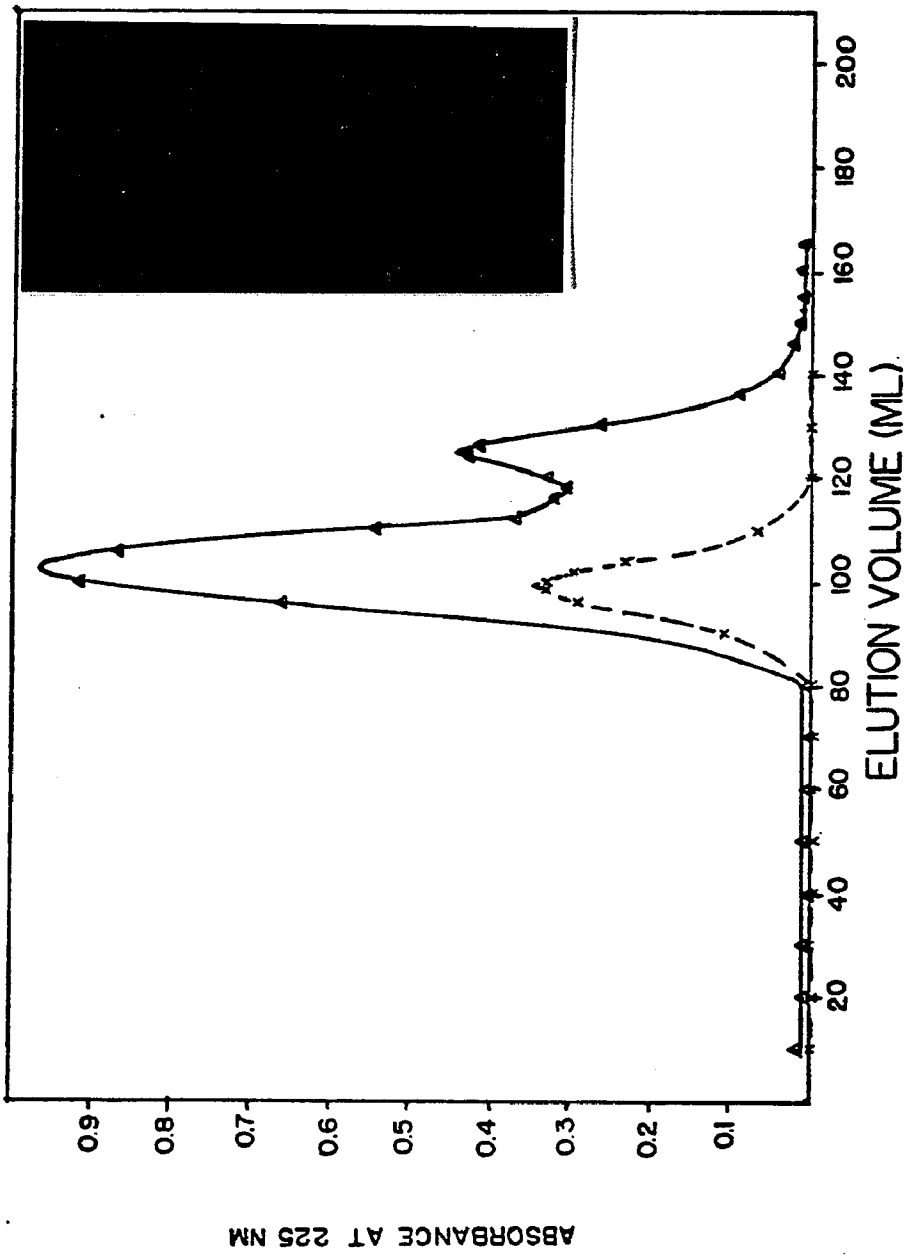
A. Reaction Conditions and Products. It was stated above that cysteine 31 and 32, which form the intermolecular disulfide bonds, can be selectively reduced with DTT (181). Since the glutathione regeneration of reduced-denatured seminal RNase also results in an active monomer, it seemed important to determine the susceptibility of the intermolecular disulfide bonds to low levels of GSH used in the regeneration. If these bonds were cleaved under conditions used in the optimal glutathione regenerations, then it would not be possible to reform native dimer under the conditions we used in the regeneration studies.

To determine the susceptibility of these bonds to GSH reduction, native enzyme at a protein concentration of 0.1 mg/ml was incubated with several glutathione concentrations and redox states for 9-24 hours at 30°C. The reaction was stopped with a 10 fold excess of NEM (over total thiol concentration), and SDS-PAGE was performed on the products. Figure 27 shows the results with 60mM GSH/0-12mM GSSG, 3mM GSH/0-12mM GSSG, 0.3mM GSH/0-12mM GSSG. All conditions caused some reduction of intermolecular disulfide bonds. Three mM GSH produced about 30% monomer. Higher and lower GSH concentration produced more and less monomer, respectively. Interest-

Figure 28. G-75 Sephadex elution pattern of native and selectively reduced seminal RNase. Native seminal RNase (0.1 mg/ml) was incubated at 30°C in 0.1M tris-acetate, 1mM EDTA, pH 8.2 containing 3mM GSH/0.6mM GSSG. After 19 hours the protein was concentrated and applied to a G-75 Sephadex column (80 x 1.5 cm) as in Figure 18.

Inset: SDS-PAGE of native seminal RNase and reduction products. Electrophoresis of native enzyme (A), selective reduction monomer (B), and dimer (C) was performed in the absence of 2-ME.

FIGURE 28


ABSORBANCE AT 225 NM
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20
40
60
80
100
120
140
160
180
200
ELUTION VOLUME (ML)

ingly, there was very little effect of GSSG concentration from 0 ot 12mM.

However, the technique employed did not permit one to determine if the free monomer was formed upon reduction or if the product remained as a noncovalent dimer. To obtain this information, 5 to 10 mg of native seminal RNase were incubated for 19 hours with 3mM GSH/0.6mM GSSG at a protein concentration of 0.1 mg/ml. The products, which possessed an activity of 120 to 150% of the native enzyme, were passed through a Sephadex G-75 column after concentration by ultrafiltration. The column was equilibrated with either 0.1M acetic acid; 0.1M, pH 5.0 sodium acetate; or 0.05M tris-Cl, 1mM EDTA, pH 7.2. The results were independent of the buffer employed. The elution pattern is shown in Figure 28. It can be seen that approximately 30% of the enzyme dissociated to a free monomer while the remaining enzyme stayed in the dimeric form. SDS gel electrophoresis of the products, shown in Figure 28 inset, revealed that the slow moving peak from G-75 was essentially noncovalent while the more rapidly moving peak was essentially covalent dimer.

To verify that the slower peak from G-75 was indeed a monomer and not a noncovalent dimer, crosslinking experiments were performed. The protein in the two G-75 peaks was crosslinked with dimethylsuberimidate (DMS),

Figure 29. SDS-PAGE of selective reduction products crosslinked with dimethylsuberimidate. Purified selective reduction monomer (0.1 mg/ml) (A) and dimer (0.13 mg/ml) (B), and hemoglobin (0.2 mg/ml) (C) were crosslinked at room temperature in 0.1M phosphate buffer at pH 9.0 containing 1 mg/ml dimethylsuberimidate. After 2 hours protein was denatured with SDS in the presence of 0.25M 2-ME. Electrophoresis was performed as described by Weber and Osborn (176) in the presence of 2-ME.

FIGURE 29

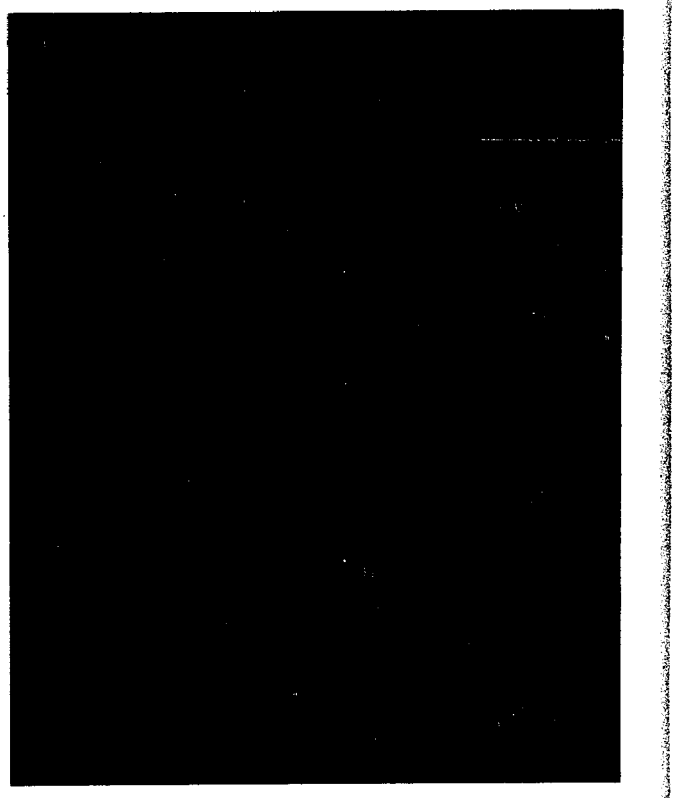


Figure 30. SDS-PAGE of selective reduction products as a function of time. Native enzyme was incubated at 30°C in 0.1M tris-acetate, 1mM EDTA, pH 8.2 containing 3mM GSH/0.6mM GSSG for 5 minutes (A), 15 minutes (B), 30 minutes (C), 1 hour (D), and 22 hours (E), and the reaction was stopped with 30mM NEM. Electrophoresis was performed on the trapped products in the absence of 2-ME.

FIGURE 30

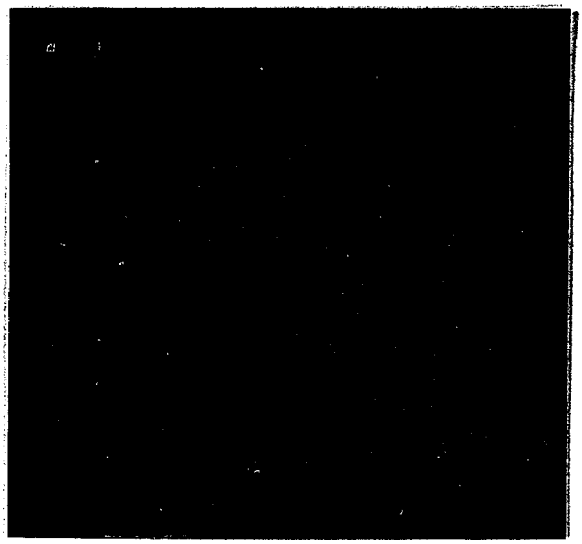
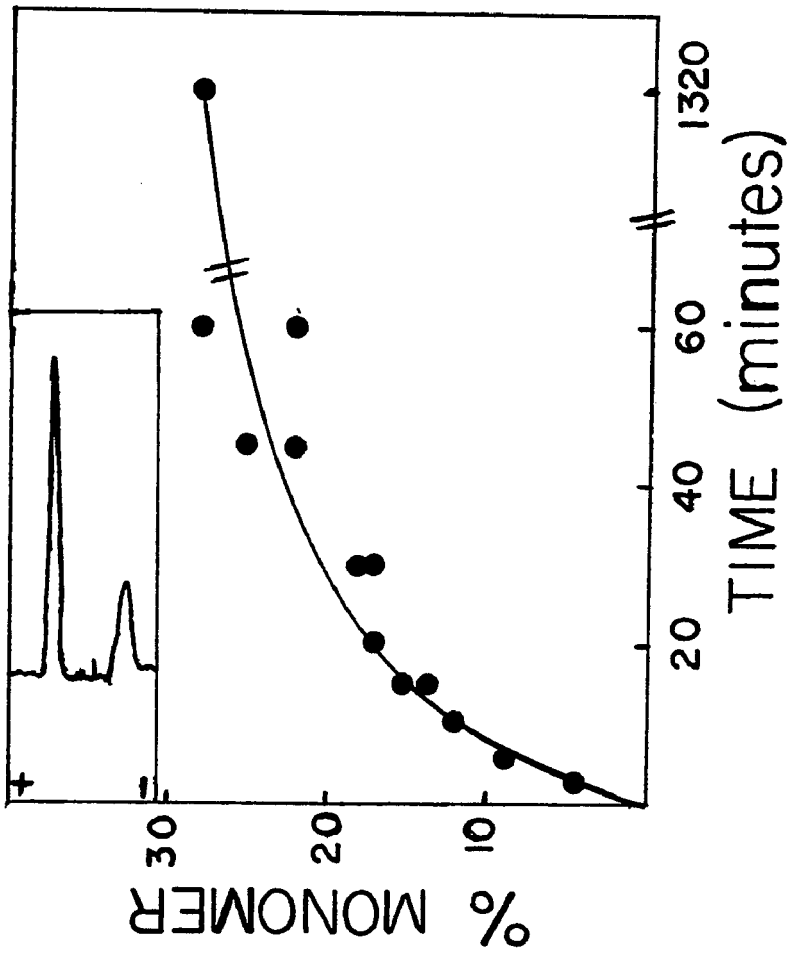


Figure 31. Kinetics of selective reduction. SDS-PAGE gels from Figure 30 (Plus gels for 2, 10, 20, and 45 minutes not shown in Figure 30) were scanned with an ISCO densitometer. Peaks were integrated by cutting out and weighing each. All points represent an average of 4 integrations.

