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QUANTITATION OF mRNAs FOR α_1-ACID GLYCOPROTEIN AND FOR SERUM ALBUMIN IN LIVERS OF NORMAL, STRESSED, FASTED AND REFED RATS

by

Linda Jean Harris

Submitted in Partial Fulfillment

of the

Requirements for the Degree

Doctor of Philosophy

Supervised by Dr. L. L. Miller

Department of Radiation Biology
and Biophysics

The School of Medicine and Dentistry

University of Rochester

Rochester, New York

1973

DISCLAIMER

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rb

To my parents

Vitae

The author was born in [redacted]. She resided in Great Neck, New York and attended Great Neck North Senior High School. Upon graduation, she enrolled at the Massachusetts Institute of Technology. She graduated with a B.S. in Life Sciences in 1973. Since that time, she has been a graduate student at the University of Rochester in the Department of Radiation Biology and Biophysics. She received her M.S. in January, 1976. From then until the present time she has been a predoctoral candidate under the supervision of Dr. Leon L. Miller.

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And last, thanks to my HP 25 for its unfailing service in doing my calculations.

Abstract

A new procedure for determining the relative levels of a specific mRNA species has been developed and applied to mRNA for rat serum albumin (RSA) and α_1-acid glycoprotein (α_1-AGP) in rat liver. The method is a radioimmunoassay for the completed protein, but which also detects antigenic determinants in nascent polypeptide chains on polysomes synthesizing the specific protein.

Results show that 24 hours after stressing the rat by turpentine injection the total number of polysomes per mg DNA has increased by 20 to 25%; however, the number of RSA synthesizing polysomes per mg DNA has decreased slightly. These results are in contrast to those from isolated in vitro rat liver perfusion studies, which show that following turpentine treatment both the absolute rate of RSA synthesis and its rate relative to total serum protein synthesis fall to approximately 30% of normal values. This discrepancy indicates that the translation of RSA mRNA may be slower following turpentine treatment. One might speculate that this is due to either a decrease in initiation rate or elongation rate (which may then slow initiation) caused by competition from other mRNAs for initiation and/or elongation factors.

In rats fasted for 6 days, the number of RSA synthesizing polysomes per mg polysomal RNA is only slightly below normal, but the total number of RSA synthesizing polysomes per mg DNA has decreased by 40%. Results from liver perfusion studies show that the rate of synthesis of almost all (not α_1-AGP) serum proteins is reduced to 20 to 25% of normal rates. Again, it is seen that RSA mRNA levels do not decrease as sharply as the rate of RSA synthesis. Twelve hours after refeeding rats which had been fasted for 6 days, the number of RSA synthesizing polysomes (on both a per mg DNA and per mg RNA basis) begins to increase, reaching a peak two to three times normal levels 24 to 48 hours after commencement of refeeding.

During the first 24 hours after turpentine injection, there is a linear increase in the number of α_1-AGP synthesizing polysomes (on both a per mg DNA and per mg RNA basis). The increase is smaller during the next 24 hours and there is a small decrease between 48 and 72 hours. The serum concentrations of α_1-AGP following turpentine treatment reflect these changes in polysome levels.

It was not possible to compare the number of α_1-AGP synthesizing polysomes in livers of normal, fasted and refed rats because the levels detected were only slightly higher than

those seen in rabbit liver and rat kidney polysome controls. It may be possible to eliminate this background activity by using only the F_{ab} fragment of the antibody. This background activity must be eliminated before the technique can be applied to quanatitating mRNA for proteins synthesized in very small quantities.

This technique offers several advantages over other procedures commonly used to quantitate mRNA-it requires very little purified antigen and antibody, is not dependent upon efficiency of translation of a particular mRNA, and it uses gamma counting instead of liquid scintillation counting.

The polysome isolation procedure used gives 70% recovery of polysomal RNA from all livers except those from animals fasted and then refed for 12 to 48 hours. Using assays sensitive to less than 1 ng protein, no free serum RSA has been detected isolating with these polysomes.

