ISOLATION OF SPECIFIC POLYRIBOSOMES BY IMMUNOCHEMICAL METHODS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
WILLIAM HERMAN ESCHENFELDT
1975

ABSTRACT

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By

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The total polysome population was isolated from the myeloma cell line MOPC-21 which synthesizes and secretes an IgG1-like molecule. The polysomes were purified by gel filtration on columns of Sepharose 2B, 4B or 6B. This procedure separates the polysomes from the smaller intracellular material. Isolation of the polysomes synthesizing the myeloma protein (IgG1) was attempted through the use of immunochemical techniques. Antibody directed against the myeloma protein was shown to bind specifically to the purified polysomes. Specific immune precipitation of the polysomes was demonstrated, although nonspecific background precipitation was consistently high. An affinity chromatography system using the myeloma--anti-myeloma system was shown to be specific for those proteins. However, repeated attempts to bind polysomes to affinity chromatography columns were unsuccessful. Nonspecific binding was high and no significant difference in specific binding could be demonstrated.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1975

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. Ronald J. Patterson for his guidance and encouragement throughout this study. I also wish to thank the Department of Microbiology and Public Health for financial assistance.

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INTRODUCTION

Since the messenger RNA-ribosomal aggregate model for protein synthesis was first described (Warner *et al.*, 1962, 1963; Wettstein *et al.*, 1963; Staehelin *et al.*, 1963), isolation of the polyribosomal fraction of cells has been widely used as a tool for the study of protein synthesis. Initially, polyribosomes were isolated by fractionating crude cell lysates on sucrose gradients and collecting the material in the 100-300 S region. In order to study synthesis of specific proteins, however, it was necessary to isolate only specific polysomes from the total population. For certain proteins such as myosin and gamma globulin this could be accomplished in part by isolating polysomes on the basis of their size (Heywood *et al.*, 1967; Shapiro *et al.*, 1966). Since cells synthesize proteins of various sizes and many different proteins of similar size, this method of polysome isolation still yielded a diverse population.

Evidence for the presence of specific protein fragments (nascent or growing polypeptides) on ribosomes led to attempts to isolate polysomes synthesizing particular proteins by precipitation with specific antiserum (Kaneyama *et al.*, 1960; Cowie *et al.*, 1961; Warren and Goldthwaite, 1962; Duerre, 1964). Some workers reported binding of antiserum to polysomes without precipitation (Warren and Peters, 1965; Voss and Bauer, 1966). Indirect precipitation of

polysomes was also reported, using antiserum to antibodies (anti-gamma globulin) (Delovitch *et al.*, 1972, 1973a, 1973b).

An established method for the isolation of specific proteins involves interaction with other proteins bound to an insoluble matrix (affinity chromatography). In 1971, Miller, Cuatrecasas and Thompson reported a partial purification of ribosomes synthesizing tyrosine amino transferase using affinity chromatography. This method relied upon the affinity of the enzyme for its substrate or substrate analog.

This study reports attempts to isolate heavy and light chain synthesizing polysomes from myeloma cells (MOPC-21). Myeloma protein, bovine serum albumin (BSA), rabbit anti-myeloma protein and rabbit anti-BSA were coupled to Sepharose via cyanogen bromide activation. The specificity of these immunoadsorbents was demonstrated in binding studies utilizing ^{14}C-labeled proteins. Polysomes were isolated from MOPC-21 cells and analyzed on sucrose gradients. Binding and precipitation studies were performed using ^{14}C-labeled rabbit anti-myeloma and unlabeled anti-myeloma, respectively. Several methods were tried to demonstrate binding of myeloma producing polysomes to the Sepharose-protein preparations.

REVIEW OF THE LITERATURE

Description of Polyribosomes

The function of ribosomal aggregates in protein synthesis was described simultaneously by two groups of workers. Wettstein, Staehelin and Noll (1963) and Staehelin *et al.* (1963) called this functional unit an "ergosome" and postulated that it consisted of five or more 73 S ribosomal particles attached to a strand of messenger RNA. Warner *et al.* (1962, 1963) described essentially the same thing, calling them polyribosomes (or polysomes). Shortly thereafter, other groups described polyribosomes in bacteria and plants, as well as in animal cells (Howell *et al.*, 1964; Gierer, 1963; Clark *et al.*, 1963).

Evidence that the size of the polyribosome corresponded to the size of the peptide synthesized provided one method for partial purification of specific polysomes (Lazerides and Lukens, 1971). Heywood *et al.* (1967) reported the identification of myosin synthesizing polysomes. They found that myosin was synthesized on polysomes containing 50 to 60 ribosomes. Shapiro *et al.* (1966) reported on gamma globulin synthesis in mouse plasma cell tumors. This system had the advantage that 20-30% of the protein synthesized by the cell was gamma globulin. They reported light chain synthesis by

polysomes of about 190 S and heavy chain synthesis by polysomes of approximately 270 S.

Binding of Specific Antibody to Polyribosomes

The discovery of growing nascent peptide chains on polyribosomes offered another possibility for specific polysome isolation and purification. In 1965, Warren and Peters reported binding antibody directed against rat serum albumin to rat liver polysomes. However, polysome precipitation did not occur. In 1966, Voss and Bauer reported the binding of antibody directed against the F_c region of heavy chains to lymphocyte polysomes. More recently, Palacios *et al.* (1972) reported binding of ^{125}I-labeled anti-ovalbumin to polysomes and Taylor and Schimke (1974) reported binding of labeled anti-albumin to rat liver polysomes, both without precipitation.

Precipitation of Polyribosomes with Specific Antibodies

Direct Precipitation

In 1967, Williamson and Askonas reported the isolation of polysomes synthesizing immunoglobulin light and heavy chains by precipitation with specific antiserum. They found that while they could precipitate polysomes in the 300 S region with antibody to the F_c portion of heavy chain, precipitation of polysomes with anti-light chain antiserum was more difficult to demonstrate. They suggested that this might be due to attachment of light chain to nascent heavy chain, or simply due to the small size of the nascent

light chain. Scherr and Uhr (1969) also reported direct precipitation of immunoglobulin synthesizing polysomes. Takagi and Ogata (1971) reported direct precipitation of serum albumin synthesizing polysomes from rat liver. Precipitation of polysomes with specific antisera was also reported in other systems: β-galactosidase (Kaneyama and Novelli, 1960; Cowie *et al.*, 1961); triose phosphate dehydrogenase (Warren and Goldthwaite, 1962); glutamic dehydrogenase (Duerre, 1964); and ovalbumin (Palmiter *et al.*, 1972; Palacios and Schimke, 1973). In 1972, Delovitch *et al.* reported the isolation of light chain synthesizing polysomes by direct precipitation. In a series of papers (Delovitch *et al.*, 1972, 1973a, 1973b) this technique was used to obtain specific messenger RNA. It is interesting to note that Delovitch found that the use of the $F_{(ab')2}$ portions of the antibody significantly reduced nonspecific binding and precipitation, suggesting a nonspecific affinity of the F_c region for the polysomes. This problem has not been mentioned in recent reports by other workers. Sarkar and Moscona (1973) reported direct precipitation of glutamine synthetase synthesizing polysomes with no difference in results using pepsin treated antibody ($F_{(ab')2}$) and whole antibody. It should be pointed out, however, that nonspecific precipitation was high in both cases.

Indirect Precipitation

Delovitch *et al.* (1972, 1973b) also used a double antibody technique (indirect precipitation) for isolation of polysomes. In this method, polysomes are first incubated with specific antibody

followed by anti-gamma globulin. This has the advantage that the "secondary antigen" (the specific anti-protein) serves as a larger collection of antigenic determinants than the nascent peptide, thus facilitating cross-linkage and precipitation by the anti-antibody. Schechter (1973, 1974a, 1974b) has used this indirect procedure for precipitation of kappa (k) light chain synthesizing polysomes from myeloma cells. He has reported that the technique can be used to process large amounts of polysomes (up to 25,000 A_{260} units per batch). Indirect immune precipitation of polysomes synthesizing ovalbumin (Shapiro *et al.*, 1974; Shapiro and Schimke, 1974), catalase (Uenoyama and Ono, 1972), and rat serum albumin (Shapiro *et al.*, 1974) have also been reported.

Isolation of Polyribosomes by Affinity Chromatography

The use of affinity chromatography for the purification of proteins is a standard and widely utilized technique (Cuatrecasas, 1970; Cuatrecasas and Anfinsen, 1971; Turkova, 1974). Specific antibodies can be purified by binding to antigen attached to an insoluble matrix. Antigen can be isolated by binding to an insolubilized antibody. The affinity does not have to be an antigen-antibody interaction. Any protein-protein interaction such as enzyme-substrate may be utilized in affinity chromatography.

In 1971, Miller, Cuatrecasas and Thompson reported the isolation of tyrosine amino transferase polysomes by affinity chromatography. A substrate analog was attached to Sepharose. When the polysome solution was passed over the material, the nascent peptides of the enzyme synthesizing polysomes were bound to the substrate. After

washing the columns, the specifically bound polysomes were eluted. An enrichment for tyrosine amino transferase synthesizing polysomes was reported.

Le Goffic, Baca and Moreau (1974) recently reported isolating *E. coli* ribosomes by affinity chromatography. The intended use of the columns (coupled with gentamicin or streptomycin) was to isolate intracellular enzymes which inactivate these antibiotics. It was noted, however, that ribosomes were also bound by the columns. It should be pointed out that this binding occurs via the ribosomal subunits and not the nascent peptides of the polysomes. Thus, this technique did not isolate specific ribosomes, but merely separated ribosomes in general from the remainder of the intracellular material.

Palacios *et al.* (1973b) have reported isolating ovalbumin synthesizing polysomes by first incubating the polysome preparation with specific antibodies in solution followed by incubation with an ovalbumin matrix formed by treating the protein with glutaraldehyde. The antibodies form cross-links between the polysomes and the insoluble matrix. The entire procedure was done batchwise, with no column chromatography. Palacios *et al.* (1973b) also reported that a matrix of anti-gamma globulin can be used to bind the polysome-antibody complex. However, attempts to bind polysomes directly with a matrix of specific antibody were unsuccessful.

Sidorova *et al.* (1974) have reported recently the isolation of rat liver albumin and mouse gamma globulin polysomes through the use of affinity chromatography. The specific protein (albumin or

gamma globulin) was attached to aminocellulose. The matrix was saturated with specific antibody and then incubated with the polysomes. Specific polysomes were bound to the matrix, forming a "sandwich" of polysome-antibody-protein-aminocellulose.

MATERIALS AND METHODS

Cells

The cells used in this study were mouse plasmacytes obtained from myeloma tumors. The MOPC-21 line of cells (P3.6.2.8.1) is an IgG1 producing line with light chains of the k class. These cells secrete whole immunoglobulin and, although they produce excess light chains intracellularly, they secrete no free light chain (Baumal and Scharff, 1973). Also used in this study were the XC.1 cell line and the S49.1 cell line. The XC.1 line is a synthesis variant of another myeloma line, C1. XC.1 cells neither secrete nor contain intracellular immunoglobulin. The line was derived from an IgG2 producer. The S49.1 line is a lymphoma cell line. The cells are thymus-derived (T cells) which do not produce detectable amounts of gamma globulin.

The MOPC-21 cell line was maintained initially as solid tumors in female BALB/c mice. The tumors were a kind gift of the Cell Distribution Center, Salk Institute. Solid tumors were carried subcutaneously and transferred every 14 days into tumor-free mice. Later, the tumor was carried in an ascites form. The ascites tumor was started by injecting a cell suspension from solid tumors intraperitoneally into female BALB/c mice. Cells were withdrawn from

the peritoneum and reinjected into new mice at approximately 7- to 10-day intervals.

The S49.1 and XC.1 cell lines, as well as the MOPC-21 line, were maintained *in vitro* in tissue culture. These tissue culture cell lines were also provided by the Salk Institute. Cells were maintained in Dulbecco's Modified Eagle Medium (GIBCO) supplemented with antibiotics (75 µg/ml streptomycin, 100 units/ml penicillin, 40 units/ml mycostatin) and 10% serum. Initially, gamma globulin free horse serum was used, but this was later replaced with fetal calf serum. Tissue cultures were maintained in plastic flasks and roller bottles. Flasks were incubated in a CO_2 incubator at 37°C with a humid atmosphere of 85% air, 15% CO_2. Roller bottles were flushed with 95% air, 5% CO_2, sealed and incubated at 37°C at a rotation speed of one-half revolution per minute. Cells were routinely grown to a density of 1×10^6 cells per ml in both flasks and roller bottles and diluted 1:10 with fresh medium. The generation time at maximum cell growth was about 16-18 hours.

Protein Preparation

Myeloma Protein

Mice bearing subcutaneous tumors were bled through the tail vein daily, for several days before they were sacrificed. The sera were pooled and the gamma globulin fraction prepared by ammonium sulfate precipitation. Ice cold saturated ammonium sulfate was added to the serum slowly with continuous stirring, to a final concentration of 40% (v/v). This solution was centrifuged at 10,000

rpm (12,000 x g max.) in a Sorvall RC2-B centrifuge for 15 minutes at 4°C. The pellet was resuspended in distilled water (usually about half the original volume). The entire precipitation procedure was then repeated. The final pellet was resuspended in distilled water and dialyzed overnight at 4°C against buffer (10 mM Tris, pH 7.4, 150 mM NaCl). Protein concentration was determined by spectral absorbance at 280 nm, using an extinction coefficient of 13.6 for gamma globulin (Small and Lamm, 1966).

Myeloma protein was also obtained from cells grown in tissue culture. MOPC-21 cells were labeled for 24-36 hours with 1 μCi/ml ^{3}H-proline. After the cells had been pelleted by centrifugation, the supernatant fluid was precipitated with ammonium sulfate as above, with the exception that the first precipitation was 50% saturated ammonium sulfate instead of 40%. Protein concentration was determined as above.

Rabbit Antiserum

New Zealand White rabbits were immunized with myeloma protein or bovine serum albumin (BSA, Cohn Fraction V). Protein at a concentration of approximately 5 mg/ml was added to an equal volume of Freund's Complete Adjuvant and mixed until an emulsion was formed. Rabbits were injected subcutaneously with 2 ml of the emulsion, followed 7 days later by a second injection. The rabbits were bled through the marginal ear vein 7-10 days following secondary injections. The animals were periodically given booster injections and bled 7-10 days hence. Normally, about 30 to 50 ml of whole blood was obtained from one rabbit in a single bleeding. A single bleeding

was also taken from each rabbit prior to the first antigen injection (normal serum). Serum was stored at -20°C. The gamma globulin fraction was obtained in the same manner as for myeloma protein.

Anti-Globulin

Goat anti-rabbit gamma globulin (GARGG) was the kind gift of Dr. L. F. Velicer and Anthony Conley, Department of Microbiology and Public Health, Michigan State University. GARGG was also purchased from GIBCO. The lyophilized serum preparation was reconstituted in 5 ml distilled water according to the company's specifications. The gamma globulin fraction was obtained by ammonium sulfate precipitation.

Bovine Serum Albumin

BSA (Cohn Fraction V) was purchased from Sigma. The protein was used without further purification.

^{14}C-Labeling of Proteins

Proteins were labeled *in vitro* with ^{14}C-formaldehyde by the procedure of Rice and Means (1971). ^{14}C-formaldehyde was purchased from New England Nuclear in ampules containing 50 µCi of isotope in approximately 3 µl water at a specific activity of 55 mCi per millimole formaldehyde.

The complete procedure was done at 0°C. One-tenth milliliter sodium borate buffer (50 mM, pH 9.0) was added to the ampule containing the ^{14}C-formaldehyde. The protein was then added in a volume of 20 µl, the total amount of protein being about 300 µg. This was followed 30 seconds later by 4 sequential 2 µl additions of sodium

borohydride at 5 mg/ml. One minute after the final 2 µl addition, an additional 10 µl sodium borohydride was added. The solution was then brought to 1.0 ml with the sodium borate buffer and dialyzed overnight against 10 mM Tris (pH 8.0).

