1983

Hydrodynamic studies of ribosomal protein S4 from Escherichia coli

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HYDRODYNAMIC STUDIES OF RIBOSOMAL PROTEIN S4 FROM ESCHERICHIA COLI

By

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B.A., University of Montana, 1978

Presented in partial fulfillment of the requirements for the degree of

Master of Science

UNIVERSITY OF MONTANA

1983

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5-23-83

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Dodd, Jonathan A., M.S., June, 1983 Biochemistry

Hydrodynamic Studies of Ribosomal Protein S4 from Escherichia Coli (88 pp.)

Director: Dr. Walter E. Hill

Protein S4 was isolated using denaturing conditions and then studied under reconstitution conditions using hydrodynamic methods. Sedimentation velocity experiments gave an average value of 1.69S for the sedimentation coefficient. Intensity fluctuation spectroscopy was used to measure the diffusion coefficient and gave an average value of 7.95×10^{-7} cm^2/sec. The average molecular weight calculated from the Svedberg equation was 21,200. Sedimentation equilibrium studies were used to analyze sample quality and to obtain a molecular weight, which was 23,200.

The hydrodynamic measurements on S4 were used to calculate its frictional coefficient. If a prolate ellipsoid model was used, the axial ratio of S4 was calculated to be not less than 4.5:1 and not greater than 7:1. Using this model, the radius of gyration expected from scattering studies would be between 26 Å and 30 Å.

Initial attempts were made to isolate S4 by high salt extraction and column chromatographies, however the quality and efficiency of this method was judged to be unsatisfactory. To circumvent this, samples of S4 were prepared by acetic acid extraction and purified by column chromatography which included steps using 6 M urea. Two-dimensional gel electrophoresis and immunoprecipitation assay were used to identify the protein as S4 and showed the sample to be highly purified.

ACKNOWLEDGEMENTS

The author would like to express his appreciation to Dr. Walter E. Hill for his advice and support in this work. The author would also like to thank Dr. Victor Bloomfield, Dr. Larry Kahan, Dr. Jason Wei and Dr. Ming Tam for their assistance.

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LIST OF ABBREVIATIONS

Bis = N,N^{1}-Methylene-bis-acrylamide

CM-Sephadex = carboxymethyl Sephadex

DRSQ = difference in radius squared

EDTA = Ethylenediaminetetraacetic acid

EM = Electron microscopy

Recon = reconstitution

RTIC = Remote temperature indication and control

TEMED = N,N,N^{1},N^{1}-tetramethylethylenediamine

Tris = Tris(hydroxymethyl)aminomethane

UPβ = Urea phosphate β-mercaptoethanol

Chapter I

INTRODUCTION

Escherichia coli Ribosome

As early as 1943, electron microscope studies of Escherchia coli lysates showed granules on the order of 100-150 Å in diameter (32). Early ultracentrifugal studies by Siegal et al. (50) showed that extracts of Escherichia coli gave ultracentrifuge patterns similar to those found by Schachman et al. (47) in studies of various bacterial extracts. Schachman had shown that the particles which gave uncorrected sedimentation coefficients of 40S, 29S and 5S in ultracentrifuge patterns contained the bulk of cellular RNA. His lysates were made using several methods of disruption in a variety of buffers, ranging from 0.02 to 0.05 M NaCl. In 1957, Chao (8) demonstrated that magnesium ion was required for stability of these particles and that in its absence they would dissociate into two components of unequal sedimentation coefficients. Although this work was done on yeast particles it demonstrated a very fundamental phenomenon. Namely, magnesium was essential for particle integrity.

In 1958 and 1959 Tissiéres and Watson (57) and Tissiéres et al. (58) published the results of early studies carried out on purified Escherichia coli ribosomes. Under various buffer conditions, particles could be isolated which gave sedimentation coefficients of 100S, 70S, 51S and 32S. The 100S particle appeared to be a dimer of 70S particles and the 70S particles appeared to be composed of one 51S particle and one 32S

particle. The association of the particles was demonstrated to be dependent on the magnesium ion concentration and to be reversible between the range of 10^{-4} M and 2 x 10^{-2} M magnesium. The particles were stable for more than 2 months in the cold in the presence of appropriate magnesium ion concentration. They contained 60-65% RNA and 40-35% protein. Molecular weights were estimated for the particles from their sedimentation coefficients, diffusion coefficients, and partial specific volumes. The molecular weight of the 32S component was reported to be 0.95 x 10^6, the 51S component 1.85 x 10^6 and the 70S component 2.8 x 10^6. These were the first chemical and physical studies on purified Escherichia coli ribosomes, and they outlined detailed conditions for their isolation and stability.

Since that time the Escherichia coli ribosome has been characterized by many different groups using various techniques. The results of early studies are excellent in many respects but difficult to correlate as a whole. For a more complete discussion see Van Holde and Hill (63).

The Escherichia coli ribosome has a molecular weight of ~2.6 x 10^6 daltons as determined by both sedimentation equilibrium (19) and sedimentation velocity and diffusion measurements (58). The intact ribosome has a sedimentation coefficient of 70S (18), a partial specific volume of 0.606 cm^3/gm (19), and a diffusion coefficient of 1.83 x 10^{-7} cm^2/sec (58). Studies of the shape of the ribosome by x-ray scattering (18), light scattering (46), and electron microscopy (64) show poor agreement. There are a number of reasons for the inconsistencies shown in shape studies of the intact ribosome. These inconsistencies are fully discussed by Van Holde and Hill (63), and yet, unresolved.

The 70S ribosome of Escherichia coli can be made to dissociate into subunits of 50S and 30S by decreasing the magnesium ion concentration to less than 4 mM. Early research on the subunits gave molecular weights of 1.65×10^6 daltons and 1.0×10^6 daltons for the 50S and 30S subunits, respectively (17). Physical studies and modeling techniques also gave rise to a variety of shapes for these molecules (63). Much evidence has come from electron microscopy studies which ideally should give a best model by observing the shapes directly. The drawback has been that electron microscopy studies are made on samples exposed to uranyl acetate, low pH and extensive drying. Thus the particles so studied may be quite distorted from their shape in solution.

Models for the 50S subunit which have been developed mainly from interpretation of electron micrographs have been compared with results of solution scattering and show essentially similar structure. The 50S subunit structure is best described as a hemisphere which is slightly elongated and has three distinct protruberances. Scattering curves calculated for uniform ellipsoidal models do not agree well with the experimental scattering curves, suggesting that the 50S subunit is of an irregular shape. Recent neutron scattering studies also give data which are consistent with the asymmetric models proposed by the electron microscopy studies (36).

Using x-ray scattering data of Hill et al. (18), Hill and Fessenden (20) have shown that an oblate ellipsoid model with dimensions of 55 Å x 230 Å x 230 Å generates a curve which fits very well with the 30S ribosomal subunit experimental scattering curve. The models for the 30S subunit which have been proposed by electron microscope studies have

also been used to generate scattering curves but these do not show good correlation with the experimental scattering data (36,13).

Although the structure of the Escherichia coli ribosome is better understood by these physical studies, the resolution is still not sufficient for an understanding of the molecular events of translation. Thus, research has intensified in the study of the constituents of the subunits themselves.

Reconstitution Studies

Perhaps the most dramatic findings of the last two decades are that active 30S and 50S subunits can be reconstituted in vitro from their isolated components. This was initially accomplished by Traub and Nomura (60) for the 30S subunit and more recently by Nierhaus and Dohme (37) for the 50S subunit. Not only have active subunits been reconstituted, but several intermediates in their formation have been isolated and studied. The reconstitution process has been shown to be very sensitive to buffer composition as well as total ionic strength.

Reconstitution studies were initiated by reconstituting subunits which had been partially disassembled under various salt conditions. The total reconstitution of the subunits from RNA and constituent proteins showed that all of the information necessary for self-assembly is contained in the RNAs and proteins. The process is truly a self-assembly process and not dependent on cellular systems.

Much information on the reconstitution process has been derived from partial reconstitution studies and physical studies on isolated intermediates. To a degree, researchers can now define which proteins are

interacting with other proteins and the rRNAs. Detailed assembly maps are emanating from such binding studies. Physical and chemical studies on intermediates and whole subunits yield information on shapes and conformational changes that may occur. By combining the interaction data with geometrical information from physical studies, researchers will in time be able to construct a detailed model of the actual molecular structure of the ribosome. Combining this model with data from functional studies on translation will result in a concise understanding of the particular molecular events of protein synthesis.

Ribosomal RNA

The Escherichia coli ribosome contains three species of RNA. The 50S subunit contains a 23S and a 5S RNA molecule and the 30S subunit contains a 16S RNA molecule. The characterization of the 23S and 16S RNA molecules was begun by Kurland (28) and Stanley and Bock (49). The 5S RNA was identified earlier by Rosset and Monier (45).

The 5S RNA molecule is a chain of 120 nucleotides and its primary structure was deduced by Brownlee, Sanger and Barrell (3). The 16S RNA molecule contains 1541-2 nucleotides and 23S RNA contains 2904 nucleotides, both of which have been sequenced (38). The sequencing has been done using the gene sequencing techniques on the DNA cistron for the 16S rRNA (2) and the Maxam and Gilbert method using cloned DNA restriction fragments for the 23S RNA (38). The RNAs have also been characterized by hydrodynamic methods (53,54) which have yielded molecular weights roughly in agreement with chemical molecular weights when effects of salt binding are taken into account.

Physical studies of the 16S and 23S RNAs show that they are fairly extended in solution, but the structure is quite dependent on ionic conditions (53,54). Modeling techniques from base pairing analysis by the method of Tinoco (56) have given rise to proposed secondary structures for the ribosomal RNAs. Experimental data using several other methods have also contributed to determining the structure of the models proposed (13). The models are based on studies of chemical modification, crosslinking, and partial nuclease digestion of the ribosomal RNAs and, in general, do not vary greatly from one another. Secondary structure also seems to be conserved and shows little variation among different species (13).

The tertiary structures of RNAs have been probed by immune electron microscopy and crosslinking on the isolated RNAs and the RNAs in situ (4,52). Some electron microscopy studies on the 16S and 23S RNAs indicate that these molecules have a structure which will fit inside the proposed dimensions of their respective subunits (65,66). Hydrodynamic studies (53,54) and other EM studies (1) are in direct disagreement with this concept. It seems likely that the RNAs require some interaction with ribosomal proteins in order to form their in situ structures, as the later studies indicate. Studies on the isolated RNA structure do not give solution structures which have a physiological significance. Thus, the study of ribosomal RNAs, while contributing to our overall knowledge of the ribosome, has not yet contributed greatly to our knowledge of ribosome structure.

Ribosomal Proteins

Prior to 1961, ribosomal proteins were generally considered to be

rather uniform in size and shape with primarily a structural function. In 1961, Waller and Harris (67) published starch gel electrophoresis studies of Escherichia coli ribosomal proteins which clearly showed them to be a heterogeneous mixture. In 1964, Waller (68) published further studies which showed the presence of at least 24 different proteins as fractionated on carboxymethyl-cellulose columns. Waller also showed that the proteins from 30S subunits were different from those of 50S subunits, as suggested by Tissiéres et al. (58). This seemed to put to rest the concept that two 30S particles formed the 50S subunit.

The pioneering work of Waller and Harris created a whole new area of research within the ribosome field. In 1967, several research groups (21,33,34,59,60) published data supporting the heterogeneity of ribosomal proteins and in 1969, Hardy et al. (16) published a straightforward method for purifying 20-21 distinct proteins from the 30S subunit of Escherichia coli. The chemical and physical properties of these proteins were described by Craven et al. (9). The ribosomal proteins were found to be a group of mostly basic proteins which varied in molecular weight from 11,000 to 61,000 daltons. Kaltschmidt and Wittmann (22) developed a two-dimensional gel electrophoresis system which resolved all the proteins of the Escherichia coli ribosome and provided a system of nomenclature based on their position. For a detailed review on early isolation procedures and studies see (69).

The small subunit of Escherichia coli ribosomes contains 21 proteins, denoted as S1-S21, while the large subunit contains 34 proteins, denoted as L1-L34. Proteins S20 and L26 were found to be the same and protein L8 is a complex of L10 and two copies each of L7 and L12. The

chemical and physical characterization of these proteins has been a major thrust of research in the last decade. During this time the primary structures of all the Escherichia coli proteins have been determined primarily by Wittmann-Leibold and her coworkers (13). These sequences can be found in recent reviews (13,70,71). Using the primary structures, secondary structure predictions have been made. When these predictions are compared with actual physical data taken from CD studies of the ribosomal proteins, good agreement is found. The 30S proteins seem to have a high content of α-helix or β-sheet with some exceptions (eg. S5, S6, S19) while the 50S proteins show mostly β-sheet or are unstructured with some exceptions (eg. L9, L29, L17, L11, L30, L1) (11).

The tertiary structure of ribosomal proteins have been extensively studied by traditional physical methods. A problem which has persistently plagued researchers is obtaining protein samples suitable for physical studies. Traditional isolation methods have employed the use of concentrated urea solutions in the protein fractionation. Once the protein is denatured in urea, the question remains as to whether it totally regains its native conformation in solution. Two approaches have been used to circumvent spurious results due to the denaturation. One is to isolate the proteins without such harsh denaturation using high salt washes after which further purification is carried out on large gel filtration columns (31). The second method is to allow the protein to go through a careful renaturation step after purification in urea. This is accomplished by gradual dialysis into a high salt buffer at low protein concentrations and a temperature incubation step. The proton magnetic resonance spectrum of the protein serves as a method of comparison

of sample quality, and in several comparative studies, both techniques yielded samples of equal quality (23,24,30,39).

Detailed physical studies using various techniques have been conducted on ribosomal proteins extracted by one or both methods and have been reviewed (70,71). The proteins have been found to range in molecular weight from about 61,000 to 5,300 with most of them in the 10,000-25,000 range. Ribosomal proteins are difficult to purify, show a marked tendency to aggregate, are not readily soluble in aqueous buffers and are therefore difficult to study. However, there have been enough studies on these proteins to draw some general conclusions. Proteins L17, L25, L28, L29 and L30 are compact, L1, L4, L5, L6, L13, L16, L19 and L24 are moderately extended and L2, L3, L9, L11, L15, L23, L27, L32 and L33 are quite extended. The L7/L12 complex is also quite extended with an axial ratio estimated to be 10:1. Proteins S6, S8, S13, S15 and S16 appear to be globular in shape. Proteins S2, S3, S5 and S21 show more extended structures, having axial ratios in the range of 4:1 or 5:1. Of the remaining proteins S1, S18 and S20 appear to be quite elongated while studies of S4 and S7 are inconclusive (13).

Proposal

Protein S4 has been scrutinized more than any of the other 20 proteins of the 30S subunit. Although widely studied its shape *in situ* and in solution is still subject to question.

S4 has a molecular weight of 23,138 (from sequencing (71)), making it one of the larger proteins on the 30S subunit. The primary sequence of S4 was deduced by Rheinbolt and Schiltz (43) in 1975. It has been

shown to protect almost one-third of the 16S RNA from nuclease digestion.

Structural studies on S4 have given a wide range of results. Paradies and Franz (40) conducted the only comprehensive hydrodynamic studies. However, their sedimentation and diffusion coefficients give a molecular weight of about 45,000 from the Svedberg equation, suggesting that they were studying a dimer. Gulik et al. (15) used x-ray scattering to obtain a radius of gyration, Rg, of 26 Å which suggests a moderately extended structure. However, Österberg et al. (39) also used x-ray scattering and obtained an Rg of 42 Å and proposed a triaxial ellipsoid model which was highly extended. Serdyuk et al. (49) obtained an Rg of 18.5 Å using neutron scattering which suggests that S4 is quite globular in conformation. From these disparate results, no conclusion on the shape of S4 could be drawn.

In an effort to resolve the controversy surrounding protein S4, this study has been made. Using hydrodynamic techniques, careful measurements of the sedimentation and diffusion coefficients were made. These values, coupled with the apparent specific volume, give a molecular weight which can be compared with values obtained experimentally from sedimentation equilibrium or sequencing methods.

These results are valuable not only in resolving the S4 controversy, but in pointing out the necessity of monitoring sample quality as the proteins are being characterized. It is apparent that similar studies are needed on many of the proteins to produce results of sufficient validity to be useful in preparing working models of ribosomes.

Chapter II

MATERIALS AND METHODS

Table 1.

List of Buffers.

Buffer A: 10 mM Tris-HCl pH 7.5, 0.1 M KCl, 20 mM $MgCl_2$, 6 mM β-mercaptoethanol.

Buffer C: 10 mM Tris-HCl pH 7.5, 70 mM KCl, 1 mM $MgCl_2$, 6 mM β-mercaptoethanol.

Buffer D: 50 mM sodium acetate pH 5.6, 6 mM β-mercaptoethanol, 0.05 mM phenylmethylsulfonylflouride.

Buffer E: 50 mM sodium acetate pH 5.6, 0.4 M LiCl, 6 mM β-mercaptoethanol, 0.05 mM phenylmethylsulfonylflouride.

Recon Buffer: 30 mM Tris-HCl pH 7.4, 0.35 M KCl, 20 mM $MgCl_2$, 10 mM β-mercaptoethanol.

70S Buffer: 0.01 M Tris-HCl pH 7.6, 0.1 M KCl, 15 mM $MgCl_2$.

65S Buffer: 0.01 M Tris-HCl pH 7.4, 0.5 M NH_4Cl, 1.5 mM $MgCl_2$.

30-50 Buffer: 0.01 M Tris-HCl pH 7.4, 0.1 M KCl, 1.5 mM $MgCl_2$.

UPβ Buffer: 0.05 M NaH_2PO_4 pH 6.5, 6 M urea, 12 mM methylamine 0.9 mM β-mercaptoethanol.

Isolation of Ribosomes

Escherichia coli, strain MRE600, harvested in 3/4-log phase were used for all preparations. The bacteria were purchased from Grain Processing, Inc., Muscatine, Iowa, and stored at -70° until ready for use.

All cells used in these studies were disrupted by mechanical grinding of a slurry of cell paste and 0.25-0.3 mm diameter glass beads in either 70S buffer or 65S buffer as described by Hill et al. (18) or in Buffer A as described by Dijk and Littlechild (10) in salt extraction procedures.

The cells were disrupted using two Bead Beaters (™Biospec Products). A stainless steel cup with a volume of approximately 275 ml was filled about two-thirds full with glass beads. Fifty grams of washed cell paste was added to the cup and it was filled to volume with buffer. The cup screws onto a blender base which has a plastic water jacket attached. A salted ice water bath was used in this water jacket for cooling. It was found that if the bead beater was allowed to run for more than thirty seconds, isolated ribosomal subunits showed degradation when analyzed in the Model E analytical ultracentrifuge. This breakdown was attributed to heat. By trial and error it was found that a cooling period of two and one-half minutes between grinding periods was adequate to compensate for the heat generated from thirty seconds of grinding. A Lindburg Enterprises Model CT-4 Chrontrol ™ interval timer was used to control the cycles of two Bead Beaters in tandem. An aggregate grinding time of one hour was found to give yields of 1.0-1.2 grams of crude ribosomes per 100 grams of bacteria. This amount is comparable

to the yield obtained by use of the Gifford-Wood minimill used by Hill et al. (18).