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I. INTRODUCTION

During the 1960's, there was much interest in the changes which occured in RNA and protein concentrations in the liver and other organs in response to such stimuli as stress, glucocorticoids and other hormones, and changes in diet. During the 1970's, immunochemical techniques, specifically immunoprecipitation, have been applied to the identification, isolation, and quantitation of polysomes synthesizing specific proteins. Affinity chromatography has allowed the isolation of pure antibodies and antigens, which are used in substantial quantitities in the immunoprecipitation techniques. Immunoprecipitation of polysomes (Ikehara and Pitot, 1973; Shapiro et al, 1974; Taylor and Tse, 1976) is used by some investigators, although not validly (see below), as a means of quantitating polysomes synthesizing a specific protein. It is also used as a source of specific mRNA which is needed as a template for the synthesis of complementary DNA; this cDNA is then used to quantitate a specific mRNA (Shapiro and Schimke, 1975; Yap et al, 1978a,b) in a population containing many mRNA species. Immunoprecipitation is also being used to identify and quantitate proteins synthesized in cell-free protein synthesizing systems (Schutz et al, 1973; Sippel et al, 1975;

Nardacci et al, 1975; Chan et al, 1973; Woo et al, 1975; Green et al, 1975; Peterson, 1976) in response to added mRNA.

During the past few years, I have developed a radioimmunoassay (RIA) to quantitate rat liver mRNA in polysomes synthesizing rat serum albumin (RSA) and α_1 acid glycoprotein (α_1-AGP). The antibodies used in the radioimmunoassay recognize both nascent chains and completed protein. In order for the assay to quantitate mRNA and not nascent chains, it is necessary that all polysomes synthesizing a specific protein be antigenically identical. This is accomplished by 1) using cycloheximide, an inhibitor of chain elongation, in buffers so that each specific mRNA contains close to a maximum number of ribosomes, and therefore the same number of antigenic determinants, and 2) covalently attaching the antibodies to an insoluble support in such a way that a polysome (or mRNA) can be bound by only one antibody molecule.

The assay procedure begins with the preincubation for 24 hours of polysomes or antigen standard with a fixed amount of antibody covalently coupled to Sephadex. ^{125}I labelled antigen is then added and incubation continued for another 24 hours. The immunoadsorbent is then washed and the amount of radioactivity bound to it is determined. The amount of specific

polysomes is determined on a relative scale, using a standard curve made with pure antigen.

The importance of this procedure is best seen after reviewing other procedures commonly used to quantitate mRNA. All general methods rely on immunoprecipitation or immuno-adsorbtion of polysomes in order to set up the assay (cDNA-RNA hybridization), or to perform the assay (simple immuno-precipitation), or immunoprecipitation to quantitate the specific protein made in response to mRNA (cell-free protein synthesizing systems). Polysomes can be specifically isolated by either immunoprecipitation or immunoadsorption (Palacios et al, 1973a,b). In either case, polysomes are first incubated with a first antibody directed against the completed protein. These antibody-polysome complexes are soluble. They can be separated from polysomes not bound by anbtibody by immuno-precipitation (either excess antigen or a second antibody directed against the first antibody can be added in order to precipitate the first antibody and polysomes bound by it), or by immunoadsorption (an insoluble matrix containing either antigen or the second antibody can adsorb out the first antibody and polysomes bound by it). Both of these methods result in up to a 250 fold (but usually 20 to 50 fold) purification of the specific polysomes (Groner et al, 1977; Schechter, 1973). At

least one investigator has also attempted to adsorb the specific polysomes directly with an insoluble matrix with only the first antibody coupled to it. This method left a substantial portion of the specific polysomes still in solution (Palacios, 1973b). The percentage of polysomes synthesizing a specific protein is determined by the percentage of polysomes removed from solution by immunprecipitation or immuncadsorption.

DNA can be transcribed from a specific mRNA species by the reverse transcriptase enzyme. This DNA is referred to as complementary DNA or cDNA. When cDNA is incubated with a population of mRNA species, the rate of hybridization of cDNA will be determined by the concentration of the specific mRNA in the total mRNA population and by the concentration of cDNA. Therefore, if the cDNA concentration is held constant, the rate will be proportional to the mRNA concentration. A standard curve can be created using samples with known specific mRNA concentrations and this curve can be used to determine the specific mRNA concentration in an unknown sample.