Pepsin Digestion of Antibody

Peptic digestion of antisera was performed essentially as described by Utsumi and Karush (1965). Antisera were dialyzed overnight against 0.2 M acetate buffer (pH 4.0). The final concentration after dialysis was between 15 and 35 mg/ml and the pH was usually between 4.0 and 4.5. The pH was adjusted to 3.5-4.0 with 2 N HCl. To 1.0 ml of the protein solution was added crystalline pepsin to a final concentration of 2.0-2.5% (w/w). This solution was then incubated at 37°C for 20 hours. At the end of this time, samples were placed on ice and the pH was adjusted to 8.0-8.5 with 1.0 N NaOH. The solutions were then dialyzed overnight against buffer (usually 10 mM sodium phosphate, pH 8.0). The protein was analyzed by sucrose gradient centrifugation and sodium dodecyl sulfate (SDS) gel electrophoresis. The gradients were 5-20% sucrose (w/v) in 50 mM potassium phosphate buffer. Samples were centrifuged at 40,000 rpm (286,000 x g max.) in a Beckman SW41 rotor for 35 hours at 4°C. Gradients were collected on an ISCO density gradient fractionator, with continuous monitoring of absorbance at 280 nm and recorded on a Gilford model 2400S recorder.

SDS gel electrophoresis was done by the method of Fairbanks *et al.* (1971). Gels were 9% acrylamide and 1% SDS. Sample volumes were 50 µl or less. Gels were electrophoresed at a constant voltage

of 8.2 volts per cm gel length for 2-3 hours, until the dye marker (Pyronin-Y) reached the bottom of the gels. The gels were scanned at 280 nm on a Gilford model 2400 recording spectrophotometer. After staining with Coomassie Blue, the gels were scanned at 540 nm.

Reduction and Alkylation of Antibody

Antisera were reduced and alkylated by a modification of the procedure of Nies *et al.* (1973). Protein solution at a concentration of 15-35 mg/ml was added to solution A (1.0 M Tris, pH 8.1, 0.2 M 2-mercaptoethanol, 2% (w/v) SDS) at a ratio of one part protein solution to four parts solution A. The sample was then placed in a boiling water bath for 5 minutes. An equal volume of solution B (1.0 M Tris, pH 8.1, 2% SDS, 0.4 M iodoacetamide) was then added and the sample incubated at room temperature for 5 minutes. The protein was precipitated by adding 5 volumes of absolute ethanol, followed in 5 minutes by centrifugation at 10,000 rpm for 5 minutes. Pellets were redissolved by adding solution C (10 mM Tris, pH 8.1, 1 mM ethylenediaminetetra-acetate (EDTA), 1% SDS, 1% (v/v) 2-mercaptoethanol) and submerging in a boiling water bath for 3 minutes. The protein was analyzed on SDS gels as described above. Heavy and light chains were separated on 5-20% (w/v) linear SDS sucrose gradients in 50 mM sodium phosphate buffer (pH 8.0). Samples were centrifuged at 40,000 rpm in a Beckman SW 41 rotor for 72 hours at 4°C.

Affinity Chromatography

Coupling of Protein to Sepharose

Protein was coupled to Sepharose by the method of Cuatrecasas (1970). Sepharose 6B was obtained from Sigma. Sepharose 4B and 2B were obtained from Pharmacia. Cyanogen bromide was purchased from Aldrich. Thirty milliliters Sepharose was washed with approximately 500 ml distilled water, then resuspended in 30 ml distilled water. Six to nine grams of cyanogen bromide finely ground by mortar and pestle were added at once with continuous stirring. The pH was adjusted to 11.0 with 5 N sodium hydroxide and maintained by subsequent additions. Temperature was maintained at 20-22°C by the addition of ice. This was continued until the pH stabilized (about 20 minutes, depending upon how rapidly the cyanogen bromide dissolved). A large amount of ice was then added, the mixture was vacuum filtered and washed with one liter of ice cold sodium bicarbonate buffer (0.1 M, pH 7.0). The activated Sepharose was then resuspended in 30 ml of the bicarbonate buffer, protein added (30-45 mg) and the pH adjusted to 6.5-7.0 with 2 N HCl. The mixture was allowed to incubate overnight at 4°C with stirring.

The coupled Sepharose was vacuum filtered and washed with one liter of buffer (usually 10 mM sodium or potassium phosphate, pH 8.0) and resuspended in 30-50 ml of the same buffer plus .02% (w/v) sodium azide. The first 100 ml of the wash buffer was monitored for protein concentration in order to estimate the amount of protein coupled to the Sepharose. The degree of coupling was 750-1000 µg protein per ml Sepharose.

Affinity Chromatography Columns

Unless otherwise noted, all columns were poured in disposable pasteur pipettes to a final packed volume of 0.2-0.3 ml Sepharose. Columns were washed with 1-2 ml 10% normal rabbit serum or horse serum to saturate nonspecific binding sites. They were then washed with 5-8 ml buffer. (Specific buffers are described in Results.) Protein was applied to columns in volumes no larger than 0.1 ml and incubated at room temperature for approximately 5 minutes, unless noted otherwise. This was followed by washing with 5-10 ml buffer, collecting fractions and precipitating with 10% trichloroacetic acid (TCA). Precipitated fractions were collected by vacuum filtration on Whatman GF/C glass fiber filters (2.4 cm) and counted in 10 ml toluene-Omnifluor (New England Nuclear) scintillation fluid. In some cases, columns were washed with elution buffers to remove specifically bound proteins. Fractions were collected and precipitated in the same manner as above.

Isolation of the Total Polysome Population from Cells

Separation of Membrane-Bound and Free Polysomes

Cells were grown in roller bottles to a density of 5-8 x 10^5 cells/ml. If labeled polysomes were desired, ^{3}H-uridine was added at a concentration of 1 μCi/ml during the last 2-3 hours of incubation.

All isolation procedures were performed on ice or at 4°C unless noted otherwise. Cells were washed once in normal saline and once in RSB (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) and then resuspended in an equal volume of RSB (or to a total volume of 1.0 ml,

whichever was greater). Following incubation on ice for 10 minutes, the cells were lysed in a glass Dounce homogenizer and the nuclei were pelleted at 2000 rpm (900 x g max.) in an International refrigerated centrifuge. The supernatant fluid was removed and centrifuged at 16,000 rpm (30,000 x g max.) for 10 minutes in a Sorvall RC2-B centrifuge. The supernatant fluid contained free polysomes. The pellet was resuspended in TKM buffer (50 mM Tris, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$) (Miller *et al.*, 1971) and treated with sodium desoxycholate (DOC) and Triton X-100, final concentrations 0.5% (w/v) and 0.5% (v/v), respectively. This solution was then centrifuged at 30,000 x g max. for 10 minutes. The supernatant contained membrane-bound polysomes.

Total Polysome Population

Cells were poured over frozen crushed saline to facilitate rapid cooling. This procedure was first employed late in the study and seemed to yield a better population of polysomes. Pelleted cells were washed once in saline, resuspended in RSB and incubated on ice for 5 minutes. The cells were lysed by adding non-ionic detergent Nonidet P-40 (NP-40, Shell) to the suspension to a final concentration of 0.5% (v/v). Nuclei were pelleted and the supernatant treated with DOC-Triton X-100 as above. This solution contained both free and membrane-bound polysomes and is referred to as crude polysomes. In later experiments, the pelleted nuclei were washed in a small volume of RSB, recentrifuged and the supernatant fluid combined with the original supernatant before treatment with DOC-Triton X-100.

Purification of Total Polysomes

Pelleting

In this procedure, the crude polysome fraction was transferred to polyallomer centrifuge tubes, brought to tube volume with RSB and pelleted by centrifugation for 2 hours at 50,000 rpm (226,000 x g max.) in a Type 50Ti rotor in the Beckman Model L5-50 ultracentrifuge. The pellet was washed once with saline and either frozen at -80°C or resuspended in buffer.

Discontinuous Sucrose Gradients

Another procedure used was the method of Schimke *et al.* (1974). Crude polysomes brought to 6 ml with polysome buffer (25 mM Tris, pH 7.6, 25 mM NaCl, 5 mM $MgCl_2$) were layered over a discontinuous sucrose gradient consisting of 4.0 ml 1.0 M sucrose over 2.0 ml 2.5 M sucrose, both in polysome buffer. This was then centrifuged at 41,000 rpm for 1.5 hours in a Beckman SW 41Ti rotor. The polysomes banded at the interphase between the 2.5 M sucrose and the 1.0 M sucrose. They were removed by puncturing the side of the tube about 3 mm below the interphase with an 18-gauge needle and withdrawing 0.8 to 1.0 ml in a 1 ml syringe. The sample was then dialyzed overnight at 0°C against polysome buffer to remove the sucrose.

Sarkar and Moscona (1973) modified Schimke's technique for the SW 50.1 rotor which has smaller capacity tubes (5.5 ml). This technique uses 1.0 ml 2.5 M sucrose, 2.0 ml 1.0 M sucrose and 2.0 ml polysome preparation, centrifuging for 2 hours at 46,000 rpm

(250,000 x g max.). We modified Sarkar's technique as follows. The 2.5 M sucrose pad was omitted, allowing the polysomes to pellet. The reasons for this were twofold. First, resuspending the pellet avoided the long dialysis with its subsequent loss of polysomes and, second, it was hoped that by pelleting through sucrose the polysomes would not be packed as firmly in the pellet, thus permitting easier resuspension. In another modification, Sarkar's technique was followed as reported except that dialysis was replaced by passage over a Sephadex G-25 column. The column was 3 ml packed volume of Sephadex in a 5 ml glass syringe. Approximately 0.7 ml of the polysome-sucrose solution was loaded onto the columns. The polysomes were eluted with polysome buffer in the void volume while the sucrose was retarded by the Sephadex.

Sepharose Columns

See Results, Section I.

Analysis of Polysomes

Polysomes were analyzed on 15-45% (w/v) linear sucrose gradients in either the SW 50.1 rotor (5.2 ml sucrose per gradient) or the SW 41Ti rotor (12.4 ml sucrose per gradient). The former were centrifuged at 45,000 rpm (250,000 x g max.) for 35 minutes, while the latter were centrifuged at 38,000 rpm (246,000 x g max.) for 75 minutes. Gradients were collected from the top with continuous monitoring of absorbance at 254 nm.

Isolation of Specific Polysomes

Specific Binding of Labeled Antiserum

Binding of ^{14}C-labeled antibody to purified polysomes was done as described by Taylor and Schimke (1974). Varying amounts of polysomes (2-25 A_{260} units) were incubated with 10-15 µg labeled antibody for 1 hour at 0°C. These were then layered onto sucrose gradients and centrifuged as described in Analysis of Polysomes. In competition studies, polysomes were incubated with 500 µg unlabeled antibody for 30 minutes followed by 10-15 µg labeled antibody for 30 minutes.

Direct Precipitation of Polysomes

This was done essentially as described by Delovitch *et al.* (1972, 1973b). Polysome fractions from sucrose gradients were incubated with antiserum (gamma globulin fraction) for 5 minutes at 37°C. Specific antigen was added, followed by incubation at 37°C for 5 minutes and 4°C for 2-4 hours (or overnight). Tubes were centrifuged for 15 minutes at 1000 x g max., the pellets washed once with polysome buffer, and centrifuged again. The pellets were dissolved in 1 N NaOH. All samples were dissolved in Bray's scintillation fluid (Bray, 1960) and counted in a Packard Tri-Carb liquid scintillation counter.

Direct and indirect precipitation was also done on purified total polysomes. The procedure was modified from Shapiro *et al.* (1974). Polysomes were incubated with whole antibodies or pepsin treated antibodies for 45 minutes at 0°C. Then either specific

antigen or GARGG was added. This mixture was then incubated an additional 60 minutes at 0°C. Pellets were washed as above and dissolved with 5% SDS. All fractions were precipitated with 10% TCA, collected on Whatman GF/C filters and counted in toluene-Omnifluor. Volumes and concentrations of reagents used in specific experiments are reported in Results.

Affinity Chromatography of Polysomes

The technique of affinity chromatography of polysomes was modified from the method reported by Palacios *et al.* (1973b) and Schimke *et al.* (1974). Briefly, it involves specific antigens or anti-antibodies bound to Sepharose, cross-linked to polysomes which have specific antibodies bound to them.

Purified total polysomes were incubated with rabbit antibodies (specific or control) for 45 minutes at 0°C. Then Sepharose coupled with protein (specific or control antigens, GARGG) was added and the mixture incubated an additional 60 minutes at 0°C. The mixtures were then transferred to pasteur pipettes at 0°C and washed with column buffer (polysome buffer plus 0.5 M sucrose, 1% DOC, 1% Triton X-100, 100 µg/ml heparin) (Schimke *et al.*, 1974). Columns were then washed with polysome buffer plus heparin, followed by elution buffer (10 mM Tris, pH 7.5, 50 mM EDTA). The EDTA causes dissociation of polysomes with subsequent release of ribosomal subunits and messenger RNA. Details of specific experiments are reported in Results.

RESULTS

Section I

Article

Polysome Isolation by Sepharose Column Chromatography

submitted for publication to

Biochimica et Biophysica Acta

POLYSOME ISOLATION BY SEPHAROSE COLUMN CHROMATOGRAPHY

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SUMMARY

Polysomes from the mouse myeloma MOPC-21 were purified by gel filtration on Sepharose 6B, 4B and 2B columns. All three columns eliminated nearly all intracellular material smaller than 40 S subunits. In addition, passage through 4B and 2B columns substantially reduced the amount of subunits and monosomes in the preparations. Purified polysomes retained structural integrity when stored at -85°C for at least 9 weeks.

INTRODUCTION

In studies involving polysomes, it is often necessary that the polysomes be free from contaminating intracellular material. A common method for achieving this is by pelleting the polysomes by ultracentrifugation. This procedure requires several hours of centrifugation, and resuspension of the polysome pellets is often difficult due to their aggregation and degradation. A procedure using discontinuous sucrose gradients has been reported [1] in which the polysomes band at or just below the interphase between 1.0 and 2.5 M sucrose. This method requires dialysis of the polysomes to remove the sucrose. Recently, Palmiter [2] has reported the use of magnesium precipitation for the isolation of polysomes. Sephadex G-100 columns have been used to isolate ribosomal subunits [3], and the purification of polysomes on hydroxyapatite has been reported [4,5]. Tangen *et al.* [6] have reported the use of Sepharose gel filtration for the isolation of microsomes, and Darnbrough *et al.* [7] have reported purification of polysomes using Sepharose column chromatography. We have extended the original observations of

Darnbrough *et al.* [7] and have rapidly purified polysomes on columns of Sepharose 6B, 4B and 2B.

MATERIALS AND METHODS

Preparation of Polysomes: The mouse myeloma line MOPC-21 (kindly provided by The Cell Distribution Center, Salk Institute) was carried as an ascites tumor in female BALB/c mice. For polysome isolation, the mice were sacrificed by cervical dislocation and ascites fluid was withdrawn with a sterile syringe. Cells were pelleted at 500 x g for 10 minutes, then resuspended to a density of 5-6 x 10^6 cells/ml in Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% fetal calf serum (Flow Laboratories). After 30-60 minutes incubation at 37°C in an atmosphere of 95% air, 5% CO_2 [5,6-^{3}H]uridine (New England Nuclear, specific activity 45 Ci/mmole) was added to a concentration of 1-2 μCi/ml. Incubation was continued for 2-3 hours. The cells were then rapidly cooled by pouring over crushed frozen saline. All subsequent procedures were performed at 0-4°C. The cells were pelleted by centrifugation for 10 minutes at 500 x g, washed twice with RSB (10 mM Tris, pH 7.4, 10 mM NaCl 3 mM $MgCl_2$), resuspended in RSB and lysed by the addition of one-tenth volume of 5% (v/v) Nonidet P-40 (Shell). After 5 minutes, nuclei were removed by centrifugation for 5 minutes at 900 x g and the supernatant fluid was treated with one-tenth volume of a solution of 5% (w/v) sodium desoxycholate (Nutritional Biochemicals), 5% (v/v) Triton X-100 (Packard). This preparation is referred to as crude polysomes.