Inset: Densitometer scan of 22 hour gel.

FIGURE 31



reduced with 2-ME, and subjected to SDS-PAGE (191,192). Hemoglobin served as a control. The results are shown in Figure 29. It can be seen that all four hemoglobin bands are clearly visible, and the covalent dimer exhibits two bands as expected; however, only one band is present for the second peak from G-75 which confirms that it is a free monomer.

To further characterize this reduction, the reaction rate was determined. Native enzyme was incubated at 30°C in 0.1M tris-acetate, 1mM EDTA, 3mM GSH/0.6mM GSSG, pH 8.2 at a protein concentration of 0.1 mg/ml. Aliquots of 0.05 ml were removed at predetermined times and quenched with a 10 fold excess of NEM over total thiol. SDS-PAGE was performed on the products. The results at 5, 15, 30, 60, and 1320 minutes are shown in Figure 30. Each gel was scanned with an ISCO densitometer, and the peaks were integrated by cutting out and weighing four replicates of each scan. The rate of the reduction was plotted in terms of percent monomer and is shown in Figure 31. The inset shows the scan of the 1320 minute gel. It can be seen that at least one hour was required for completion; this is in contrast to the selective reduction with DTT, which was complete in less than ten minutes (181).

Since this selective reduction only proceeded to 30% monomer, it was necessary to determine if there was

Figure 32. SDS-PAGE of selectively reduced monomer attempted dimerization. Purified selectively reduced monomer was incubated at 30°C in 0.1M tris-acetate, 1mM EDTA, pH 8.2 containing 3mM GSH/0.6mM GSSG. After 5 minutes (A), 30 minutes (B), 1 hour (C), 5 hours (D), and 24 hours (E) the reaction was stopped with 30mM NEM. Electrophoresis was performed on the trapped products in the absence of 2-ME.

FIGURE 32

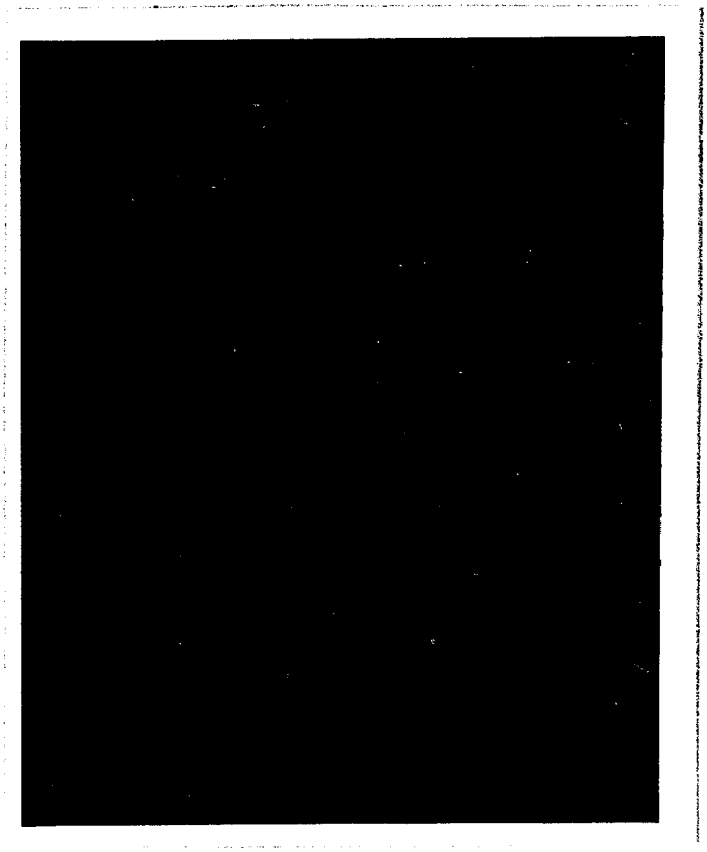


TABLE II

Partial amino acid composition of selective reduction dimer and monomer.[a]

Amino Acid	Native Protien	Reduction products	
		Dimer	Monomer
Alanine	8	8.22	8.01
Aspartate	11	11.1	11.3
Glutamate	11	11.1	12.8
Glycine	6	6.08	7.95
Isoleucine	3	2.54	3.10
Leucine	2	2.05	2.29
Phenylalanine	3	2.84	3.11

[a]Compositions are expresses as residues per subunit. Only stable amino acids from the long column are included.

a 70:30 equilibrium between the selectively reduced monomer and dimer. Selectively reduced monomer purified by G-75 Sephadex chromatography was incubated at a protein concentration of 0.1 mg/ml in 0.1M tris-acetate, 1mM EDTA, 3mM GSH/0.6mM GSSG, pH 8.2 at 30°C. Aliquots were removed at predetermined times and quenched with a ten fold excess of NEM over total thiol. SDS-PAGE was performed on the products, and points from 5 minutes, 30 minutes, 1 hour, 5 hours and 24 hours are shown in Figure 32. The results show that the selectively reduced monomer is not in equilibrium with the dimer at a 70:30 ratio under these conditions. Thus, the reduction is irreversible in the presence of glutathione. The apparent failure of this irreversible reaction to proceed to 100% monomer, as expected, is discussed later.

B. Characterization of Selectively Reduced Monomer. Affinity chromatographic purification of the selectively reduced monomer and the remaining covalent dimer, yields species with 180 and 90% activity, respectively. Each shows a single band on PAGE in the presence or absence of SDS when the leading half of the dimer or trailing half of the monomer peaks are pooled.

Partial amino acid analysis (Table 2) of the selectively reduced monomer indicates incorporation of two moles of glutathione per chain. Thus, the selectively reduced monomer, like the regenerated monomer, is a

glutathione mixed disulfide. In contrast, the remaining dimer showed no evidence of glutathione incorporation (Table 2). This is in agreement with the SDS-PAGE results of the dimer, which show that it is covalent.

Circular dichroism spectra of the selective reduction monomer and dimer (each purified by affinity chromatography) were virtually identical to the spectra for the regenerated monomer and native dimer, respectively. Thus, selective reduction of seminal RNase with glutathione to yield an active monomer causes the same C.D. changes as between native enzyme and refolded monomer. In summary, the structure assumed by the refolded monomer appears to be identical to the enzyme state formed when native enzyme is incubated with the optimal regeneration system.

V. Ivestigation into the In Vivo Form of Seminal RNase.

It is shown above that the intermolecular disulfide bonds of seminal RNase are reduced by glutathione levels normally found in cells (114,115). Thus, it became of interest to determine if the dimeric form of the enzyme was the native form found in vivo or if it was an artifact of purification. To determine this, the enzyme was purified according to the usual procedure; however, all thiols in the semen were blocked with NEM prior to the purification.

Ten ml of semen were incubated at pH 6.5 with 100mM NEM at room temperature for 15 minutes; at this pH all free thiols react within the first few minutes while lysine residues are not modified (193). Following the reaction the plasma was desalted on G-25 Sephadex at 4°C in the presence of EDTA; the EDTA drastically reduces any disulfide exchange (99). Following these preliminary steps, the original purification procedure was followed; however, until the SP-Sephadex step 1mM EDTA was maintained in the solutions to prevent disulfide interchange.

If the enzyme occurred in vivo as the dimer no change in the purified enzyme should have been seen. However, if the enzyme existed as a monomer with the free thiol at 31 and 32, the purified enzyme should have also remained monomeric. However, if the enzyme occurred in vivo as the glutathione mixed disulfide, NEM could not block disulfide exchange, and intermolecular disulfide formation could still occur after the NEM was removed by G-25. Thus, EDTA was included to effectively eliminate air oxidation, particularly during the heating step where disulfide exchange would be most likely to occur (194). Further, the mixed disulfide species is quite stable and resists dimerization as shown above. Thus, the glutathione mixed disulfide species should remain intact throughout the purification procedure.

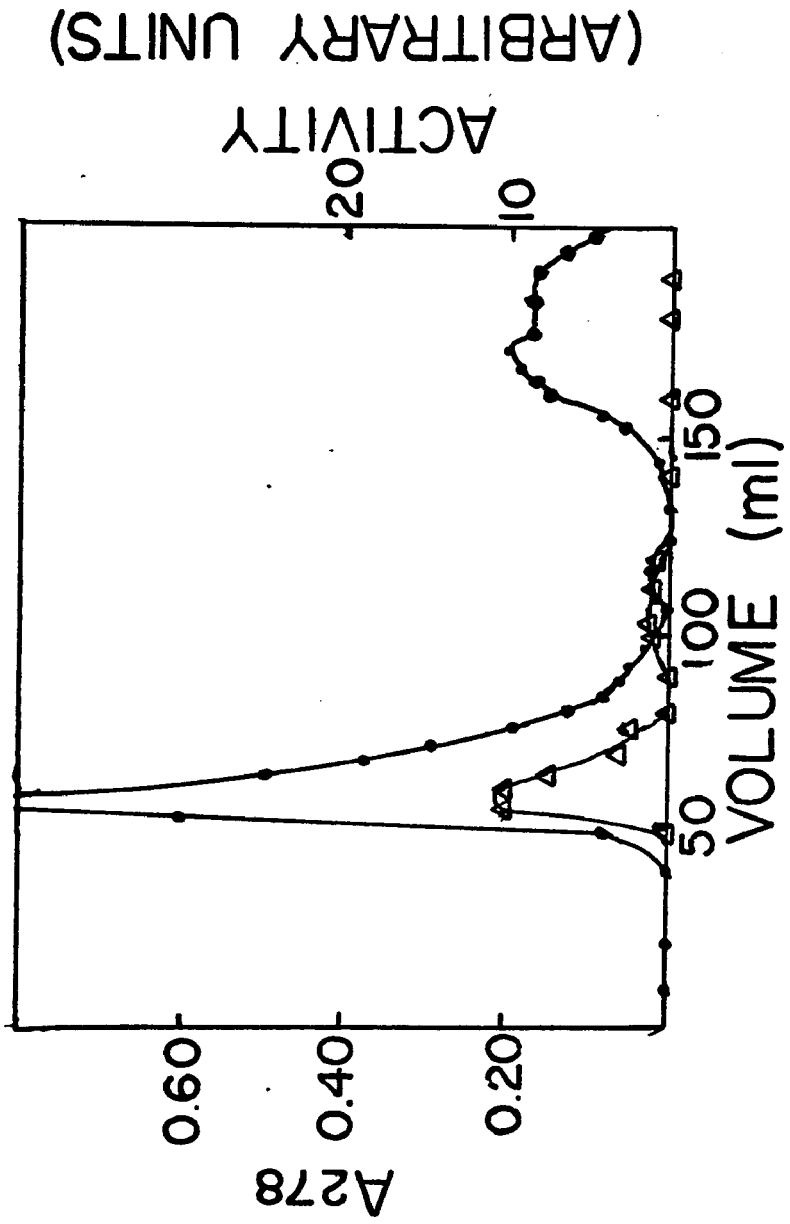
Column chromatography with SP-Sephadex and Cellex-P gave typical elution patterns, except the minor active peak from SP-Sephadex seemed to be reduced in size. From the original 10 ml of seminal plasma, 6.5 mg of seminal RNase were recovered, a good yield. Thus, a large percentage of the enzyme was not modified by the NEM treatment and lost during purification. Characterization of the enzyme showed that it eluted from G-75 with the native enzyme, migrated on SDS gel electrophoresis identical to the native enzyme, had activity equal to the native enzyme, and regenerated identical to the native enzyme. Thus, although the mixed disulfide species can not be completely ruled out, it appears as though seminal RNase occurs in vivo as the covalent dimer.