Although the cell-free protein synthesizing systems do not require the isolation of a specific mRNA, the translation product is immunprecipitated. Either mRNA or total celllular RNA can be added to a cell-free protein synthesizing system, and

the mRNA will be translated into a protein product. The amount of specific protein synthesized is determined by measuring the amount of radioactive amino acids incorporated into the immunoprecipitated product.

All of these systems have the drawback that immunoprecipitation requires large amounts of antibody or antigen. The RIA uses very small amounts of both (e.g. 64 ng pure RSA and 17 μg pure anti-RSA to assay 9 batches of polysomes). They also all require liquid scintillation counting, while the RIA uses gamma counting. Scintillation cocktails are expensive and sample preparation can be time consuming. The cell-free protein synthesizing systems rely on the constant efficiency of translation of a specific mRNA. It is well known that efficiencies vary with incubation conditions (Tse and Taylor, 1977; Marcu and Dudock, 1974; Peterson, 1976; Taylor and Schimke, 1973; Benveniste et al, 1976; Hunter et al, 1977). The immunoprecipitation method actually measures the number of ribosmes engaged in the synthesis of a specific protein, and not the mRNA concentration, since rRNA accounts for 99% of polysomal RNA. Hybridization can be used to measure the specific mRNA in a total RNA extract or just in the polysome extract. When the total RNA extract is used, non-functional RNA will also be measured. Comparison of results from RIA, hybridization, and

cell-free protein synthesis experiments should offer insight into transcriptional and translational control mechanisms.

In these studies, mRNA levels for albumin and α_1-acid glycoprotein have been measured in livers of untreated rats, rats stressed by turpentine injection, fasted for 6 days, and refed for up to 3 days after a 6 day fast. These proteins were chosen because of the pronounced changes in their rates of protein synthesis which occur under these conditions as measured in vivo (Neuhaus et al, 1966; Morgan and Peters, 1971; Peters and Peters, 1972; Weimer and Coggshall, 1967) and in the isolated rat liver perfusion system (John and Miller, 1969; Miller and John, 1970). RSA mRNA concentrations (Pain et al, 1978; Yap et al, 1978b) have also been measured by some of the methods just described. The fact that the rates of synthesis of these two proteins respond differently to these experimental conditions may be due to any number of things,including: 1) changes in mRNA levels; 2) differences in affinity of the various mRNA species for initiation and/or elongation factors; and 3) changes in supply of initiation and/or elongation factors. Results from these experiments show that there are large changes in specific mRNA levels, but the RSA mRNA levels do not change sufficiently to account for the total changes in rate of protein synthesis. The latter two possibilities are

only speculation- RSA mRNA may be a poor competitor for initiation factors, so that when these factors are in a limited supply, the translational efficiency of RSA mRNA may also decrease.

A. POLYSOME ISOLATION PROCEDURES

Since 1963, when the role of polysomes in protein synthesis was first established, (Noll, 1969) there has been an enormous number of polysome isolation procedures published. Each procedure has been developed with a specific purpose in mind. Most isolation procedures are based on either of two principles: 1) precipitation of ribonucleoprotein particles from the post-mitochondrial supernatant (PMS) with high Mg^{++} concentrations (100mM) (Palmiter, 1974; Clemens, 1974) or 2) ultra-centrifugation of the PMS through a sucrose gradient (Morton et al, 1975; Ramsey and Steele, 1976b). Isolation procedures can also be divided into those which isolate both membrane-bound and free polysomes together (Morton et al, 1975) and those which separate the two classes of polysomes (Blobel and Potter, 1967; Ramsey and Steele, 1976b).

Before choosing an isolation procedure, one must decide which of many qualities of the isolated polysomes is most important. Some of the factors which should be considered are:

1) whether isolation of total polysomes or separation of free and membrane-bound polysomes is desired;
2) whether it is necessary to obtain either total recovery of polysomes from the homogenate or a

representative sample with a known recovery;

3) whether the polysomes must be undegraded by ribonucleases, shearing forces, and protected from translational run-off of the ribosomes from the mRNA;

4) whether the polysomes must be free from contamination by other subcellular components;

5) whether the polysomes or mRNA or ribosomes isolated from them must be biologically active.