Sepharose Chromatography: All columns were poured and maintained at 4°C. Columns (1.5 x 15 cm) of Sepharose 6B, 4B and 2B (Pharmacia) were poured and washed with at least ten bed volumes of polysome buffer plus heparin [8] [25 mM Tris, pH 7.6, 25 mM NaCl, 5 mM $MgCl_2$, 100 µg/ml sodium heparin (Sigma)]. Crude polysomes were applied to the columns in volumes up to 2.5 ml and eluted at a flow rate of approximately 10 ml/hr with polysome buffer. Polysomes were eluted in the void volume and were visible as cloudy white fractions. Following thorough washing with polysome buffer, columns could be reused. One of our columns has been in use for over three months. The columns were periodically treated with a solution of 0.1% diethylpyrocarbonate (Calbiochem) in polysome buffer.

Sucrose Gradients: Polysomes were analyzed on 15-45% (w/v) linear sucrose gradients in polysome buffer plus heparin. The gradients were centrifuged at 250,000 x g for 40 minutes at 4°C in a Beckman SW 50.1 rotor in the Beckman Model L5-50 ultracentrifuge. Fractions of 0.2 ml were collected from the top using an ISCO Model 640 density gradient fractionator. Absorbance at 254 nm was monitored continuously with the ISCO Model UA-4 absorbance monitor and recorded on a Gilford Model 2400-S recorder. Fractions were collected directly into scintillation vials and counted in 5 ml of Bray's scintillation fluid [9] at 4°C.

RESULTS

Aliquots from a single preparation of crude MOPC-21 polysomes were purified on Sepharose 2B, 4B and 6B columns. The absorbance

and radioactivity profiles from sucrose gradients are shown in Figure 1. A large peak of material smaller than 40 S subunits is evident in the crude polysomes which is absent in the purified polysomes. Sepharose 6B, with an exclusion limit of approximately 4×10^6 Daltons for globular proteins, excludes polysomes and subunits, while retarding the remainder of the intracellular material. Sepharose 4B and 2B, with approximate exclusion limits for globular proteins of 20×10^6 Daltons and 40×10^6 Daltons, respectively, retard subunits and monosomes. Sepharose 2B also retards the smaller polysomes. Thus the void volume is enriched for the larger polysomes.

Table 1 compares the percentage of radioactivity in the polysome region and the nonpolysome region of the gradients. The polysome region is defined as that region larger than monosomes. The percentage of nonpolysomal radioactivity in the crude fraction is high due to the presence of unincorporated [^{3}H]uridine. The data from both 4B and 2B Sepharose fractions show a substantial increase in the percentage of polysomal radioactivity over that in the 6B fraction.

Purified polysomes from the columns were stored frozen without further preparation. Figure 2 shows the absorbance profiles of MOPC-21 polysomes purified over a 6B column. Figure 2A is a sucrose gradient profile of the polysomes analyzed on the day of isolation. Figure 2B is a profile of the same preparation after storage at -85°C for 9 weeks. Slight degradation is evident. The 40 S and 60 S subunit peaks have increased with corresponding decrease in the 80 S monosome peak. The percentage of subunits and monosomes has

increased from 26.6% on the day of isolation to 32.9% after 9 weeks storage.

DISCUSSION

Passage of crude polysome preparations over any of the Sepharose columns substantially reduces the contamination by intracellular material smaller than the 40 S subunits. Columns of Sepharose 4B and 2B enrich for the polysomal fraction, retarding subunits and monosomes, while Sepharose 2B also retards the smaller polysomes, yielding a population enriched for the largest polysomes. Furthermore, the polysomes remained structurally intact. The gel filtration technique yields polysomes that are diluted during purification. Crude preparations applied to the Sepharose 6B column at about 100 A_{260} units/ml are eluted as purified polysomes at about 25-30 A_{260} units/ml. For our purposes this dilution of purified polysomes is acceptable.

Preliminary results indicate that the polysomes are active in an endogenous cell-free protein synthesizing system. We have also used the columns to purify polysomes from the post-nuclear supernatant from cells which have been lysed without the use of detergents. Under these conditions, the eluted fraction contains intact microsomes, as well as free polysomes permitting subsequent separation of membrane-bound and free polysomes.

This procedure offers a relatively simple and rapid technique for the purification of total polysomes. Fractions from the columns need not be monitored continuously, as the fractions containing polysomes are readily visible. Also, stability of the purified

polysomes when stored at -85°C is excellent, probably due to the reduction of intracellular nucleases achieved through the gel filtration procedure.

ACKNOWLEDGMENTS

We wish to thank William Chaney for helpful discussions during the initial stages of this study. This investigation was supported by USPHS grant AI-11493. This is journal article no. 7073 from the Michigan Agricultural Experiment Station.

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Table 1. Distribution of radioactivity in crude polysomes and Sepharose purified polysomes

Polysomes[a]	A_{260}/A_{280}	Radioactivity[b] in non-polysomal area	Percentage	Radioactivity[b] in polysomal area	Percentage
Crude	1.69	537,400	80.0	134,200	20.0
6B	1.71	97,200	40.4	143,200	59.6
4B	1.72	23,300	15.6	126,100	84.4
2B	1.71	10,700	9.0	108,000	91.0

[a]Polysomes isolated from MOPC-21 ascites cells after 3.0 hours *in vitro* labeling with [^{3}H]uridine.

[b]Measurements include both soluble and insoluble radioactivity, thus the excessively high percentage in the crude, non-polysomal region is due to unincorporated [^{3}H]uridine.

Figure 1. Sucrose gradient profiles of crude and Sepharose purified polysomes prepared as in Materials and Methods. Arrow indicates monosomes (80 S). A_{254} (——); [^{3}H]uridine (●——●).

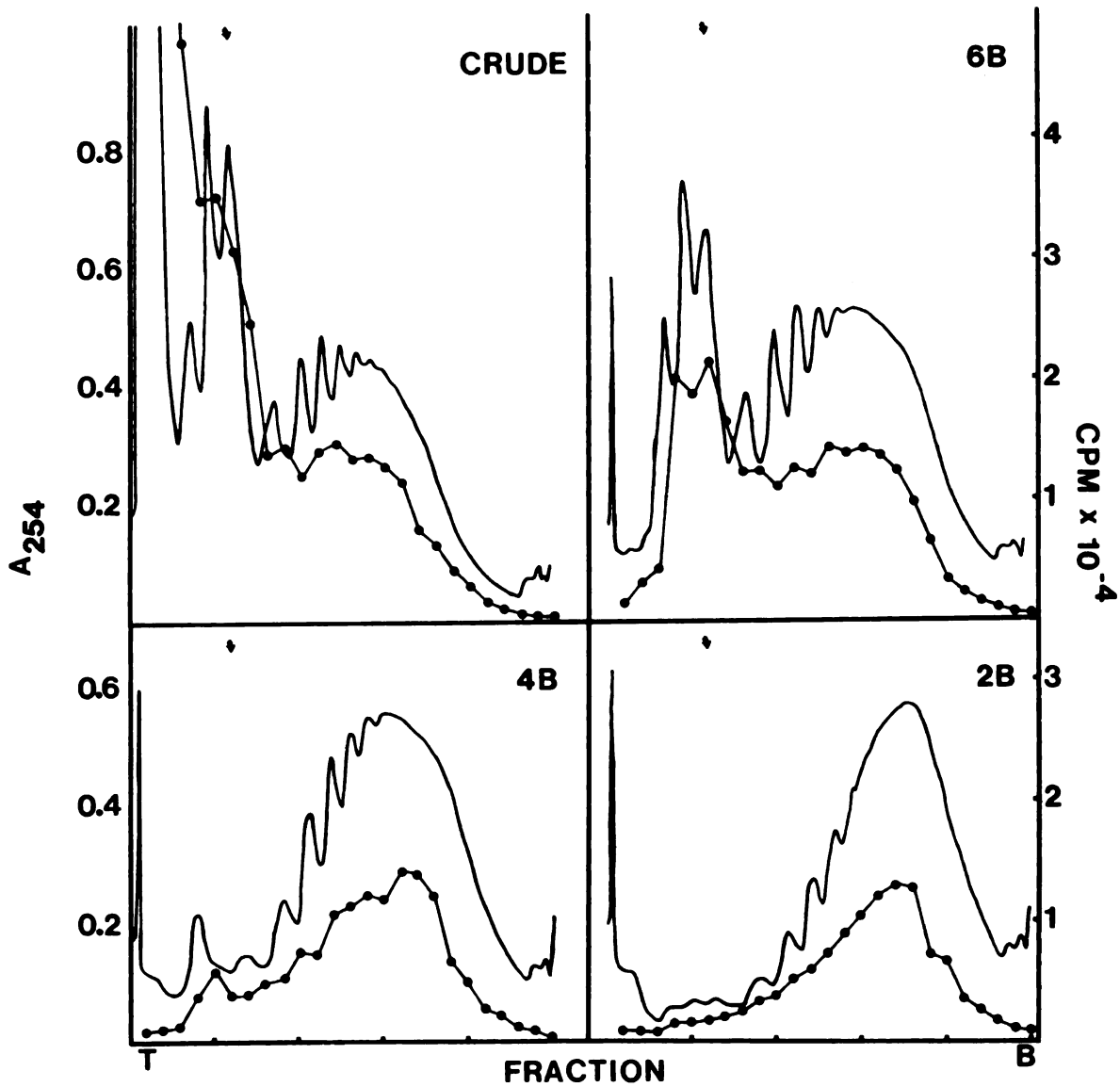

CRUDE
6B
4B
2B
0.8
0.6
0.4
0.2
0.6
0.4
0.2
4
3
2
1
3
2
1
A254
CPM x 10-4
T
FRACTION
B

Figure 1

Figure 2. Sucrose gradient profiles of Sepharose 6B purified polysomes. The crude polysomes were prepared from MOPC-21 tissue culture cells maintained *in vitro* in Dulbecco's Modified Eagle Medium, supplemented with 10% fetal calf serum, in an atmosphere of 85% air, 15% CO_2. Cells were labeled with [^{3}H]uridine and harvested at 5-8 x 10^5 cells/ml. The remainder of the isolation procedure was identical to that described in Materials and Methods. A. Analyzed on day of isolation. B. After storage at -85°C for 9 weeks. Arrow indicates monosomes (80 S).

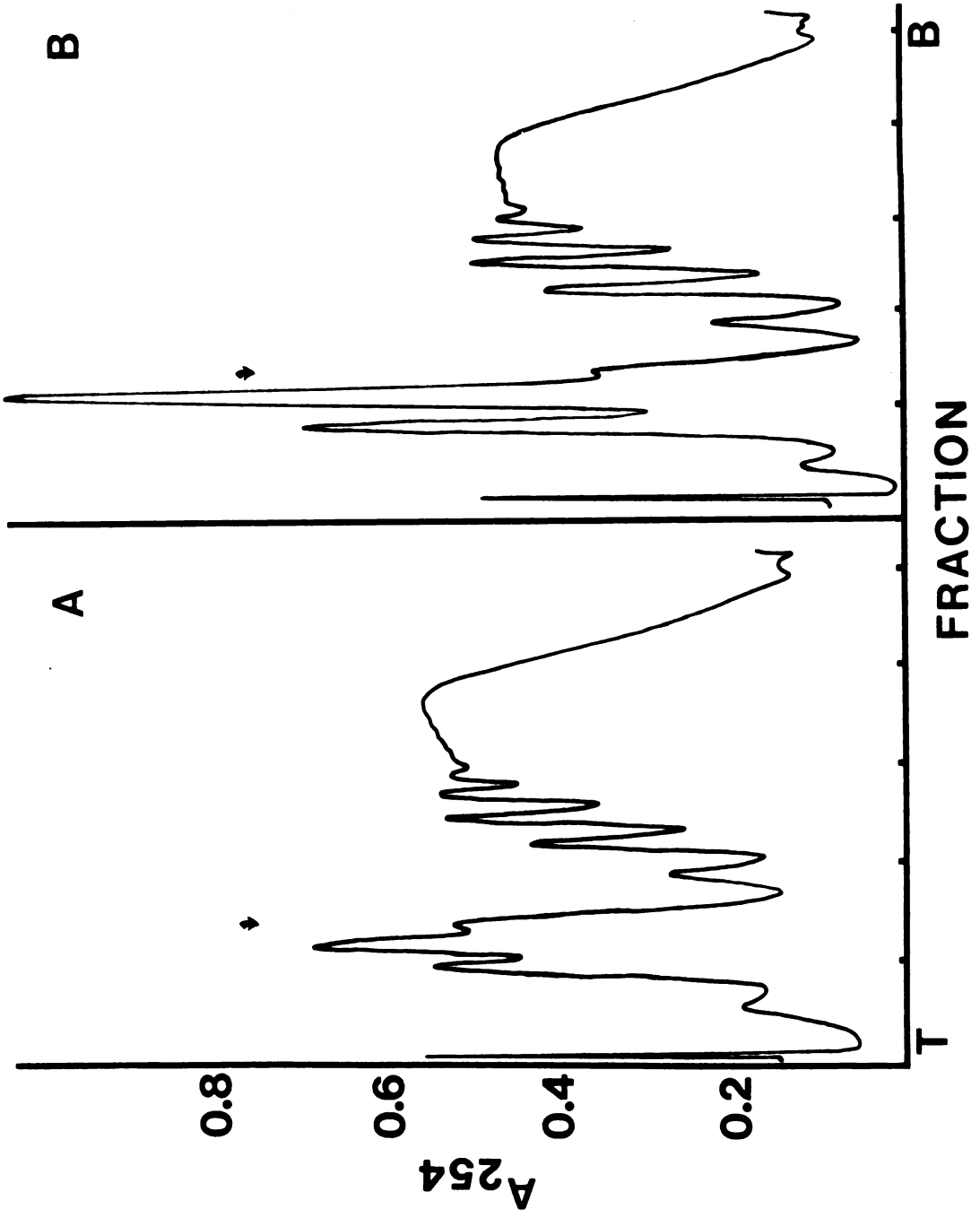


Figure 2

RESULTS

Section II

Isolation of Free and Membrane-Bound Polysomes

Tissue culture cells were lysed without the use of detergent and the postnuclear supernatant fraction was applied to a Sepharose 6B column. The purified polysomes were pooled and centrifuged at 30,000 x g max. for 5 minutes to fractionate free and membrane-bound polysomes. The pellet was resuspended in polysome buffer plus 0.5% (w/v) DOC, 0.5% (v/v) Triton X-100, which released the bound polysomes from the membrane. Figure 3A is the sucrose gradient profile of the free polysomes; 3B is the profile of the membrane-bound polysomes.

To test the effect of the 30,000 x g centrifugation step, crude polysomes prepared with the use of detergents to release all membrane-bound polysomes were centrifuged at 30,000 x g for 5 minutes. Sucrose gradient profiles of the supernatant fraction and the pellet are shown in Figure 4A and 4B, respectively. The absorbance profile in 4A decreases rapidly in the region of the larger polysomes, while the profile of the pelleted material shows a significant peak in the region of the largest polysomes. This latter peak is probably due to aggregation of some of the polysomes. It should be noted that in Figure 4B the absorbance profile falls below the baseline in the

Figure 3. Sucrose gradient profiles of free and membrane-bound polysomes. A. Free polysomes. B. Membrane-bound polysomes. A_{254} (———).