VI. Attempts to Induce Dimerization of the Regenerated Monomer with Seminal Vesicle and Seminal Plasma Extracts

Based on the above results the native form of the enzyme appears to be dimeric; however, regeneration of reduced-denatured seminal RNase with glutathione leads to the formation of an active monomer. Thus, it is of interest to determine if there is some factor in seminal vesicles or seminal plasma which allows the formation of this dimer. Preliminary experiments to this end have not been successful.

Figure 33. G-75 Sephadex of attempted monomer dimerization with a seminal vesicle extract. Reaction products were applied to a (80 x 1.5 cm) G-75 Sephadex column equilibrated with 0.05M phosphate buffer containing 0.85% NaCl at 4°C, pH 7.5. Elution was followed by A_{278} (—●—●—) and RNase activity (—▲—▲—). Flow rate was 1 drop/3 seconds, and 2.5 ml fractions were collected.

FIGURE 33

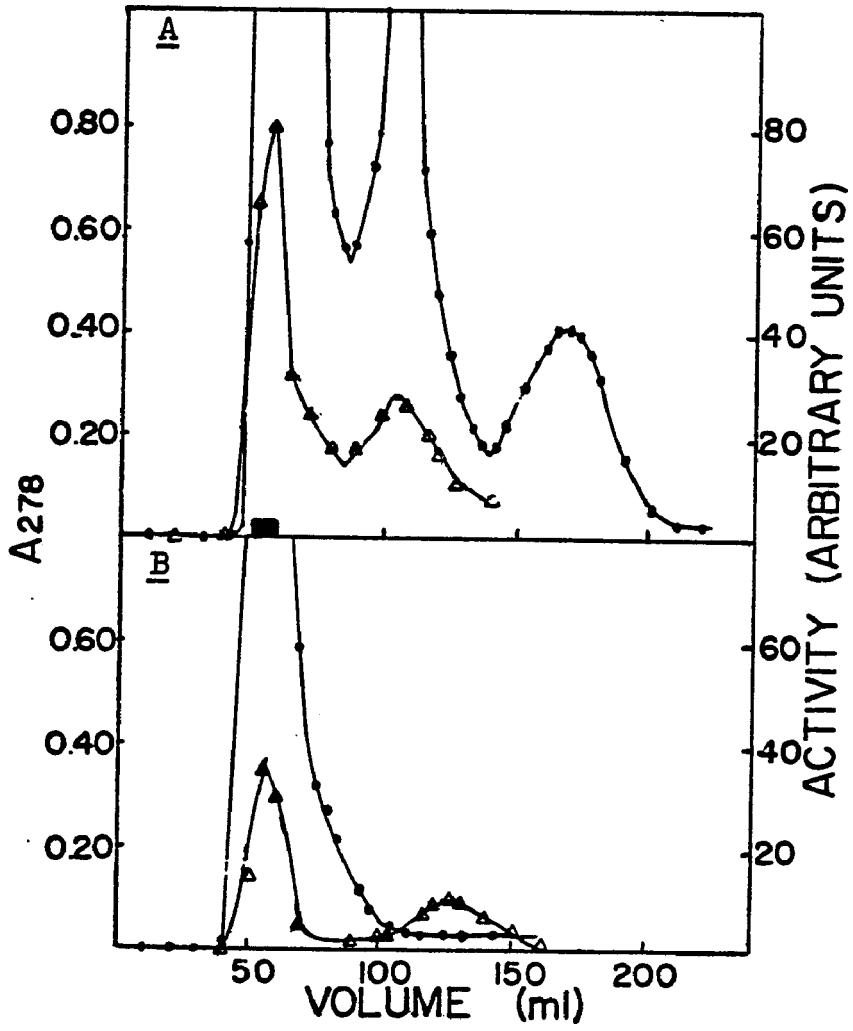

A278
0.60
0.40
0.20
VOLUME (ml)
50
100
150
ACTIVITY
(ARBITRARY UNITS)
20
10

Seminal vesicles from two steers were removed and homogenized according to Goldberger, et al. (100). The 17,000 X G supernatant was used for the dimerization experiments. RNase activity in the homogenate was very low and not in agreement with data from Farina, et al. on bull seminal vesicles (162). Two ml of the seminal vesicle extract at pH 8.0 were incubated with 2 ml of regenerated monomer (mixed disulfide species) in 0.05M tris-Cl, pH 8.0. Activity decreased from 155 to 95% in 50 minutes and was constant for the next 10 minutes. After 60 minutes of incubation the products were eluted through G-75 Sephadex at 4°C. Figure 33 shows the elution pattern. It can be seen that virtually all of the activity eluted in the high molecular weight fraction; thus, it was not possible to determine if the monomer had been converted to the dimer. Since it was not certain that seminal vesicles had been obtained (only tentative identification could be made); the RNase activity was low; and steer (rather than bull) seminal vesicles, which may have not been actively synthesizing, were used, the experiments were discontinued.

Similar experiments were also performed with seminal plasma. The seminal plasma had more activity than the monomer added; thus, it was not possible to follow activity accurately. However, no significant activity change was observed over 55 minutes at 30°C.

Figure 34. A: G-75 Sephadex of attempted monomer dimerization with seminal plasma. Reaction products were applied to and eluted from a (80 x 1.5 cm) G-75 column as in Figure 33. Reaction was performed as described in text. B: Rechromatography of high molecular weight fraction after incubation with DNaseI. High molecular weight fraction from Figure 34A was pooled as indicated by black bar and incubated with DNaseI as described in text. Products were then reapplied to the same G-75 Sephadex column and eluted as in Figure 33.

FIGURE 34


A
B
0.80
0.60
0.40
0.20
A278
80
60
40
20
ACTIVITY (ARBITRARY UNITS)
50
100
150
200
VOLUME (ml)

The products were eluted from G-75 Sephadex, and the results are shown in Figure 34A. They indicate that most of the activity bound to the high molecular weight fraction analogous to the seminal vesicle experiment, and a small fraction eluted at the dimer position.

It has been shown that RNaseH type of RNases (RNases which specifically cleave double stranded RNA) bind tightly to DNA (195). Thus, it was thought that the enzyme may have bound to high molecular weight DNA. To determine if this was the case, the high molecular weight fraction from Figure 34A was pooled and incubated with 2 mg of DNaseI and a few mg of $MgCl_2$ at 30°C for 30 minutes. The products were then passed through G-75 Sephadex again as shown in Figure 34B. The results show that some RNase was liberated from the high molecular weight fraction and was likely bound to DNA. It is interesting that the liberated enzyme eluted later than the free enzyme in 34A. Thus, the enzyme was likely in the monomeric form and had not dimerized in the semen.

Considerable work remains to be done with the regeneration of this enzyme. The most important problem to be answered is how the dimer is formed <u>in vivo</u>. It would also be of interest to determine if the rat liver disulfide interchange enzyme will accelerate the regeneration rate and cause dimerization.

Further characterization of the thermal stability differences of RNaseA, seminal RNase, and its modified analogues would also be important. Such a characterization may lead to a better understanding of the factors involved in protein stability.

DISCUSSION

I. Affinity Chromatography

Affinity chromatography makes use of an immobilized competitive inhibitor for a specific enzyme (in this case RNase) to retain that active enzyme on a solid support while excluding inactive enzyme and other proteins. Thus, affinity chromatography is the best technique for purification of refolding products since it will not bind inactive enzyme.

Previous studies have generally utilized one affinity material, the commercially available Sepharose coupled 5'-(4-amino-phenylphosphoryl)uridine 2'(3')-phosphate (Sepharose-aPhpUp) for RNase affinity chromatography (175,187,188,196,197). The present study was undertaken to characterize 5' UTP agarose, another affinity material. The results clearly indicate the biospecificity of RNase binding to 5' UTP-agarose under the conditions employed.

The buffer system used most commonly for RNase affinity chromatography employs low ionic strength, pH 5.2, acetate buffer to bind the enzyme to the column and higher ionic strength acetic acid, a deforming buffer, to elute it from the column. However, it has been shown that this sytem exhibits considerable non-specific adsorption of other proteins (175). Two other

buffer systems have been shown to reduce nonspecific binding to Sepharose-aPhpUp. These alternatives employ either a higher ionic strength acetate buffer or a low ionic strength buffer of the heterocyclic cation piperazine to bind the enzyme (175,188). Both systems decrease nonbiospecific ionic interactions. The piperazine buffer, which was chosen here, was originally employed by Stewart and Stevenson (175) to bioadsorb bison-bison pancreatic RNase to Sepharose-aPhpUp. They suggested that nonspecific binding to that matrix was due to ionic interactions (175,188,198) and more specifically to anionic, probably carboxyl groups on the agarose support (198). They postulated that piperazine competed for these anionic groups more efficiently than the sodium ions derived from the sodium acetate buffer (187). The results presented here are in direct agreement with this hypothesis since it was found that piperazine-HCl eluted nonbiospecific proteins carrying a positive charge at pH 5.3 more efficiently than the sodium acetate buffer (Figures 5,8).

Elution from RNase affinity columns is usually accomplished with a "deforming buffer," that is, a buffer which distorts the enzyme active site to reverse binding (186). The most commonly used deforming buffers are acetic acid or low pH phosphate buffer (175). However, neither system is required to remove RNase from 5' UTP-

agarose since greater than 95% recovery is possible with pH 5.45 phosphate buffer. Indeed most efficient elution occurred at pH 5.45. This type of elution is preferable to protect the enzyme from acid denaturation.

Biospecific binding to and elution of RNaseA from 5'-UTP-agarose was clearly demonstrated in the competitive elution experiments. The competitive inhibitor 2'(3') UMP not only eluted the enzyme, but a plot of $1/(V-V_o)$ versus soluble inhibitor concentration was linear as predicted by equation (1) for competitive elution (page 90). Conversely, if the insoluble ligand had been a noncompetitive inhibitor of RNase and bound somewhere other than the active site, $1/V-V_o$ versus soluble inhibitor would not have been linear and would have obeyed equation (2) (see appendix for derivation):

$$\frac{1}{V-V_o} = \frac{K'}{V_o\,[IM] + [I](V_o)[IM]/zK} + \frac{I\ zK'}{V_o\,[IM]zK + [I]V_o[IM]} \qquad (2)$$

where K = the dissociation constant for (RNase-soluble inhibitor) complex, K' = the dissociation constant for (RNase-insoluble inhibitor) complex, and z = K"/K' where K" is the dissociation constant for binding of (RNase-soluble inhibitor) to the insoluble inhibitor. All other terms are identical to those defined for equation (1). Clearly this equation predicts nonlinear asymptotic elution curves when z is greater or less than 1, and that elution is independent of I when z=1.