The crude ribosomal fraction was separated by differential centrifugation. After grinding, the glass beads were allowed to settle. The aqueous phase was poured off and the glass beads were washed with fresh buffer which was combined with the agueous phase. This mixture was subjected to centrifugation at 16,000 rpm for 1 hr in a Beckman JA-17 rotor at 4°C to remove undisrupted cells and the cell wall fraction. The supernatant was then centrifuged at 50,000 rpm for 3 hours at 4°C in a Beckman Ti-60 rotor. The pellet from this centrifugation, which contains the ribosome fraction, was resuspended overnight in 200 ml of buffer. The suspension was then subjected to a second spin at 16,000 rpm for 1 hr in a JA-17 rotor at 4°C followed immediately by a second spin at 50,000 rpm for 3 hours at 4°C in a Beckman Ti-60 rotor. The resulting pellets constitute the crude ribosomal fraction. They were resuspended in 35-50 ml of 30-50 buffer (18), or Buffer C (10). The sedimentation pattern of the resuspended fraction was routinely checked in the Beckman Model E analytical ultracentrifuge for quality.

Isolation of Ribosomal Subunits

The crude ribosomal pellets obtained from the second high-speed centrifugation were resuspended in buffer 30-50 or buffer C. It was then allowed to stir a minimum of three hours after resuspension to insure complete dissociation of subunits. Sample concentrations of approximately 30 mg/ml were used.

Ribosomal subunits were separated by zonal centrifugation using the method of Eikenberry et al. with minor modifications.

Sucrose solutions were made from stock sucrose solutions of C & H ® pure cane sugar which is more free of ribonuclease than beet sugar. The stock sucrose solution was pretreated with 0.1% v/v of diethylpyrocarbonate to insure against ribonuclease contamination. The diethylpyrocarbonate was removed by boiling the solution under vacuum for 8 hours. Appropriate salts were added to the stock sucrose solution and the solution was diluted with double-distilled water to make a working stock solution of approximately 50% sucrose w/v in the appropriate buffer system.

A Beckman Ti-15 zonal rotor equipped with a B-29 core was used in a Beckman Model L8-70 centrifuge for the zonal separation. Approximately 250 ml of buffer was put into the rotor before starting the centrifuge. The sample was then made 5% in sucrose by the addition of one-tenth volume of 50% working sucrose stock solution and loaded into the outside of the rotor using a peristaltic pump. This was followed by a 10-30% exponential sucrose gradient of one liter prepared in an International Equipment Co. gradient pump. The gradient was followed by 200 ml of 50% working stock sucrose solution. The rotor was then spun for 14-1/2 hrs at 31,000 rpm at 4°C.

The sample was recovered by displacing the rotor contents with cold, double-distilled water pumped into the center of the rotor with a peristaltic pump. The effluent was collected in 10 ml fractions using a Gilson Escargot fraction collector after being monitored at 280 nm using a Chromatronix Model 220 absorption detector. A plot of the absorption versus tube number was obtained using an Omniscribe recorder (Houston Instruments) interfaced to the detector and fraction collector. The recorder trace was used to pool appropriate fractions of subunits (Fig. 1).

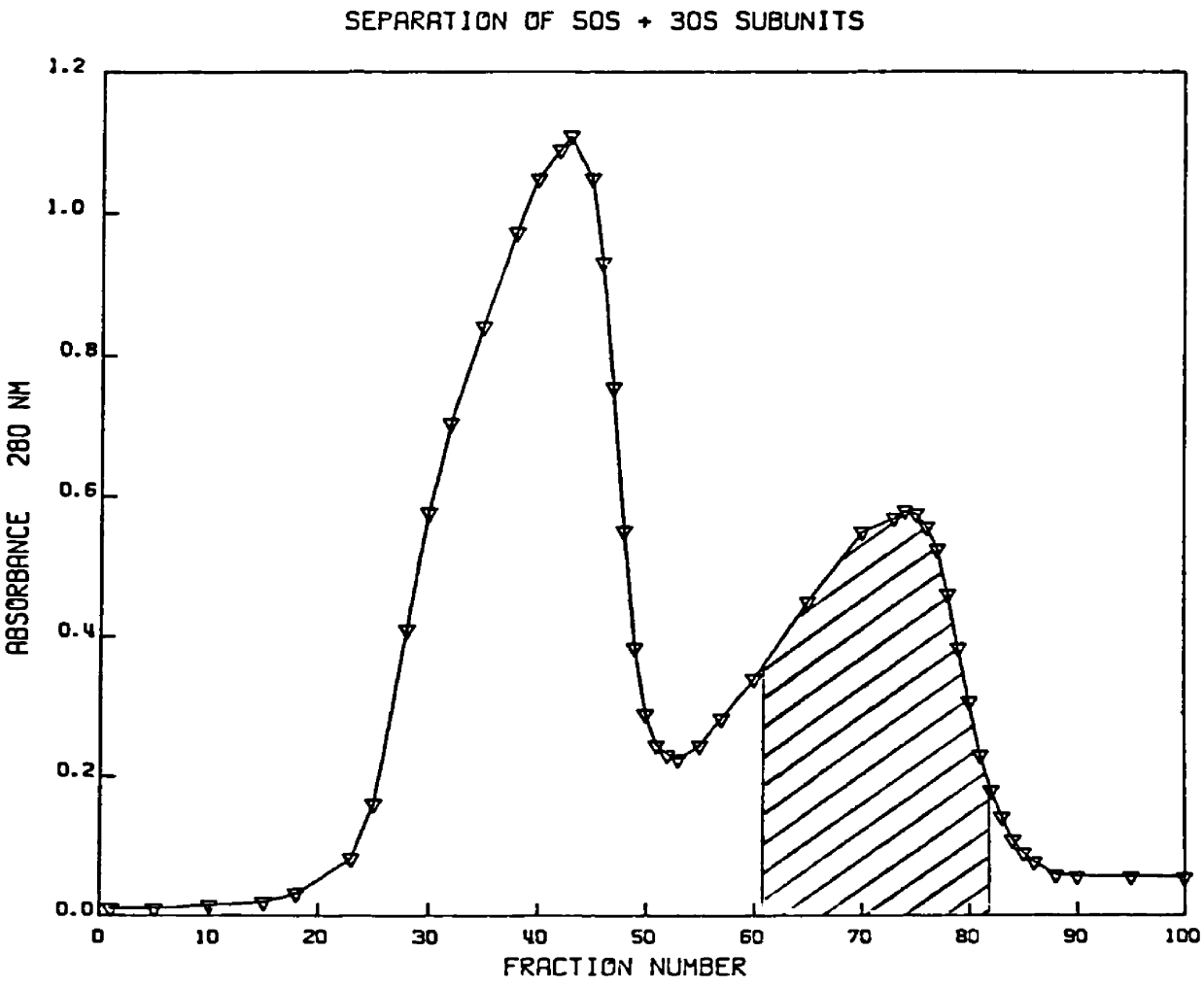


Figure 1. Plot of the absorbance at 280 nm versus fraction number from a typical zonal separation of 50S and 30S subunits. Shaded portion represents the fractions of 30S subunits normally pooled for use.

Subunits were recovered by ethanol precipitation. Pooled fractions were raised to 0.01 M magnesium ion by adding one-hundredth volume of a 1 M $MgCl_2$ solution and 0.001 molar in dithiothreitol by adding solid dithiothreitol. Two volumes of cold 95% ethanol were added to the pooled fractions to precipitate the subunits. The solutions of subunits were allowed to stir at 4°C for at least 30 minutes and then centrifuged at 8000 rpm for 30 min in a Sorvall GSA rotor. The pelleted subunits were

resuspended to concentrations of about 20 mg/ml, estimated from A_{280} profile, in 30-50 buffer or Buffer C and dialyzed at 4°C against 100 volumes of buffer for 24 hrs. Subunits were used immediately or stored at -70°C.

Protein Extraction

Salt Extraction Method

A solution of 30S subunits in Buffer C was diluted to a concentration of 100 OD A_{260} units/ml. This solution was made 0.01 M in EDTA by slow addition of 1/100 volume of 1 M stock EDTA solution to the stirred subunits. An equal volume of 2 M LiCl, pretreated with activated charcoal and mixed bead ion exchange resin No. AG 501-8x10 from BioRad, in Buffer C was then added slowly to the stirred mixture. The mixture was allowed to stir overnight (10 hrs) at 4°C. The mixture was then centrifuged 100,000 x g for 10 hrs in either a Beckman Ti-60 or Ti-70 rotor to remove the core particles. The supernatant was then diluted with an equal volume of Buffer D and dialyzed against 1.67 vol of Buffer D + 0.085 M LiCl with 3 changes of buffer over a 48 hr period. Aggregates were removed by spinning the dialyzed solution at 8000 rpm for 30 min in a Sorvall GSA rotor. Spectrapore ™ 6 dialysis membrane with a molecular weight cutoff of 2000 was used throughout for dialysis of protein solutions.

Acetic Acid Extraction Method

Proteins extracted by acetic acid were treated as described by Hardy et al. (16). A solution of subunits of concentration ~10 mg/ml was made 0.01 M in magnesium by adding solid $MgCl_2$. Two volumes of glacial acetic acid were then added to this mixture and the extraction

was allowed to proceed for at least 30 min with stirring at 4°C. The RNA was pelleted at 8000 rpm for 30 min in a Sorvall GSA rotor. The supernatant containing the proteins was then precipitated with 5 volumes of acetone and spun down at 8000 rpm for 30 min in a Sorvall GSA rotor. The precipitate was resuspended in a volume of UPβ buffer equivalent to the starting volume and then dialyzed against UPβ buffer for 48 hrs. These were then subjected to pre-reduction prior to column chromatography.

Protein fractionation by ion exchange chromatography

Salt-extracted preparations

Salt-extracted proteins were fractionated on a CM-Sephadex C-25 column. Quantity of sample for the columns was adjusted so as to use the same ratio of protein to column volume as Littlechild and Malcolm (31). Protein solutions were applied to the columns at rates of either 10 ml/hr or 25 ml/hr. The columns were then washed with Buffer D and 0.085 M LiCl until the A_{230} difference was zero. Columns were eluted with linear gradients of LiCl (0.085 M to 0.8 M) in Buffer D. Gradient volumes were decreased by the same ratio as of protein to column volume ratio. Fractions (2-5 ml) were collected by a Pharmacia Frac-300 fraction collector and A_{235} readings were made on a Beckman DU-8 spectrophotometer.

Acetic-Acid-Extracted Preparations

Proteins to be fractionated in urea buffers were first subjected to pre-reduction. The protein solution pH was raised to 8.4 with NaOH and 1% v/v β-mercaptoethanol was added. The mixture was incubated to 37°C for 30 min after which the pH was lowered to 6.5.

The reduced protein sample was applied to a P11 phosphocellulose column at a rate of 45 ml/hr. Sample loads ranged from 400 to 800 mg total protein. The column was then allowed to wash overnight with UPβ buffer. The column was eluted with a linear gradient of NaCl, 0.0 to 0.6 M in UPβ buffer, having a total volume of 7 L. Fractions of 10-15 ml were collected using a Pharmacia Frac-300 fraction collector. Protein concentration was monitored at A_{230} using a Beckman DU-8 or by using a Model 100-10 Hitachi Altex UV-Vis variable wavelength detector equipped with an Altex model 155-00 flow cell.

Gel filtration chromatography

Protein solutions were further purified on G-100 or G-75 Sephadex superfine columns having diameters of 2.5 cm and varying in length from 60 to 120 cm. Sample volumes ranged from 5-10 ml and flow rates were 10-20 ml/hr. Fractions (1-2 ml) were collected and protein concentrations were monitored by absorption at 280 nm.

Hydrophobic gel chromatography

Some fractions from CM-Sephadex chromatography which could not be purified completely by gel filtration chromatography were subjected to chromatography on Phenyl-Sepharose-4B. The protein samples were dialyzed against Buffer D + 2.5 M LiCl. These proteins were then applied to a column of Phenyl-Sepharose. The column was eluted with a 200 ml linear gradient of LiCl, 2.5 M to 0.0 M in Buffer D. Fractions of 1 to 2 ml were collected and protein concentration monitored by absorbance at 230 nm.

Protein renaturation

Samples of pooled column fractions were checked for an A_{280} of less than 0.08 which corresponds to a concentration of less than 0.1 mg/ml. Protein samples of less than 0.1 mg/ml were renatured by dialyzing the samples into reconstitution buffer. Samples were dialyzed for 72 to 96 hours with 2 or 3 changes of buffer. The samples were then allowed to come to room temperature before concentration.

Protein concentration

Protein concentration of the samples was achieved by one of two methods. Some samples were concentrated by ultrafiltration in a Model 52 Amicon stirred cell. Diaflo UM2 membranes were used at a pressure of 40 psi of N_2.

Samples were also concentrated using Millipore CX-10 immersible filters. These filters were attached to a vacuum using silicon tubing and immersed in the sample. These samples were stirred constantly while filtration was proceeding.

Physical Characterization

Sedimentation velocity

Sedimentation velocity experiments were performed using a Beckman Model E analytical ultracentrifuge equipped with RTIC, schlieren optics, and a photoelectric scanner system with multiplexer. Sedimentation velocity experiments were carried out using a type AN-H titanium rotor and a double sector cell with sapphire windows. The rotor was spun at 68,000 rpm, 4°C and scans were taken at 32 minute intervals. The

scanner was set at 280 nm with a slit width on the photomultiplier of 0.1 to 0.16 mm. The photomultiplier was set to scan at 3.02 cm/min and the strip chart recorder was set on the fast setting for high magnification.

A sedimenting macromolecule in a centrifugal field obeys the relationship $\frac{dr}{r} = s\omega^2 dt$

where s = the sedimentation coefficient

ω^2 = the angular velocity

r = radius from axis of rotation

t = time in sec

Integration yields

$$\ln \frac{r}{r_o} = s\omega^2 (t-t_o)$$

A least-squares plot of $\ln r/r_o$ vs time yields the slope $s\omega^2$ and dividing by ω^2 gives the sedimentation coefficient.

The sedimentation coefficients were corrected to 20°C in water using the equation

$$S_{20,w} = S_{T,B} \times \frac{\eta_4}{\eta_{20}} \times \frac{(1-\bar{v}\rho)_B}{(1-\bar{v}\rho)_w}$$

where η = viscosity

$\bar{v}$ = partial specific volume

ρ = density

Temperature changes in $\bar{v}\rho$ were neglected because they are about 2 orders of magnitude less than viscosity changes.

In these studies the $S_{20,w}$ was assumed to be the $S^\circ_{20,w}$ since the concentration used was judged to be low enough for negligible

concentration dependence.

Diffusion coefficient measurements

Diffusion coefficients were measured by the method of intensity fluctuation spectroscopy. Samples were prepared and concentrated at the University of Montana and then conveyed immediately to the laboratory of Dr. Victor Bloomfield at the University of Minnesota. Dr. Bloomfield's laboratory is equipped with a Lexel 2W Argon ion laser which is tunable to 700 mw at 4880 Å. Data were collected using a 64 channel Model 1096 Langley Ford single clipped digital autocorrelator which was interfaced to a Digital Equipment Corp. Minc minicomputer for immediate data analysis. Actual scattering experiments and data processing were done by Dr. Jason Wei. The data were analyzed by computer programs written by Dr. Warren Gallegher based on the cummulants (25) and histogram methods (15) to obtain diffusion coefficients in a polydisperse sample. Data were collected for various delay times and analyzed.

The first order electric field correlation function for a monodisperse solution is

$$|g^1(\tau)| = e^{-DK^2\tau} \qquad (25)$$

where D = diffusion coefficient

$K = (\frac{4\pi}{\lambda_o})\ n_o\ \sin(\frac{\theta}{2})$

n_o = retractive index

θ = scattering angle

τ = delay time MT = sample time

mT = delay time ↑ Number of channels

For a polydisperse system the function must be generalized for a number

of species $$|g^1(\tau)| = \int_o^\infty G(\Gamma)\, e^{-\Gamma\tau}\, d\Gamma \qquad (25)$$

$$\Gamma = DK^2$$

$G(\Gamma)$ = normalized distribution of decay rates

A single clipped digital autocorrelator measures

$$c_m = \sum_{i=M}^{N} \eta(t_i)\, \eta_K(t_{i-m}) \qquad (41)$$

where N = number of sample intervals

t_i = sample interval time

M = # channels in the autocorrelator

$\eta(t_i)$ = # photons detected in the $i^{\underline{th}}$ time interval

$\eta_K(t_{i-m}) = 1$ if $\eta(t_i) \geq k$

$= 0$ if $\eta(t_i) < k$

k = clipping level

For a large number of experiments, the measured c_m will be close to the average, $c_m = \langle c_m \rangle$, which is related to the normalized electric field correlation function

$$\langle c_m \rangle = N \langle \eta \rangle \langle \eta_k \rangle \left[1 + \left(\frac{1+k}{1+\langle \eta \rangle}\right) \beta |g^1\tau|^2\right]$$

$$\beta = \sim 1 \qquad (41)$$

a function which takes into account

incomplete spatial coherence and

noise in the detector

The experimental values measured, $\langle c_m \rangle$, are then the equivalent of a second order equation of the first order normalized correlation function and may be expressed as

$$\langle c_m \rangle \equiv g^2(\tau) = 1 + A|g^1(\tau)|^2 \qquad (25)$$

A = measurable background and effects of spatial coherency

A plot of $\ln|g^2(\tau)-1|$ vs τ then yields a line with slope proportional to D.

$$\ln|g^2(\tau)-1| = \ln A + {}^{-}2DK^2\tau$$

In the cummulant analysis technique, the logarithmic expression is expanded in a MacLaurin series

$$\ln|g^2(\tau)-1| = \ln A - 2\bar{\Gamma}\tau + \frac{\mu^2}{\bar{\Gamma}^2}(\bar{\Gamma}\tau)^2 - \frac{2}{3!}\cdot\frac{\mu 3}{\bar{\Gamma}^3}(\bar{\Gamma}\tau)^3 \cdots$$

For a simple system only the first two terms are needed but for a polydisperse sample the higher order terms come into use in curve fitting procedures using a weighted polynominal fit. This analysis gives a Z-average diffusion coefficient (41).

The histogram method of analysis expresses the first order correlation function in the form of

$$|g^1(\tau)| = \sum_{j=1}^{M} G(\Gamma_j) \int_{\Gamma_j-\Delta\Gamma/2}^{\Gamma_j+\Delta\Gamma/2} e^{-\Gamma\tau} d\Gamma \qquad (14)$$

M = # of steps in the histogram.

The net signal autocorrelation function then has the form

$$g^2(\tau) = A\beta\Big(\sum_{j=1}^{M} a_j\left(\frac{-1}{\tau}\right)\{e^{-(\Gamma_j + \frac{\Delta\Gamma}{2})\tau} - e^{-(\Gamma_j - \frac{\Delta\Gamma}{2})\tau}\}\Big)^2$$

$$a_j = G(\Gamma_j)$$

Then a least squares analysis is used to minimize χ^2, the goodness of fit, with respect to all a_j's simultaneously. The a_j's are adjusted until the χ^2 values are within the statistical error of the measured

data (14).

Dr. Jason Wei used both methods of analysis in order to provide an internal check of the analysis techniques. Histograms of 15-30 steps were used and gave good agreement up to the fourth order cummulant.