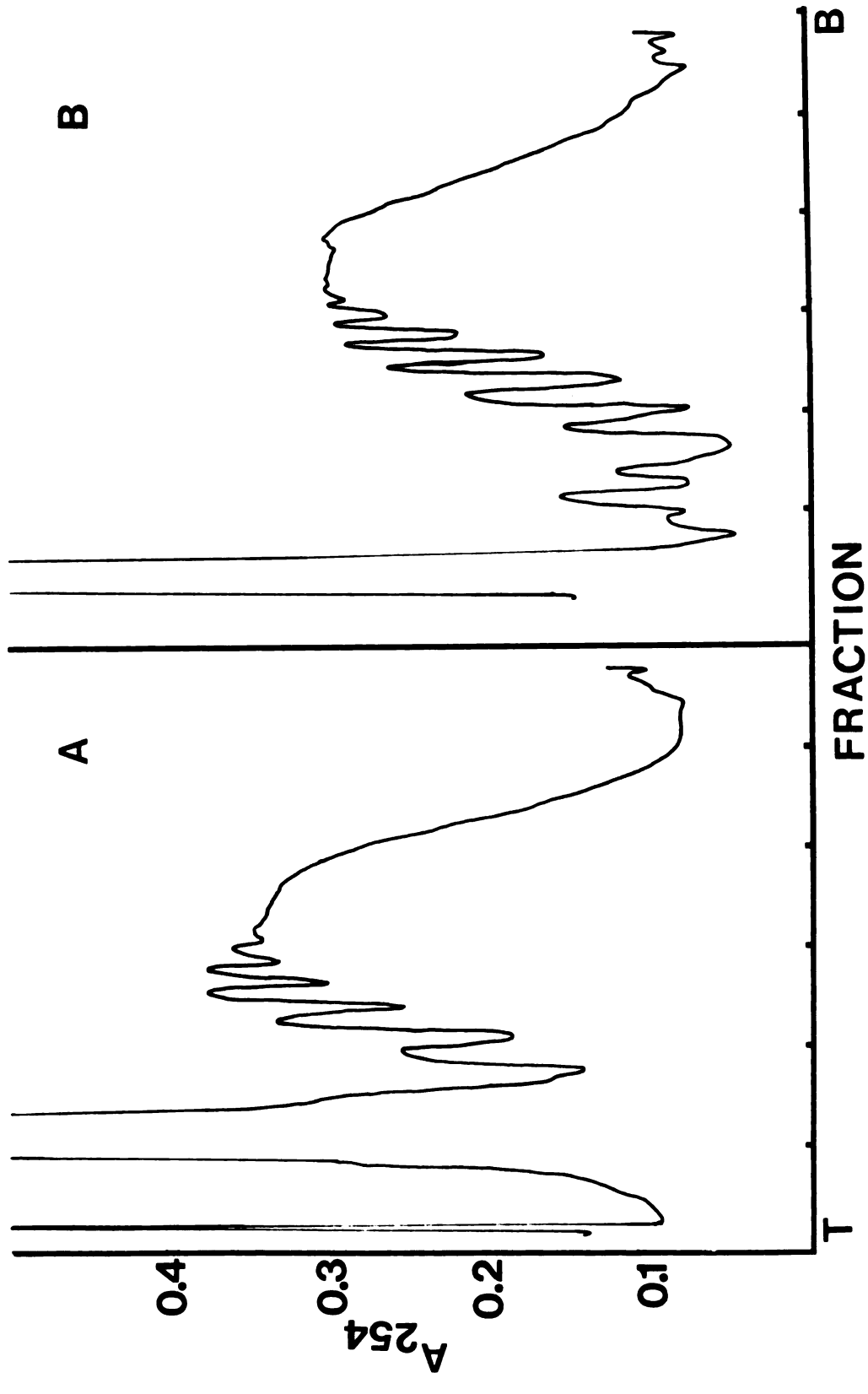


Figure 3

Figure 4. Sucrose gradient profiles of total polysomes following 30,000 x g centrifugation. A. Supernatant. B. Pellet. A_{254} (———).

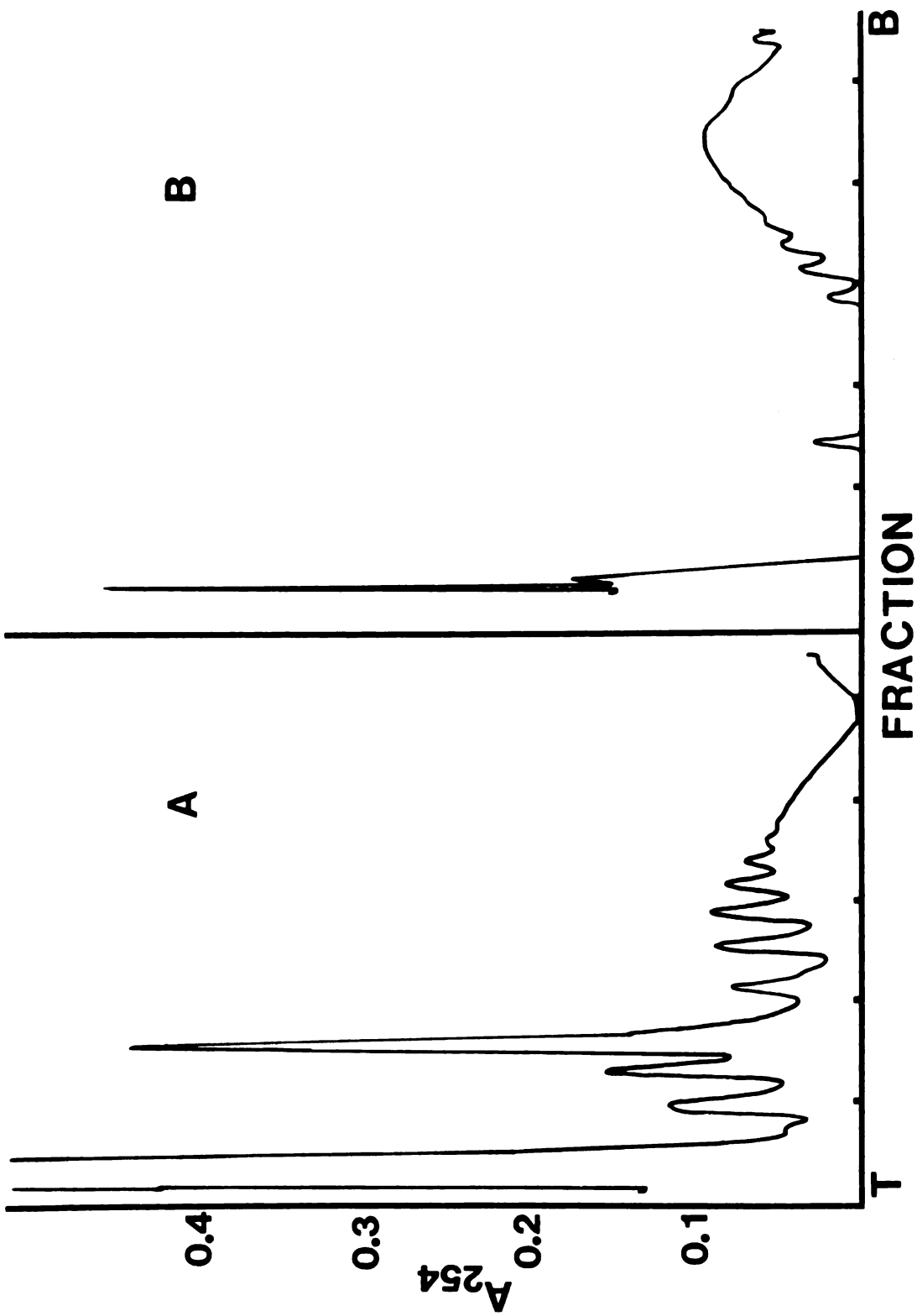


Figure 4

upper half of the gradient, obscuring some of the detail. However, this pellet from presumptive unbound polysomes was a consistent finding following 30,000 x g centrifugation, raising questions as to the validity of the technique for separation of free and membrane-bound polysomes.

Binding of Specific Antibodies to Polysomes

To a sample of MOPC-21 polysomes from ascites cells (labeled for 6 minutes with ^{3}H-amino acids; 4 A_{260} units) was added 5 µg ^{14}C-antibody against the myeloma protein (anti-MOPC). This was incubated for 1 hour at 0°C. To another sample at 0° was added 230 µg unlabeled anti-MOPC, followed 30 minutes later by 5 µg labeled anti-MOPC. This was incubated at 0° for a further 30 minutes. The samples were then layered onto 15-45% linear sucrose gradients over a 2.0 M sucrose cushion and centrifuged in an SW 50.1 rotor as described in Materials and Methods. Figure 5 shows the ^{3}H and ^{14}C profiles from the gradients. The counts have been normalized and are plotted as the percentage of the total counts in each tube. Since the ^{3}H-amino acid profiles of the polysomes from the 2 tubes were nearly identical, only one is shown. The profiles of the ^{14}C counts are substantially different, however. The sample which was pre-incubated with unlabeled antibody has much less label in the polysome region (2.8%) than the sample which was not pre-incubated (17.9%). (The polysome region for this experiment is defined as fractions 10-29.) It is also noted that both samples have peaks at the bottom of the gradient in the 2.0 M sucrose cushion, probably due to cross linking by the antibody. In the sample which

Figure 5. Binding of labeled anti-MOPC to myeloma polysomes. Percent CPM calculated as

$$\frac{\text{CPM/fraction}}{\text{CPM total}} \times 100\%$$

^{3}H-uridine (polysome profile) (●——●). Incubated with ^{14}C-anti-MOPC only (O——O). Pre-incubated with unlabeled anti-MOPC followed by ^{14}C-anti-MOPC (□——□).

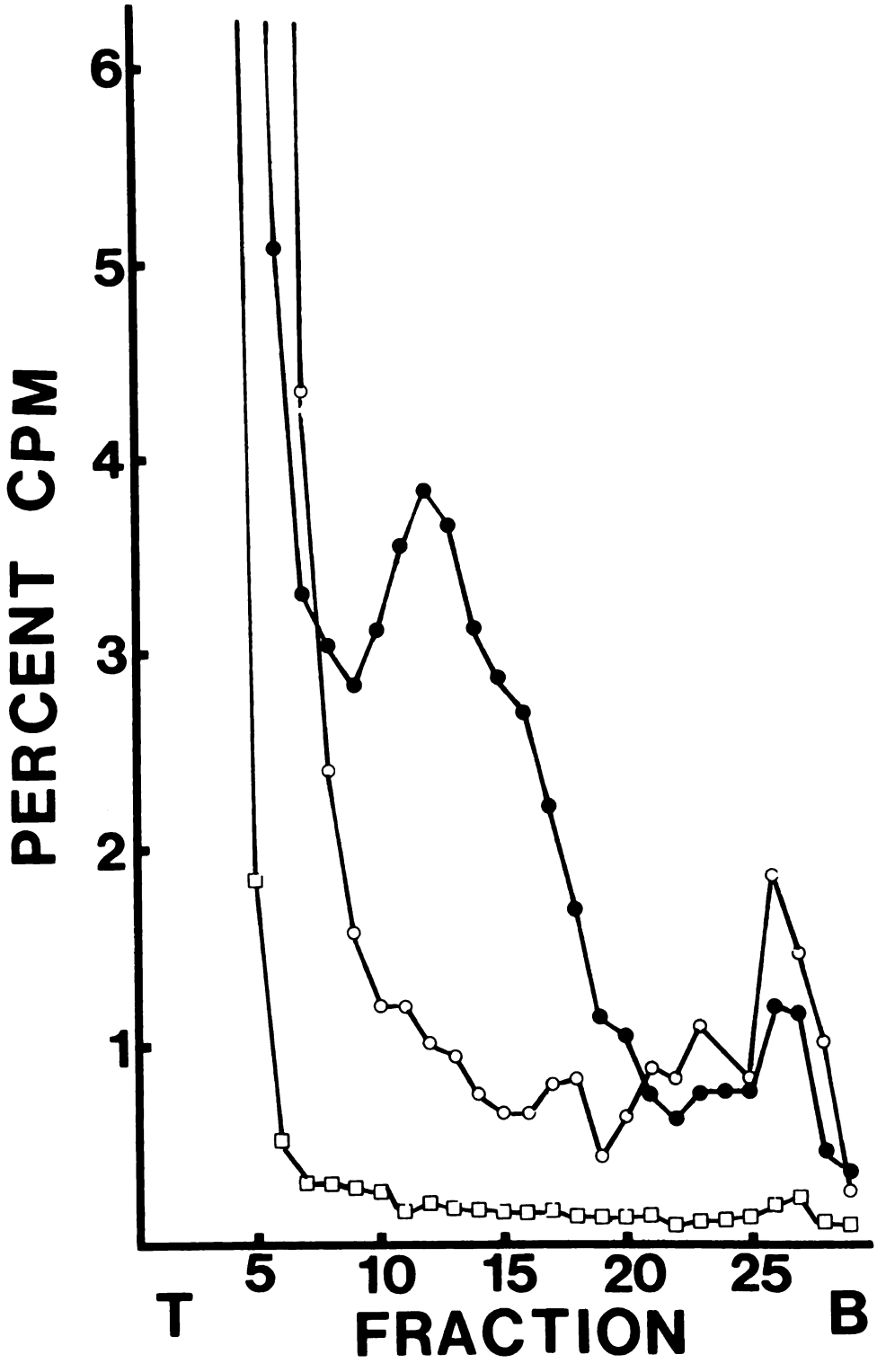


Figure 5

was not pre-incubated with unlabeled anti-MOPC, 6.1% of the labeled antibody is found in the cushion region (fractions 24-29) while only 0.8% of the labeled antibody from the pre-incubated sample is in this region. ^{3}H-amino acid counts in this region were 4.7% and 4.6%, respectively.

MOPC-21 polysomes labeled with ^{3}H-uridine and purified by passage over a Sepharose 2B column were incubated with ^{14}C-anti-MOPC or with unlabeled antibody followed by ^{14}C-anti-MOPC. Sucrose gradient profiles are shown in Figure 6. Figure 6A shows the profiles of the sample pre-incubated with unlabeled antibody and 6B the sample incubated with labeled antibody only. There is a peak of labeled antibody coinciding with the polysome peak in 6B; this is not seen in 6A. The pre-incubated sample had 2.1% of the labeled antibody in the polysome region; the other sample contained 14.3% of the labeled antibody in the polysome region.

Table 2 shows the results of a number of binding experiments, including the two discussed above. The non-polysomal region is defined as the region containing monosomes and smaller material. Although the amount of labeled antibody bound in the polysomal region varies among the experiments, within individual experiments binding is always greater to MOPC-21 polysomes than to 549.1 or XC.1 polysomes and binding to MOPC-21 polysomes is inhibited by pre-incubation with unlabeled antibody. In experiment 4, it can be seen that pre-incubation with normal rabbit gamma globulin (NRGG) does not inhibit binding of labeled antibody. These results, along with the low binding to polysomes from cells not synthesizing the

Figure 6. Binding of labeled anti-MOPC to Sepharose 2B purified polysomes. A. Pre-incubated with unlabeled anti-MOPC followed by ^{14}C-anti-MOPC. B. Incubated with ^{14}C-anti-MOPC alone. ^{3}H-uridine (polysomes) (O——O). ^{14}C-anti-MOPC (●——●).