The competitive elution experiments are also supported by the excellent agreement between the K_I calculated here for 2'(3') UMP and the literature value calculated from difference spectra (189).

Evidence for biospecific binding of seminal RNase was given for binding of native and monomeric forms. Twice as much dimer as monomer binds to the column by weight. There are two possible explanations for this phenomenon. The enzyme could be exhibiting half-of-the-sites binding in the dimer, as has been suggested for the binding of this enzyme to small molecular weight inhibitors at this pH (199). Alternatively, the 5' UTP could be bound to the matrix such that when one active site binds, the second site is spacially prevented from binding another 5' UTP moiety. Both of the alternatives will lead to twice as much dimer (by weight) on the column as monomer. Further, both explanations require bio-specific binding.

Similarly, RNase BS-2 purification by 5' UTP-agarose supports biospecific binding. Only 2 bands were found on SDS-PAGE after the enzyme was passed through the affinity column, and these bands were coincident with the mobility of the seminal RNase monomer and dimer.

Since the column did not bind extraneous proteins or inactive RNase but did bind RNaseA, seminal RNase monomer, and dimer tightly, it effected good purifications

in the refolding experiments. Further, since this is a previously unrecognized RNase affinity material, it should prove quite useful in conjunction with other resins, for RNase ligand binding studies.

II. Regeneration Studies

It is clear that the kinetics and extent of seminal RNase regeneration are dependent upon the regeneration conditions used, in good agreement with the results from a variety of proteins (99,113,122). In particular, the similarities to the regeneration of RNaseA are quite striking. Both processes were affected by the glutathione concentration and redox state, and the temperature of regeneration (99). Maximal regeneration occurred under reducing conditions and at moderate temperatures. In similarity to other enzymes the rate and extent of regeneration decreased when reduced glutathione concentration was increased to 30mM or decreased to 0.3mM (113). Changes in oxidized glutathione had less of an effect since the yield of products was similar when GSH was fixed at 3mM and GSSG was varied from 0.03 to 1.2mM; however, in agreement with other proteins, when the GSSG concentration was 3mM or greater, rates and extent of regeneration decreased (99,113).

The occurrence of optimal regeneration under reducing conditions is in good agreement with the redox conditions found in vivo. It has been shown that

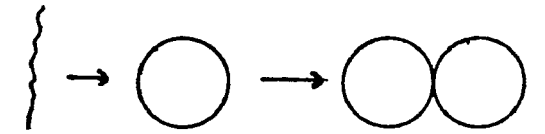
GSH/GSSG ratio in rat liver is approximately 3mM/0.06mM (114). Further, several authors have shown that the protein synthetic apparatus is protected by thiols (200); thus, if proteins fold on or near the ribosomes, they must fold in a reducing environment. Lastly, the effect of glutathione on regeneration is in good agreement with the proposed mechanism of glutathione enhanced regeneration. The first disulfides to form in RNaseA are thought to be at least partially random (121). Thus, these wrong disulfide bonds must be reduced again before formation of correct disulfides can occur. Reducing conditions facilitate this reduction and increase the rate of folding.

The regeneration of seminal RNase was found to be independent of protein concentration indicating that any slow step must be unimolecular. This was not the case in air regeneration of either seminal RNase or RNaseA where a dramatic effect of protein concentration was seen and inferred some intermolecular slow step (98,201).

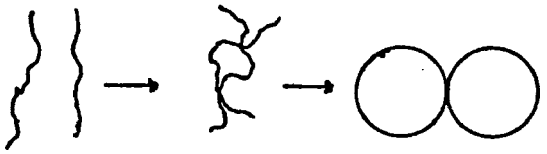
The regeneration of seminal RNase was expected to be highly dependent upon protein concentration (reduced seminal RNase) since the monomeric species exhibits twice the activity of the dimer toward yeast RNA. At higher concentration it was reasoned that dimer formation would be favored and that recovery of enzymatic activity would be less than at lower protein concentrations where

the monomer would be favored. This was not the case. Indeed, seminal RNase refolding kinetics did not show any effect of protein concentration when regeneration proceeded in a solution containing 3mM GSH and 3mM GSSG (Figure 14). Under these conditions refolding led to extensive noncovalent association (Figure 19). Thus, although subunit association clearly did occur under these more oxidizing conditions, intramolecular folding must have proceeded first, rather than a concerted intermolecular subunit folding-association. This is shown schematically below (Scheme II).

Scheme II



(1) Two step



(2) Concerted

Mechanism 2 would be expected to have a clear protein concentration effect. The concerted folding step would require intermolecular interactions which would be less frequent at lower protein concentration. Thus, the regeneration would be slower at lower concentration. This is in good agreement with results obtained under optimal, more reducing conditions where subunit folding occurred without association. It is not possible to

determine which step in mechanism 1 is rate limiting under oxidizing conditions since the activity of the monomer folded under these conditions is not known. (It cannot be assumed that it has the same activity as the optimally folded monomer.) In contrast, it is clear that under more reducing conditions, subunit association is rate limiting and indeed precluded.

A mechanism involving first subunit folding followed by association is in good agreement with results from other multisubunit proteins. Pig heart or muscle lactate dehydrogenase, rabbit muscle aldolase, Saccharomyces carlsbergensis pyruvate kinase, and rabbit IgG have all been shown to fold within the individual subunits before association occurs (127,134,137,141-143). The rate limiting step in these proteins is different in each case and depends upon protein concentration. Lactate dehydrogenase (heart and muscle) refolding has been shown to have a slow association step (142). However, at higher concentrations, it has been shown that the rate determining step is unimolecular (141). At these high concentrations Tenenbaum-Bayer and Levitski found 100% regeneration of activity (141), but Rudolph, et al. have shown that poor regenerations occurred (presumably due to aggregation) (142). Aldolase folding also depends upon protein concentration. Association is slow at low concentration and proceeds spontaneously at high

concentrations (202). The rate limiting step of S. carlsbergensis pyruvate kinase appears to be unimolecular based upon first order refolding kinetics (127). The rate limiting step in IgG folding has not as yet been reported; however, it is clear that subunit folding precedes association, and the latter occurs smoothly following intramolecular folding (117,137,139).

The effect of protein concentration upon the folding of other multimeric proteins suggests that there is a critical concentration above which association proceeds spontaneously and below which association is slowed. It could be argued that the reason for the poor association with seminal RNase is because the studies have been done below this concentration. However, it is clear that air oxidative refolding (Figure 20) does proceed to a covalent dimer at concentrations used in some of the glutathione refolding experiments. Thus, the reason for poor dimerization is unlikely related to protein concentration.

The effect of pH upon regeneration also agrees with that of RNaseA and other proteins (99,113). It suggests a systematic titration of similar group(s) in all cases. In this study the pK of this group appears to be about 7.5. Positive identification of the group is not possible; however, protein thiol groups are likely candidates (pK approximately 8.3). Enzyme cysteines, rather than the GSH cysteine, are likely responsible since the

effect was also seen in air oxidation of RNaseA (98).

The effect of pH is clearly an effect on the reaction kinetics. At pH 6.85 where the reaction is very slow, the product eventually reaches the same activity as the products of the pH 8.2 reaction. Thus, titration of this group does not cause the enzyme to reach any wrong conformation by breaking some critical salt bridge, rather the search for the correct conformation is merely slowed. This is in agreement with the view that the titrated group is a thiol. Incorrect disulfide bonds must be formed and broken during the structure search, and this disulfide exchange is slowed when the sulfhydryl is protonated (113).

The products of regeneration of seminal RNase and RNaseA are also quite similar. Both processes proceed to a monomeric product which exhibits similar structural properties as characterized by circular dichroism. The spectra have similar general shapes, troughs, crossover wavelengths, and maximum ellipticities (170). In contrast, the spectra are different from that of the native seminal RNase. Thus, the refolded enzyme has a slightly different structure from the native enzyme but is similar to RNaseA.

Disulfide bonds exhibit significant negative ellipticities in the far UV (203). Thus, it could be argued that the difference between the circular dichroism spectra

of native and refolded seminal RNase arises from breaking the intermolecular disulfide bonds and replacing them with the mixed disulfide of glutathione. However, the similarities between the spectra of the seminal RNase monomer and RNaseA argues against this. Further, disulfide contributions to the spectra would be small (203). Indeed, Bewley (204) has found no difference between the far UV circular dichroism of human pituitary growth hormone (a protein whose secondary structure remains intact upon reduction of its two disulfides) and that of the reduced or reduced, alkylated protein hormone. Thus, the disulfide bond contribution to the far UV CD is negligible in a protein molecule compared to the overall peptide bond ellipticities. Similarly, the CD differences between the refolded seminal RNase monomer and dimer are likely due to secondary structural differences.

The above discussion suggests similarities in the folding mechanism of seminal RNase and RNaseA. There are, however, clear differences between the two regenerations. First, total yield of the major product in seminal RNase folding is lower than in RNaseA folding (99). RNaseA refolding yields greater than 90% native activity while seminal RNase regeneration yields approximately 65% of the 200% active monomer. Second, based upon recovery of 130% final activity (65% recovery of

active monomer) in seminal RNase, the $t_{1/2}$ for seminal RNase refolding is approximately 90 minutes while $t_{1/2}$ for RNaseA refolding equals 75 minutes (99). In addition, seminal RNase kinetics do not fit a simple first order plot while RNaseA kinetics do (99). Third, both enzymes exhibit maximal rates of regeneration in the range 25 to 30°C, and both processes are similarly slowed at lower temperatures (14°C) (99). However, the effect of high temperature is larger on seminal RNase than RNaseA. The latter reaches 75% activity recovery at 42°C in 240 minutes while the former never rises above 33% of the native activity under similar conditions. These differences suggest slightly different molecular folding mechanism.

The difference in the folding mechanisms at high temperatures lies in the different thermal stabilities of the two enzymes. RNaseA maintains 100% activity upon incubation at 42°C while seminal RNase loses activity, and the loss is only partially reversible. This indicates that one of the several differences between the sequences of RNaseA and seminal RNase must cause a rather large difference in the thermal stabilities of the two enzymes.

Eighty percent of the residues from RNaseA are conserved in seminal RNase. The most obvious changes between them are four extra lysines, two extra cysteines,

and one extra proline in seminal RNase (per chain). It is especially interesting that 35% of the potential hydrogen bond forming amino acids in RNaseA are either replaced by non hydrogen bonding groups or inserted into different places in the seminal RNase monomer. On the other hand, hydrophobicity is largely conserved in seminal RNase. The only change of a hydrophobic group occurs in gln 28 in RNaseA, which becomes leu 28 in seminal RNase. The stability and folding differences may be caused by one of these differences.

Dimerization does not occur during the regeneration of seminal RNase at either moderate or high temperatures; thus, one can modify cysteines 31 and 32 to investigate their role in the mechanism of folding. If either refolding kinetics or yield of the modified enzyme are different from that of the unmodified enzyme under a given set of conditions, it seems reasonable to assume that the residues are important in the folding mechanism. Clearly, this appears to be the case for the high temperature regeneration of seminal RNase since modification of cysteine 31 and 32 reverses much of the temperature dependence. Thus, the conversion of lysine 31 and serine 32 of RNaseA to cysteine 31 and 32 of seminal RNase causes a significant change in the temperature dependence of regeneration. Since it is clear that these residues do not change the regeneration by forming intermolecular

disulfide bonds, because the unmodified product was monomeric, the change must occur intramolecularly. Further, the percent yield and kinetics of regeneration at moderate temperatures do not change significantly; thus, the modification of cysteine 31 and 32 do not cause an observable change in the optimal regeneration mechanism.