Sedimentation equilibrium

Sedimentation equilibrium experiments were carried out using a Beckman Model E analytical ultracentrifuge. An ANH-type titanium rotor was used with a 12 mm length double sector cell with sapphire windows. The temperature used was 4°C controlled to ± 0.1°C by the RTIC system of the Model E. The high speed method of Yphantis (62,72) was used. Samples were run at 40,000 rpm and the interference pattern was photographed at 20-24 hrs using Kodak type II-G spectroscopic plates. Fringe displacement was measured on a Nikon 6C microcomparator equipped with IKL digital micrometers interfaced directly to an Imsai 8080 microcomputer. Five fringes were read and data was analyzed by computer program for standard deviation. Any point which gave a deviation of greater than 0.01 fringe was rejected. Number-, weight- and Z-average molecular weights were determined by computer programs written by Robert Dyson and modified by Donald Blair.

Partial specific volume and density increment

The apparent partial specific volume, $\bar{v}$, of a protein in solution can be calculated from its amino acid composition (48) or measured directly (26).

To determine the apparent partial specific volume of a protein from its amino acid composition the weight fractions of each amino acid are

multiplied by the individual partial specific volumes (48). These are summed and divided by the sum of the weights

$$V_p = \frac{\sum_i w_i v_i}{\sum_i w_i}$$

V_p is the apparent partial specific volume of the protein.

Alternatively, the apparent specific volume of a macromolecule in solution may be measured by determining the density increment, $d\rho/dc$. In 1964 Cassasa and Eisenberg (6) outlined a procedure for determining the density increment which is related to the apparent partial specific volume by $\frac{d\rho}{dc} = 1 - \bar{v}_2\rho_o$

The density increment is obtained by determining the slope of a density versus concentration plot.

The density of a sample was determined using a Paar DMA-02C precision digital density meter (26). This instrument measures the time required for a hollow oscillator filled with solution to oscillate for a predetermined number of oscillations. The time for a standard of known density is related to the time of the unknown and its density by

$$\rho_1 - \rho_2 = \frac{1}{A}(T_1^2 - T_2^2)$$

A = instrument constant

ρ_i = density of solution i

T_i = time for oscillations of solution i

Glass double distilled water was used as the standard. The density of water at 4°C is 0.999973 according to the Handbook of Chemistry and Physics.

The temperature of samples was controlled to 4° (±0.01)°C using a thermostat assembly consisting of a Laude K4/RD refrigerated circulating bath and a Haake FS pump which was controlled by a Tronac Model 1040 precision temperature controller. The density meter was interfaced to a Wang 600 programmable calculator which started, reset, and recorded the clocked time for the preset number of oscillations (10^4 in all experiments).

Several hours were needed to warm up the equipment. The sample was dialyzed prior to use for 72 hrs with two changes of buffer. A quantity of dialysate was used to make dilutions of the stock solution. The A_{280} of each sample was determined using a Beckman DU-8 spectrophotometer. A linear least squares program was used to determine the slope of the density versus concentration plot.

The experimental protocol of Cassassa and Eisenberg calls for each sample in a concentration series to be dialyzed to equilibrium in order to satisfy the requirement of chemical equilibrium. In practice, however, the common method of determining concentration of a macromolecule in solution is by spectroscopy. The error resulting from spectroscopy measurements is greater than the errors resulting from a small deviation from chemical equilibrium and so a weight dilution series made with an analytical balance is the method employed for determining concentration once an initial absorbance reading is made.

Extinction Coefficient

The extinction coefficient was determined using a protocol similar to that outlined by Kupke and Dorrier (27) for dry weight measurements.

Ground glass weighing vials 7.5 x 15 mm were heated to constant weight using a Thelco vacuum oven at 100°C. An aluminum block with holes drilled to accommodate 8 vials was used as a holder. Wire posts were set around the holes for the vial tops to rest on which allows for adequate gas exchange while excluding dust.

The protein samples were run in triplicate. A volume of 250 µl of either sample or dialysate was put into the vials. The buffer used was recon buffer without β-mercaptoethanol as it was anticipated that the absence of reducing agent would not seriously affect the absorbance of the protein but its volatility might complicate the weighing process. The sample and dialysate vials were then frozen by placing them in the holder which was then put in a Revco Ultra Low freezer at -70°C for 1 hr. The samples and dialysate were then put under a high vacuum and lyophilized for 24 hrs.

The vials were then heated at 100°C under vacuum repeatedly until they remained at constant weight (± 1.0 µg) for 2 successive weighings.

The absorbances of solutions were determined at 280 nm on a Beckman DU-8 spectrophotometer. The volume was calculated from the density of the solution measured as previously described. A Mettler H20T analytical balance was used for all weighings.

Protein Identification and Purity

Protein samples were routinely identified by the method of two-dimensional polyacylamide gel electrophoresis (22). To prepare a protein sample, an aliquot of a column fraction was precipitated using 5 volumes of acetone. The precipitate was spun down at 10,000 xg for

30 min and then aspirated to remove residual acetone. The precipitate was then resuspended in 50 µl of 8 M urea and 0.04 M Tris pH 8.2.

The first dimension gels were run in flint glass tubing, 3 mm id., cut in 8 cm lengths. A separation gel of 5 cm was used and was composed of:

Urea, ultra pure	36% w/v	
Acrylamide	4% w/v	
Bis	0.13% w/v	
EDTA, disodium salt	0.8% w/v	pH 8.6
Boric acid	3.2% w/v	
Tris base	4.87% w/v	
TEMED	0.3% v/v	

The solution was degassed for about 10 minutes prior to use. Polymerization was initiated by addition of 20 µl of a 10% w/v solution of ammonium persulfate.

A stacking gel of 1 cm was used and was composed of:

Urea, ultra pure	48% w/v	
Acrylamide	4% w/v	
Bis	0.2% w/v	
EDTA, disodium salt	0.085% w/v	pH 8.2
Boric acid	0.32% w/v	
Tris base	0.45% w/v	
TEMED	0.45% v/v	

The stacking gel was poured on top of the separation gel after degassing for about 10 minutes. Polymerization was initiated by addition of 25 µl of 10% w/v ammonium persulfate.

Eight to ten gels were usually prepared at a time which requires 5 ml of separation gel solution and 2 ml of stacking gel solution. For less volume the amount of ammonium persulfate added must be reduced. Two sets of gels were then run simultaneously with equal volumes of sample on each. One set was run from cathode to anode using bromophenol blue as an indicator. The other set was run from anode to cathode using pyronine G as an indicator. The gels were run at 3 mA per gel constant amperage for 4.5 hours on a Buchler Model 3-1155 power supply. The buffer solution consisted of 0.725% w/v Tris base, 0.48% w/v boric acid and 0.24% w/v EDTA disodium salt, pH 8.2.

When running a single protein, approximately 0.2 mg was dissolved in ~50 µl of 8 M urea, 0.04 M Tris HCL pH 8.2. When looking for impurities as much as 1 mg of a single protein was applied.

The gels were removed from the tubing after 4.5 hours using a syringe and distilled water or buffer. A 22 G 1.5 inch needle was found to be best for injecting the water or buffer in removing the gels.

The pairs of gels from the first dimension were then placed at the top of an 11 cm x 0.15 cm gel slab with the stacking gels toward the center. The gel solution for the second dimension was composed of:

Urea, ultra pure	36% w/v	
Acrylamide	18% w/v	
Bis	0.2% w/v	pH 4.6
Glacial acetic acid	5.2% w/v	
Potassium hydroxide	0.27% w/v	
TEMED	0.58% v/v	

Approximately 22 ml of gel solution was usually prepared per gel. After 10 min degassing the polymerization was initiated by addition of 0.8 ml of 10% w/v ammonium persulfate per gel. The buffer used for the second dimension consisted of 1.4% w/v glycine, 0.15% v/v glacial acetic acid, pH 4.0. Electrophoresis was carried out from anode to cathode using pyronine G as an indicator. The gels were allowed to run until the dye front was 1-2 cm from the bottom of the gel. Using a Buchler Model 3-1155 power supply, a running time of 8-9 hrs at 160 V constant voltage was adequate.

Proteins were visualized by staining the slabs in a solution of methanol, water and glacial acetic acid, ratio of 4.5:4.5:1, with 0.2% w/v coomassie brilliant blue R dye. They were destained using the solution without dye.

A sample containing all the 30S proteins was run with each set of gels as a control. Approximately 1 mg of proteins was dissolved in 100 µl of 8 M urea; 0.04 M Tris-HCl, pH 8.6. Fifty µl of this solution was applied to one of a set of gels. Only 20 µl of the single protein samples were needed per one-dimension gel. A direct comparison of the migration was used to identify the protein present. By overloading the gel, impurities were detected.

Immunoprecipitation was carried out in order to determine the purity of the sample. Dr. L. Kahan at the University of Wisconsin graciously performed these tests in his lab. The protein sample was tested against antiserum from ribosomal proteins S3, S5, and S7 which are the most frequent contaminents of S4. This sample was also compared to Dr. Kahan's standard S4.

Hydrodynamic properties and relationships

Throughout this work an evaluation of the molecular weights from sedimentation equilibrium data was the primary tool used for sample quality. A macromolecule which is at sedimentation equilibrium obeys the fundamental equation,

$$c(r) = c(a)\, e^{\omega^2 M(1-\bar{v}\rho)(r^2-a^2)/2RT}$$

or rearranging and taking the logarithm

$$\ln \frac{c(r)}{c(a)} = \frac{\omega^2 M(1-\bar{v}\rho)(^2-a^2)}{2RT}$$

where

$c(i)$ = concentration at i

r = radial distance from the center of rotation

a = meniscus distance from center of rotation

ω^2 = angular velocity $(\frac{2\pi rpm}{60})^2$

M = molecular weight of solute

$\bar{v}_2$ = partial specific volume of solute

ρ = density of solvent

R = gas constant

T = the absolute temperature

A plot of ln c(r) vs r^2 yields a straight line for a homogeneous monodisperse sample. The slope will be directly related to the molecular weight.

The hydrodynamic properties of a macromolecule are also related to the molecular weight by the Svedberg equation:

$$M = \frac{sRT}{D(1-\bar{v}\rho)}$$

where

s = the sedimentation coefficient

D = the diffusion coefficient and the others are as stated previously.

A comparison of the molecular weight obtained by both methods serves as an internal reference.

The frictional coefficient of a macromolecule in solution is related to the experimentally determined sedimentation coefficient by

$$f = \frac{M(1-\bar{v}\rho)}{NS}$$

and to the diffusion coefficient by

$$f = \frac{RT}{ND}$$

where f = frictional coefficient

N = Avagadros number

The frictional coefficient of a sphere, f_o, is given by Stokes law

$$f_o = 6\pi\eta R_o$$

η = viscosity of the medium

R_o = radius of the hydrated sphere

$$= \left(\frac{3M}{4\pi N}(\bar{v}_2 + \delta v_1)\right)^{1/3}$$ (δ is the amount of hydration, $^{gms}H_2O/gm$ protein)

The ratio f/f_o is then a measure of the deviation of a molecule from a sphere of equivalent volume (55). For comparisons of a molecule's asymmetry the ratio f/f_{min} is defined as

$$\frac{f}{f_{min}} = \frac{f}{f_o}\left(\frac{\bar{v}_2 + \delta\bar{v}_1}{\bar{v}_2}\right)^{1/3}$$

and is the ratio of the frictional coefficient of a macromolecule to that of an anhydrous sphere of equivalent volume. This quantity is a maximum value for the asymmetry of the molecule if the hydration is set

to 0. If a hydration factor is known, the value f/f_{min} can be used to obtain the f/f_o value and a value for the asymmetry can be determined.

The f/f_{min} value is related to the experimentally determined properties, the sedimentation coefficient and the diffusion coefficient, by

$$\frac{f}{f_{min(s)}} = \frac{(4/3)^{1/2}\ (1-\bar{v}_2\rho)\ M^{2/3}}{6\eta(\pi N)2/3\ \bar{v}_2\ 1/3\ S^{\circ}_{20,w}}$$

$$\frac{f}{f_{min(D)}} = \frac{(\frac{4N}{3})^{1/3}\ kT}{6\eta\pi^{2/3}\ D^{\circ}_{20,w}\ (M\bar{v}_2)^{1/3}}$$

k = Boltzman's constant

The volume of a hydrated macromolecule v_h is

$$v_h = \frac{M}{N}\ (\bar{v}_2 + \delta\bar{v}_1)$$

The effective hydrodynamic radius, R_h, of a macromolecule is

$$R_h(sed) = \frac{M(1-\bar{v}\ )}{6\eta\pi Ns} \qquad R_h(Diff) = \frac{RT}{N6\pi\eta D}$$

The radius of gyration of a particle is defined as

$$Rg = \left(\sqrt{\frac{\Sigma_i M_i r_i^{\ 2}}{\Sigma_i\ M_i}}\right)^{1/2}$$

for a sphere of uniform density

$$Rg = \sqrt{3/5\ R^2}$$

for a prolate ellipsoid

$$Rg = \sqrt{\frac{b^2 + 2a^2}{5}}$$

b = semi-major axis

Chapter III

RESULTS

Protein isolation

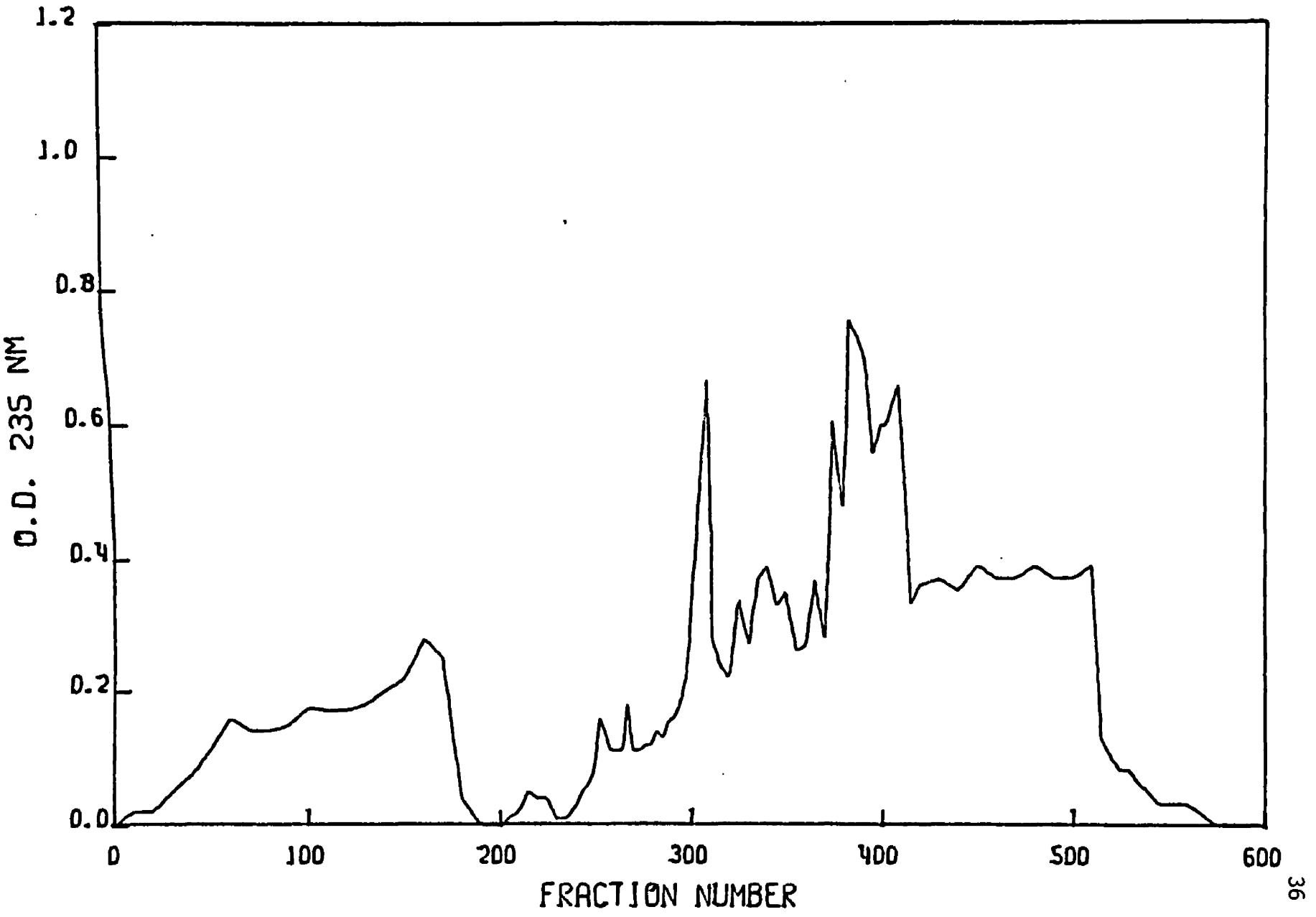
Salt-extracted proteins

Attempts to purify the 30S ribosomal subunit proteins were initially made following the protocol of Littlechild and Malcolm (31) and Dijk and Littlechild (10). These attempts were scaled down by a factor of approximately one-tenth from those described previously (10,31). The protocol was followed exactly, except that benzamidine was omitted from the buffers since it was found to absorb strongly at 230 nm and 280 nm making the spectrophotometric determination of protein difficult.

The first extraction was made using 1.2 gm of 30S subunits. The elution profile is shown in Figure 2. In comparing the experimental profile with published profiles it was noted that the experimental profile appeared somewhat compressed and that peaks in the profile did not directly correspond to specific proteins. The most striking problem encountered in this procedure was the poor yield of protein from the 30S subunits. The extraction process was very inefficient and considerable loss of protein occurred during sample dialysis due to aggregation.

To minimize the loss of protein upon dialysis, a batch binding assay was performed with total 30S proteins in buffer D and LiCl concentrations of between 0.01 and 0.1 M. It was found that the majority of sample would still bind CM-Sephadex at a concentration of 0.085 M LiCl and that the increased ionic strength significantly decreased loss due to

Figure 2. Protein elution profile A_{235} versus fraction number of first extraction of 30S subunits with 1M LiCl from a CM-Sephadex C-25 column having 42 ml volume and dimensions of 1.1 x 30 cm. Flow rate was 10 ml/hr. Sample size was approximately 20-25 mg, eluted with a 750 ml linear gradient of LiCl, 0.15 M to 0.8 M, in Buffer D. The fractions collected were 2-3 ml.


PROTEIN ELUTION PROFILE 30S CM-SEPHADEX
1.2
1.0
0.8
0.6
0.4
0.2
0.0
O.D. 235 NM
0
100
200
300
400
500
600
FRACTION NUMBER

aggregation during dialysis. Subsequent to this assay, salt-extraction preparations were always dialyzed against buffer D + 0.085 M LiCl to equilibrium as determined by conductivity measurements. This was a significant deviation from the published experimental protocol.