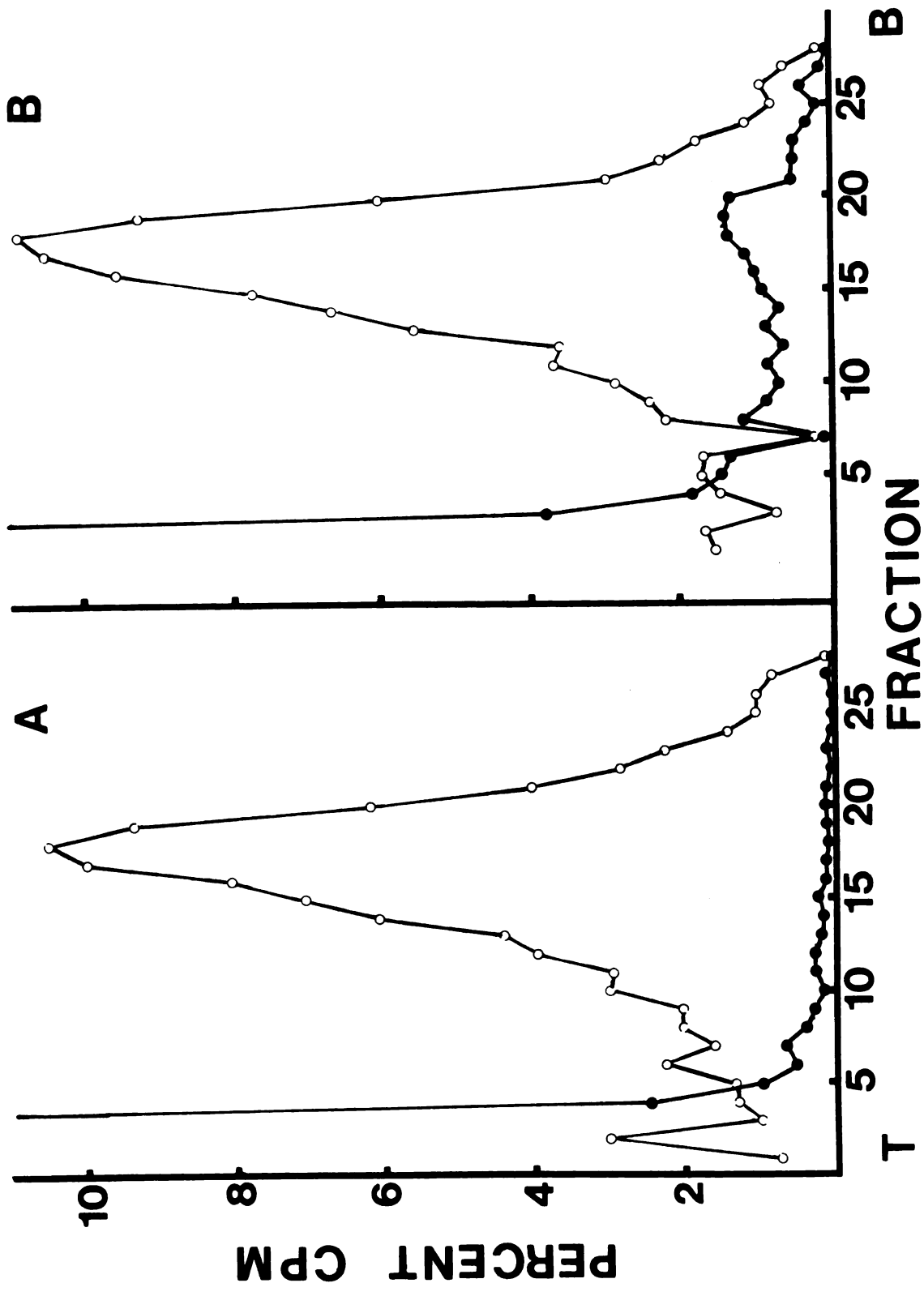


Figure 6

Table 2. Binding of ^{14}C-anti-MOPC-21 to polyribosomes

Experiment	Polysomes[a]	Unlabeled competing protein	µg ^{14}C-Ab per A_{260} polysomes	Percent CPM in non-polysomal region[b]	Percent CPM in polysomal region[b]
1	MOPC-21(6B)	none	1.25	82.1	17.9
	MOPC-21(6B)	anti-MOPC	1.25	97.2	2.8
2	MOPC-21(6B)	none	1.16	73.5	26.5
	MOPC-21(6B)	anti-MOPC	1.16	92.1	7.9
3	MOPC-21(2B)	none	1.34	85.7	14.3
	MOPC-21(2B)	anti-MOPC	1.34	97.9	2.1
4	MOPC-21(6B)	none	2.03	88.0	12.0
	MOPC-21(6B)	anti-MOPC	2.03	97.4	2.6
	MOPC-21(6B)	NRGG[c]	2.03	88.2	11.8
5	MOPC-21(6B)	none	0.43	93.5	6.5
	MOPC-21(6B)	none	0.35	94.1	5.9
	S49.1(6B)	none	0.68	97.7	2.3
	XC.1(6B)	none	0.68	97.8	2.2
6	MOPC-21(6B)	none	0.61	83.0	17.0
	MOPC-21(6B)	none	1.22	83.2	16.8
	MOPC-21(6B)	none	2.44	77.7	22.4
	XC.1(6B)	none	1.14	93.1	6.9

[a] Value in parentheses denotes the Sepharose size used for polysome purification.

[b] Polysome region is material larger than monosomes (80 S) as analyzed on linear sucrose gradients.

[c] NRGG is normal rabbit gamma globulin.

myeloma protein, indicate that the binding is a specific antigen-antibody interaction.

Direct Immune Precipitation of Polysomes

Free and membrane-bound fractions of crude MOPC-21 polysomes (labeled with ^{3}H-uridine) were centrifuged on 15-45% linear sucrose gradients in an SW 41 rotor as described in Materials and Methods. Fractions of 0.4 ml were collected. One-tenth milliliter samples of each fraction were precipitated with TCA and collected on Whatman GF/C filters to determine the total radioactivity of each fraction. To the remaining fractions was added approximately 50 µg of pepsin-treated anti-MOPC. Following incubation for 5 minutes at 37°C, tubes were placed on ice and 1.0 µg unlabeled myeloma protein was added per tube. After incubation overnight at 4°C, fractions were pelleted, washed once with TKM buffer and the pellets were solubilized with one drop of 1.0 N NaOH. Protein was TCA precipitated and collected on GF/C filters. The samples were counted in toluene-Omnifluor. The results are shown in Figure 7. Precipitation is heaviest in the region of the larger polysomes in the membrane-bound fraction (7B) while precipitation in the free fraction (7A) is more generalized. Of the radioactivity in the polysome region, 7.8% was precipitated in the free fraction and 6.6% in the membrane-bound fraction. Since myeloma protein is synthesized on membrane-bound polysomes, one would not expect to find specific precipitation with free polysomes. There is no way of determining from this experiment how much of the precipitation is specific. If the precipitation is primarily specific, then the results would indicate that the

Figure 7. Direct immune precipitation of polysomes. The sucrose gradient profiles are plotted as percent CPM per fraction calculated as in Figure 5. ^{3}H-uridine polysomes (●——●). Immune precipitation is plotted as the percent of radioactivity in each fraction precipitable by antiserum (✪——✪).

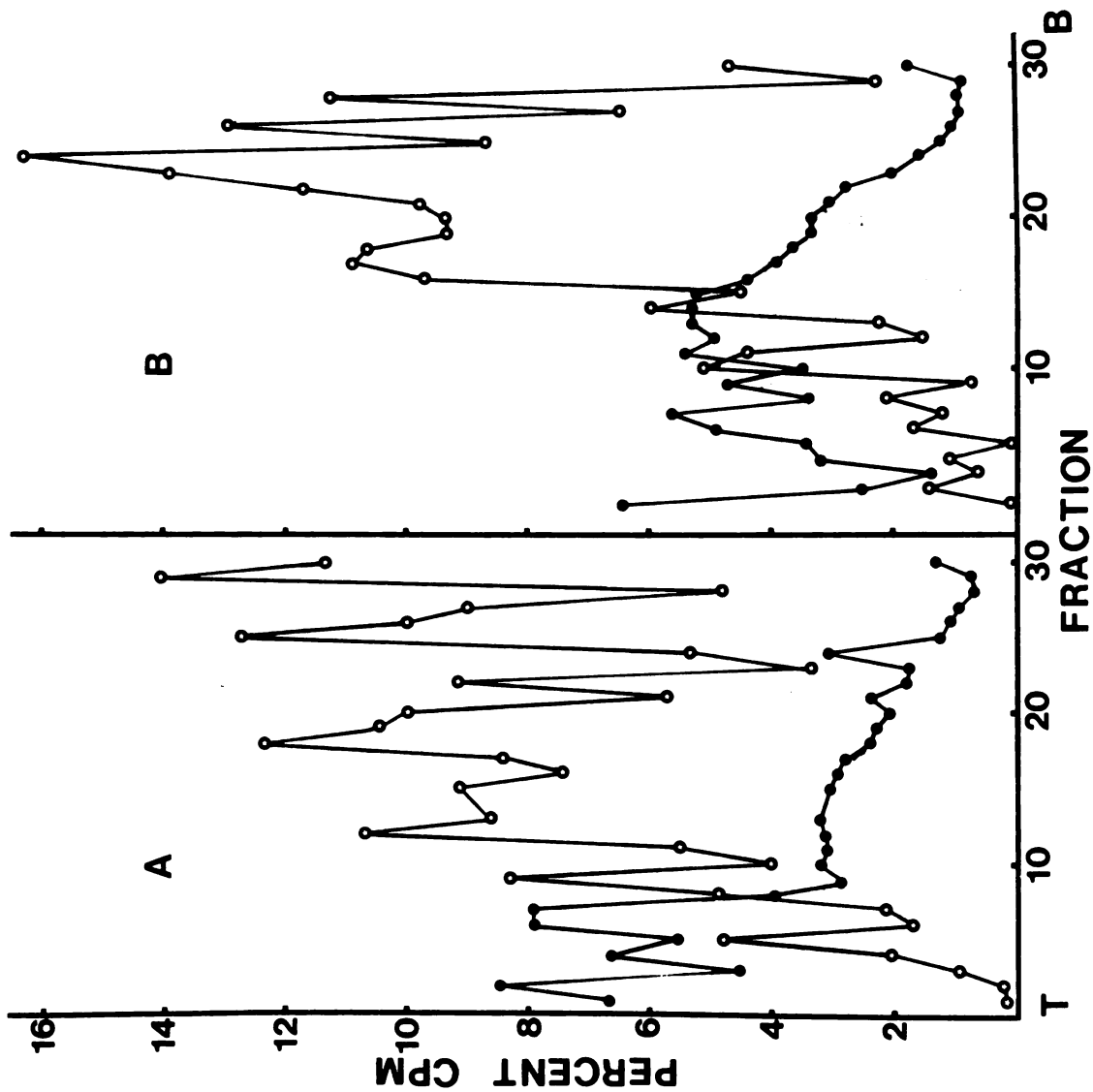


Figure 7

separation of free and bound polysomes was not complete. This would be in agreement with the results discussed earlier, concerning separation of free and membrane-bound polysomes.

Indirect Immune Precipitation of Polysomes

Titration of the goat anti-rabbit globulin (GARGG) indicated that 1 mg of the antibody would precipitate approximately 40 µg of rabbit gamma globulin (data not shown). Therefore, in all indirect precipitations, GARGG was added in a 25-fold excess (w/w) over rabbit antibody. To determine the optimum amount of rabbit anti-MOPC needed for precipitation of polysomes, the following experiment was done. To equal amounts of polysomes (4.85 A_{260} units, purified over Sepharose 6B) were added varying amounts of rabbit anti-MOPC. Following incubation at 0°C for 45 minutes, GARGG was added and the incubation continued for 1 hour. A 25 µl sample was removed from each tube for determination of total radioactivity and then DOC-Triton X-100 was added to a final concentration of 1%. Samples were pelleted at 1000 x g for 15 minutes, washed once with polysome buffer, pelleted again and resuspended in 0.5 ml 0.5% SDS. Samples of 0.1 ml were removed for determination of radioactivity and the remainder was frozen at -20°C. Controls were run using equivalent amounts of normal rabbit gamma globulin in place of anti-MOPC. The results are shown in Table 3 and Figure 8. Nonspecific precipitation is relatively high. Subtraction of the nonspecific from the specific precipitation yields a curve with maximum precipitation at about 12 µg antibody per A_{260} unit of polysomes.

Table 3. Indirect immune precipitation of MOPC-21 polysomes[a]

Anti-MOPC (mg)	NRGG (mg)	GARGG (mg)	Total CPM reaction	CPM precipitated	Percent precipitated
--	--	4.43	84,300	2,900	3.4
.012	--	0.29	96,300	5,600	5.8
--	.012	0.29	100,100	3,900	3.9
.030	--	0.74	96,200	8,000	8.3
--	.030	0.74	94,900	5,900	6.2
.060	--	1.48	96,000	11,900	12.5
--	.060	1.48	100,400	6,400	6.3
.090	--	2.22	97,200	11,600	12.0
--	.090	2.22	93,900	6,600	7.1
.120	--	2.90	92,700	10,900	11.8
--	.120	2.90	87,900	6,500	7.4
.180	--	4.43	84,600	8,700	10.2
--	.180	4.43	86,400	5,400	6.3

[a]Per reaction, 4.85 A_{260} units of MOPC-21 polysomes labeled with ^{3}H-uridine and purified over Sepharose 6B.

Figure 8. Indirect immune precipitation of polysomes: titration of specific antisera. Polysomes were incubated with varying amounts of anti-MOPC (●——●) or NRGG (O——O) followed by GARGG. Specific precipitation (✪——✪) was calculated by subtracting NRGG precipitation from anti-MOPC precipitation.

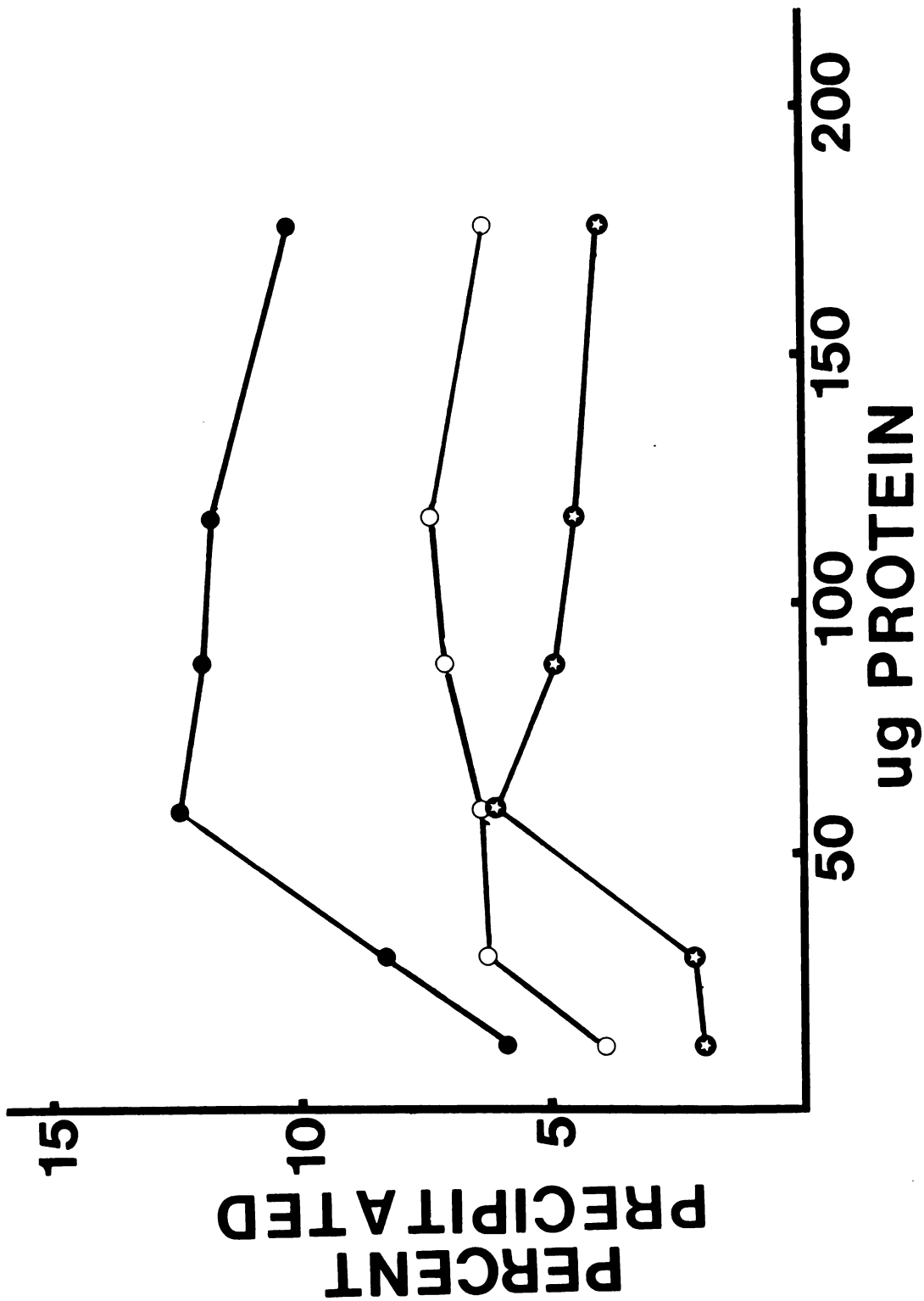


Figure 8

The results of other indirect immune precipitation experiments are shown in Table 4. The amount of precipitation varies among the experiments, but the specific precipitation is consistently higher than the nonspecific. XC.1 polysomes, which do not produce myeloma protein, showed no difference between antibody and normal gamma globulin precipitation.