The change in regeneration upon modification apparently arises from the increased thermal stability of modified enzyme. RNaseA maintains 100% activity upon incubation with glutathione for two days at 42°C while the native seminal RNase loses greater than 75% of its activity. Conversion of the cysteine residues 31 and 32 to aminoethylcysteine (with ethyleneimine) decreases this activity loss; however, the mechanism of this increased stability is unclear. The problem, in part, arises from the irreversible character of the denaturation. If the temperature is decreased to 25°C after 24 hours of native enzyme <u>regeneration</u> at 42°C, over 100% native activity is recovered; however, a similar decrease in temperature after 48 hours of <u>degeneration</u> at 42°C results in the recovery of only 60% activity. Longer degeneration times result in less activity return upon lowering the temperature. Clearly, there is more than one process occurring which results in reversibility under some conditions and irreversibility under others (apparently longer time). Thus, one cannot apply denaturation thermodynamics to

the process since it requires complete reversibility (6-8).

The partial reversibility of this thermal inactivation has been noted in the high temperature glutathione facilitated regeneration of lysozyme (144). However, temperatures above 60°C were required to achieve the poor regenerations. Although this temperature stopped the regeneration, a subsequent temperature decrease caused activity to return. Lysozyme recovered most of its activity in one hour at 37°C while seminal RNase required over 24 hours to recover its activity at 25°C. As pointed out by those authors (144), the spontaneous regeneration upon a temperature decrease proves that high temperature inactivation does not have to depend upon irreversible changes in the protein.

Rather, the high temperature species must be a high entropy stabilized species in equilibrium with other refolding intermediates. When the heat necessary to maintain the species is removed, it decays to the native enzyme which is more stable at lower temperatures. This equilibrium is further suggested by the smooth (though large) temperature dependence of the regeneration kinetics. At 36°C (lower temperature) the equilibrium is shifted toward the native species and more activity is recovered in the regeneration (approximately 90%).

Irreversible inactivation likely occurs from aggregation of this species or some other intermediate, similar to irreversible denaturation of other proteins (23-27).

The modification results allow us to say something about the molecular mechanism of folding. Creighton (118,145-148) has pointed out that there is a required folding mechanism for bovine pancreatic trypsin inhibitor (BPTI). This protein possesses three native disulfide bonds and will refold to its native structure following reduction and denaturation. Further, the disulfide bond formation scheme has been determined. The last disulfide bond to form along the pathway is between cysteine 14 and 38. However, one of these residues must first form a wrong disulfide intermediate with one other half cystine before folding can proceed correctly (see scheme 1, page 41). Modification of residues 14 and 38 with iodoacetamide or iodoacetic acid prevents formation of the two disulfide intermediates which would normally lead to native protein. Clearly, cysteine 31 and 32 in seminal RNase are not involved in any such mechanism. The enzyme with cysteine 31 and 32 modified exhibits optimal regeneration kinetics (moderate temperature) identical to the unmodified enzyme. Thus, if there is a required folding mechanism for seminal RNase at moderate temperatures, cysteine 31 and 32 are not involved. Only

the half cystines conserved from RNaseA can be involved; this supports the similarities observed in the kinetics and product characterization of seminal RNase and RNaseA, and the statement that the mechanisms therefore appear to be similar.

Conversely, cysteine 31 and 32 are involved in the folding of seminal RNase at higher temperatures. The folding mechanism at 42°C appears to change when cysteine 31 and 32 are modified and takes on characteristics similar to RNaseA folding. In support of the high temperature mechanistic similarities between RNaseA and modified seminal RNase is the effect of modifier group charge. It was seen that although all modifying groups gave better refolding at 42°C, the positive and neutral groups gave results more similar to RNaseA while the negatively charged groups showed kinetic profiles between both RNases. This difference is likely due to the fact that RNaseA possesses a lysine and serine at residues 31 and 32, respectively, and, thus, a positive charge. On the other hand, seminal RNase has cysteines at these positions which would be negatively charged at pH 8.2. One might speculate that unmodified seminal RNase and seminal RNase modified with a negatively charged group could form some internal salt with some positively charged group of the protein. The neutral and positively charged modifying groups could not form such a salt bridge and

would likely behave like lysine on RNaseA.

The regenerations with the negatively charged groups were not identical to native seminal RNase folding at 42°C. This implies that the formation of this hypothetical salt bridge can not be the sole cause of the high temperature effect. This is also reflected in the thermal degeneration of RNaseA and seminal RNase. Although the modification reversed much of the thermal denaturation of seminal RNase, they did not cause the stability to be as great as RNaseA at 42°C. Thus, there are other factors besides cysteine 31 and 32 which are involved in the thermal stability difference between the two enzymes.

Thermal denaturation studies of RNaseA with its disulfide bonds intact have been performed (8). The results are consistent with a mechanism involving increased conformational entropy and solvent clathrate melting, leading to hydrophobic bond disruption. However, it has been shown that RNaseA assumes random structure when its disulfide bonds are reduced and alkylated (205); thus, these crosslinks are also important in maintaining native structure. If these two stabilizing factors are all that maintain RNaseA structure, a change in thermal stability would not be expected if internal hydrogen bonds are lost while hydrophobic and disulfide bonds are maintained.

Aminoethyl seminal RNase and RNaseA have identical disulfide bonds and half cystines, and they have very similar hydrophobic bonding; however, the two proteins have different hydrogen bonding capacities. The above theory implies that they should have similar thermal stabilities. Although regenerations at 42°C are similar, thermal stabilities appear different. RNaseA maintains its activity at 42°C while aminoethyl seminal RNase loses some of its activity.

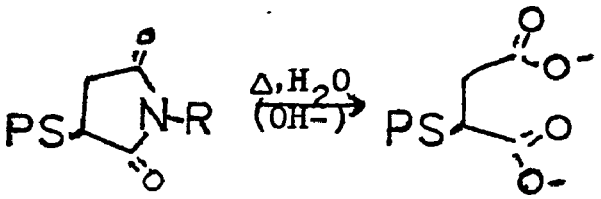
One wonders if the different thermal stabilities arise from the hydrogen bonding changes. If this is the case, thermal stability and denaturation is not adequately described by the above theory.

There are also a number of other individual changes between the two enzymes such as 5 prolines per chain in seminal RNase and 4 in RNaseA (161,165). One of these changes may also play a role in the thermal stability differences. Thus, the above discussion is not meant as proof for or against any thermal stability models. Rather, it was meant to point out what appear, on the surface at least, to be discrepancies with the popular theory.

It was noted in the high temperature regenerations of both maleimide and iodoacetamide modified seminal RNase that the regenerated activity was not stable at 42°C. This, however, was not seen in the iodoacetic

acid or ethyleneimine blocked species. It is likely the reason for this is the hydrolysis of the succinimide product of maleimide modification and of the carboxamidomethyl product of iodoacetamide modification to the corresponding carboxylic acids. This is shown in scheme 3 a and b, respectively:

Scheme 3:



(a)

(Succinimide)

$$P\text{-}S\text{-}CH_2CONH_2 \xrightarrow[(OH^-)]{\Delta,\ H_2O} P\text{-}S\text{-}CH_2COO^- \qquad (b)$$

(Carboxamidomethyl)

There is considerable evidence for these reactions and the kinetics of activity loss are consistent with kinetics of hydrolysis (206,209). If indeed this did occur, loss of activity could then be traced directly to the formation of negatively charged carboxyls, which would be similar to regeneration of seminal RNase modified with the other negatively charged groups. This reaction would also explain results found by Anderson (210) with lysozyme. In his studies Anderson found a loss of ^{14}C-NEM

from modified lysozyme as a function of time. Since the ^{14}C label is on the #1 - C ethyl group from commercial sources, this reaction would indeed lead to a loss of the label.

These reagents (maleimides and iodoacetamide) were not used for the thermal denaturation studies since they are hydrolyzed to other groups. It is seen in Figure 26 that seminal RNase thermal denaturation at 42°C is slow. A significant effect was not seen for 24 hours, and by this time considerable hydrolysis should occur.

A reaction leading to loss of the aminoethyl or carboxymethyl labels of the ethyleneimine or iodoacetate modified seminal RNase, respectively, would not be expected to occur since these modifications are stable to protein hydrolysis in strong acid (193). Thus, the aminoethyl modified seminal RNase could be used for the slow degeneration experiments.

In conclusion, it has been shown that the regenerations of RNaseA and seminal RNase are similar. The major difference in their regenerations was found to be in the temperature dependence. This difference was qualitatively explained by decreased thermal stability of seminal RNase arising, in part, from the conversion of lysine 31, serine 32 in RNaseA to cysteine 31 and 32 in the seminal enzyme.

Further, product characterizations indicate that the structure of the refolded seminal RNase monomer is similar to RNaseA and different from native seminal RNase. The results suggest a structural change when seminal RNase associates.

III. Selective Reduction

The accumulation of glutathione mixed disulfides during regeneration of proteins under optimal conditions is rare. Indeed, such accumulation obviously will not allow formation of correct protein disulfide bonds and preclude native structure formation. However, it is clear that two mixed disulfide bonds with glutathione do in fact form in seminal RNase during regeneration, and reactivation only occurs because the individual subunits are active.

The mixed disulfides proved to be very stable and indeed the mixed disulfide protein was purified and thoroughly characterized. Based upon this characterization it is concluded that cysteines 31 and 32, which normally form the intermolecular disulfide bonds, are involved in the mixed disulfides. Only peptide mapping experiments could verify this conclusion completely, but several pieces of indirect evidence strongly support the conclusion. First, the monomer has twice the activity of the native dimer. Second, the circular dichroism spectra of the refolded monomer, selectively reduced

monomer, and RNaseA are all quite similar. Thus, the active site is intact, and the secondary structure is as expected when compared to RNaseA. On the other hand, if the thiols normally involved in intramolecular disulfide bonds were involved in the mixed disulfides, severe active site distortion and inactivity would be expected. Further, the circular dichroism spectrum of RNaseA with wrongly paired disulfide bonds is quite different from the native enzyme (211).

The glutathione mixed disulfides prevent formation of the native intermolecular disulfide bonds. This is indicated by the lack of significant dimerization under either more reducing or oxidizing conditions while air oxidative regeneration at the same protein concentration(s) readily produces covalent dimer (although the activity and stability of the air regenerated dimer were low suggesting that it was not native). D'Alessio has found that at lower protein concentrations (0.1 mg/ml and below), the percent of dimer formed during air regeneration is decreased; however, at concentrations as low as 0.020 mg/ml greater than 50% covalent dimer is produced (personal communication).

Two facts suggest that dimerization cannot occur in the presence of glutathione: first, the lack of covalent dimerization under the optimal conditions of regeneration and, second, formation of the stable mixed disulfide. To

verify this hypothesis the susceptibility of the intermolecular disulfide bonds in the native enzyme to reduced glutathione was investigated. The results clearly indicate that the intermolecular disulfide bonds are selectively reduced under regeneration conditions. Thus, it is impossible to accomplish 100% dimerization under the conditions employed in the regenerations. Although more oxidizing conditions were shown to prevent reduction of the intermolecular disulfides (Figure 27), poor regenerations occur under these conditions and cannot be used to produce regenerated dimer.

It could be argued that although selective reduction occurs, dissociation is a pH phenomenon and only occurs at pH 3.0 where most gel filtration studies were performed. Such results have been seen with IgG. Below pH 4.8, molecules selectively reduced and alkylated at the intermolecular disulfide bonds readily dissociate to the free subunits. However, when the pH is increased above pH 4.8 they reassociate (212,213). This is clearly not the case with seminal RNase, since gel filtration studies at pH 3.0, 5.0, and 7.2 yield a 70:30 mixture.