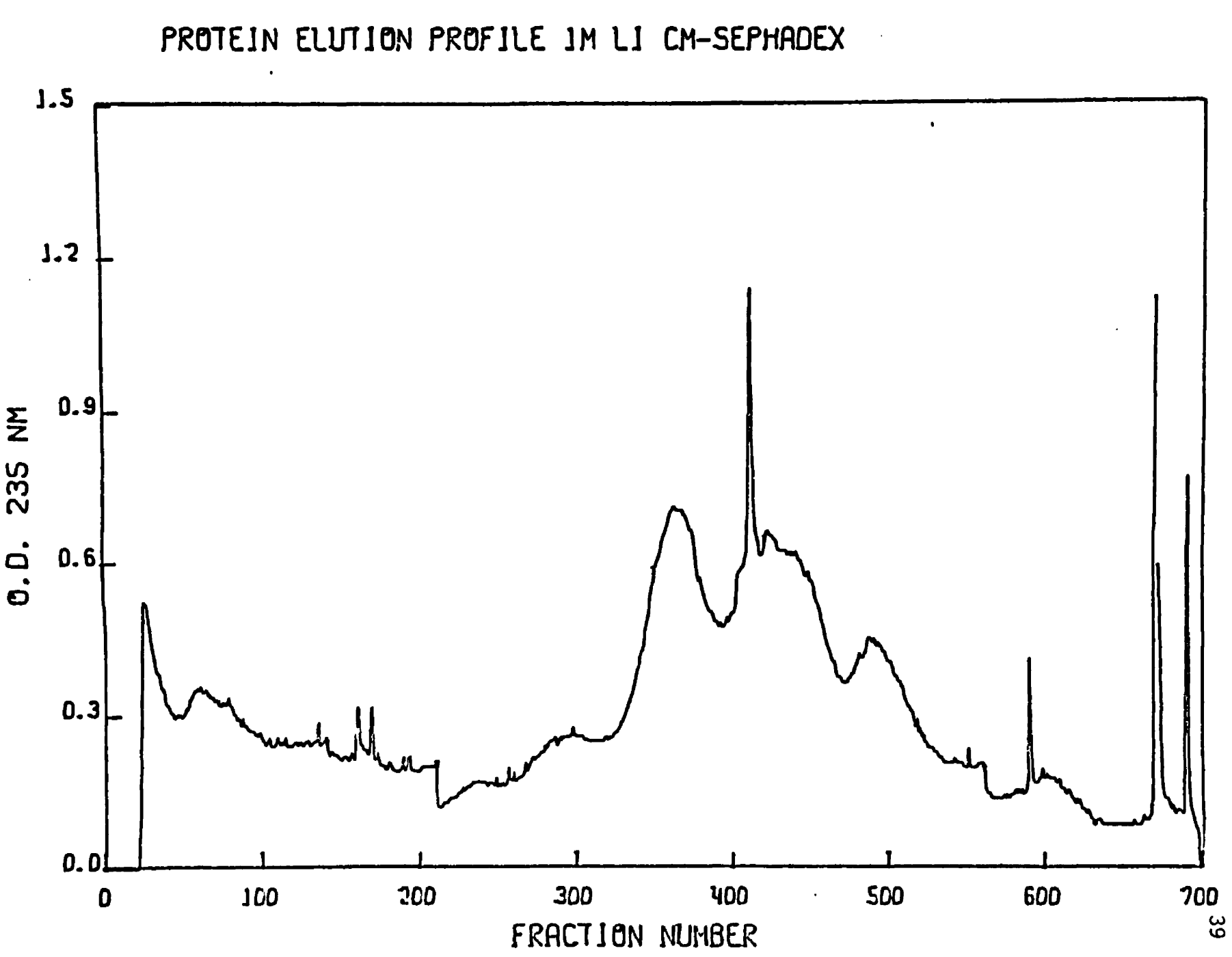
Another attempt was made using the salt extraction technique, but on a larger scale. The elution profile, shown in Figure 3, was more similar to that of Dijk and Littlechild (10). Two-dimensional gel analysis gave the following results:

Fraction No.	Proteins present
25	S5,S7,S8,S10
40	S5,S8,S15,S16,S17
60	S3,S5,S8
80	S3,S5,S8
365	S3,S4,S5,S15,S16,S17
410	S3,S4,S5,S16
450	S3,S4,S5
480	S3,S4,S5,S7,S9,S10,S19,S20
589	S3,S4,S5,S9,S20
600	S3,S4,S5,S9,S14,S20

No fractions came off which contained S4 without S3 and S5 present. This is not what the description of Dijk and Littlechild (10) would lead one to expect. They reported that S4 could be obtained in a homogeneous preparation. However, they also noted that large amounts of S4 were isolated in a complex with S3 and S5. It was suspected that the S4 in our preparations was in the form of this complex.

An attempt was made to separate S3, S4 and S5 upon a hydrophobic

Figure 3. Protein elution profile A_{235} versus fraction number of 30S proteins extracted by 1 M LiCl. The protein from about 2.5 gms of 30S subunits was extracted and applied to a CM-Sephadex column, 2.6 x 30 cm, with a bed volume of 60 ml. Sample was eluted with 2 L linear gradient 0.15 to 0.8 M LiCl in Buffer D, and 3 ml fractions were collected.


PROTEIN ELUTION PROFILE 1M LI CM-SEPHADEX
1.5
1.2
0.9
0.6
0.3
0.0
O.D. 235 NM
0
100
200
300
400
500
600
700
FRACTION NUMBER

column system using Phenyl-Sepharose 4B. Fractions from the previous CM-Sephadex column rich in these proteins were pooled and used as a sample for the hydrophobic column system. Figure 4 shows the elution profile obtained and two-dimensional gel analysis showed no separation.

Another extraction of 2.5 gms of 30S subunits was made for use on a CM-Sephadex column using the conditions of the previous separation but increasing the column volume. The elution profile, shown in Figure 5, was similar to the previous isolation and two-dimensional gel analysis confirmed that the separation was no better.

Since no S4 could be isolated from S3 and S5 on the ion exchange column, an attempt was made to separate them on a G-75 Sephadex gel filtration column. The elution profile shown in Figure 6 was the result of this experiment, using pooled fractions from the previous CM-Sephadex column rich in S3, S4 and S5 for a sample. Two-dimensional gel analysis showed no resolution of S4 from S3 and S5.

With all the above efforts over a two-year period still not giving quality samples, it was decided to attempt the acetic acid-urea preparation method (16) and use great care in renaturing these samples.

Acetic Acid-Urea extracted proteins

The first acetic acid-urea protein extraction and column were run according to the protocol of Hardy et al. (16) with modifications of Rhode et al. (44). About 1.1 grams of 30S subunits were extracted with acetic acid giving a protein sample of approximately 400 mg. The 30S subunits used for this extraction consisted of a mixture of subunits isolated either in Buffers A and C as per Dijk and Littlechild (10) or in Buffers 65S and 30-50 as per Hill et al. (18). Approximately 500 ml

Figure 4. Protein elution profile, A_{235} versus fraction number, of S3, S4, S5 fractions pooled from CM-Sephadex column and applied to 17 ml of Phenyl-Sepharose 4B in a 1.1 x 30 cm column. Sample was eluted with a 200 ml linear gradient, 2.5 M to 0.0 M LiCl in Buffer D, and 0.5 ml fractions were collected.

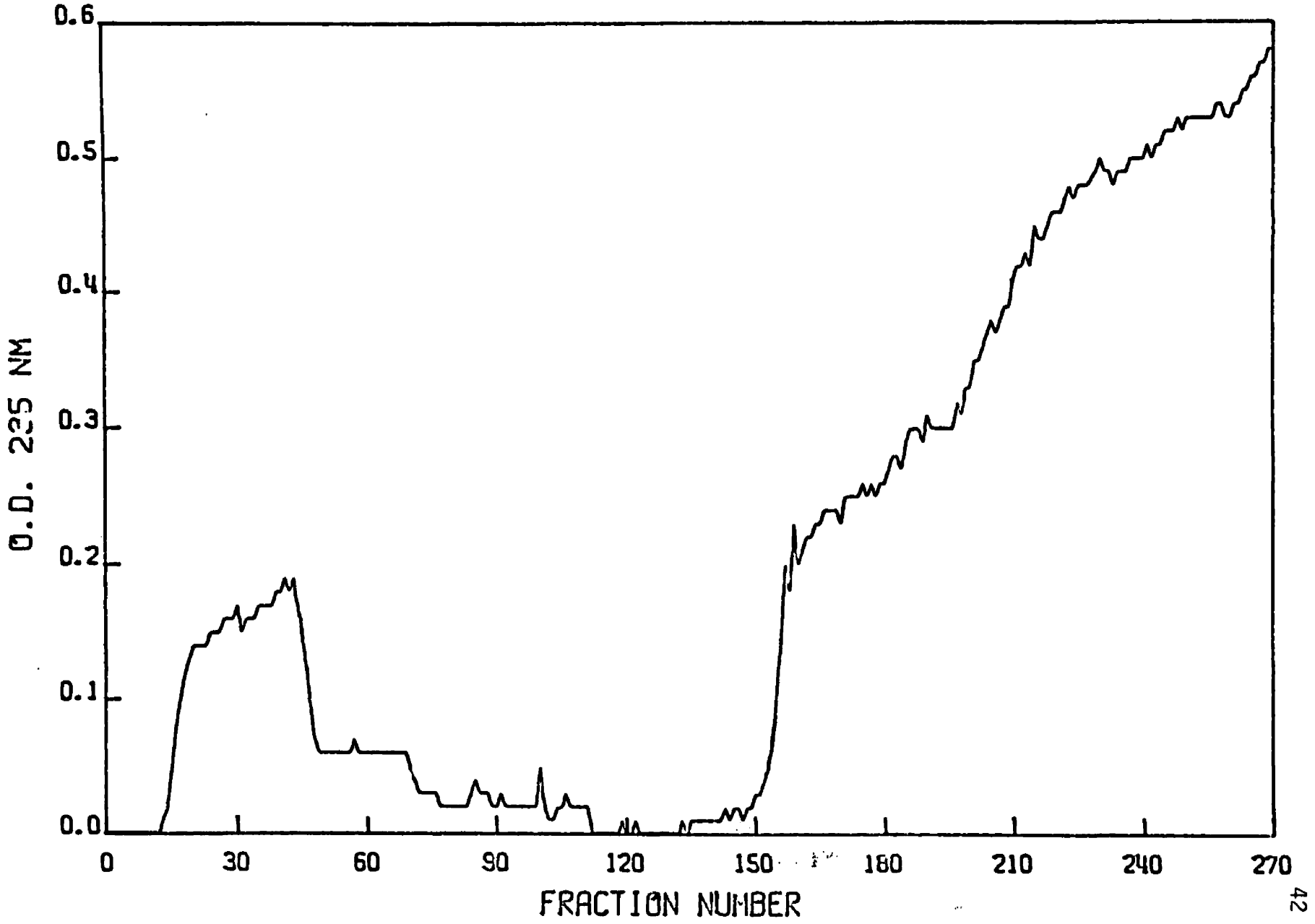

PHENYL-SEPHAROSE PROFILE OF S3-4-5
0.6
0.5
0.4
0.3
0.2
0.1
0.0
O.D. 235 NM
0
30
60
90
120
150
180
210
240
270
FRACTION NUMBER

Figure 5. Protein elution profile, A_{235} versus fraction number, of 1M LiCl extracted 30S subunits. The protein from about 2.5 gms of subunits was extracted and applied to a CM-Sephadex column, 2.6 x 30 cm, having a bed volume of 170 ml. The sample was eluted with a 2 L linear gradient, 0.15 M to 0.8 M LiCl in Buffer D, and 4 ml fractions were collected.

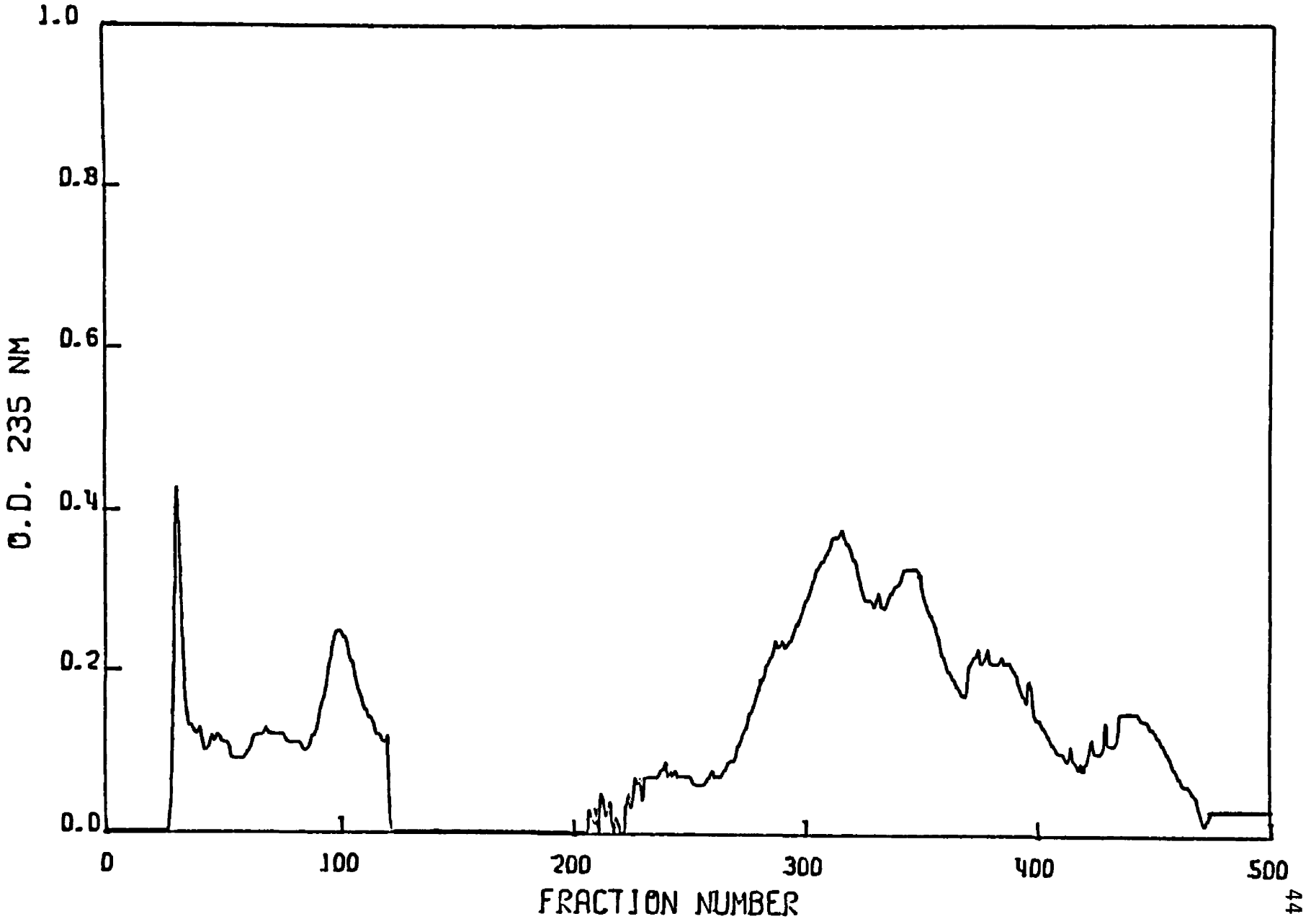
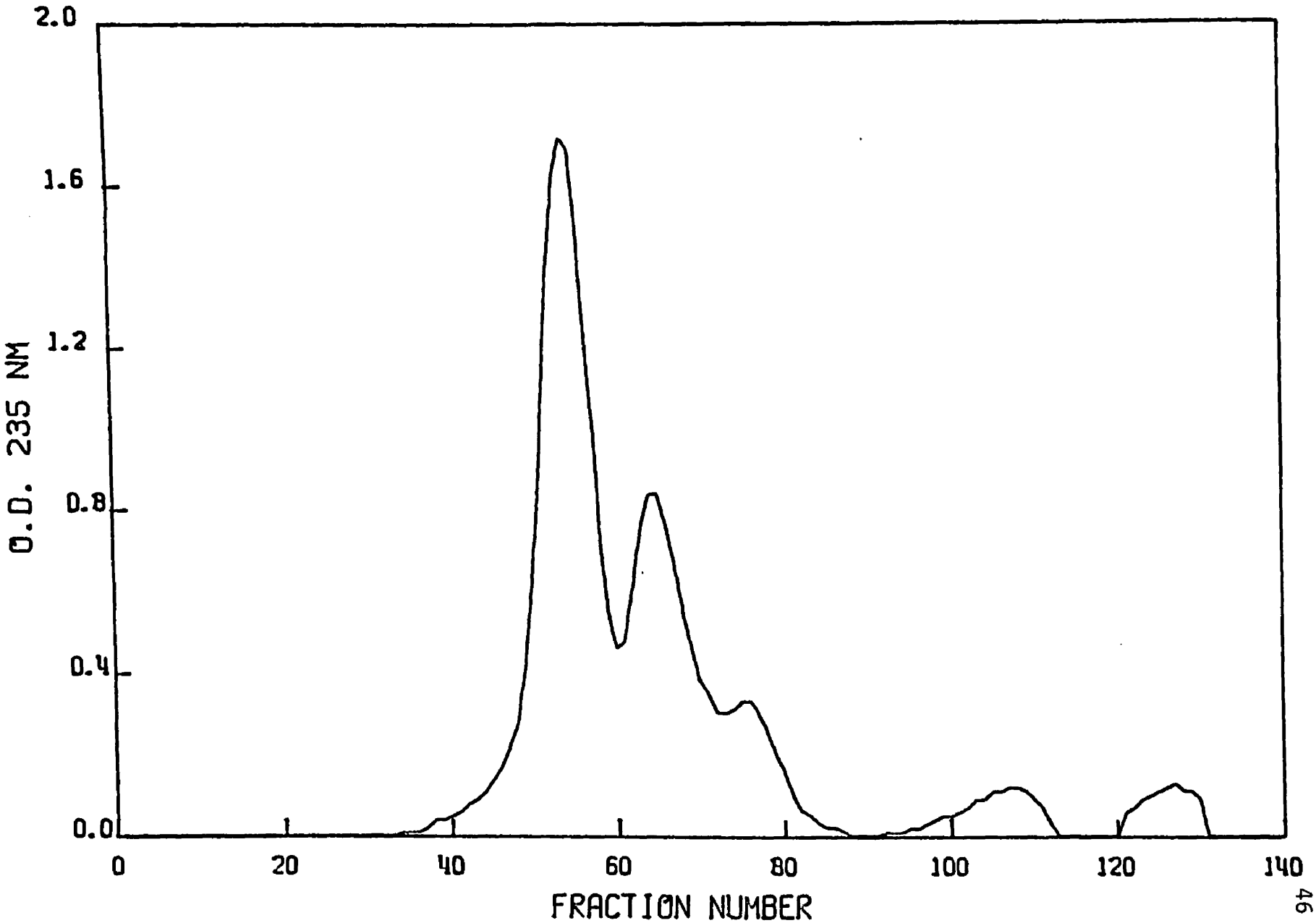

COLUMN ELUTION PROFILE 30S CM-SEPHADEX
1.0
0.8
0.6
0.4
0.2
0.0
O.D. 235 NM
0
100
200
300
400
500
FRACTION NUMBER

Figure 6. Protein elution profile, A_{235} versus fraction number of S3, S4, S5 fractions pooled from CM-Sephadex column and applied to G-75 Sephadex column in Buffer E. Column size was 1.6 x 100 cm and a flow rate of 5 ml/hr was used. Fractions of 1.25 ml were collected.