Characterization of Affinity Columns with Protein

Affinity chromatography material consisted of Sepharose 6B and 4B coupled directly to rabbit anti-MOPC, anti-BSA, myeloma protein, GARGG, and pepsin treated anti-MOPC. Anti-MOPC was also coupled through spacer molecules of diamino hexane (DAH) and ε-amino caproic acid (EACA). The specificity of these preparations was tested with specific and nonspecific proteins, using pasteur pipette columns as described in Materials and Methods. Table 5 shows the results of an experiment in which anti-MOPC Sepharose and anti-BSA Sepharose were pretreated with unlabeled BSA or myeloma protein. Pretreatment of the columns with the specific protein inhibits binding of the labeled protein while pretreatment with the nonspecific protein has little or no inhibitory effect.

Table 6 shows the results of binding of ^{14}C-labeled BSA and myeloma protein to anti-MOPC and anti-MOPC (pepsin treated) attached to Sepharose through spacer arms of either DAH or EACA. The control columns were composed of Sepharose with the spacer arms attached but without antibody. Binding and specificity are excellent. It was necessary to treat the DAH columns with high salt buffer (1.0 M potassium phosphate, pH 7.6) to remove nonspecific binding. This

Table 4. Indirect immune precipitation of ^{3}H-uridine labeled polysomes[a]

Polysomes[b]	Anti-MOPC (μg/A_{260})	NRGG (μg/A_{260})	Total CPM per reaction	CPM Precipitated	Percent precipitated
MOPC-21 (2B)	38	--	87,400	9,000	10.3
"	--	37	91,100	5,000	5.5
"	--	--	88,800	700	0.8
MOPC-21 (4B)	17	--	110,000	7,100	6.4
"	17	--	116,100	5,800[c]	5.0
"	--	21	112,600	2,700	2.4
"	--	21	114,300	2,100[c]	1.8
"	--	--	79,000	600	0.8
"	--	--	73,900	200[c]	0.2
XC.1 (6B)	12	--	51,100	1,100	2.2
"	--	12	51,300	1,100	2.0
"	--	--	48,800	300	0.6

[a]All reactions contained 25 μg GARGG per μg rabbit gammaglobulin. Control reactions without anti-MOPC or NRGG contained the same amount of GARGG as the other reactions in that experiment.

[b]Value in parentheses denotes Sepharose size used for purifcation.

[c]Precipitates were pelleted through discontinuous sucrose gradients (Shapiro *et al.*, 1974).

Table 5. Protein specificity of immunoadsorbents

Immunoadsorbent[a]	Unlabeled competing protein	Added ^{14}C-protein	Percent ^{14}C-protein bound
anti-MOPC Sepharose	--	MOPC-21	93.3
"	1.2 mg MOPC-21	"	24.7
"	1.0 mg BSA	"	88.5
anti-BSA Sepharose	--	BSA	79.4
"	1.2 mg MOPC-21	"	77.5
"	1.0 mg BSA	"	-5.3

[a]Antiserum coupled directly to cyanogen bromide activated Sepharose.

Table 6. Specificity of immunoadsorbents prepared using six carbon spacers

Antiserum coupled to matrix	Spacer	^{14}C-protein added	^{14}Percent C-protein bound
None	EACA	BSA	-10.1
Anti-MOPC	"	"	9.0
Anti-MOPC$_{pepsin}$	"	"	3.4
None	EACA	MOPC-21	-12.0
Anti-MOPC	"	"	70.7
Anti-MOPC$_{pepsin}$	"	"	61.8
None	DAH[a]	BSA	3.2
Anti-MOPC	"	"	-5.0
Anti-MOPC$_{pepsin}$	"	"	15.1
None	DAH[a]	MOPC-21	10.8
Anti-MOPC	"	"	81.9
Anti-MOPC$_{pepsin}$	"	"	74.0

[a]DAH immunoadsorbents were washed with 1.0 M potassium phosphate (pH 7.6) to remove nonspecific binding.

was probably due to a large number of unreacted amino groups which would be positively charged at neutral or slightly alkaline pH.

Since the antibody was prepared against myeloma protein isolated from the serum of tumor bearing mice and, in initial studies at least, polysomes were obtained from cells of a tissue culture line of the tumor, it was necessary to demonstrate that the antibody would react with the myeloma protein produced by the tissue culture cells. A typical set of results obtained with extracellular protein isolated as described in Materials and Methods from tissue culture cells is shown in Table 7. Antibodies were coupled directly to Sepharose 6B.

Table 7. Affinity chromatography of extracellular protein from MOPC-21 cells

Sepharose	Total CPM	CPM eluted in wash	% Bound
anti-BSA	5200	5500	-6.6
anti-MOPC	5200	1400	72.8
anti-MOPC (pepsin)	5200	1700	66.6

Also, since the antibody against the myeloma protein was raised by immunization with the complete gamma globulin molecule, an attempt was made to demonstrate specificity to individual heavy and light chains. Therefore, ^{3}H-meyloma protein was reduced and alkylated, and H and L chains were separated on a 5-20% sucrose gradient (0.5% SDS) as described in Materials and Methods. Figure 9 shows the sucrose

Figure 9. Sucrose gradient profiles of heavy and light chains. A_{280} (●——●).

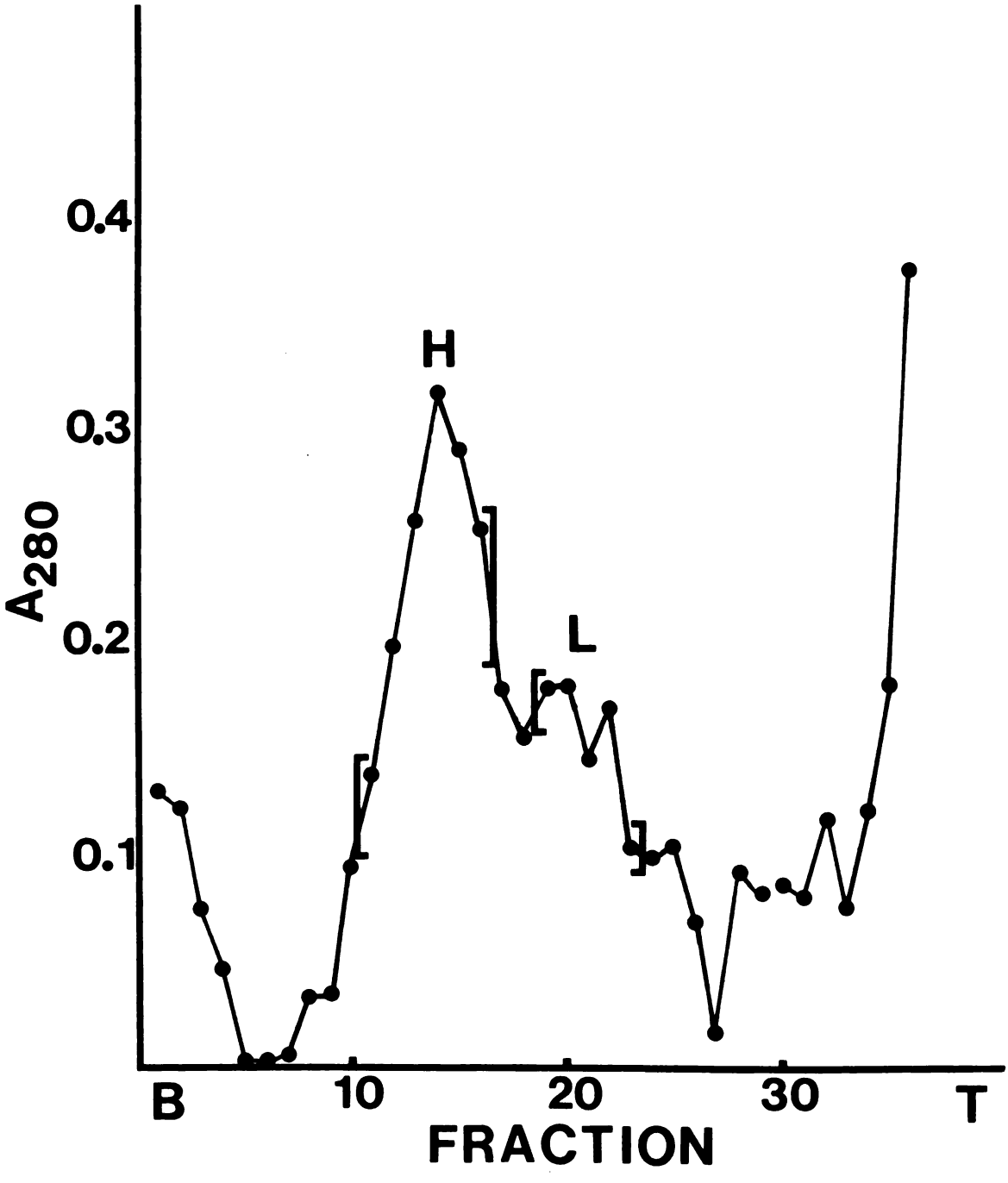


Figure 9

gradient profile after centrifugation. Fractions 11-16 (putative H chains) and 19-23 (putative L chains) were pooled and dialyzed against 10 mM Tris (pH 8.1). SDS polyacrylamide gel electrophoresis of the samples showed one major band containing about 90% of the material in each sample. The bands were approximately the molecular weight of H and L chains when compared with marker proteins. The samples were clarified by centrifugation and then analyzed for binding to Sepharose anti-BSA and Sepharose anti-MOPC columns. The results are shown in Table 8.

Table 8. Affinity chromatography of isolated heavy and light chains

Sepharose	Protein	Total CPM	CPM Eluted	% Bound
anti-MOPC	H-chain	1090	650	39.9
anti-BSA	H-chain	1090	1150	-5.5
anti-MOPC	L-chain	1140	920	19.1
anti-BSA	L-chain	1140	1080	5.2

Attempts were made to demonstrate binding of isolated nascent chains from myeloma polysomes to specific antibody columns. The nascent chains were prepared by treating a solution of MOPC-21 polysomes (labeled for 6 minutes with ^{3}H-amino acids) with Na EDTA (pH 7.0) at a final concentration of 33 mM. After 10 minutes at 0°C, the sample was layered onto a 10-30% (w/v) linear sucrose gradient (10 mM Tris, pH 7.4) and centrifuged at 50,000 rpm for 2 hours at 4°C in

an SW 50.1 rotor. Absorbance of ribosomal subunits was monitored at 254 nm and 0.2 ml fractions collected and assayed for radioactivity. Figure 10 shows a typical profile. From this experiment, fractions 2 and 3 (labeled nascent chains) were pooled and dialyzed to remove sucrose. Table 9 shows the results of 2 typical binding experiments with these nascent chains. Nonspecific binding is high, for the myeloma protein as well as for the nascent chain. Although this high background of nonspecific binding was seen consistently in all of the nascent chain binding experiments, the binding to specific columns was always greater, usually on the order of 10-20% higher than the binding to nonspecific adsorbents.

Table 9. Binding of nascent chains to immunoadsorbents

Protein coupled to Sepharose	Protein added	Percent protein bound
anti-MOPC	MOPC-21	82.5
anti-BSA	MOPC-21	20.0
anti-MOPC	nascent chains[a]	25.2
anti-BSA	nascent chains[a]	17.1
anti-MOPC	nascent chains[a]	45.7
NRGG	nascent chains[a]	25.6

[a]Nascent chains isolated from ^{3}H-amino acid mix pulse-labeled MOPC-21 polysomes as described in text.

Figure 10. Isolation of nascent chains from MOPC-21 polysomes. MOPC-21 polysomes labeled with ^{3}H-amino acids were treated with Na EDTA and separated on 10-30% linear sucrose gradients. A_{254} (——); ^{3}H-amino acids (●——●).

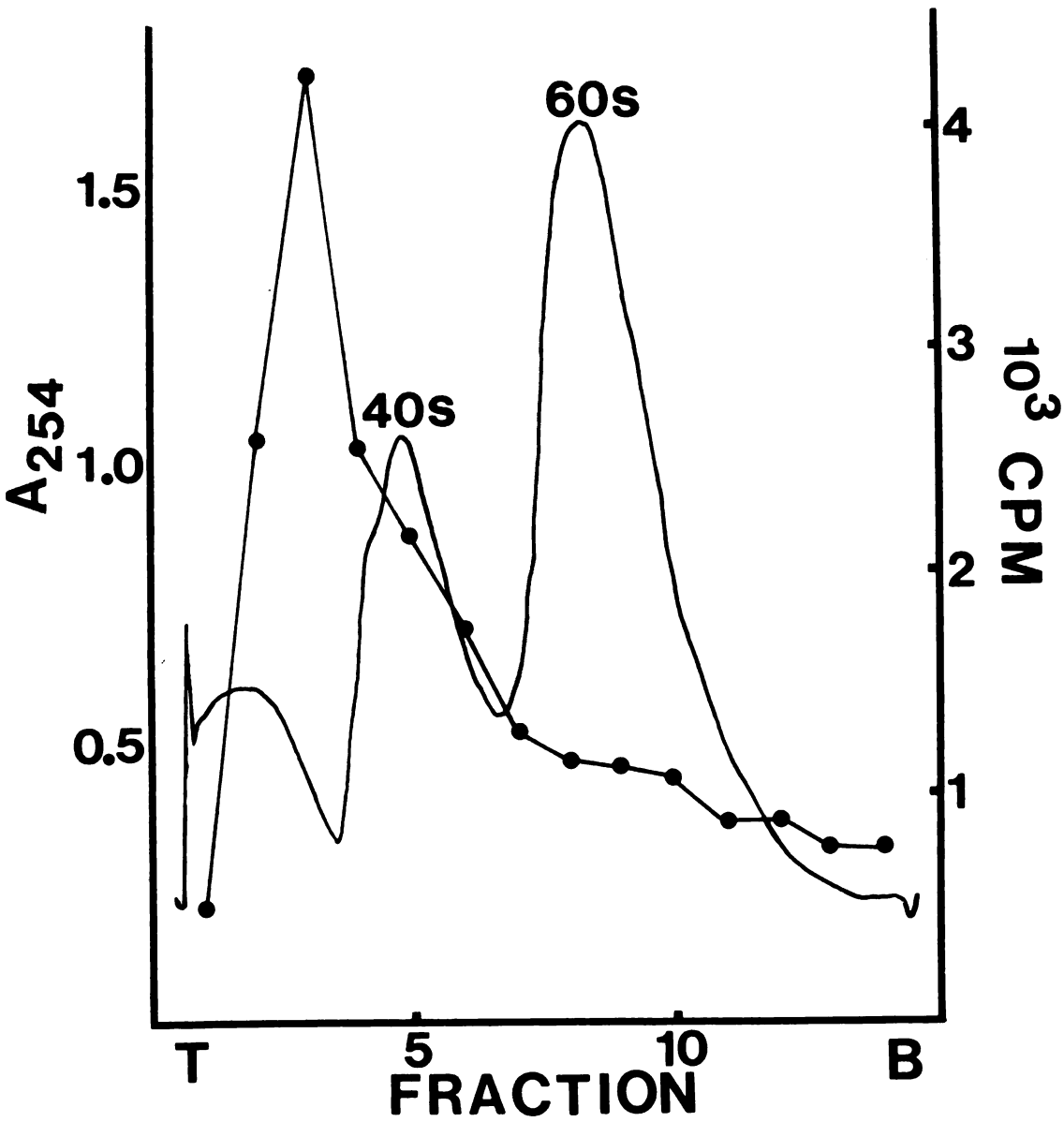


Figure 10

Direct Affinity Chromatography of Polysomes

Binding of polysomes to antibody attached to a Sepharose backbone was attempted both in solution and in pasteur pipette columns. The ^{3}H-uridine labeled polysomes were incubated for 1 hour at 0-4°C with Sepharose-anti-MOPC or Sepharose-NRGG, either in the columns or in solution with stirring. After incubation, the batch samples were transferred to columns. Columns were then washed with polysome buffer and detergent solution (polysome buffer plus 0.15 M NaCl, 0.5 M sucrose, 1% DOC, 1% Triton X-100). The detergent solution was removed by washing again with polysome buffer. Bound polysomes were eluted by washing with either 10 mM Tris, pH 7.4, 50 mM EDTA, or with 0.5% SDS, 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA. Table 10 shows the results of 2 such experiments. Later experiments showed that treating the columns with the SDS solution released more radioactivity, indicating that the EDTA treatment was not sufficient to release all of the bound polysomes. The results in Table 10 show no difference in binding between MOPC-21 and XC.1 polysomes or between binding to specific and nonspecific matrices.