By all of our criteria (circular dichroism, activity, amino acid analysis, SDS-PAGE, Reisfeld-PAGE, and G-75 gel filtration) the regenerated and selectively reduced monomers are identical. Thus, it is evident that part of the reason for poor regeneration of native enzyme

is selective reduction of intermolecular disulfide bonds under optimal regeneration conditions. However, DTT also causes selective reduction of these bonds to yield the active monomer (181), but the products reoxidize to a covalent dimer when DTT is removed (D'Alessio, personal communication). The difference between these two reducing agents is that DTT yields the free thiol while the GSH reaction proceeds to the glutathione mixed disulfide. Thus, the mixed disulfide must prevent reassociation-oxidation.

The production of the mixed disulfide monomer must be irreversible since neither the selectively reduced nor regenerated monomers will reform covalent dimer. The basis for this preferential production of monomer during regeneration and irreversible reduction of native enzyme becomes apparent upon examination of the reactions involved in the conversion of the mixed disulfide monomers to the native covalent dimer. This is shown in scheme 4:

Scheme 4:

$$P_{SSG}^{SSG} + 2\,GSH \rightleftarrows P_{SH}^{SH} + 2\,GSSG \qquad (1)$$

$$2\,PSH + 2\,GSSG \rightleftarrows P_{SS}^{SS}P + 2\,GSH \qquad (2)$$

In this scheme P_{SSG}^{SSG}, P_{SH}^{SH}, and $P_{SS}^{SS}P$ represent mixed disulfide monomer, free thiol monomer, and native enzyme. The striking feature of these reactions is the complex dependence upon glutathione redox state. In reaction (1)

GSH will reduce P^{SSG}_{SSG} to P^{SH}_{SH}, and formation of $P^{SS}_{SS}P$ from P^{SH}_{SH} would require GSSG as can be seen in reaction (2). Thus, the redox potential of the two protein disulfide species (P^{SSG}_{SSG} and $P^{SS}_{SS}P$) will control the direction of the overall reaction provided the protein concentration is high enough to allow association. Several lines of evidence indicate that P^{SSG}_{SSG} is the favored species, that reaction (1) favors its formation under the optimal regeneration conditions, and that once it is formed the bulky glutathione groups prevent even noncovalent dimerization. First, attempts to dimerize P^{SSG}_{SSG} in the presence of reduced and/or oxidized glutathione all failed; however, dimerization readily occurs from seminal RNase monomers with the free thiol at cysteine 31 and 32. Second, the intermolecular disulfide bonds are selectively reduced at the same glutathione redox state used in the regenerations. Third, P^{SSG}_{SSG} accumulates during regeneration or selective reduction. These data suggest that $P^{SS}_{SS}P$ is more easily reduced than P^{SSG}_{SSG}. Thus, in a more reducing environment adequate to reduce P^{SSG}_{SSG} to P^{SH}_{SH}, $P^{SS}_{SS}P$ would also be reduced. Under less reducing conditions found optimal for regeneration, P^{SSG}_{SSG} is favored.

It is interesting that the selective reduction only proceeds to 30% monomer and yet the reaction is irreversible. This is an apparent dichotomy since all

irreversible reactions proceed to completion. However, the conflict is easily resolved. Native seminal RNase gives two bands on Reisfeld-PAGE. D'Alessio has shown that the more anionic component is an amide hydrolysis product of the cationic component (manuscript in preparation). In our hands this anionic component comprises approximately 30% of the total native enzyme. Further, the selective reduction dimer does not contain this anionic component. Likewise, the monomers (regenerated and selectively reduced) are homogeneous to Reisfeld-PAGE suggesting that they do not contain a mixture of deamidated and amidated components. Thus, it is likely that the selective reduction stops at 30% because the reaction only proceeds with the anionic component. This is also supported by work of D'Alessio, et al. (181) who found that only 30% of the DTT selectively reduced seminal RNase dissociates to the free monomer although virtually 100% of the intermolecular disulfide bonds are cleaved. The results suggest that the amide hydrolysis causes the protein to dissociate more readily. Further, it appears that the reduction, denaturation process causes all the protein to be converted to the component produced in selective reduction. However, more work is required to clarify this issue.

IgG is the only other protein with intermolecular disulfide bonds to be regenerated with the glutathione

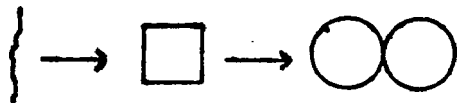
system (117). The intermolecular disulfide bonds readily reform from the dissociated monomers with either air oxidation or the glutathione redox system, while intermolecular disulfide bonds of seminal RNase will only reform under air oxidation conditions. This suggests a difference between the intermolecular forces in the two proteins and is supported by the following data. Selective reduction with DTT (which yield free thiols) causes partial dissociation of seminal RNase subunits but not IgG subunits (117,181). Seminal RNase monomers alkylated at cysteine 31 and 32 do not associate while alkylated IgG H and L chains do (D'Alessio, personal communication,214). It follows from these data that the intermolecular forces maintaining seminal RNase noncovalent dimer are weaker than those maintaining the IgG subunits.

In conclusion, it has been shown that seminal RNase can be selectively reduced. The products are a free monomer and covalent dimer, which are identical to the regenerated monomer and native enzyme, respectively. Thus, regeneration leads to a structure which is available to the native enzyme.

The difference in the circular dichroism spectra between the native dimeric and monomeric species of seminal RNase suggest that structural adjustments during refolding are not finished until intermolecular events

are complete. Thus, in summary, the overall folding scheme would appear to be:

Scheme 5:



where ⟆ is the reduced, denatured monomer, □ is the active monomer, and ∞ is the native enzyme. This is supported by the large activity difference between monomer and dimer. All monomeric species (modified or free thiol at cysteine 31 and 32) have more activity than the dimer. This structural adjustment upon association likely accounts for the decreased activity of the dimer. This scheme is also supported by different proteolytic susceptibilities of the monomer and dimer (215) and a higher molar absorptivity for the monomer compared to the dimer (182) suggesting structural changes on dissociation. The scheme is especially attractive since it has been postulated that the individual subunits exchange N-terminal ends upon association (D'Alessio, personal communication).

REFERENCES

1. Wetlaufer, D. B., and Ristow, S., Ann. Rev. Biochem., 42, 132-158 (1973).

2. Anfinsen, C. B., Science, 181, 223-230 (1973).

3. Baldwin, R. L., Ann. Rev. Biochem., 44, 453-475 (1975).

4. Habeeb, A.F.S.A., in Immunochemistry of Proteins, 1, 163-229, M. Z. Atassi, Ed. (Plenum Press, N.Y. 1977).

5. Tanford, C., Adv. Protein Chem., 24, 1-94 (1970).

6. Brandts, J. F., J. Am. Chem. Soc., 86, 4291-4301 (1964).

7. Brandts, J. F., J. Am. Chem. Soc., 86, 4302-4314 (1964).

8. Brandts, J. F., and Hunt, L., J. Am. Chem. Soc., 89, 4826-4838 (1967).

9. Edelhoch, H., and Osborne, J. C., Jr., Adv. Protein Chem. 30, 183-250 (1976).

10. Wetlaufer, D. B., Malik, S. K., Stoller, L., and Coffin, R. L., J. Am. Chem. Soc. 86, 508-514 (1964).

11. Kauzmann, W., Adv. Protein Chem. 14, 1-63 (1959).

12. Klotz, I. M., and Farnham, S. B., Biochemistry 7, 3879-3887 (1968).

13. Kreschneck, G. C., and Klotz, I. M., Biochemistry 8, 8-12 (1969).

14. Clothia, C., Nature, London 248, 338-339 (1974).

15. Bello, J., Reise, H. C. A., and Vinograd, J. R., J. Phys. Chem. 60, 1299-1306 (1956).

16. Flory, P. J., J. Am. Chem. Soc. 78, 5222-5235 (1956).

17. Scheraga, H. A., in The Proteins, Vol. 1, 2nd Ed., H. Neurath (New York: Academic Press, 1963) p. 477-594.

18. Anfinsen, C. B., and Scheraga, H. A., Adv. Protein Chem. 29, 205-300 (1975).

19. Johnson, R. E., Adams, P., and Rupley, J. A., Biochemistry 17, 1479-1484 (1978).

20. Tanford, C., Adv. Protein Chem. 23, 121-282 (1968).

21. Warren, J. R., and Gordon, J. A., J. Biol. Chem. 245, 4097-4104 (1970).

22. Franks, F., and Eagland, E., CRC Criti. Rev. Biochem. 3, 165-219 (1975).

23. Kauzmann, W., and Simpson, R. B., J. Am. Chem. Soc. 75, 5154-5157 (1953).

24. Fernsdorf, H. K., Watson, M. T., and Kauzmann, W., J. Am. Chem. Soc. 75, 5157-5166 (1953).

25. Fernsdorf, H. K., Watson, M. T., and Kauzmann, W., J. Am. Chem. Soc. 75, 5167-5172 (1953).

26. Steinhardt, J., Zaiser, E. M., and Beychok, S., J. Am. Chem. Soc. 80, 4634-4644 (1958).

27. Warner, R. C., and Levy, M., J. Am. Chem. Soc. 80, 5735-5744 (1958).

28. Acampora, G., and Hermans, J., Jr., J. Am. Chem. Soc. 89, 1543-1547 (1967).

29. Tsong, T. Y., Baldwin, R. L., and McPhie, P., J. Mol. Biol. 63, 453-469 (1972).

30. Carlsson, U., Aasa, R., Henderson, L. E., Jonson, B-H., and Lindskog, S., Eur. J. Biochem. 52, 25-36 (1975).

31. Anfinsen, C. B., Schechter, A. N., and Taniuchi, H. Cold Spring Harbor Symp. Quant. Biol. 36, 249-255 (1971).

32. Jardetzky, O., Thielmann, H., Arata, Y., Markley, J. L., and Williams, M. N., Cold Spring Harbor Symp. Quant. Biol. 36, 257-261 (1971).

33. Nozaki, Y., and Tanford, C., J. Biol. Chem. 246, 2211-2217 (1971).

34. Herskovits, T. T., Gedegbeku, B., and Jaillet, H., J. Biol. Chem. 245, 2588-2598 (1970).

35. Schrier, E. E., Ingwall, R. T., and Scheraga, H. A. J. Phys. Chem. 69, 298-303 (1965).

36. Jirgensons, B., J. Biol. Chem. 242, 912-918 (1967).

37. Conio, C., Patrone, E., Brighetti, S., J. Biol. Chem. 245, 3335-3340 (1970).

38. Singer, S. J., Adv. Protein Chem. 17, 1-69 (1962).

39. Izumi, T., and Inoue, H., J. Biochem. 81, 1769-1779 (1977).

40. Ben-naim, A., and Baer, S., Trans. Faraday Soc. 60, 1736-1741 (1964).

41. Jirgensons, B., Biochim. Biophys. Acta 434, 58-68 (1976).

42. Jirgensons, B., Optical Activity of Proteins and Other Macromolecules, 2nd Ed., Springer-Verlag, NY (1973).

43. Rosenberg, R. M., Crespi, H. L., and Katz, J. J., Biochim. Biophys. Acta, 175, 31-40 (1969).

44. Lee, P. K. J., and Jirgensons, B., Biochim. Biophys. Acta, 229, 631-641 (1971).

45. Jirgensons, B., and Shimazaki, K., Makromol. Chem., Suppl. 1, 275-282 (1975).

46. Su, Y-Y. T. and Jirgensons, B., Arch. Biochem. Biophys., 181, 137-146 (1977).

47. Mirsky, A. E., and Pauling, L., Proc. Nat. Acad. Sci., USA, 22, 439-447 (1936).

48. Robinson, D. R., and Jencks, W. P., J. Am. Chem. Soc. 87, 2462-2470 (1965).

49. Bonner, O.D., Bednark, J. M., and Arisnan, R. K., J. Am. Chem. Soc., 99, 2898-2902 (1977).

50. Gordon, J. A., and Jencks, W. P., Biochemistry 2, 47-57 (1965).

51. Nozaki, Y., and Tanford, C., J. Biol. Chem. 238, 4074-4081 (1963).

52. Muller, N., and Platko, F. E., J. Phys. Chem. 75, 547-553 (1971).

53. Tanford, C., Kawahara, K., and Lapanje, S., J. Am. Chem. Soc. 89, 729-736 (1967).

54. Tanford, C., Kawahara, K., Lapanje, S., Hooker, T. M. Jr., Zarlengo, M. H., Salahuddin, A., Aune, K. C., and Takagi, T., J. Am. Chem. Soc. 89, 5023-5029 (1967).