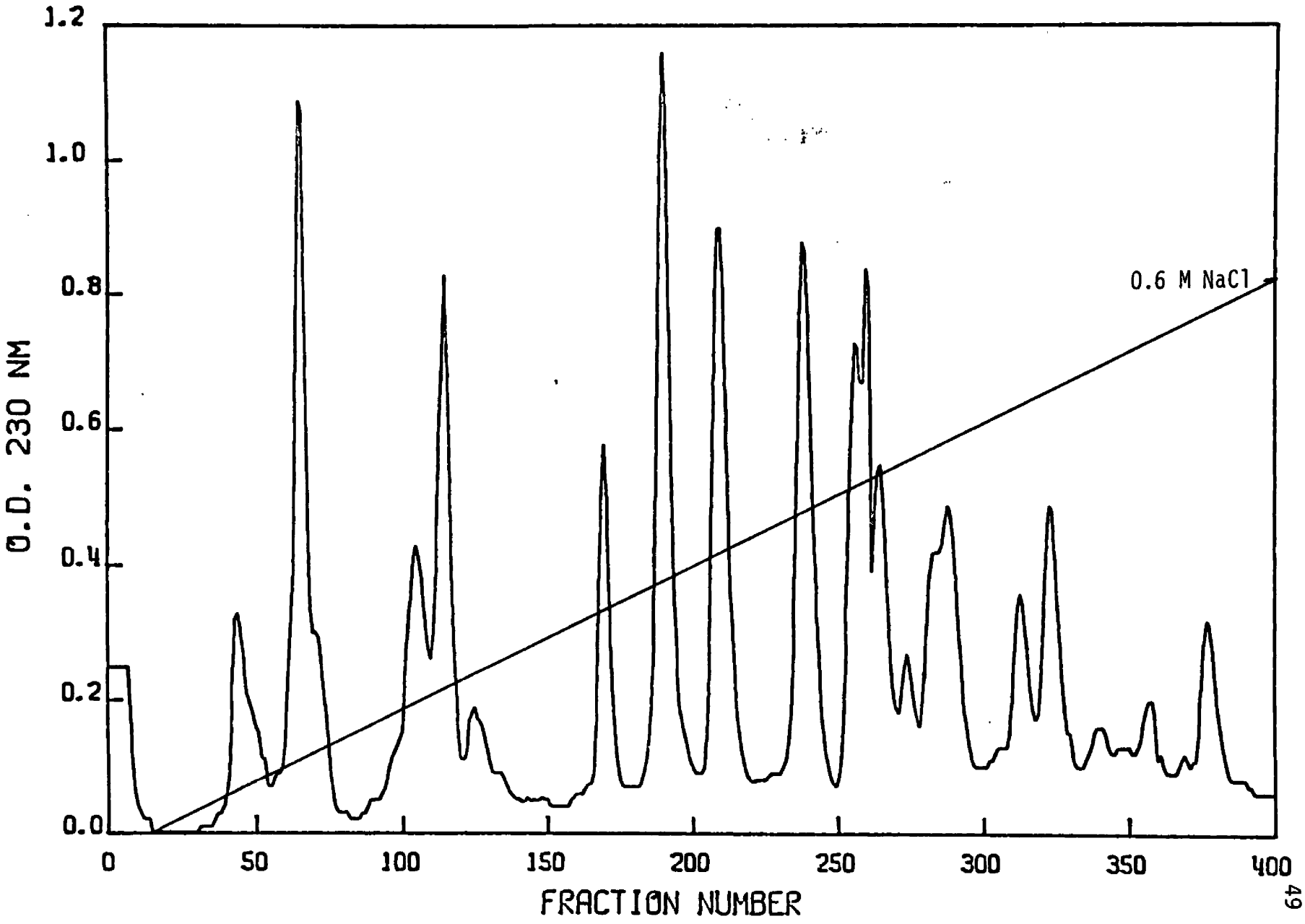

ELUTION PROFILE OF TUBES 339-370 ON G-75
2.0
1.6
1.2
0.8
0.4
0.0
O.D. 235 NM
0
20
40
60
80
100
120
140
FRACTION NUMBER

of a 60% v/v solution of NaOH/HCl washed P11 phosphocellulose in UPβ buffer was poured in this column and packed at a flow rate of ~45 ml/hr using a peristaltic pump. The settled bed volume was approximately 300 ml when flowing, which corresponds roughly to that reported by Hardy et al. (16). The acetic acid-extracted protein was precipitated with acetone and resuspended in 300 ml of UPβ buffer which is a deviation from other methods (16,44). After dialysis, the protein sample was reduced with 1% β-mercaptoethanol and applied to the phosphocellulose column at a rate of approximately 45 ml/hour. The absorbance of the sample at 230 nm prior to application was A=3.56/ml. The absorbance of column eluate, or flow-through, showed an average of 0.49 A_{230} units/ml. The column was eluted with a 6 liter linear gradient, 0.0 to 0.6 M NaCl in UPβ buffer, and 15 ml fractions were collected. The elution profile indicated very discrete separation (Figure 7). Two-dimensional gel electrophoresis analysis of column fractions gave the following results:

Fraction number	Proteins present
65	S6
115	S5, S10
170	S16, S17
190	S7, S17
209	S3
238	S4
261	S9, S18
288	S14, S17

The resolution appeared to be excellent on this column. Fraction numbers 235-245 were combined on the basis of their A_{230} readings. The

Figure 7. Protein elution profile, A_{230} versus fraction number, of acetic acid extracted proteins in UPβ buffer. About 400 mg of protein was applied to a phosphocellulose column, 2.6 x 60 cm, with a bed volume of 300 ml. The sample was eluted with 6 L of linear gradient 0.0 to 0.6 M NaCl in UPβ buffer. Flow rate was ~45 ml/hr and 15 ml fractions were collected.


PROTEIN ELUTION PROFILE TP30 P11
1.2
1.0
0.8
0.6
0.4
0.2
0.0
O.D. 230 NM
0.6 M NaCl
0
50
100
150
200
250
300
350
400
FRACTION NUMBER

preparation, which was designated Sample #1, was initially dialyzed into 15% acetic acid and frozen as described by Hardy et al. (16). Precipitate was evident after dialysis. Sample #1 was subsequently thawed and dialyzed into Recon buffer at low concentration to allow for renaturation. The sample was then concentrated at room temperature to a concentration of about 1 mg/ml using Amicon UM2 ultrafiltration membranes. The yield at this point was about 8 mg of protein S4, which was applied to a G-75 Sephadex column 2.5 X 70 cm just prior to use.

The yield estimated for this column was based on the extinction coefficient of $A_{280}^{1\%} = 0.87$ as reported by Serdynk et al. (49). It should be pointed out that the dialysis into acetic acid (which was not necessary) resulted in a loss of protein. Freezing the sample resulted in additional loss due to precipitation and aggregation.

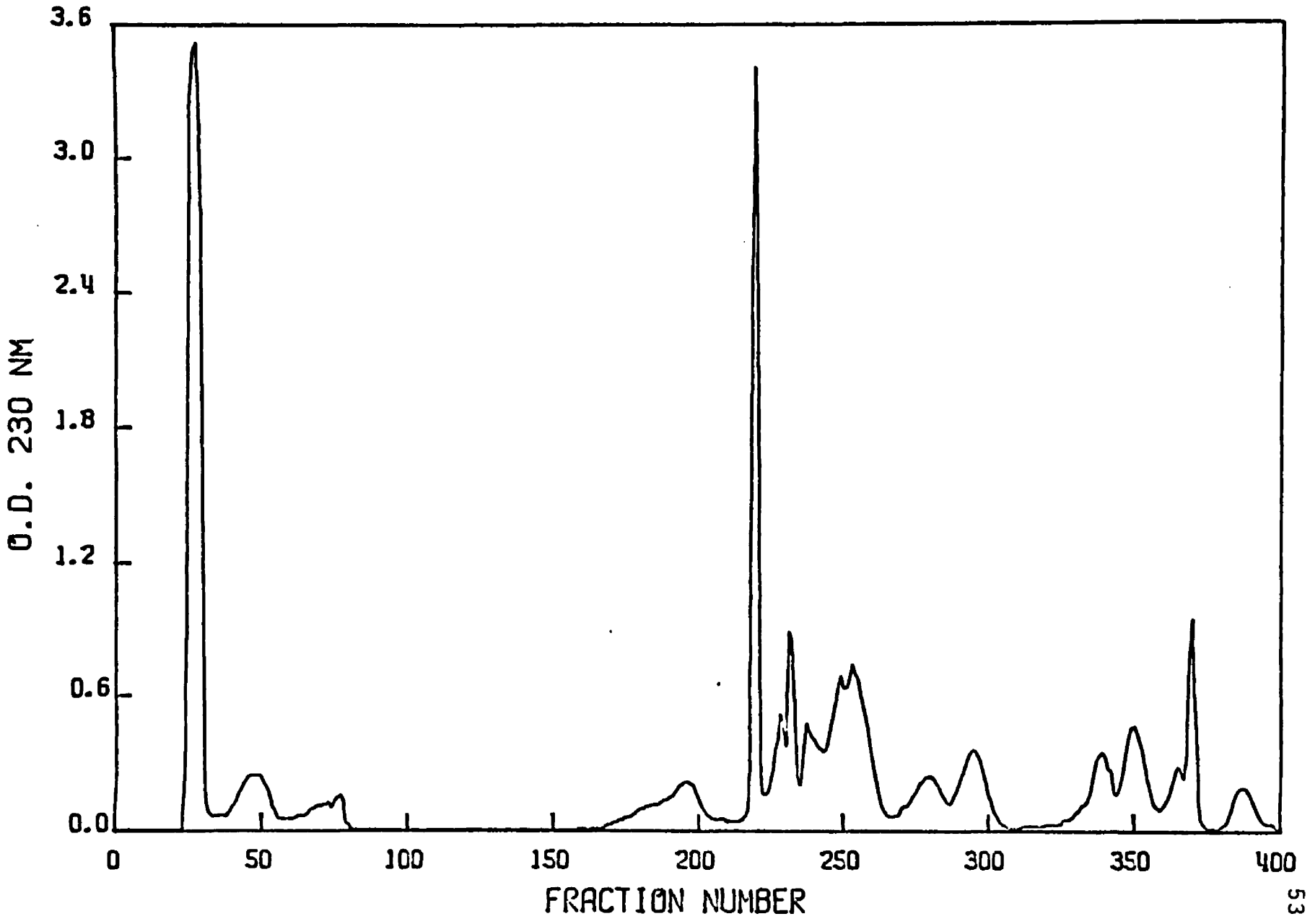
Sample #2 was prepared under similar extraction and isolation conditions. The initial protein extract was about 500 mg of total 30S proteins. An Hitachi-Altex UV-Vis absorbance monitor equipped with a flow cell and recorder was used to create an elution profile for this sample. The profile of the second extraction strongly resembled the elution profile of the previous profile. It was only necessary to analyze two fractions by two-dimensional gel electrophoresis in order to identify the S4 fraction. Fractions 301-315 were combined to give Sample #2 which was initially frozen. After dialysis into Recon buffer the yield of S4 from the second extraction was about 25 mg. A routine sedimentation equilibrium experiment showed the sample to be degraded which was corraborated by sedimentation velocity and diffusion experiments and so it was discarded.

A third extraction was performed on 2 gms of 30S subunits. This sample was first extracted with 1 M LiCl as per Dijk and Littlechild (10). The supernatent from the high speed spin was then treated with $MgCl_2$ and acetic acid as per Hardy et al. (16) to remove residual RNA. The mixture was then dialyzed into UPβ buffer and subjected to chromatography. The purpose was to enhance the amount of S4 relative to other 30S proteins which would not be extracted by 1 M LiCl. This was the result, although the actual yield of S4 was far below the theoretical yield due to the inefficiency of the 1 M LiCl extraction. Fractions #360-373 were pooled and dialyzed into Recon buffer. The total yield of S4 for this extraction was about 10 mg, and was designated Sample #3. Sample #3 was stored at 4°C.

Sample #4 was prepared from 1 gm of 30S subunits according to the protocol of Sample #1 and Sample #2. The elution profile from the P11 column gave an atypical pattern (Figure 8). During the column elution, the gradient former malfunctioned and this resulted in salt concentration surges instead of a smooth gradient. The salt surges caused the hyperspikes at tube numbers 25-35 and 215-220. In order to obtain any useful S4 from this preparation, extensive two-dimensional gel analysis was performed.

Fraction number	Proteins present
219	S3, S4, S5
223	S3, S4
224	S4
225	S4
226	S4

Figure 8. Protein elution profile, A_{230} versus fraction number, of acetic acid extracted proteins from a 1 M LiCl wash of 2 gms of 30S subunits. Column was 2.6 x 60 cm of phosphocellulose in UPβ buffer. Sample was eluted with a 6 L linear gradient, 0.0 to 0.6 M NaCl in UPβ buffer at a rate of 45 ml/hr and 15 ml fractions were collected.


PROTEIN ELUTION PROFILE TP30 P11
3.6
3.0
2.4
1.8
1.2
0.6
0.0
O.D. 230 NM
0
50
100
150
200
250
300
350
400
FRACTION NUMBER

Fraction number	Proteins present
227	S4
228	S4
229	S4
230	S4, S9
231	S4, S9

Fractions 225-229 were combined to make up Sample #4. The fractions were dialyzed into Recon buffer and then concentrated to $\geq$ 1 mg/ml and redialyzed. The total yield was about 5 mg. Sample #4 was stored at 4°C.

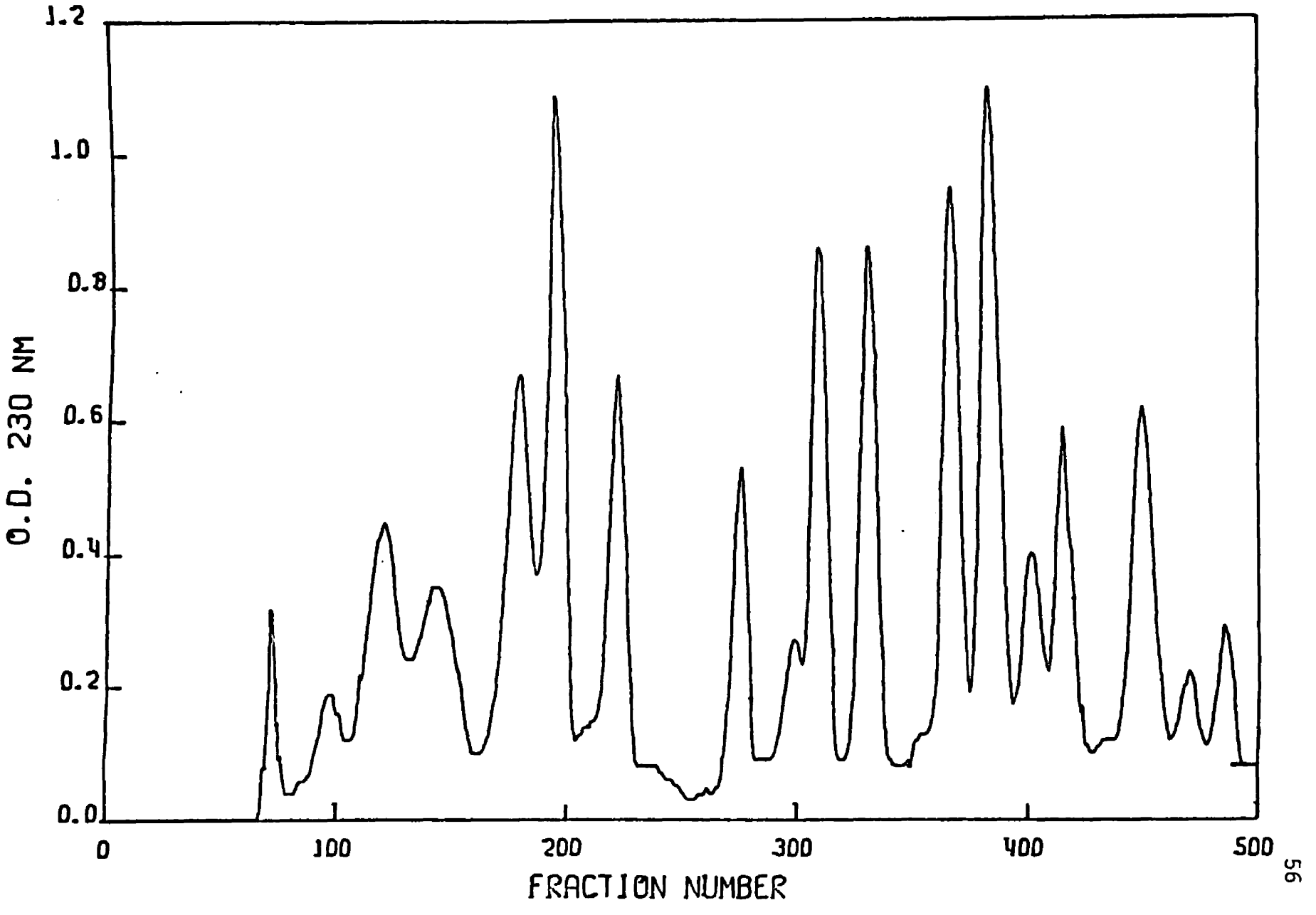
A fifth extraction was made using 1.6 gm of 30S subunits using the preparation conditions of Sample #1. The elution profile of this column was similar to previous columns (Figure 9) and only two two-dimensional gels were required to identify the S4 fractions. Fractions #361-372 were combined to give Sample #5 which gave an estimated yield of 25 mg of S4 after dialysis and concentration. Some of this sample was used immediately and the rest stored at 4°C.

Physical Studies of S4

Sedimentation velocity

Sedimentation velocity experiments were carried out on Samples #1, #2, and #4 of protein S4. All three experiments were conducted under the same conditions, although for sample #4 a cylindrical lens had been installed on the photoelectric scanning system on the Beckman Model E analytical ultracentrifuge which increased the light intensity and thereby reduced noise in the scanner trace. The data obtained from

Figure 9. Protein elution profile, A_{230} versus fraction number, of acetic acid extract of 1.6 gms of 30S subunits which was applied to a phosphocellulose column, 2.6 x 60 cm. Sample was eluted with a 6 L linear gradient, 0.0 to 0.6 M NaCl in UPβ buffer at a rate of 45 ml/hr and 15 ml fractions were collected.


PROTEIN ELUTION PROFILE 30S P11
1.2
1.0
0.8
0.6
0.4
0.2
0.0
O.D. 230 NM
0
100
200
300
400
500
FRACTION NUMBER

samples #1 and #4 are shown in Figures 10 and 11 and a line generated by linear least squares analysis overlays the data. The sedimentation coefficients calculated for these data were 1.66S for sample #1 and 1.72S for sample #4. Sample #2 had been judged to be degraded from sedimentation equilibrium experiments and diffusion experiments and the $S^{\circ}_{20,w}$ value obtained (1.45S) corroborated this finding. The data for samples #1 and #4 showed good linearity.

Diffusion coefficient measurements

Attempts were made to obtain diffusion coefficients on samples #1, #2, and #4. An aliquot of sample #1 was freshly dialyzed and carried on wet ice to Dr. Bloomfield's laboratory at the University of Minnesota. The histogram and cummulants analysis of this sample gave a $D_{20,w}$ of 7.9×10^{-7} cm^2/sec. However, the concentration was very low, estimated at 0.25 mg/ml, and much aggregate was shown to be present, probably due to the sample having been previously frozen.

An aliquot of sample #2 was sent on wet ice, but diffusion experiments indicated this sample to be degraded, which was corraborated by sedimentation equilibrium and velocity experiments.