Indirect Affinity Chromatography of Polysomes

Two types of indirect binding were attempted. In both procedures, polysomes were incubated for 45 minutes at 0°C in solution with either anti-MOPC or NRGG at a ratio of 35-50 µg protein per A_{260} unit of polysomes. These samples were then mixed with Sepharose-MOPC, Sepharose-NRGG or Sepharose-GARGG, and incubated 1 hour at 0°C. Washing and elution procedures were identical to direct technique.

Table 10. Direct binding of polysomes to immunoadsorbents

Protein coupled to Sepharose	Polysomes	Polysomal CPM added	Polysomal CPM bound	Percent polysomes bound
Anti-MOPC[a]	MOPC-21	54,700	5,400[b]	9.8
NRGG[a]	MOPC-21	45,700	3,300[b]	7.3
Anti-MOPC[a]	XC.1	18,400	2,000[b]	10.9
NRGG[a]	XC.1	12,300	1,300[b]	10.9
Anti-MOPC[c]	MOPC-21	105,800	30,200[d]	28.5
NRGG[c]	MOPC-21	105,800	33,300[d]	31.4
Anti-MOPC[c]	MOPC-21	87,000	14,600[d]	16.8
NRGG[c]	MOPC-21	87,000	15,000[d]	17.3

[a]Polysomes incubated with immunoadsorbent in solution.

[b]Bound polysomes eluted with 10 mM Tris HCl (pH 7.4), 50 mM EDTA.

[c]Polysomes incubated with immunoadsorbent in pasteur pipettes.

[d]Bound polysomes eluted with 0.5% SDS.

Table 11 shows the results of several experiments. The nonspecific background binding in all is very high and there was no consistent pattern of greater specific binding than nonspecific binding.

To determine if passage over the affinity columns affected the structural integrity of the polysomes, a 0.25 ml sample of MOPC-21 polysomes (6 A_{260} units; purified over Sepharose 6B) was applied to a column of Sepharose-NRGG (0.25 ml packed volume) and incubated for 30 minutes at 0°C. The polysomes were then eluted with 1.0 ml polysome buffer and divided into 2 equal fractions. One fraction was incubated with 2.5 µg ^{14}C-anti-MOPC for 1 hour at 0°C. Two samples of the same polysomes which had not been passed over an affinity column were used as controls. One was incubated with 2.5 µg labeled antibody. All 4 samples were analyzed on sucrose gradients. Sucrose gradient profiles of the samples incubated with labeled antibody are shown in Figure 11. The polysome profiles of the samples not incubated with labeled antibody were identical to those which were. Figure 11A is the control polysome profile while 11B is the profile of the polysomes passed over the Sepharose-NRGG. No degradation in the polysome region is evident. There is a significant decrease in monosomes and subunits in the sample passed over the affinity column. The percentages of radioactivity in the polysomal and non-polysomal regions are given in Table 12. Since the column material is Sepharose 4B, monosomes and subunits will be retarded somewhat, eluting slightly later than the void volume. However, since the total volume of the sample and wash was five-fold greater than the bed volume of the column, there should have been no

Table 11. Indirect binding of polysomes to immunoadsorbents

Experiment	Protein coupled to Sepharose	Polysomes[a]	Protein[b]	Polysomal CPM bound[c]	Percent polysomes bound
1	MOPC-21	MOPC-21	anti-MOPC	2,200	4.6
	NRGG	"	"	4,500	8.4
	MOPC-21	XC.1	"	1,800	9.1
	NRGG	"	"	1,400	7.5
2	GARGG	MOPC-21	anti-MOPC	10,400	12.0
	GARGG	"	NRGG	13,800	15.8
3	GARGG	MOPC-21	anti-MOPC	17,400	12.6
	NRGG	"	"	11,100	8.0
4	GARGG	MOPC-21	anti-MOPC	2,700	37.1
	NRGG	"	"	4,500	62.1
5	GARGG	MOPC-21	anti-MOPC	28,400	26.8
	GARGG	"	NRGG	20,000	18.9

[a]Experiment 1, polysomes incubated with protein and immunoadsorbent in solution; in all others the polysomes were incubated with protein in solution and then poured over immunoadsorbent in pasteur pipettes.

[b]Protein with which polysomes were preincubated.

[c]Experiment 1, bound polysomes eluted with EDTA buffer; in all others the bound polysomes were eluted with SDS buffer.

Figure 11. Effect on polysomes of passage over affinity columns. A. Control polysomes incubated with ^{14}C-anti-MOPC. B. Polysomes passed over Sepharose-NRGG column; incubated with ^{14}C-anti-MOPC. ^{3}H-uridine (polysomes) (O——O); ^{14}C-anti-MOPC (●——●).

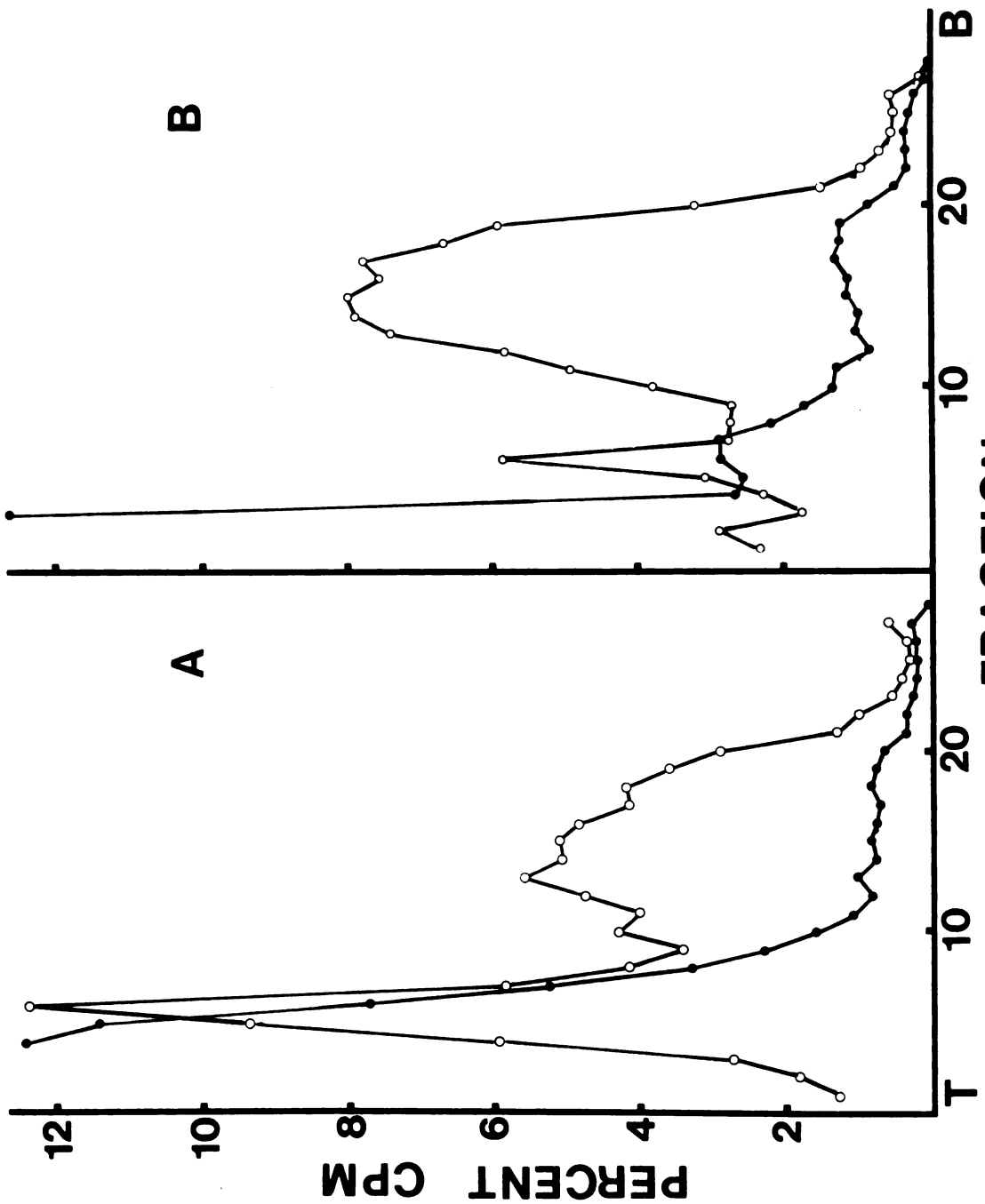


Figure 11

Table 12. Effect on polysomes of passage over affinity columns

Polysomes	Label	CPM in non-polysome region	Percent CPM in non-polysome region	CPM in polysome region	Percent CPM in polysome region
control	^{3}H-amino acids	15,100	47.0	17,000	53.0
control	^{14}C-antibody	4,600	88.4	600	11.6
eluted from column	^{3}H-amino acids	5,600	26.5	15,400	73.5
eluted from column	^{14}C-antibody	3,800	85.4	600	14.6

separation on the basis of size. These results would indicate, then, that monosomes and subunits may bind nonspecifically to the immunoadsorbent. Binding of antibody to the polysomes does not appear to have been affected by passage over the columns, although it is interesting to note that there appears to be antibody binding to the monosomes and subunits. In Figure 11B, with the reduced monosome-subunits peak, the antibody levels decrease four fractions earlier than in 11A. There is a small peak of antibody coinciding with the monosome-subunits peak.

A sample of the same polysomes used in the previous experiment was treated with EDTA, final concentration 33 mM, for 10 minutes at 0°C. One-tenth milliliter of this solution was applied to a Sepharose-NRGG column (0.25 ml packed volume) and incubated for 30 minutes at 0°C. The column was then washed with 2 ml polysome buffer and the eluted radioactivity was determined by TCA precipitation. Of 101,700 cpm applied, 22,800 (22.5%) were eluted. Thus, 77.5% of the radioactivity was bound to the column. A second sample of the EDTA treated polysomes was incubated with 2.5 μg ^{14}C-anti-MOPC for 1 hour at 0°C, then analyzed on a 10-30% (w/v) linear sucrose gradient (10 mM Tris, pH 7.4, 10 mM NaCl, 10 mM EDTA) by centrifugation at 50,000 rpm for 105 minutes at 4°C in an SW 50.1 rotor. The profile is shown in Figure 12. The EDTA treatment has reduced the polysomes to subunits. Thus, the sample which was passed over the column had been completely converted to 40 S and 60 S subunits. A significant amount of the labeled antibody has sedimented with the subunits. The two peaks of antibody fall just to the heavy side of

Figure 12. Binding of ^{14}C-anti-MOPC to ribosomal subunits. EDTA treated MOPC-21 polysomes labeled with ^{3}H-uridine (O——O) were incubated with ^{14}C-anti-MOPC (●——●) and analyzed on 10-30% linear sucrose gradients.

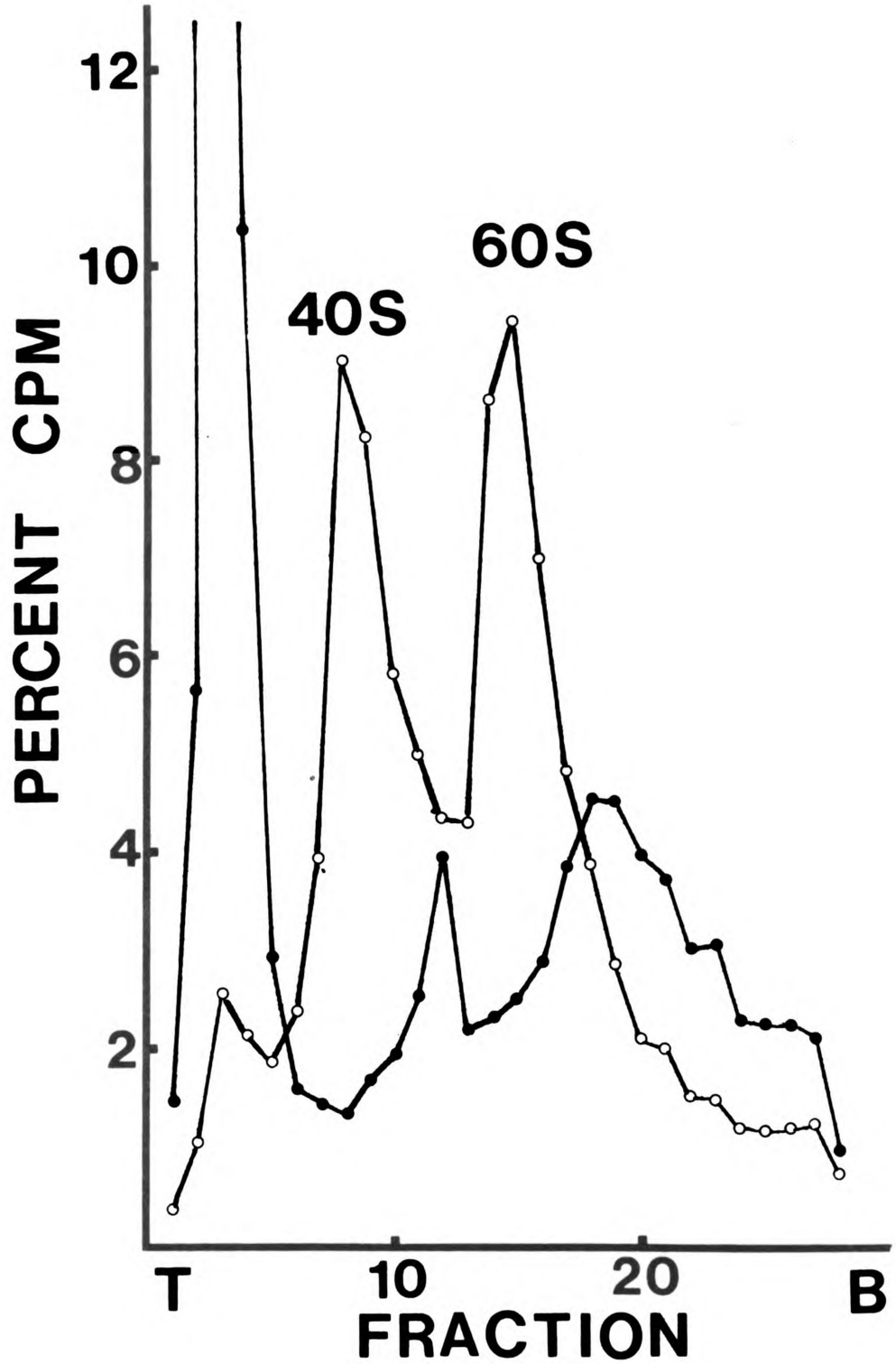
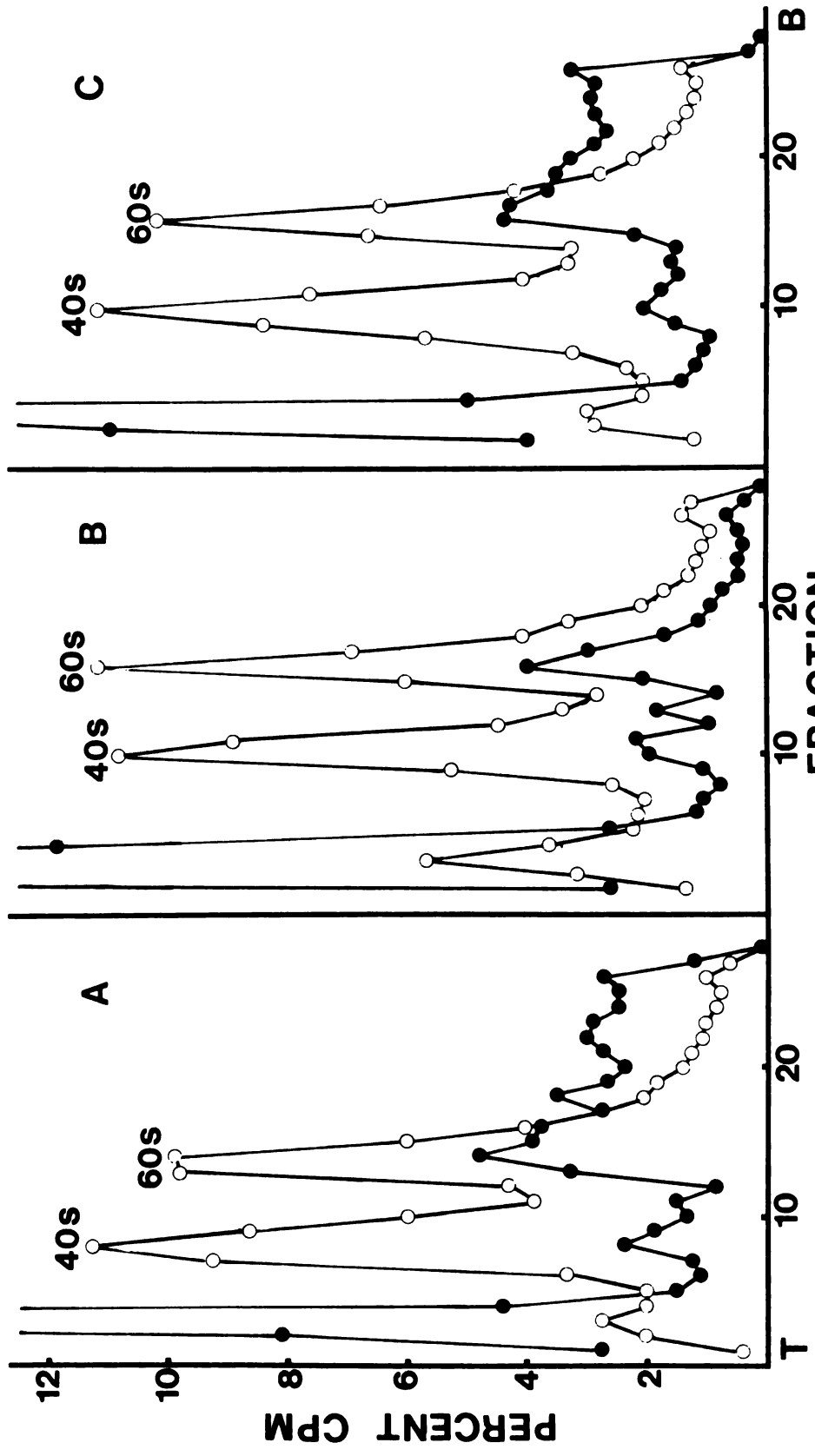


Figure 12

the subunit peaks. The shoulders on the heavy sides of the subunit peaks may be due to cross-linking by the antibody. With the subunits region of the gradient defined as fractions 6-28, 60.9% of the labeled antibody is in the subunits region.