Usually, some but not all of the above parameters have been measured for a particular procedure. It should be remembered also that a procedure that is suitable for one tissue may not necessarily be suitable for another tissue.

It is very difficult to get a clean separation of membrane-bound and free polysomes and at the same time to get a good recovery of undegraded polysomes (Blobel and Potter, 1967; Ramsey and Steele, 1976a,b; Venkatesan and Steele, 1972; Dissous et al, 1978a,b). Therefore, unless there is a specific need to separate the two classes, it is much easier to isolate total polysomes. Isolation of total polysomes usually involves treatment of a homogenate with Triton X-100 to detach the endoplasmic reticulum from the nucleus, followed by centrifugation to sediment nuclei and mitochondria. Polysomes

can then be isolated from the post-mitochondrial supernatant (PMS). Separation of free and membrane-bound polysomes (Venkatesan and Steele, 1972; Ramsey and Steele, 1976b) usually calls for centrifugation (high speed) of the homogenate to pellet nuclei, mitochondria, microsomes and lysosomes. Free polysomes can then be isolated from the supernatant (Blobel and Potter, 1967) and bound polysomes can be isolated from the pellet after detergent treatment. Other procedures call for differential centrifugation of the PMS through discontinuous sucrose gradients. The two classes of polysomes band at different densities.

Few papers document the recovery of polysomes. For tissues such as liver, which are rich in membrane-bound polysomes, recoveries are generally poor unless the homogenate is treated with Triton X-100 to free the endoplasmic reticulum from the nuclear membrane. Another cause of poor recovery is insufficient centrifugation time so that only heavier polysomes are pelleted (Blobel and Potter, 1967; Ramsey and Steele, 1976b). If incomplete recoveries are obtained and quantitative comparisons are to be made, then it is necessary to show that the polysomes isolated are representative of the total population and to determine the yield.

Polysomes can become degraded for several reasons. Endonucleases may degrade mRNA. This is usually controlled for by the presence of ribonuclease inhibitors: heparin (Zollner and Fellig, 1953), diethyl pyrocarbonate (Jones, 1976; Rosen and Fedorcsak, 1966), rat liver supernatant ribonuclease inhibitor (Ramsey and Steele, 1976b; Dissous et al, 1978b; Gribnau et al, 1970; Rahman, 1966), bentonite (Cheng et al, 1974), proteinase K (Cheng et al, 1974; Mach et al, 1973), macaloid (Stanley and Bock, 1965; Jones, 1976) and polyvinyl sulfate (Cheng et al, 1974) or by addition of excess RNA (yeast RNA) (Morton et al, 1975; Dissous et al, 1978b; Gribnau et al, 1970; Rahman, 1960) to the homogenate to serve as a competitive inhibitor of the ribonuclease, and either low (pH 5.5) (Ilan, 1976) or high pH (pH 8.5) (Davies et al, 1972), high Mg concentrations (Gribnau et al, 1972; Rahman, 1966) and sulfhydryl reducing agents (Morton et al, 1975; Ramsey and Steele, 1976b; Venkatesan and Steele, 1972; Roskam et al, 1976; Dissous et al, 1978b; Gribnau et al, 1970) and by working at cold (0-4 C) temperatures.

There are at least three known ribonucleases in rat liver. The ribonuclease inhibitors mentioned are only effective under certain conditions of ionic strength, pH, etc.; therefore, depending upon the conditions used to test ribonuclease

activity, one inhibitor may be found to be more or less effective than another inhibitor.

Shearing forces, such as those caused by repeated pipetting through a narrow bore pipette, homogenization with a tight fitting pestle, and sonication can all cause degradation of polysomes (Noll, 1969).

Polysomes can be protected from translational run-off by slowing the rate of elongation with respect to the rate of initiation; this can be accomplished with cycloheximide (Palmiter, 1973; Lodish et al, 1971). Care must be taken when using either translational inhibitors or ribonuclease inhibitors that biological activity of the mRNA will not be destroyed.