An aliquot of sample #4 which had never been frozen was prepared for diffusion experiments. As soon as renaturation was completed the sample was concentrated to about 1.25 mg/ml. The sample was then immediately dialyzed for 36 hrs against recon buffer. After a low-speed spin to remove precipitate the concentration of the sample was about 1 mg/ml. This sample was immediately packed on wet ice and sent to Dr. Bloomfield's lab for diffusion analysis. The histogram results are shown in Figure 12. The increased concentration greatly enhanced the

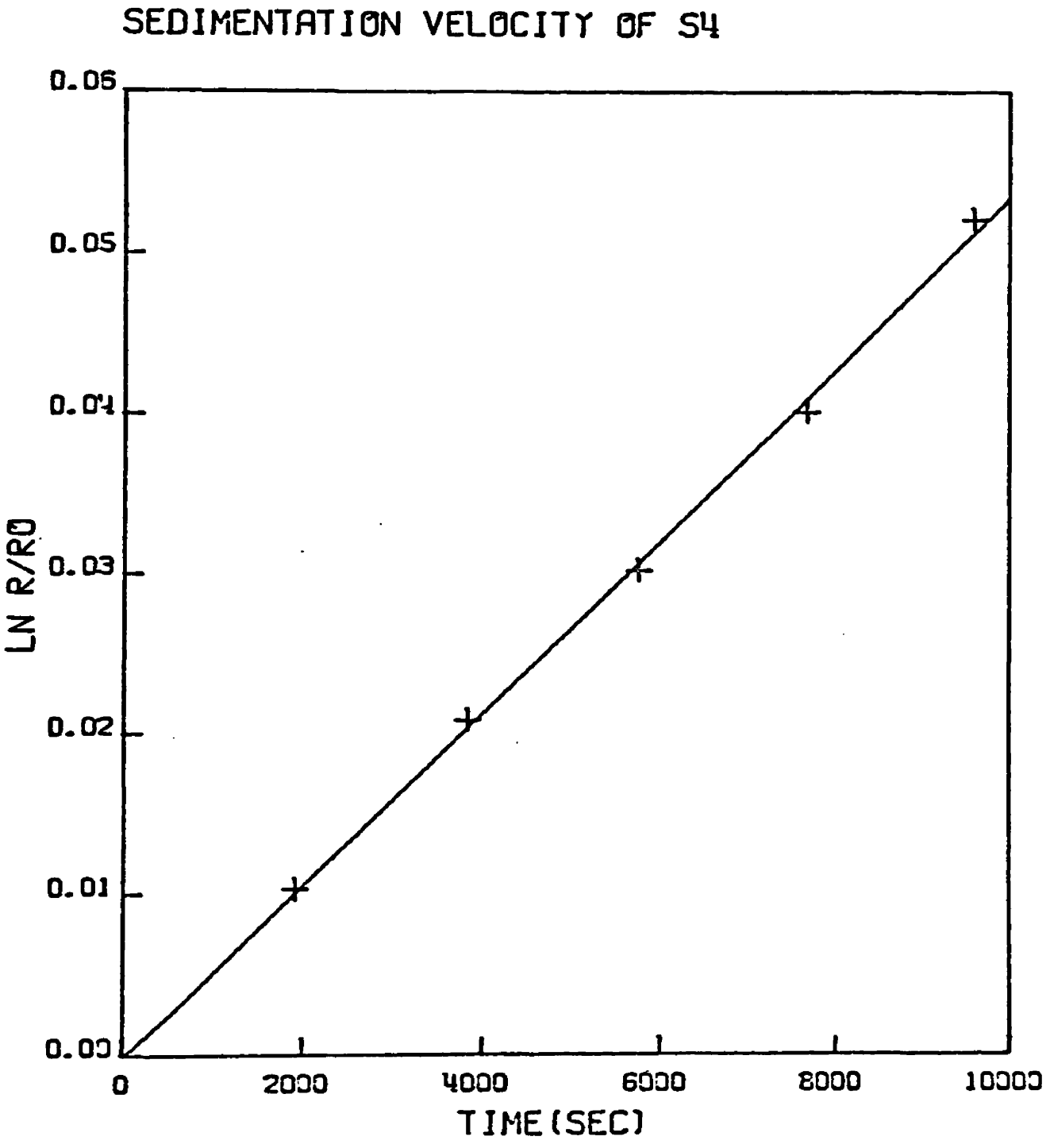


Figure 10. Sedimentation velocity data from Sample 1 of S4. Experimental points are overlayed with a linear least squares plot. Sample concentration was ~0.25 mg/ml. The rotor was spun at 68,000 rpm, 4°C, and scanned at 32 min. intervals.

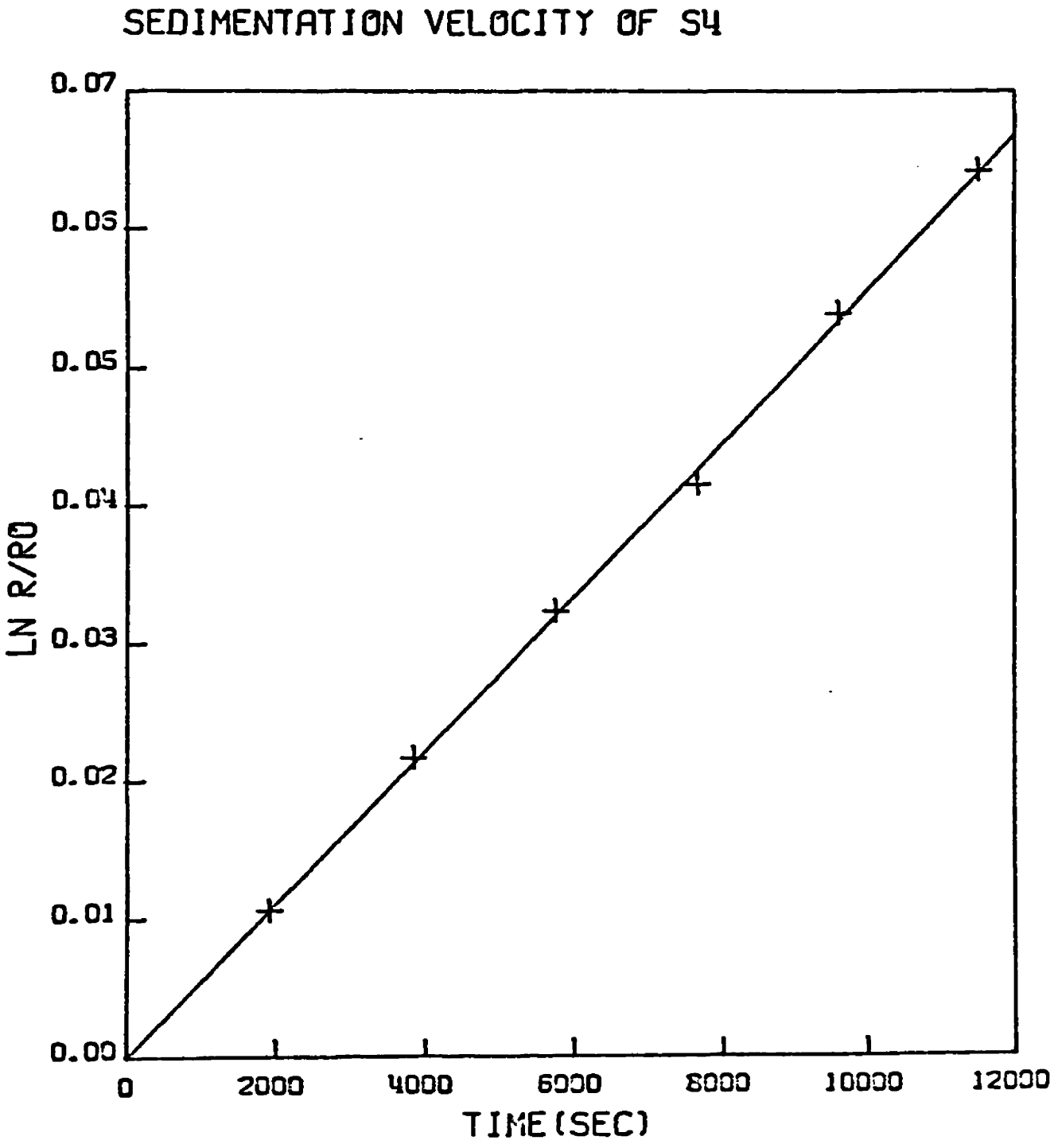
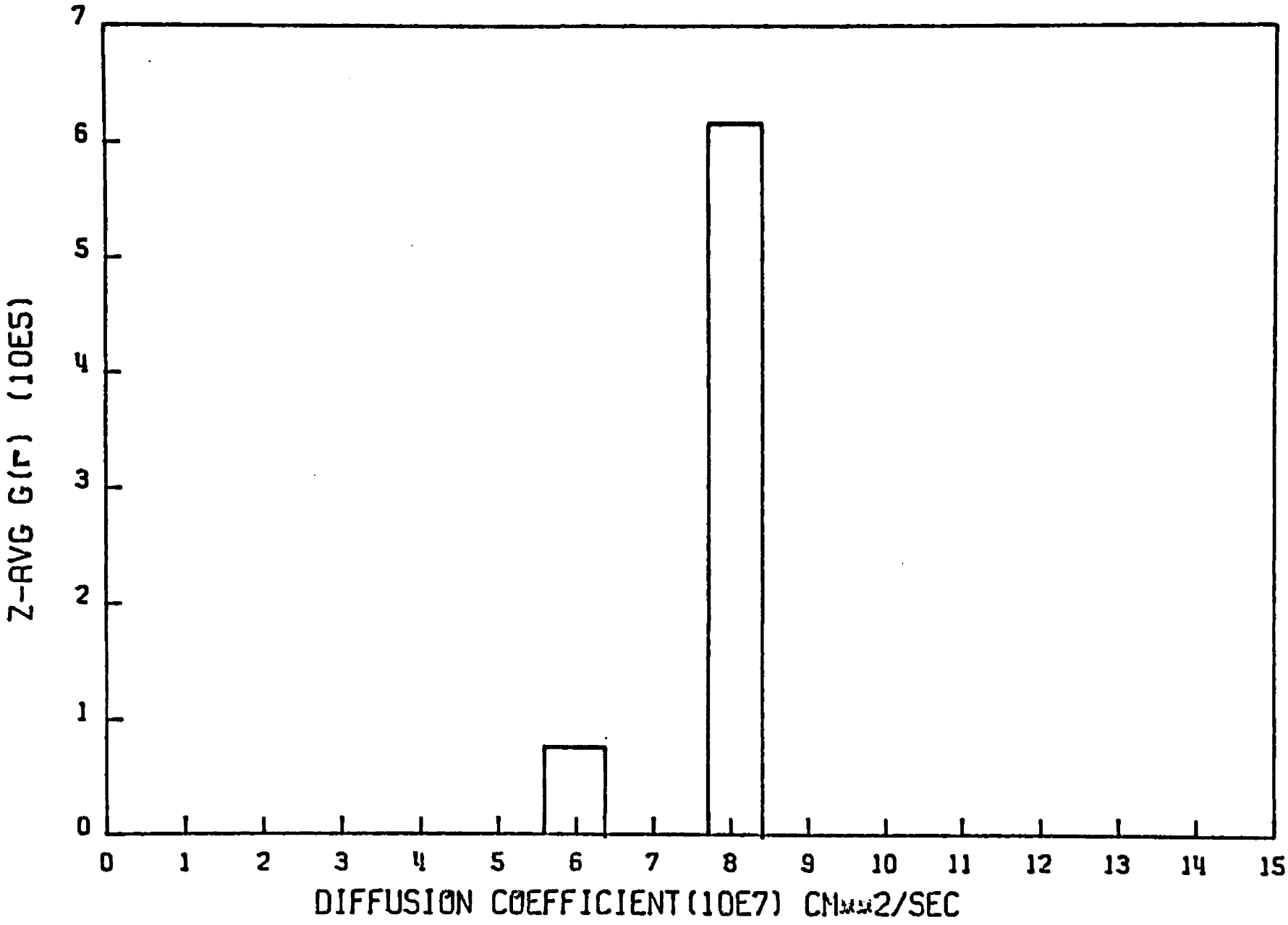


Figure 11. Sedimentation velocity data of Sample 4 of S4. Data points are overlayed with a linear least squares plot. Sample concentration was ~0.75 mg/ml. The rotor was spun at 68,000 rpm, 4°C, and scanned at 32 min. intervals.

Figure 12. Histogram plot of the diffusion coefficient analysis done on Sample 4 of S4. Plot is of the scattering intensity over the range of the diffusion coefficients indicated.


HISTOGRAM ANALYSIS OF S4
Z-AVG G(r) (10E5)
0
1
2
3
4
5
6
7
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
DIFFUSION COEFFICIENT(10E7) CM**2/SEC

quality of the data and the speed in handling and absence of any freezing reduced the amount of aggregate present. The $D_{20,w}$ for Sample #4 was 8.0×10^{-7} cm^2/sec and histogram analysis terminated after 194 iterations showed good agreement with the second order cummulants analysis.

Extinction coefficient

The dry weight analysis technique showed great variations between samples that had been prepared identically. Sample #5 of S4 was used as the sample after it had been dialyzed into recon buffer without β-mercaptoethanol. The β-mercaptoethanol was left out because of its volatile nature.

The average value for the $E_{280}^{0.1\%}$ was 0.69 O.D. ml/mg (±0.13) for six samples using two blanks. If the high and the low readings were dropped from the six samples used, the $E_{280}^{0.1\%}$ increased to 0.73 O.D. ml/mg. The error for these readings was still ± 20%.

The high error encountered in this procedure was due to a number of uncontrollable conditions. First of all, the balance used, a Mettler H20T, did not have sufficient precision, but is the best available locally. The room environment introduced great variations due to vibration, temperature, humidity and pressure. While corrections were made for atmospheric conditions, the small amount of sample available and the low concentrations made exact measurements impossible.

Density increments and apparent partial specific volume

Attempts were made to obtain a density versus concentration plot on samples #4 and #5. The problem of protein solubility in recon buffer made this determination very difficult. At concentrations of

approximately 1 mg/ml, the time difference for 1×10^4 oscillations when the concentration was varied by 0.25 mg/ml was 100 μsec. Since the Paar density meter is only accurate to ± 10 μsec and since a protein concentration of only 1.25 mg/ml was used the total error for a least squares plot was unacceptably high. Also, the extinction coefficient could not be determined precisely enough with the balance available. Consequently, no definitive value for the density increment could be experimentally determined. For this reason the value of $\bar{v}$ calculated by Craven et al. (9) of 0.74 ml/gm was used.

Sedimentation equilibrium experiments

Sedimentation equilibrium experiments were routinely carried out on each sample immediately after concentration and dialysis to determine sample quality. Experiments performed on sample #1 showed a high degree of aggregation and no useful numbers were obtained from this sample.

Experiments performed on sample #2 revealed it to be degraded.

Extensive equilibrium sedimentation analysis was done on sample #3. Several experiments gave data which were identical, showing a curvature to the plot of M_w versus concentration which began low in M_w at low concentration and rose to a peak and then decreased with increasing concentration. This curvature gave a large error in the average M_w. A baseline plate was needed to correct for this, but the presence of large aggregate in the sample would not allow a baseline plate to be taken. Other problems encountered during this time were the presence of some small molecular weight material and the tendency of the sample to aggregate during analysis.

Sedimentation equilibrium analysis was also carried out on sample #4. An attempt was made to obtain time-dependent information on the aggregation. The same problems which interfered with sample #3 were encountered. The data were not of sufficiently high quality to be useful.

Sample #5 provided the best results of all sedimentation equilibrium experiments. Adjustments to the equipment and the loan of photographic plates from Dr. David Teller at the University of Washington made this possible. There was also less aggregation in this sample, probably due to the speed in handling. The M_w average molecular weight calculated for this sample was 23,200 ± 200. A baseline correction plate was made for this run. Also several points were dropped in the low concentration region. A graph of the ln J versus change in radius^2 shows good linearity (Figure 13) and the plots of number-, weight-, and z-average molecular weights versus concentration (Figure 14) were excellent.

Immunoprecipitation assay

The aliquot of sample #4 which was used for diffusion measurements was used for an immunoprecipitation assay (Plate #1). The sample was tested against antibodies to S3, S5 and S7. Dr. Larry Kahan at the University of Wisconsin, Madison, carried out the experiments. The interpretation from this sample was that the S4 content was greater than 90%. The major contaminants were S3 estimated at less than 5% and S7 less than 2%. Dr. Kahan indicated that this was an excellent sample

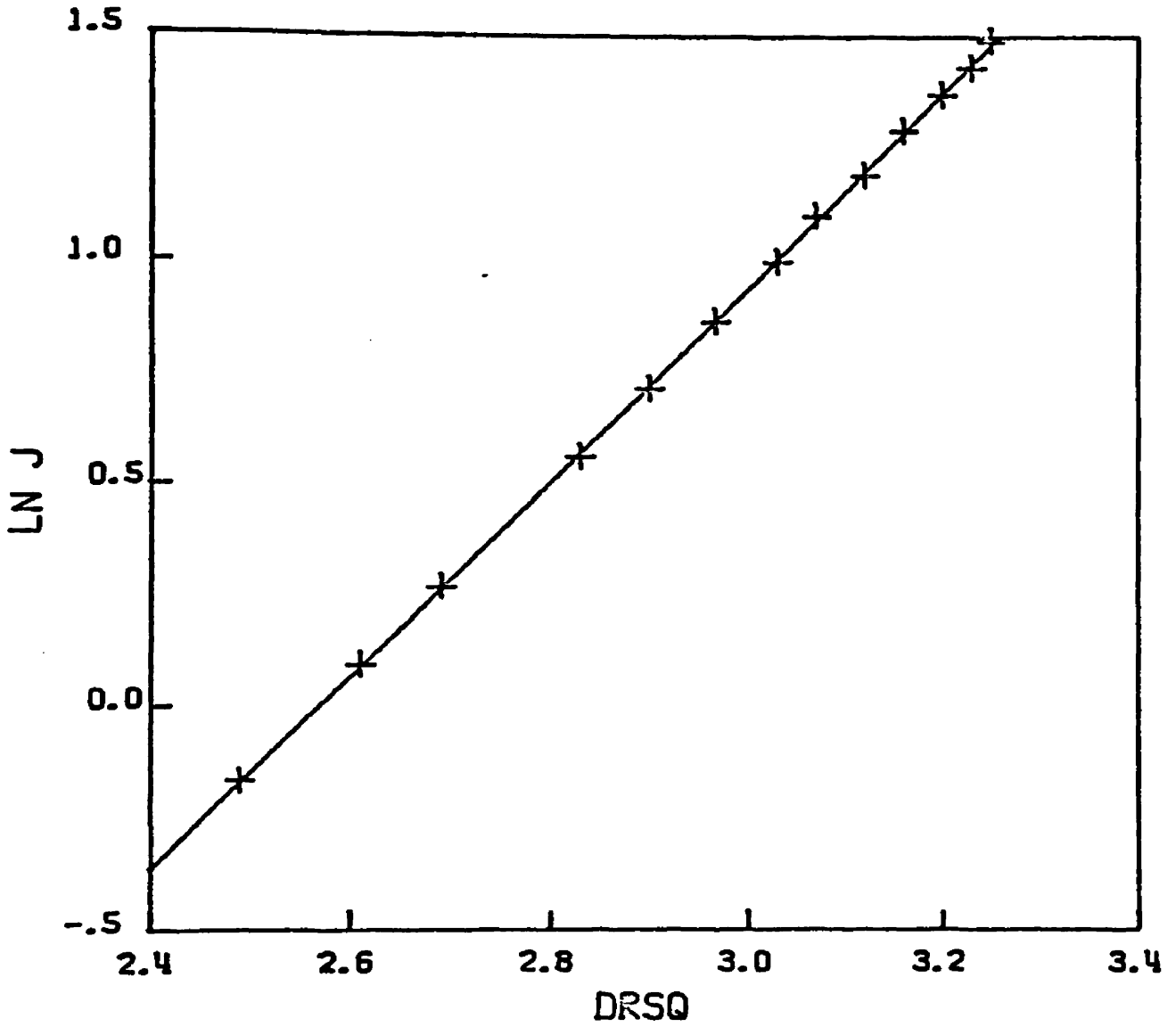


Figure 13. Plot of ln J, the average fringe displacement, versus the difference in radius squared, DRSQ. Plot shown is for Sample 5 with baseline subtracted. Five fringes were read. Sample was about 0.5 mg/ml spun at 40,000 rpm at 4°C.