The above experiment was repeated, incubating the EDTA-treated polysomes with 125 µg unlabeled anti-MOPC or unlabeled NRGG for 30 minutes. The samples were then incubated with 2.5 µg ^{14}C-anti-MOPC for 30 minutes. A third sample was incubated for 1 hour with labeled anti-MOPC. All samples were analyzed on sucrose gradients as above. Figure 13A shows the profile of the sample which was not pre-incubated with competing protein. The antibody profile is similar to that seen in Figure 12. There are peaks of antibody coinciding with the subunits peaks, as well as a large shoulder of antibody in the region larger than 60 S. The subunits region contains 54.7% of the antibody. Figure 13C is the profile of the sample pre-incubated with NRGG. The results are nearly identical to 13A. The subunits region contains 50.0% of the antibody. The profile of the sample pre-incubated with unlabeled anti-MOPC is shown in Figure 13B. Although there are antibody peaks coinciding with the subunits peaks, there is little antibody in the region larger than the 60 S subunits. Overall, only 25.4% of the antibody is in the subunits region. The addition of the unlabeled antibody to the subunits caused visible precipitation. Only 50% of the ^{3}H-uridine radioactivity of the subunits was recovered on the gradients. Presumably, the other 50% was pelleted as a precipitate. Recovery of the ^{14}C-antibody on the gradient was 100%.

Figure 13. Binding of ^{14}C-anti-MOPC to ribosomal subunits: specificity. A. EDTA treated polysomes incubated with ^{14}C-anti-MOPC. B. EDTA treated polysomes incubated with unlabeled anti-MOPC prior to incubation with ^{14}C-anti-MOPC. C. EDTA treated polysomes incubated with unlabeled NRGG prior to incubation with ^{14}C-anti-MOPC. ^{3}H-uridine (O——O); ^{14}C-anti-MOPC (●——●).


PERCENT CPM
12
10
8
6
4
2
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10
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FRACTION
A
40s
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B
40s
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C
40s
60s

Figure 13

DISCUSSION

Isolation of a specific messenger RNA involved in the synthesis of a particular protein is an important area of study. In many of the reports in the literature, purification of the message is attempted by isolation of a particular size of poly(A) containing RNA (Harrison *et al.*, 1974a, 1974b; Stavnezer *et al.*, 1974; Cowan and Milstein, 1973; Swan, Aviv and Leder, 1972; Mach *et al.*, 1973; Brownlee *et al.*, 1973; Milstein *et al.*, 1972). Other workers have reported the isolation of specific polysomes by immune precipitation (Delovitch *et al.*, 1972, 1973a, 1973b; Sarkar and Moscona, 1973; Palmiter *et al.*, 1972; Schechter, 1973, 1974a, 1974b; Shapiro *et al.*, 1974). The poly(A) containing RNA can then be isolated from the total precipitated RNA. Affinity chromatography with antigen-antibody systems utilizes the same mechanisms as immune precipitation with the advantages that a shorter reaction time is required and the antigen-antibody ratio is not as critical, as precipitation is not desired.

We have attempted to isolate those polysomes from myeloma cells specifically engaged in the production of the H and L chains of the myeloma protein. Approximately 20% of the intracellular protein synthesized by the MOPC-21 cell is myeloma protein. In order to reduce this intracellular protein so that immunologic reagents will

react only with nascent peptide chains on polysomes, a rapid technique to separate polysomes from soluble intracellular proteins was devised.

Sepharose gel filtration of crude polysome preparations substantially reduces the amount of contaminating intracellular material smaller than the 40 S subunits. Passage over Sepharose 6B gives a normal polysome profile, while passage over 4B and 2B removes monosomes and subunits, enriching for larger polysomes. The polysomes are structurally intact and preliminary results indicate they are active in an endogenous cell-free protein synthesizing system. Binding of labeled antibody indicates that the nascent peptide chains are intact.

Passage of the post-nuclear supernatant fraction over Sepharose columns yields free polysomes along with the microsomal fraction. Removal of the microsomes by centrifugation at 30,000 x g for 5 minutes consistently left a free polysome population in the supernatant which contained a high percentage of monosomes and subunits, with decreased numbers of larger polysomes. The polysomes from the microsomal pellet showed complimentary profiles--decreased monosomes and subunits with increased amounts of larger polysomes. Possibly there is some aggregation among the free polysomes. These aggregates would then pellet along with the microsomes. Centrifugation of a crude polysome preparation isolated with the use of detergents gives a significant pellet (Figure 4). This was a total polysome population in which there should have been no polysomes attached to membranes. Similar results were obtained using total polysome preparations purified over Sepharose columns. The specific binding of labeled antibody

to polysomes in the free fraction (Table 2) would suggest that there is also contamination of membrane-bound polysomes in the free fraction. There is evidence for the existence of a "tight" fraction and a "loose" fraction of membrane-bound polysomes (Rosbash and Penman, 1971a, 1971b; Harrison *et al.*, 1974a), although there is still disagreement as to the different modes of attachment of the two types. It is possible that our free fraction may be contaminated with "loose" membrane-bound polysomes, which are reported to be predominantly monosomal (Harrison *et al.*, 1974a).

Thus, it appears that our procedure for the isolation of membrane-bound and free polysomes does not give a clean separation. Since separated membrane-bound and free polysomes were not absolutely necessary in this study, the majority of the work was performed using total polysome preparations.

Rabbit anti-MOPC purified by affinity chromatography on Sepharose-MOPC and labeled with ^{14}C was shown to bind specifically to polysomes isolated from MOPC-21 cells. Generally, from 10-25% of the labeled antibody bound to the polysomes, as indicated by radioactivity in the polysomal region of analytical sucrose gradients. When incubated with XC.1 or S49.1 polysomes, which do not produce myeloma protein, significantly less binding was seen. Binding could also be reduced substantially by pre-incubating the polysomes with unlabeled anti-MOPC before addition of the labeled antibody. The unlabeled antibody competes for the binding sites, thus precluding any binding of the labeled antibody. Pre-incubation of the polysomes with normal rabbit gamma globulin does not inhibit binding of labeled

antibody, indicating that the competition is for specific binding sites rather than generalized nonspecific binding.

Binding of antibody to monosomes and subunits was also seen. This binding appears to be specific, as it can be reduced by pre-incubation with unlabeled antibody (Figures 5 and 13). It is likely that some of the monosomes will have a portion of messenger RNA attached (Baglioni *et al.*, 1971), along with a nascent peptide. If the nascent peptide is large enough to be antigenic, the labeled antibodies would be expected to bind. The antibody was also seen to bind to individual ribosomal subunits (Figures 12 and 13). It has been reported that when ribosomal subunits are isolated under low salt conditions, they tend to bind proteins nonspecifically (Moav and Harris, 1970a, 1970b). Our entire polysome isolation procedure is done under low salt conditions. It may be that the subunits are binding significant amounts of intracellular protein, the most abundant of which is the myeloma protein. Thus, we may be seeing specific binding of antibodies to myeloma protein which is bound nonspecifically to the ribosomal subunits.

Direct immune precipitation of polysomes from sucrose gradients gave equivocal results. There was a slightly higher precipitation percentage in the free polysomes than in the membrane-bound polysomes. The precipitation was primarily in the region of the larger polysomes in the membrane-bound fraction, while it was more generalized throughout the polysome region in the free fraction. The amount of precipitation which is nonspecific was not determined in this case. If the precipitation were specific (or predominantly specific), then there

again appears to be contamination of membrane-bound polysomes in the free fraction.

Indirect immune precipitation of MOPC-21 polysomes resulted in relatively high backgrounds of nonspecific precipitation. Specific precipitation was consistently higher than nonspecific, however. It has been reported that thorough washing of the pellet with detergent and sucrose is necessary to remove the nonspecific binding (Shapiro *et al.*, 1974). Nonspecific contamination of as little as 0.4% has been reported (R. T. Schimke, personal communication). We were unable to reduce significantly the nonspecific precipitation with these techniques, however.

Sucrose gradient analysis of RNA from specific and nonspecific immune precipitates of polysomes failed to reveal any differences in the RNA populations (data not shown). If the specific precipitate were enriched for myeloma producing polysomes, the RNA fraction should be enriched for myeloma messenger RNA. With the high background of nonspecific precipitation, this technique is probably not sensitive enough to detect small differences in the region of the myeloma messengers.

We believe that the differences in percentage precipitation between specific and nonspecific samples are real and that they represent precipitation of myeloma producing polysomes. The nonspecific precipitation is certainly too high to allow the isolation of a purified messenger RNA. However, even this limited specificity is sufficient to justify the possibility of binding myeloma producing polysomes to affinity columns.

Rabbit antibody to myeloma protein (both whole and pepsin treated) bound to Sepharose either directly or through six-carbon spacer arms was shown to bind myeloma protein specifically. With the diamino hexane spacer, it was necessary to wash the columns with high salt buffer to remove nonspecific binding. This was probably due to the larger number of unreacted spacer molecules with terminal amino groups. It was shown that the myeloma protein secreted by the tissue culture cells was specifically bound by the antibody columns, as were isolated heavy and light chains. Isolated nascent chains from MOPC-21 polysomes gave high nonspecific binding to the antibody columns, although the binding to specific columns was consistently greater. The reasons for this are not clear. These results, along with those from the binding and precipitation experiments, indicate that the anti-MOPC preparations--both free and bound to Sepharose--are active and specific for the nascent peptide chains of myeloma producing polysomes.

Both direct and indirect binding of polysomes to affinity columns gave high percentages of nonspecific binding. There was no consistent difference between specific and nonspecific binding. Ribosomal subunits showed very high binding to nonspecific control columns. Polysomes purified over Sepharose 2B (thus removing most of the subunits and monosomes) also showed high nonspecific binding, although they occasionally had a slightly higher percentage binding to specific columns than to nonspecific columns. Treatment of bound polysomes with 50 mM EDTA, which dissociates them into subunits, failed to release most of the radioactivity from the columns.

Preferential binding of subunits is one possible explanation for this observation. The remaining material could be eluted from the columns by washing with a 0.5% SDS solution.

There are several possible explanations for the lack of specific binding of polysomes to the affinity columns. Steric hindrance may be a factor. In comparison to antibody molecules, polysomes are extremely large and complex. With the antibody molecule immobilized on a Sepharose bead, it may be impossible for it to come into contact with the nascent chains, thus precluding a specific antigen-antibody interaction. Attaching the antibody to the Sepharose at a neutral pH helps to some extent, as Cuatrecasas and Anfinsen (1971) have shown that the antibody is bound less tightly (probably at fewer points) at the lower pH and thus retains the majority of its activity. Attaching the antibody to Sepharose through a spacer molecule can also help. Although a six-carbon spacer molecule is small compared to the size of the antibody molecule, the new mode of attachment may allow an increased flexibility for the antibody molecule. Also, if a spacer molecule with a terminal amino group is used, the antibody will be attached through free carboxyl groups, whereas attachment directly to cyanogen bromide activated Sepharose is through free amino groups. Again, this new mode of attachment might allow greater flexibility on the part of the antibody molecule. The indirect binding technique is another possible way to lessen the steric problems. By binding an antibody molecule to the nascent peptide on the polysome and using an "anti-antibody" attached to the Sepharose,

the relative size of the antigenic determinant on the polysome is increased, improving the chances of specific interaction.

A serious point of concern is the structure of the Sepharose. It consists of cross-linked agarose essentially in the form of porous beads. These beads can be thought of as having both an external and an internal surface. Since the exclusion limits of Sepharose are quite large (the smallest being approximately 4×10^6 Daltons for globular proteins for Sepharose 6B), proteins such as antibodies will be included. Therefore, when antibodies are bound to activated Sepharose, they will attach both externally and internally. The proteins with which the specificity of the Sepharose-antibody preparations are tested are also included, and thus have access to all of the bound antibodies. Polysomes, however, due to their large size, are excluded from the Sepharose beads. Even 2B, which has the largest exclusion limit (approximately 40×10^6 Daltons), will include only the smallest polysomes. Thus, the polysomes have access only to the antibody which is attached to the external surface of the Sepharose. The ratio of external to internal surface area of the various Sepharose sizes is not known. The internal surface area is almost certainly a significant percentage of the total. Thus, a significant proportion of the bound antibody is not accessible to the polysomes. If the internal surface area is the majority of the total, then specific binding might be impossible due to the lack of available antibody molecules.

This particular problem could be overcome by using a different matrix system. The matrix system of glutaraldehyde treated protein

(Schimke *et al.*, 1974) avoids this problem, but presents the alternate problem of requiring large amounts of protein. Antibody could be attached (either directly or through spacer molecules) to Sephadex G-25 or G-10. These are porous beads of cross-linked dextran, which have exclusion limits of approximately 25,000 and 10,000 Daltons, respectively, for globular proteins. Thus, antibodies would be excluded, attaching only to the external surface of the bands. Other materials such as aminocellulose (Sidorova *et al.*, 1974) or latex beads might also be used. It is likely that if the system can be made to work, it will require the use of a different matrix system and a modified method of attachment of the protein to allow for maximum flexibility.

SUMMARY

Attempts were made to isolate specific gamma globulin-synthesizing polysomes from the total polysome population of MOPC-21 cells, utilizing specific antibodies. The crude polysome preparations contain the cytoplasmic material of the cells. Approximately 20% of the intracellular protein is myeloma protein. Since this protein would compete with the nascent peptides of the polysomes for the specific antibody, it was necessary to separate the total polysome population from the remaining intracellular material. We found gel filtration on Sepharose to be the most satisfactory method of achieving this. We then demonstrated that our antibody bound specifically to the purified polysomes. Specific immune precipitation was shown, although nonspecific backgrounds were consistently high. The affinity chromatography system was shown to be functional and specific, using various specific and control proteins. Repeated attempts to bind polysomes to affinity columns yielded high nonspecific binding with no consistent increase in specific binding.

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