55. Nozaki, Y., and Tanford, C., J. Am. Chem. Soc. 89, 742-749 (1967).

56. Green, R. F., and Pace, C. N., J. Biol. Chem. 249, 5388-5393 (1974).

57. Benz, F. W., and Roberts, G. C. K., J. Mol. Biol. 91, 345-365 (1975).

58. Benz, F. W., and Roberts, G. C. K., J. Mol. Biol. 91, 367-387 (1975).

59. Schrier, M. Y., and Schrier, E. E., Biochemistry 15, 2607-2612 (1976).

60. Brandts, J. F., Olveira, R. J., and Westort, C., Biochemistry 9, 1038-1047 (1970).

61. Hawley, S. A., Biochemistry, 10, 2436-2442 (1971).

62. Zipp, A., and Kauzmann, W., Biochemistry 12, 4217-4228 (1973).

63. Hvidt, A., J. Theor. Biol. 50, 245-252 (1975).

64. Ginsburg, A., and Carroll, W. R., Biochemistry 4, 2159-2174 (1965).

65. Brandts, J. F., and Lumry, R., J. Phys. Chem. 67, 1484-1494 (1963).

66. Bradbury, J. H., and Norton, R. S., Biochim. Biophys. Acta. 328, 10-19 (1973).

67. Aune, K. C., Salahuddin, A., Zerlengo, M. H., and Tanford, C., J. Biol. Chem. 242, 4486-4489 (1967).

68. Von Hippel, P. H., and Schleich, T. Acc. Chem. Res. 2, 257-265 (1969).

69. Von Hippel, P. H., and Scheich, T., in Structure and Stability of Biological Macromolecules, Vol. 2, S. N. Timasheff and G. D. Fasman, Eds., Marcel Dekker, NY, pp 417-574 (1969).

70. LaCroute, F., and Stent, G. S., J. Mol. Biol. 35, 165-173 (1968).

71. Wilhelm, J. M., and Haselkorn, R., Proc. Nat. Acad. Sci., USA 65, 388-394 (1970).

72. Hunt, T., Hunter, T, and Munro, A., J. Mol. Biol. 43, 123-133 (1969).

73. Dintzis, H., Proc. Nat. Acad. Sci., USA 47, 247-261 (1961).

74. Levinthal, C., J. Chim. Phys. 65, 44-45 (1968).

75. Wetlaufer, D. B., Proc. Nat. Acad. Sci., USA 70, 697-701 (1973).

76. Anfinsen, C. B., Biochem. J. 128, 737-749 (1972).

77. Sternberg, M. J., and Thornton, J. M., Nature, London 271, 15-20 (1978).

78. Tanaka, S., and Scheraga, H. A., Proc. Nat. Acad. Sci., USA 72, 3802-3806 (1975).

79. Levitt, M., and Warshel, A., Nature, London 253, 694-698 (1975).

80. Kuntz, I. D., Crippen, G. M., Kollman, P. A., and Kimelman, D., J. Mol. Biol. 106, 983-994 (1976).

81. Levitt, M. J. Mol. Biol. 104, 59-107 (1976).

82. Go, N., and Taketomi, H., Proc. Nat. Acad. Sci. USA 75, 559-563 (1978).

83. Hagler, A. T., and Honig, B. Proc. Nat. Acad. Sci., USA 75, 554-558 (1978).

84. Ptitsyn, O. B, and Rashin, A. A., Dokl. Akad. Nauk. SSSR 213, 473-475 (1973).

85. Ptitsyn, O. B., and Rashin, A. A., Biophys. Chem. 3, 1-20 (1975).

86. Rose, G. D., Winters, R. H., and Wetlaufer, D. B., FEBS Letters 63, 10-16 (1976).

87. Anson, M. L., and Mirsky, A. E. J. Gen. Physiol. 9 169-179 (1925).

88. Anson, M. L., and Mirsky, A. E., J. Phys. Chem. 35, 185-193 (1931).

89. Anson, M. L., and Mirsky, A. E., J. Gen. Physiol. 17, 393-398 (1934).

90. Anson, M. L., and Mirsky, A. E., J. Gen. Physiol. 17, 399-408 (1934).

91. Mirsky, A. E., and Anson, M. L., J. Gen. Physiol. 18, 427-438 (1935).

92. Mirsky, A. E., J. Gen. Physiol. 24, 709-723 (1941).

93. Anson, M. L., Adv. Protein Chem. 2, 361-386 (1945).

94. Sela, M., White, F. H., Jr., and Anfinsen, C. B., Science 125, 691-692 (1957).

95. White, F. H., Jr., J. Biol. Chem. 383-389 (1960).

96. Anfinsen, C. B., Haber, E., Sela, M., and White, F. H., Jr., Proc. Nat. Acad. Sci., USA 47, 1309-1314 (1961).

97. White, F. H., Jr., J. Biol. Chem. 236, 1353-1360 (1961).

98. Epstein, C. J., Goldberger, R. F., Young, M. D., and Anfinsen, C. B., Arch Biochem. Biophys., Suppl. 1, 223-231 (1962).

99. Ahmed, A. K., Schaffer, S. W., and Wetlaufer, D. B., J. Biol. Chem. 250, 8477-8482 (1975).

100. Goldberger, R. F., Epstein, C. J., and Anfinsen C. B., J. Biol. Chem. 238, 628-635 (1963).

101. Venetianer, P., and Straub, F. B., Biochim. Biophys. Acta. 67, 166-168 (1963).

102. Fuchs, S., DeLorenzo, F., and Anfinsen, C. B., J. Biol. Chem. 242, 393-402 (1967).

103. DeLorenzo, F., and Molea, G., Biochim. Biophys. Acta. 146, 593-595 (1967).

104. Williams, D. J., Gurari, D., Rabin, B. R., FEBS Letters 2, 133-135 (1968).

105. DeLorenzo, F., Goldberger, R. F., Steers, E., Jr., Givol, D., Anfinsen, C. B., J. Biol. Chem. 241, 1562-1567 (1966).

106. Givol, D., DeLorenzo, F., Goldberger, R. F., and Anfinsen, C. B., Proc. Nat. Acad. Sci., USA 53, 676-684 (1965).

107. Ohba, H., Harano, T., Omura, T., Biochem. Biophys. Res. Comm. 77, 830-836 (1977).

108. Goldberger, R. F., Epstein, C. J., and Anfinsen, C. B., J. Biol. Chem. 239, 1406-1410 (1964).

109. Yutani, K., Yutani, A., and Isemura, T., J. Biochem. 62, 576-583 (1967).

110. Poulson, L. L., and Ziegler, D. M., Arch. Biochem. Biophys. 183, 563-570 (1977).

111. Ziegler, D. M., and Poulson, L. L., Trends Biochem. Sci. 2, 79-81 (1977).

112. Anderson, W. L., and Tomasi, T. B., Jr., Arch. Biochem. Biophys. 182, 705-711 (1977).

113. Saxena, V. P., and Wetlaufer, D. B., Biochemistry 9, 5015-5022 (1970).

114. Tietze, F., Anal. Biochem. 27, 502-522 (1969).

115. Jocelyn, P. C., Biochemistry of the SH Group, Academic Press, N.Y. (1972).

116. Hogle, J. M., and Liener, I. E., Can. J. Biochem. 51, 1014-1020 (1973).

117. Petersen, J. G. L., and Dorrington, K. J., J. Biol. Chem. 249, 5633-5641 (1974).

118. Creighton, T. E., J. Mol. Biol. 95, 167-199 (1975).

119. Teale, J. M., and Benjamin, D. C., J. Biol. Chem. 251, 4603-4608 (1976).

120. Sinha, N. K., and light, A., J. Biol. Chem. 250, 8624-8629 (1975).

121. Hantgan, R. R., Hammes, G. G., and Scheraga, H. A., Biochemistry 13, 3421-3431 (1974).

122. Schaffer, S. W., Ahmed, A. K., and Wetlaufer, D. B. J. Biol Chem. 250, 8483-8486 (1975).

123. Steiner, R. F., DeLorenzo, F., and Anfinsen, C. B., J. Biol. Chem. 240, 4648-4651 (1965).

124. Teipel, J. W., and Koshland, D. E., Jr., Biochemistry 10, 792-798 (1971).

125. Takagi, T., and Isemura, T., J. Biochem. 57, 89-95 (1965).

126. Friedmann, T., and Epstein, C. J., J. Biol. Chem. 242, 5131-5140 (1967).

127. Bornmann, L., Hess, B., Zimmermann-Telschow, H., Proc. Nat. Acad. Sci., USA 71, 1525-1529 (1974).

128. Wieker, H-J., and Hess, B., Hoppe-Seyler's Z. Physiol. Chem. 353, 1877-1893 (1972).

129. Parr, G. R., and Hammes, G. G., Biochemistry 15, 857-862 (1976).

130. Teipel, J. W., and Koshland, D. E., Jr., Biochemistry 10, 798-805 (1971).

131. Yazgan, A., and Henkens, R. W., Biochemistry 11, 1314-1318 (1972).

132. Wong, K-P., and Tanford, C., J. Biol. Chem. 248, 8518-8523 (1973).

133. Ko, B. P. N., Yazgan, A., Yeagle, P. L., Lottich, S. C., and Henkens, R. W., Biochemistry 16, 1720-1725 (1977).

134. Vimard, O., Orcini, G., and Goldberg, M. E., Eur. J. Biochem. 51, 521-527 (1975).

135. Kohn, L..D., J. Biol. Chem. 245, 3850-3858 (1970).

136. Cuatrecasas, P., Taniuchi, H., Anfinsen, C. B., Brookhaven Symp. Biol. No. 21: 172-200.

137. Freedman, M. H., and Sela, M., J. Biol. Chem. 241, 2383-2396 (1966).

138. Fuch, S., and Sela, M., J. Biol. Chem. 240, 3558-3567 (1965).

139. Sears, D. W., Kazin, A. R., Mohrer, J., Friedman, F., and Beychok, S., Biochemistry 16, 2016-2025 (1977).

140. Sears, D. W., and Beychok, S., Biochemistry 16, 2026-2031 (1977).

141. Tenenbaum-Bayer, H., and Levitzki, A., Biochim. Biophys. Acta. 445, 261-279 (1976).

142. Rudolph, R., Heider, I., Westhof, E., and Jaenicke, R., Biochemistry 16, 3384-3390 (1977).

143. Pauly, H. E., and Pfleiderer, G., Biochemistry 16, 4599-4604 (1977).

144. Wetlaufer, D. B., Kwok, E., Anderson, W. L., and Johnson, E. R., Biochem. Biophys. Res. Comm. 56, 380-385 (1974).

145. Creighton, T. E., J. Mol. Biol. 96, 767-776 (1975).

146. Creighton, T. E., J. Mol. Biol. 96, 777-782 (1975).

147. Creighton, T. E., J. Mol. Biol. 113, 275-293 (1977).

148. Creighton, T. E., J. Mol. Biol. 113, 313-328 (1977).

149. Ristow, S. S., and Wetlaufer, D. B., Biochem. Biophys. Res. Comm. 50, 544-550 (1973).

150. Anderson, W. L., and Wetlaufer, D. B., J. Biol. Chem. 251, 3147-3153 (1976).

151. Brown, J., Fed. Proc. 33, 1389 (1974).

152. Brown, J., Fed. Proc. 34, 591 (1975).

153. Teale, J. M., and Benjamin, D. C., J. Biol. Chem. 251, 4609-4615 (1976).

154. Teale, J. M., and Benjamin. D. C., J. Biol. Chem. 252, 4521-4526 (1977).

155. Högberg-Raibaud, A., and Goldberg, M. E., Biochemistry 16, 4014-4019 (1977).

156. Chavez, L. G., and Scheraga, H. A., Biochemistry 16, 1849-1856 (1977).

157. Haber, E., and Anfinsen, C. B., J. Biol. Chem. 236, 422-424 (1961).

158. Taniuchi, H., J. Biol. Chem. 245, 5459-5468 (1970).

159. Matheson, R. R., Jr., Dugas, H., and Scheraga, H. A., Biochem. Biophys. Res. Comm. 74, 869-876 (1977).

160. Johnson, E. R., Anderson, W. L., Atassi, M. Z., Lee, C. L., and Wetlaufer, D. B., Fed. Proc. 34 Abstr. #2140 (1975).