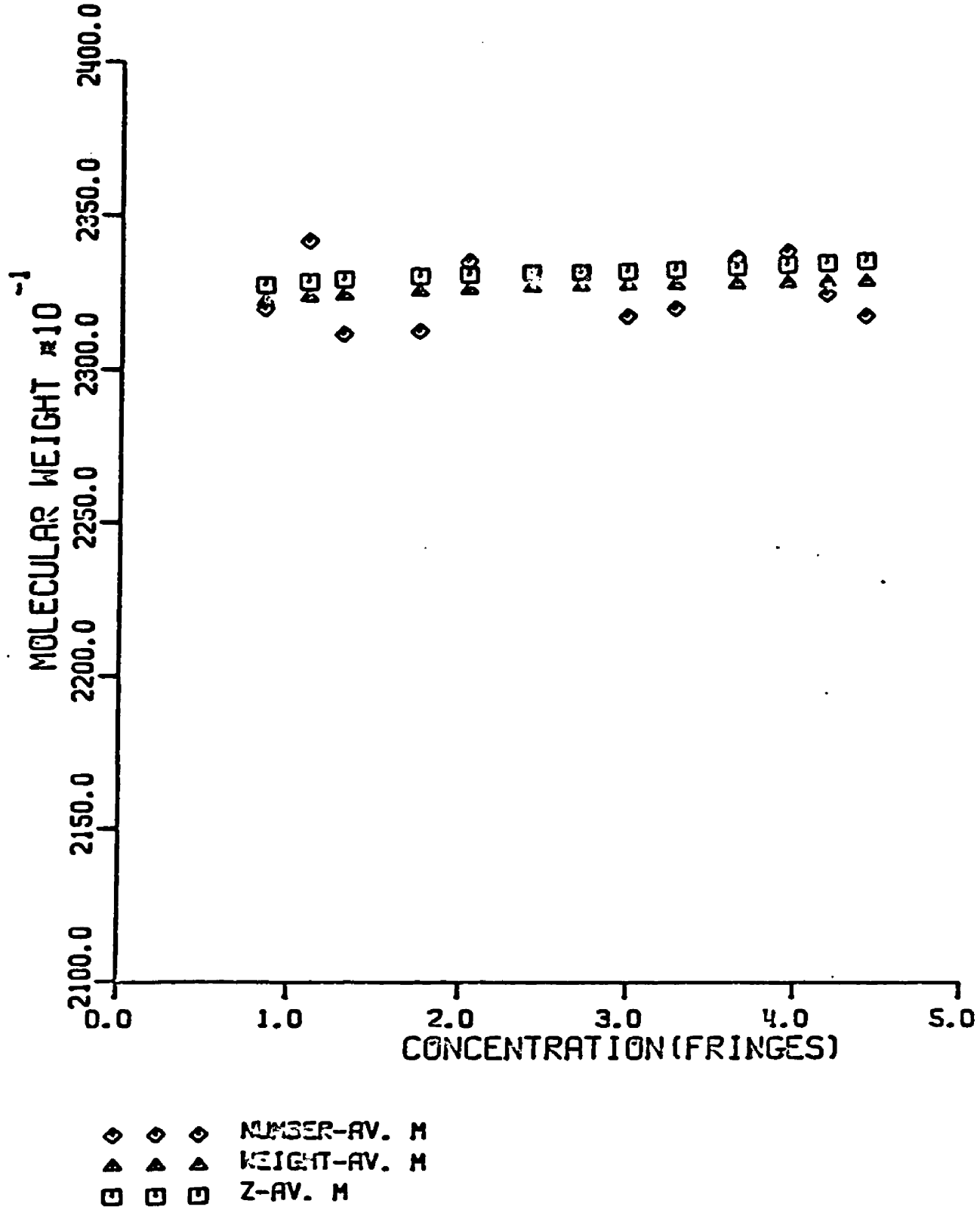
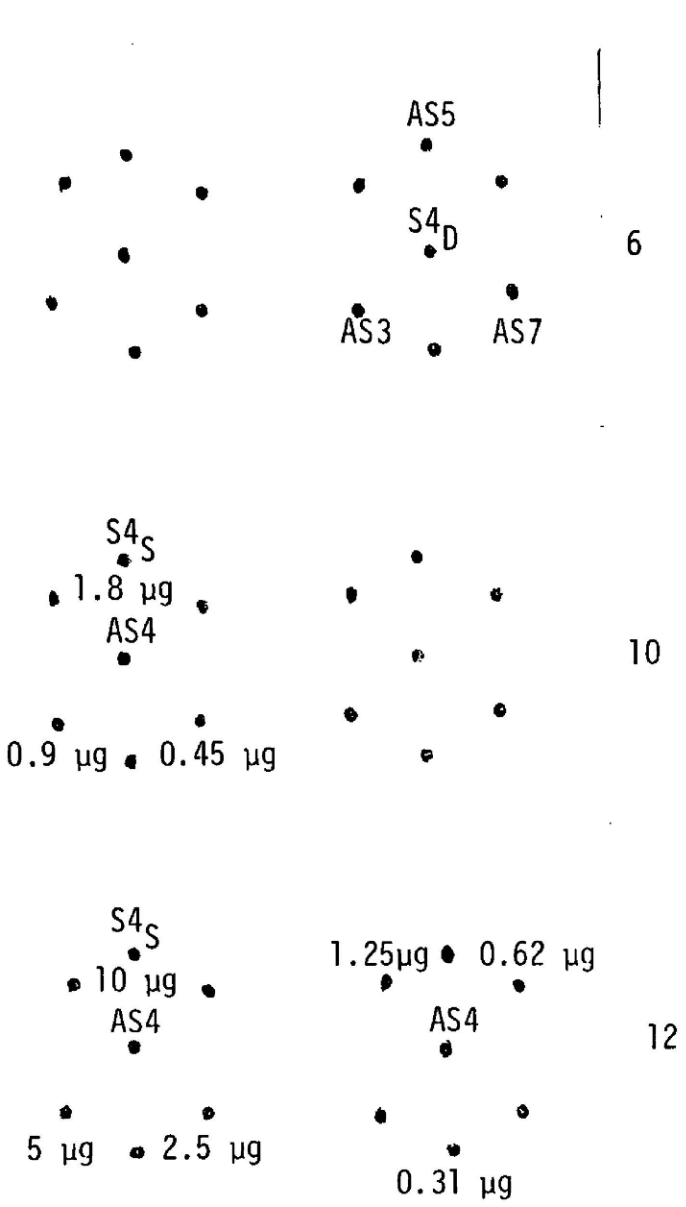
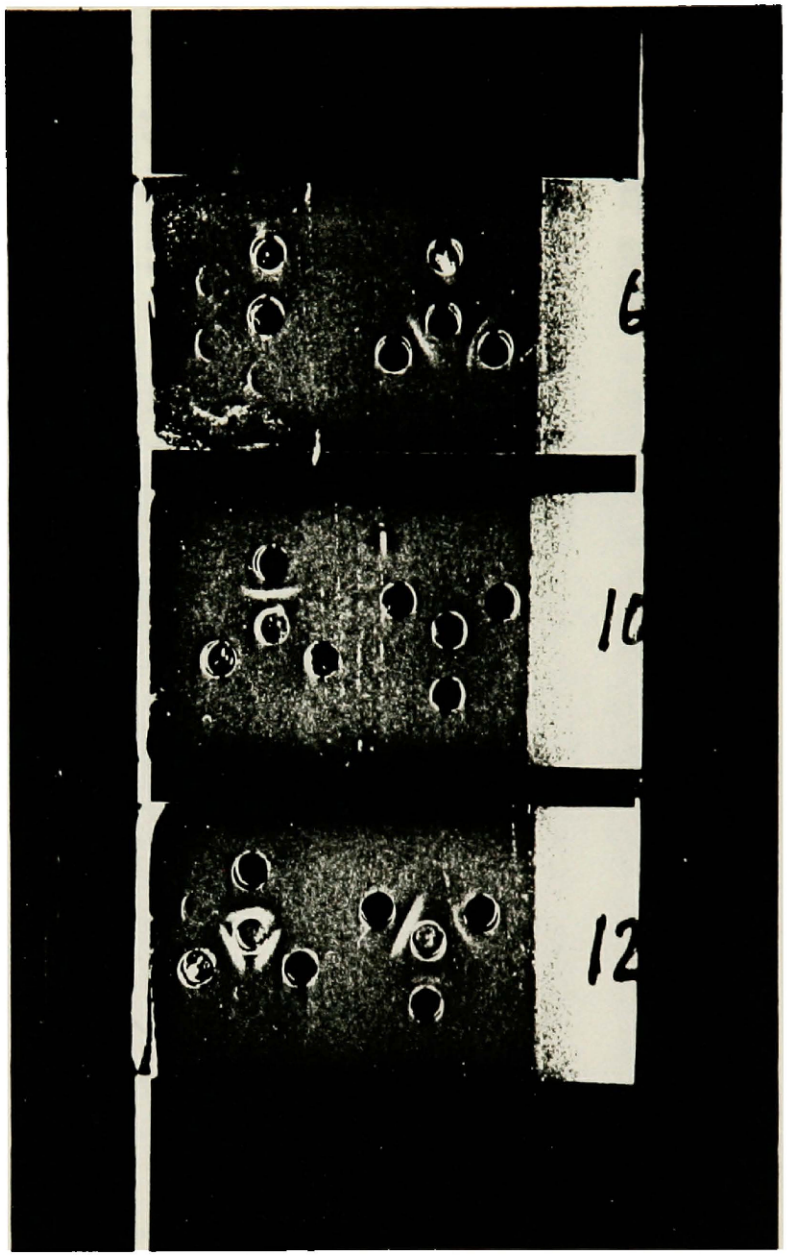


Figure 14. Point average molecular weights versus concentration in fringe displacement. The data have been smoothed considerably.


AS5
S4D
AS3
AS7
6
S4S
1.8 µg
AS4
0.9 µg
0.45 µg
10
S4S
10 µg
AS4
5 µg
2.5 µg
1.25µg
0.62 µg
AS4
0.31 µg
12


6
10
12

judging from his experience with other samples prepared similarly.

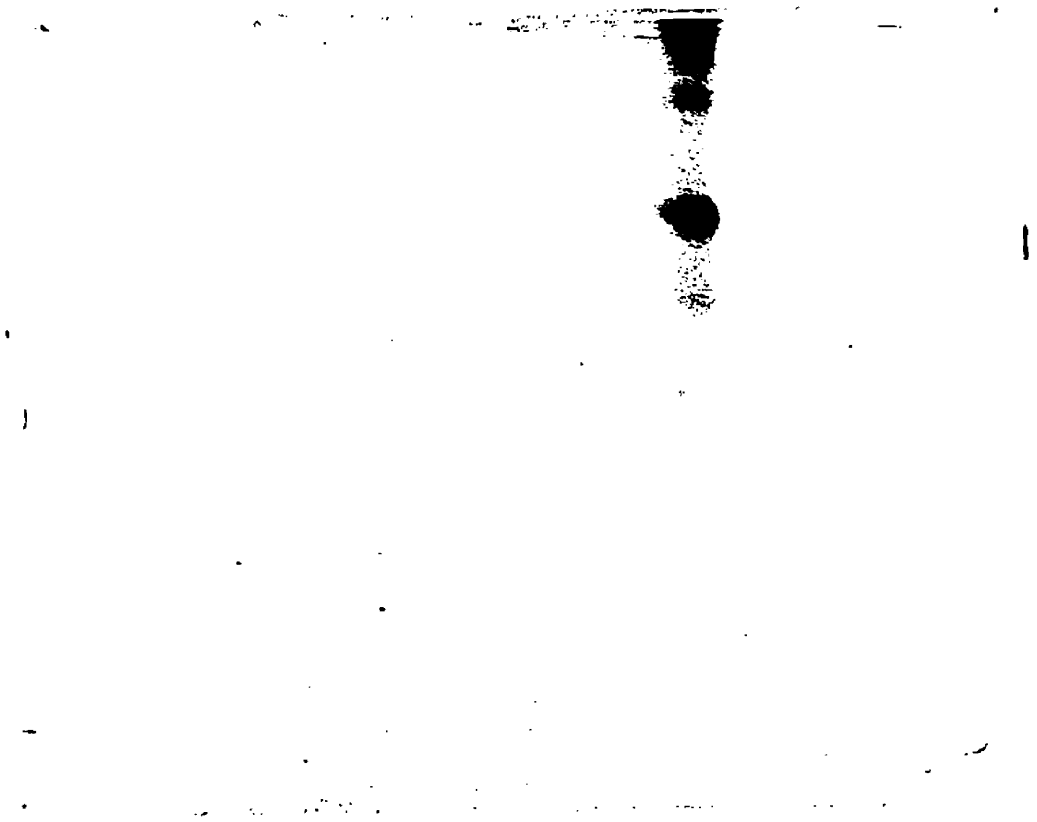
Two-dimensional gel analysis

Plate #2 shows a two-dimensional gel analysis run according to the protocol of Kaltschmidt and Wittmann (22). The S4 sample used was an aliquot of sample #5. The gel is heavily overloaded to accentuate any contaminants. For this reason there is a large amount of aggregate in the S4 lane of the second dimension which has not migrated detectably out of the first dimension. Two other lines appear approximately halfway to the S4 spot which are not easily explained, but may represent other aggregates of some kind. The S4 spot shows up very prominently and the gel shows no S3 or S5. A small amount of degradation is also evident from the lightly shaded area which has migrated further than S4. This might also contain slight contamination from S7.

Calculations

Calculations on the data from S4 were made using the standard methods based on comparisons of frictional coefficients. The values for the sedimentation coefficient and diffusion coefficient were both used to determine the frictional coefficient of the particle, f. The ratio of the frictional coefficient to that of an anhydrous sphere of equivalent volume, f/f_{min}, was then calculated. In order to try to assess the actual asymmetry of the molecule, the amount of hydration was estimated. This is necessary since there is no way to measure this value precisely. Upon assuming an appropriate range of possible hydrations, the frictional ratio, f/f_o, which reflects the axial asymmetry can be calculated. These values for S4 were then compared with values calculated for

Plate #2



ellipsoids of revolution using the Perrin function. Ellipsoids of revolution served as the models on which the shape of S4 was based.

Table #2 shows a summary of the data from the acetic acid-urea preparations of S4 and some calculated molecular weights. The average $S^{\circ}_{20,w}$ was calculated to be 1.69 (±0.03)S when the value from sample #2, which was degraded, is discarded. The average $D^{\circ}_{20,w}$ was calculated to be 7.95 (±0.02) x 10^{7} cm^{2}/sec. The $S^{\circ}_{20,w}$ value may be low since concentration dependence was ignored and the error for a particle of this size can be quite high. The $D^{\circ}_{20,w}$ value may be slightly higher than the true value, due to the method of data analysis. The sets of data

Table 2

Summary of Data and Calculated Molecular Weights

	Preparation				
	1	2	3	4	5
$S^{\circ}_{20,w}$	1.66 S (± 0.1)	1.45 S (± 0.1)	---	1.72 S (± 0.1)	---
$D^{\circ}_{20,w}$	$(7.9 \pm 0.2)(x10^{-7})\frac{cm^2}{s}$	---	---	$(8.0 \pm 0.2)(x10^{-7})\frac{cm^2}{s}$	---
$\bar{v}$	$0.74 \pm (0.01)\frac{cm^3}{g}$ (a)	---	---	---	---
ρ_o	---	---	---	1.02	1.02
MW_{SE}	---	---	---	---	23,200 ± 200
MW_{SD}	20,900	---	---	21,400	---
$E^{1\%}_{280}$	---	---	---	---	$0.73 \pm (0.14)\frac{O.D.\ ml}{gm}$
yield	8 mg	25 mg	10 mg	5 mg	25 mg

(a) Craven et al. (9) calculated from amino acid composition

obtained from sample #1 and #4 both gave calculated molecular weight values of about 21,000 which represented a 10% deviation from the molecular weights derived from the amino acid sequence and sedimentation equilibrium experiments. This was considerable error, but when working with particles of this size using hydrodynamic techniques and marginal experimental conditions on a polydisperse sample, such error is not unexpected.

The value for the ratio f/f_{min} was 1.61 when calculated from the sedimentation coefficient and 1.42 when calculated from the diffusion coefficient. Table #3 shows the values for the ratio f/f_o calculated for various hydration values. These values indicated a moderately asymmetric particle. A prolate ellipsoid was arbitrarily chosen as the model for a basis of comparison. The values of f/f_o give a probable range for the axial ratio of S4 from 4.5:1 to 8:1 (5).

Table 3

Values of f/f_o calculated from f/f_{min} and assumed hydrations

	Hydration (gm H_2O/gm protein)				
f/f_{min}	0.30	0.35	0.40	0.45	0.50
1.42	1.27	1.25	1.23	1.21	1.19
1.50	1.34	1.32	1.30	1.28	1.26[5]
1.61	1.44	1.42	1.40	1.38	1.35[5]

In order to compare results in these studies with values obtained in scattering studies, a comparison based upon spheres with equivalent frictional coefficients must be developed. The experimental values of

$S_{20,w}$ and $D_{20,w}$ can be used to calculate the radius of a sphere of equivalent frictional coefficient. These values are 29.6 Å and 27 Å, respectively. In order to compare these values to radii obtained from scattering data, an external layer of hydration of about 2.8 Å (5) must be removed, since scattering techniques will not detect this external solvent layer. In the case of protein S4, this amount of H_2O is equal to about 0.5 gm H_2O/gm protein which is sufficiently large to account for almost all hydration expected for a globular to slightly extended protein. This does not assume any internal hydration, so a value for internal hydration can only be estimated.

A graph of the radii of gyration versus axial ratios assuming a prolate model calculated at various hydrations can then be constructed (Figure 15). This graph assumes a constant volume for the protein based upon the $\bar{v}$ of 0.74 cc/gm and molecular weight of 23,138 gm plus the volume for different values of total water added. The space defined by the limits of axial ratio of 1 to 8 and hydration of 0.0 to 1.2 gms H_2O/gm protein then give all possible values for a radius of gyration.

To correlate the experimental values of the hydrodynamic experiments with these values, another set of curves must be constructed. In these, the frictional coefficient measured using either the sedimentation or diffusion experiments must be held constant. As the axial asymmetry of a proposed model increases, the volume must decrease to allow the frictional value to remain constant. This produces a set of lines that intersect the contant-volume lines as axial asymmetry is increased (see Figure 15). From these intersects an appropriate internal hydration value can be chosen which in turn will mandate the axial

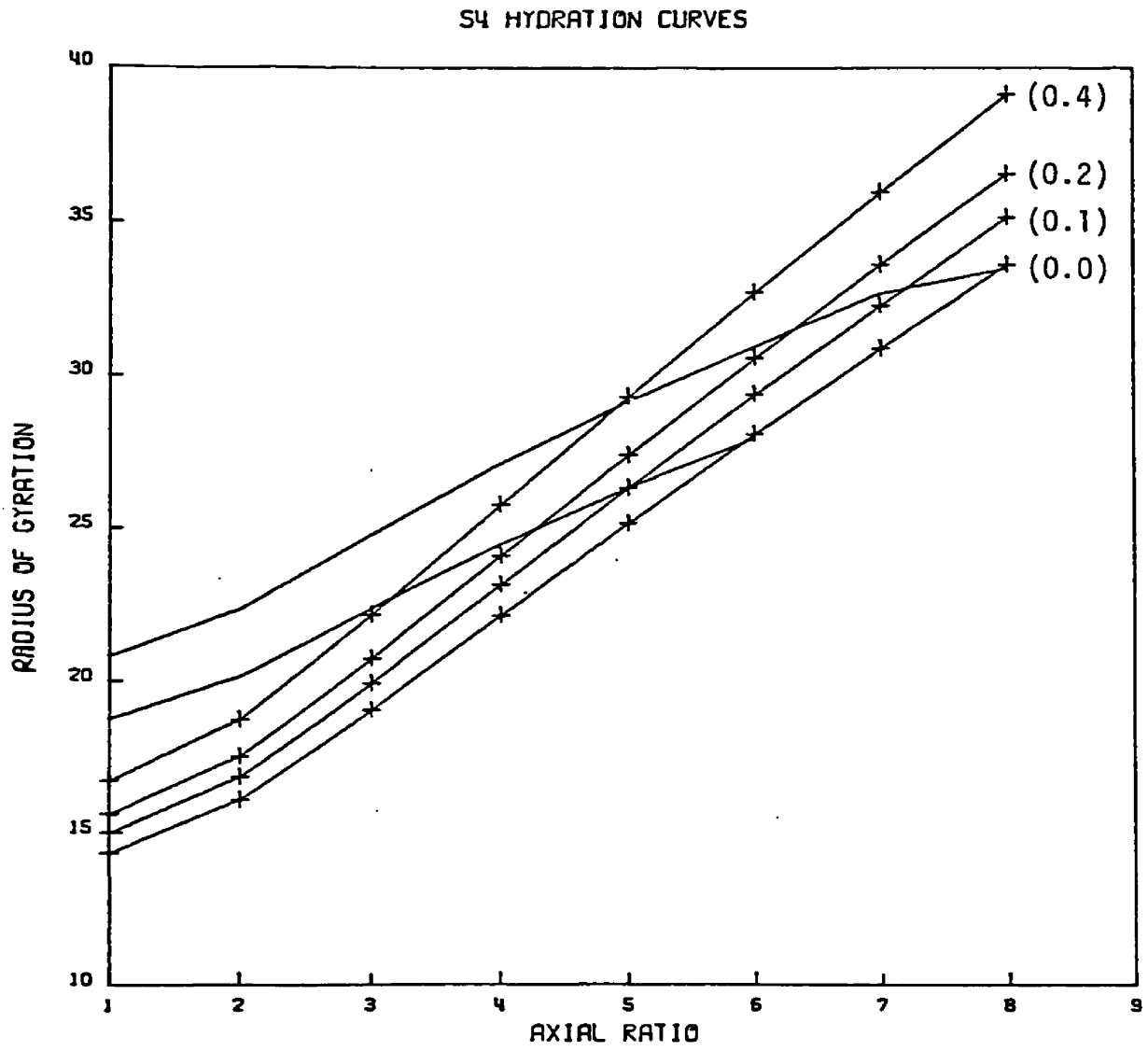


Figure 15. Plots of the radius of gyration versus the axial asymmetry for protein S4 with various hydrations in parentheses (+-+) as noted in gm H_2O/gm protein. The lower line with no symbols is representative of the Rg and axial ratios to be expected if the frictional coefficient obtained from diffusion measurements were held constant. The upper line is representative of Rg and axial ratios if sedimentation results produced the frictional coefficient.

ratio and radius of gyration expected from a solution scattering experiment. In this manner our data can be compared to those obtained by other workers (see Discussion).

From our results we can see that if we assume reasonable values for total hydration (maximum 0.7 gm H_2O/gm protein), subtracting out that assumed to be external (0.5 gm H_2O/gm protein) leaves a maximum of 0.2 gm H_2O/gm protein internal hydration allowed. Values between 0-0.2 gm H_2O/gm protein then allow the axial ratio to vary between ~4.5-6:1 and that expected radius of gyration to be between ~25-27 Å based on $D_{20,w}$ data. If $S_{20,w}$ values are used, the allowable axial ratios vary between 6.5-8:1 and the radius of gyration is expected to be between ~32-34 Å.

The values from the sedimentation velocity experiments are higher than those from diffusion data due to inherent difficulties in studying this type of particle. A small protein such as S4, studied with a photoelectric scanner at low concentrations will not sediment fast enough to produce a sharp boundary. This will produce a scanner trace with a broadening of the boundary for successive scans which makes interpretation somewhat difficult. The photoelectric scanner itself is subject to noise which will further complicate the interpretation of the scans. For this reason the diffusion studies of S4 should be weighed more heavily than the sedimentation studies.

In summary, the acceptable values based on these hydrodynamic studies for the axial ratio are a minimum of 4.5:1 and a maximum of 7:1 and for the radius of gyration are expected to be not less than 25 Å and not more than 34 Å. If a 5:1 prolate ellipsoid model is assumed, the dimensions are approximately 140 Å x 28 Å x 28 Å.

Chapter IV

DISCUSSION

The results from the hydrodynamic studies carried out on protein S4 indicate that this protein has a moderately extended conformation in solution. The axial asymmetry is estimated to be between 4.5:1 and 7:1 if a prolate ellipsoidal model is used.

Since protein S4 has been widely studied prior to this investigation, the specific conditions of this study deserve some comment. First of all, our studies were carried out in reconstitution buffer (0.03 M Tris-HCl pH 7.4, 0.35 M KCl, 0.02 M $MgCl_2$, 10 mM β-mercaptoethanol). Since S4 shows a marked tendency to aggregate in reconstitution buffer, low concentrations of protein were used for physical measurements. Samples of S4 were found to be relatively stable when kept at 4°C, while freezing was found to greatly enhance aggregation. The exclusion of any aggregation present in a particular sample of S4 was not possible in our hands. In order for a physical measurement to be made, the sample must be dialyzed to equilibrium and during this time some aggregation always took place. Therefore, the physical measurements made in this study have been made under conditions which minimize the aggregation and the analysis of raw data has employed computer fitting and filtering techniques to remove any effects caused by aggregation.

A summary of physical studies by various workers on protein S4 is presented in Table 4. It is apparent from this table that the conditions used for these studies are inconsistent with one another, making

Table 3. Values from different studies by reference number.

Parameter	1	2	3	4	5	6	7
$S_{20,w}$	1.65 S	---	1.95 S	---	---	---	1.69 S
$D_{20,w}$	---	---	3.7×10^{-7} cm^2/g	---	---	---	7.95×10^{-7} cm^2/g
$\bar{v}$	---	0.74 cm^3/g	0.725 cm^3/g	0.74 cm^3/g	---	---	0.74 cm^3/g
$E^{1\%}_{280}$	1.2	---	---	---	0.87	---	0.73
MW_{SE}	21,400	---	25,000	---	24,000	---	23,200 ± 200
SD	---	---	25,000	---	---	---	21,200
XS	---	23,000	24,000	23,800	---	---	---
NS	---	---	---	---	20000-24000	---	---
Rg	---	26 Å	33.6 Å	42 Å	18.5 Å	30.7 Å	26-30 Å
Concentration of S4	1.5 mg/ml	10 mg/ml	0.5-15 mg/ml	2-7 mg/ml	1 mg/ml	~	1 mg/ml
Buffer	1	7	1,2,3,4,5	1,6	1	1	1
Hydration	---	0.7 g/g	0.35 g/g	---	---	---	0.7 g/g
Method of Preparation	1	1	1	1,2	1	3	1

Method of preparation
1) Acetic Acid-Urea
2) LiCl extracted
3) Urea and LiCl extracted

Reference numbers
1) Rhode et al. (44)
2) Gulik et al. (15)
3) Paradies and Franz (40)
4) Österberg et al. (39)
5) Serdyuk et al. (49)
6) Ramakrishman et al. (42)
7) Dodd et al. manuscript in prep.

Buffers
1) Recon: 0.03 M Tris-HCl, pH 7.4, 0.35 M KCl, 0.02 M $MgCl_2$, 10 mM β-mercaptoethanol
2) 0.01 M Tris-HCl pH 7.0, 0.1 M KCl, 5 mM $MgCl_2$
3) 0.01 M $CH_3COONa(K)$, pH 5.5, 0.1 M NaCl(KCl), 5 mM $MgCl_2$
4) 5 mM potassium cacodylate, pH 7.5, 0.1 MKCl, 5 mM $MgCl_2$
5) 0.01 M K_2HPO_4, pH 7.5, 0.1 M KCl, 50 mM $MgCl_2$
6) 0.05 M CH_3COONa, pH 5.5, 0.4 M LiCl, 6 mM β-mercaptoethanol
7) 0.01 M KCl pH 7.0

comparisons among them difficult. This is most apparent in a comparison of the radius of gyration values. The results of this study will be compared with each of the previous studies.

The earliest studies of a purified S4 preparation are those of Rhode et al. (44). This group reported an $S_{20,w}$ of 1.65 (± 0.1)S. This gives a frictional coefficient ratio, f/f_{min}, of 1.6 which is consistent with an extended conformation and/or considerable hydration. The isolation of the sample was made using acetic acid-urea and physical measurements were made on low-concentration samples in reconstitution buffers. This result is well within experimental error of the values obtained in our studies. It is significant to note that Rhode et al. did not indicate the presence of any aggregation in their samples nor did they monitor the amount of aggregation that might have been present. The value for the molecular weight as determined by sedimentation equilibrium is ~10% below that of the value for the molecular weight calculated from the amino acid sequence and that determined in our laboratory by sedimentation equilibrium.

Comprehensive hydrodynamic and scattering studies were performed by Paradies and Franz (40). However, several inconsistensies are apparent upon examination of their data. Coupling the reported values for $S_{20,w}$ and $D_{20,w}$ in the Svedberg equation does not give the reported molecular weight. In fact, the molecular weight suggests that the particle being studied was a dimer. This fact contradicts the molecular weights which they reported from x-ray scattering studies. It is not possible to correlate their results with ours because their studies were carried out in buffers which were not similar to reconstitution buffer and the

concentrations vary drastically. Paradies and Franz state that no aggregation was observed in preparations of S4 at ionic strengths between 0.08 to 0.2 M. However, reconstitution buffer has an ionic strength of ~0.37 M. Concentrations in scattering studies were as high as 15 mg/ml using a reconstitution type buffer, conditions which are not possible without the presence of some aggregation. These inconsistencies make it difficult to accept their Rg of 33.6 Å. In turn they reported dimensions of 140 Å x 10 Å which suggest that, using their Rg value, the particle is almost entirely anhydrous. Such assumptions are not warranted.

Österberg et al. conducted x-ray scattering studies of S4 prepared by both denaturing (16) and high salt (10) methods. Their studies were carried out at 2-7 mg/ml of S4 and they noted no difference in the x-ray scattering curves between methods of sample preparation. They did note some tendency to aggregate in the urea-prepared sample but do not indicate whether this was a serious problem or not. The reported value of the radius of gyration is 42 Å and a triaxial ellipsoid model with semi-axis lengths of 90 Å x 25 Å x 4 Å or an elliptical cylinder model with dimensions of 111 Å x 20 Å and a height of 5 Å are suggested. These values do not agree with the previous hydrodynamic data or scattering studies or with the results of our studies. The fact that some aggregation is observed in the urea-prepared sample but not in the salt-extracted sample at concentrations between 2-7 mg/ml is surprising as it has been frequently noted that S4 has a marked tendency to aggregate at concentrations much greater than 1-1.5 mg/ml when prepared using the urea-extraction method. Not only did we obtain no S4 in a monodisperse form using the salt-extraction method (10), but the ribosomal proteins as

a whole seemed to show a tendency to aggregate when extracted in this way.

The radius of gyration of 42 Å as found by Österberg et al. (39) seems quite high as is evidenced by the extreme dimensions of the proposed models. They suggest triaxial ellipsoid model having a small axis of 8 Å which is not even wide enough to accommodate one thickness of α-helix secondary structure. Circular dichroism studies indicate that S4 contains 41% α-helix (11). The same is true for the cylinder model. Both models are based on a radius of gyration of 42 Å and empirical values for the monomer of S4. It seems likely that this study has also been conducted on a sample with much aggregation present which would easily account for a radius of gyration of 42 Å making any model proposed difficult to fit with realistic dimensions. Unfortunately, they did not publish Guinier-region plots showing the radius of gyration determination which may have shown curvature indicating the presence of aggregates.

Neutron scattering studies performed by Serdyuk et al. (49) have yielded a completely different result from all other studies. Using S4 isolated by the method of Hardy et al. (16) at concentrations of 1 mg/ml in reconstitution buffer, they report a radius of gyration value of 18.5 Å. This indicates a very compact globular shape for S4. Sedimentation equilibrium experiments carried out on the S4 samples gave a molecular weight between 20,000 and 24,000.

The value of the radius of gyration is difficult to explain if an attempt is made to analyze it in the same fashion as our data were analyzed (see Figure 15). The radius of gyration for a sphere with no hydration is 14.7 Å. If a value of internal hydration is estimated to

be 0.2 gm H_2O/gm of protein the maximal allowable asymmetry of S4 would be 2.9:1, assuming a prolate ellipsoid model. In order for a particle with an asymmetry of 2.9:1 to give data equivalent to our hydrodynamic data, an internal hydration of at least 0.5 to 0.6 gm H_2O/gm protein would be required. This would be an unacceptably high value for internal hydration and is inconsistent with the assumed value. If the more reasonable value of 0.2 gm H_2O/gm protein is assumed for the internal hydration as the maximal allowable internal hydration, then the minimal asymmetry that would be needed to give data would correlate with our data would be ~4.5:1 for a prolate ellipsoid giving a radius of gyration of 24-25 Å. This radius of gyration is 40% greater than that reported by Serdyuk et al. The explanation for this discrepancy may be that a degraded sample was being analyzed by Serdyuk et al. causing a spuriously low value for the Rg or that their extrapolation of the Guinier plots made on S4 samples were imprecise due to low scattering intensity.

In contrast to the studies of Österberg et al. (39) and Serdyuk et al. (49), the x-ray scattering studies by Gulik et al. (15) show good agreement with our findings. The reported radius of gyration is 26 Å which is identical to the radius of gyration calculated using the hydration and axial asymmetry data from Figure 15 (see calculations section of Chapter III). The agreement of our results with theirs is surprising since their experiments were carried out at high concentration (10.2 mg/ml) and in low-salt conditions (0.01 M KCl, pH 7.0). Gulik et al. makes note of the fact that in high ionic strength buffers, such as reconstitution buffer, aggregation is apparent even at moderate concentration (2 mg/ml) and for that reason they used the low-salt buffer.

However, they use circular dichroism studies of S4 in both high and low ionic strength media to support the hypothesis that S4 conformation does not vary with concentration or the nature and ionic strength of the buffer.

In a different type of study, Ramakrishnan et al. (42) report a radius of gyration for S4 of 30.7 Å ± 4.6 Å. The technique used by this group was neutron scattering triangulation in which the protein was studied in situ. This technique has low resolution compared to traditional scattering studies. The error inherent in this type of analysis allows sufficient lattitude in the results such that they could easily be compatible with the values obtained by Gulik et al., Paradies and Franz, and our values as well.

The greatest source of error in the various studies on S4 arises from aggregation under reconstitution conditions or at high concentrations. This creates a large error in analysis of x-ray or neutron scattering data because the radius of gyration is determined from the slope of a Guinier plot and must be extrapolated. Low intensity of scattered light from a dilute sample gives a poor signal to noise ratio while high concentration increases the likelihood of aggregation. In our studies, the technique of intensity fluctuation spectroscopy and histogram analysis circumvent these problems to a great degree. A sample of low concentration can be used for this type of analysis and the presence of aggregates can be dealt with through the histogram analysis for polydisperse samples. This technique allows the determination of the diffusion coefficient for the monomer much more precisely than other scattering techniques. Employment of this technique for a sample can give precise

values for a diffusion coefficient concommitantly with providing an assessment of sample quality.

It was the original intent of this investigation of S4 conformation to use samples prepared by the salt-extraction method (10). However, it was found to be unfeasible to isolate S4 by this method for use in hydrodynamic studies (see Results). The assertion had been made that the proteins isolated by this method showed more ordered structure when studied by proton magnetic resonance spectroscopy (70) and that the use of less harsh conditions in this method than those of the traditional isolation procedures (16) produced a more native particle (31). Recently studies of proteins prepared by both methods seem to indicate that proteins prepared by the denaturing method are capable of resuming conformations which show PMR spectra qualitatively the same as those prepared by the more gentle salt-extraction technique (13,23,24,30,35, 39). The minimum requirement for the renaturation of the denatured protein samples studied seems to indicate the requirement of concentrations below 0.1 mg/ml in reconstitution buffer and a temperature incubation of some nature. These criteria seem consistent with the conditions for the partial and total reconstitution of the 30S subunit (60) and the concepts of self-assembly and spontaneous protein folding. It should also be pointed out that conditions used in the salt-extraction method are also denaturing (29). The degree of denaturation is therefore the issue and in the case of some of the salt-extracted proteins the level of salt used for extraction may disrupt the structure of the proteins as much as urea. The superior quality of salt-prepared proteins is, therefore, not as likely as was originally proposed (31).

Since S4 has only one cysteine residue, its inability to fully renature due to a variety of cysteine pairings is not a problem. From immunoprecipitation analysis the homogeneity of the sample was noted to be exceptional.

In conclusion, this study indicates that S4 has a moderately extended conformation in solution. The axial asymmetry for a prolate ellipsoid model is estimated to be not less than 4.5:1 and not more than 7:1. The radius of gyration would be between 25 Å and 32 Å with an expected value of 26-27 Å. The dimensions for 5:1 prolate ellipsoid model would be ~140 Å x 28 Å x 28 Å, which is consistent with x-ray scattering studies (16), and neutron scattering triangulation studies (42). Further studies of S4 are recommended, especially interaction studies as a complex with S3 and S5 (see Results) as this may have great relevance to structural and functional domains.

Chapter V

SUMMARY

Ribosomal protein S4 from Escherichia coli has been studied in reconstitution buffer using hydrodynamic techniques. The sedimentation coefficient and diffusion coefficient have been determined to be 1.69 (± 0.1)S and 7.95 (± 0.1) x 10^{-7} cm^2/s, respectively. Sedimentation equilibrium experiments have been used throughout these studies to insure sample quality. The sedimentation equilibrium studies gave a weight-average molecular weight of 23,200 ± 200. Immunoprecipitation assay has shown the sample to be homogeneous, as does two-dimensional gel electrophoresis.

Calculations based on the hydrodynamic measurements indicate that S4 has a moderately extended conformation in solution. The axial ratio for a prolate ellipsoid model is not less than 4.5:1 and not more than 7:1. The expected radius of gyration is between 26 Å and 30 Å.

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