161. Richards, F. M., and Wyckoff, H. W., in The Enzymes, Vol. 4, 647-806, P. D. Boyer, Ed., Academic Press, N.Y. (1971).

162. Farina, B., Piera, Q., Leone, E., Rend. Accad. Sci. Fis. Mat., Naples 41, 303-311 (1974).

163. D'Alessio, G., Floridi, A., DePrisco, R., Pignero, A., and Leone, E., Eur. J. Biochem 26, 153-161 (1972).

164. DiDonato, A., and D'Alessio, G., Biochem. Biophys. Res. Comm. 55, 919-928 (1973).

165. Suzuki, H., Greco, L., Parente, A. Farino, B., LaMontagna, R., and Leone, E., in Atlas of Protein Sequence and Structure, Vol. 5, Suppl. 2 (1976).

166. Floridi, A., D'Alessio, G., and Leone, E., Eur. J. Biochem. 26, 162-167 (1972).

167. Libonati, M., and Floridi, A., Eur. J. Biochem. 8, 81-87 (1968).

168. Libonati, M., Malorni, M. C., Parente, A., and D'Alessio, G., Biochim. Biophys. Acta 402, 83-87 (1975)

169. Libonati, M., Furia, A., Beintema, J. J., Eur. J. Biochem. 69, 445-451 (1976).

170. Schaffer, S. W., Int. J. Peptide and Protein Res. 7, 179-184 (1975).

171. Fletcher, Q. L., and Hash, J. H., Biochemistry 11, 4274-4280 (1972).

172. Kalnitsky, G., Hummel, J. P., and Dierks, C., J. Biol. Chem. 234, 1512-1516 (1959).

173. Ellman, G. L. Arch. Biochem. Biophys. 82, 70-77 (1959).

174. Beers, R. F., Jr., and Sizer, J. W., J. Biol. Chem. 195, 133-140 (1952).

175. Stewart, G. R., and Stevenson, K. J., Biochem. J. 135, 427-441 (1973).

176. Weber, K. and Osborn, M., J. Biol. Chem. 244, 4406-4412 (1969).

177. Reisfeld, R. A., Lewis, U. J., and Williams, D. E., Nature, London 195, 281-283 (1962).

178. Moore, S., and Stein, W. H., Methods Enzymol. 6, 819-831 (1963).

179. Spackman, D. G., Methods Enzymol. 11, 3-15 (1967).

180. Cassim, J. Y., and Yang, J. T., Biochemistry 8, 1947-1951 (1969).

181. D'Alessio, G., Malorni, M. C., and Parente, A., Biochemistry 14, 1116-1122 (1975).

182. Parente, A., Albanesi, D., Garzillo, A. M., D'Alessio, G., Ital. J. Biochem. 26, in press (1977).

183. Dopheide, T. A. A., Moore, S., and Stein, W. H., J. Biol. Chem. 242, 1833-1837 (1967).

184. Schroeder, W. A., Shelton, J. R., and Robberson, B., Biochim. Biophys. Acta 147, 590-592 (1967).

185. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. J. Biol. Chem. 193, 265-275 (1951)

186. O'Carra, P., Barry, S., Griffin, T., Methods Enzymol. 34, 108-126 (1974).

187. Wilchek, M., and Gorecki, M., Eur. J. Biochem. 11, 491-494 (1969).

188. Chaiken, I. M., and Taylor, H. C., J. Biol. Chem. 251, 2044-2048 (1976).

189. Anderson, D. G., Hammes, G. G., Walz, F. G., Jr., Biochemistry 7, 1637-1645 (1968).

190. Dunn, B. M., and Chaiken, I. M., Proc. Nat. Acad. Sci., USA 71, 2382-2385 (1974).

191. Davies, G. E., and Stark, G. R., Proc. Nat. Acad. Sci., USA 66, 651 (1970).

192. Moe, O. A., and Smith, G. K., J. Chem. Ed. 54, 392-393 (1977).

193. Means, G. E., and Feeney, R. E., Chemical Modification of Proteins, Chapt. 6, Holden-Day, Inc., San Francisco, Calif. (1971).

194. Victoria, E. J., Mahan, L. C., and Masouredis, S. P., Biochemistry 16, 2566-2569 (1977).

195. Büsen, W., Hinrich, J., Hausen, P., Eur. J. Biochem 74, 203-208 (1977).

196. Gutte, B., Biochem Soc. T. 3, 897-899 (1975).

197. Gutte, B., J. Biol. Chem. 252, 663-670 (1977).

198. Porath, J., Janson, J., and Laas, T., J. Chromatogr. 61, 167-177 (1971).

199. D'Alessio, G., DiDonato, A., and Piccoli, Proc., 10th Meeting Fed. Eur. Biochem. Soc., Abstr. 637 (1975).

200. Matthaei, J. H., and Nirenberg, M. W., Proc. Nat. Acad. Sci., USA 47, 1580-1588 (1961).

201. Parente, A., Malorni, M. C., and D'Alessio, G., Boll.Soc. Ital. Biol. Sper. 68, 1-4 (1972).

202. Engelhard, M., Rduolph, R., and Jaenicke, R., Eur. J. Biochem. 67, 447-453 (1976).

203. Coleman, D. L., and Blout, E. R., J. Am. Chem. Soc. 90, 2405-2416 (1968).

204. Bewley, T. A., Biochemistry 16, 209-215 (1977).

205. Haber, E., and Anfinsen, C. B., J. Biol. Chem. 237, 1839-1844 (1962).

206. Frese, R., and Gröschel-Stewart, U., Experientia 28, 1013-1015 (1972).

207. Beutler, E., Srivastava, S. K., West, C. Biochem. Biophys. Res. Comm. 38, 341-347 (1970).

208. Flatmark, T., and Sletten, K., J. Biol. Chem. 243, 1623-1629 (1968).

209. Spero, L., Warren, J. R., and Metzger, J. F., Biochim. Biophys Acta 336, 79-85 (1974).

210. Anderson, W. L., Nucleation of Hen Egg Lysozyme under Physiological Conditions, Ph.D. Dissertation, University of Minnesota, 122 pp (1974).

211. Pflumm, M. N., and Beychok, S., J. Biol. Chem. 244, 3982-3989 (1969).

212. Palmer, J. L., and Nisonoff, A., Biochemistry 3, 863-869 (1964).

213. Bjork, I., and Tanford, C., Biochemistry 10, 1289-1295 (1971).

214. Bigelow, C. C., Smith, B. R., and Dorrington, K. J., Biochemistry 13, 4602-4608 (1974).

215. Parente, A., Branno, M., Malorni, M. C., Libonati, M., and D'Alessio, G., Biochim.Biophys. Acta 445, 377-385 (1976).

APPENDIX

Derivation of Affinity Chromatography Equations:

Dunn and Chaiken (190) have derived a similar equation for competitive elution of an enzyme; these derivations are quite analogous.

Noncompetitive Elution.

If the immobilized protein binds to both the bare enzyme and the enzyme-substrate complex then:

$$E + I \overset{K}{\triangleq} EI$$

$$E + \overline{IM} \overset{K'}{\triangleq} E\overline{IM}$$

$$EI + \overline{IM} \overset{K''}{\triangleq} EI\overline{IM}$$

where E = soluble enzyme, I = competitive inhibitor, and $\overline{IM}$ = immobilized protein.

Now if $K' \neq K''$, then $K'' = K'(z)$ where z = constant, and

$$K = (E)(I)/(EI) \text{ and } K' = (E)(\overline{IM})/(E\overline{IM})$$

$$= (EI)(\overline{IM})/z\ (EI\overline{IM})$$

Now, if a nonporous gel is used such that when the protein does not interact with the matrix $V_o = V_p$, where V_o = void volume and V_p = volume to elute half the enzyme, then the elution of E will be described by

$$V_p = V_o D + V_o$$

where D = the distribution coefficient for interaction with the matrix. Thus, if there is no interaction with the matrix then D = 0 and $V_p = V_0$. D is numerically the ratio of E interacting with the matrix divided by that not

interacting with it. Thus,

$$D = (EI\overline{IM}) + (E\overline{IM}))/(E) + (EI))$$

dividing through by (E):

$$D = \frac{(EI\overline{IM})/(E) + (E\overline{IM})/(E)}{1 + (EI)/(E)}$$

substituting for $EI\overline{IM}$ and $E\overline{IM}$

$$D = \frac{(EI)(\overline{IM})/zK'(E) + (\overline{IM})(E)/K'(E)}{1 + (EI)/(E)}$$

simplifying

$$D = \frac{(\overline{IM})/K' \ (1 + (EI)/z(E))}{(1 + (EI)/(E))} = \frac{(\overline{IM})}{K'} \frac{(1 + (I)/zK)}{(1 + (I)/K)}$$

Now, since $V_p = V_o D + V_o$, then

$$V = V_o \frac{(\overline{IM})}{K'} \frac{(1 + (I)/zK)}{(1 + (I)/K)} + V_o$$

and

$$\frac{1}{V_p - V_o} = \frac{K'}{V_o(\overline{IM}) + \frac{I(V_o)\overline{IM}}{zK}} + \frac{IK'z}{V_o(\overline{IM})zK + I\ V_o(\overline{IM})}$$

This assumes $(\overline{IM}) >> (E)$ and $(I) >> (E)$.

Now if z=1 as is often assumed:

$$V_p = V_o \frac{(\overline{IM})}{K'} + V_o$$

and

$$\frac{1}{V_p - V_o} = \frac{K'}{V_o(IM)}$$

VITA

Gary K. Smith was born August 31, 1952, in Easton, Pennsylvania, the first born of Carl J. H. and Gloria L. Smith. He was a student of the Easton Area School System and graduated from Easton Area High School in 1970.

His undergraduate training was obtained in the Chemistry Department of Lebanon Valley College. In May 1974 he received the Bachelor of Science in Chemistry.

Mr. Smith's graduate career began in September 1974 in the Chemistry Department of Lehigh University. During his graduate work he taught organic and biochemistry laboratories for a year and a half and was the holder of the Newton Wayne Buch and Constance Nancie Buch pre-doctoral fellowship for two and a half years.

His research interests extend into various areas of protein chemistry, including protein folding, protein-protein interactions, and protein modifications. He is a member of the American Chemical Society (Biological Division) and Phi Lambda Upsilon Chemistry Honor Society.