Typical contaminants of polysomes include serum proteins, cell sap and membrane proteins (Olsnes, 1971), glycogen (Ramsey and Steele, 1976a,b; Murty and Halinan, 1969), phospholipid (Ramsey and Steele, 1976a,b), cross contamination between free and membrane-bound polysomes (Venkatesan and Steele, 1972; Ramsey and Steele, 1976b), mitochondria and lysosomes (Dissous et al, 1978a,b) and contents of lysed nuclei. Sometimes these contaminants can be removed by an additional procedure that readily distinguishes between the contaminants and polysomes.

Biological activity of mRNA (i.e. as a template for translation or reverse transcription) can be interfered with reversibly by heparin (Palmiter, 1973; Waldman and Goldstein, 1973) or irreversibly by diethylpyrocarbonate (Palmiter, 1974).

B. RADIOIMMUNOASSAY

Radioimmunoassays are amongst the most sensitive assays known for the detection of polypeptides. They are based on the competitive inhibition by either standard or unknown protein of the binding of labeled, but otherwise immunochemically identical, protein to the antibody. In the most common form of the assay, the equilibrium RIA, radiolabelled antigen is mixed with either standard or sample containing an unknown amount of antigen. Antibody is then added and the mixture is incubated until the antigen-antibody reaction has reached equilibrium. Antigen-antibody complexes are usually still soluble because of the small concentrations used. Free antigen and antigen bound to antibody are separated by any of a variety of methods. The amount of free (F) and bound (B) radiolabelled antigen are determined and a standard curve is made by plotting B/F against the log of the antigen concentration.

The RIA technique was first developed by Yalow and Berson (1960) for the assay of insulin. Since that time, assays have been developed for other peptide hormones, steroid hormones, and proteins (Skelley et al, 1973). The sensitivity of the RIA is limited by the avidity of the antibody for the antigen and by the specific activity of the labelled antigen.

The three main problems in developing an assay are:

1) acquiring an antiserum with high avidity for the antigen;
2) finding a procedure to separate free and bound antigen; and
3) labelling the pure antigen to a high specific activity without altering its immunological reactivity.

Antisera usually are obtained by injection (subcutaneously, intramuscularly, into foot pad or foot web or into lymph node) of the antigen mixed with an adjuvant (usually Complete Freund's Adjuvant) into an animal (usually of a different species) which would recognize the antigen as a foreign substance. Booster injections of antigen in Incomplete Freund's Adjuvant (IFA) are given at intervals, and the serum of the injected animal tested regularly until an adequate antiserum is obtained. At this time, a large amount of antiserum is obtained from the animal. Animals of the same species can vary greatly in their response to the same antigen and immunization regimen, so that more than one animal is often immunized with the same antigen.

There are a number of commonly used methods for the separation of free and bound antigen:

1) Electrophoresis- based on the different electrophoretic mobilities of the free antigen and antigen-antibody complexes
2) Adsorption systems- charcoal is often used to adsorb free but not bound antigen
3) Salt precipitation- precipitation of all antibody (free and complexed with antigen) with Na_2SO_4. This technique cannot be used when the free antigen is also precipitated by Na_2SO_4
4) Indirect immunoprecipitation- a second antibody, directed against the first antibody is used to precipitate all of the first antibody (both free and complexed with antigen), leaving free antigen in solution
5) Solid phase- antibody (either that directed against the antigen or a second antibody directed against the first antibody) is made insoluble by coupling to an insoluble matrix (Sephadex, cellulose, filter paper, walls of a test tube). All of the first antibody is then insoluble because it is either coupled to a solid matrix or bound by a second antibody coupled to a solid matrix.

Protein antigens are usually radiolabelled by iodination with ^{125}I or ^{131}I because of the high specific acitivities attainable and because of the ease of counting γ emitters. There are many procedures available for iodination, but care must be taken that the conditions are not so harsh as to cause irreversible denaturation of the protein. Some of the more commonly used procedures are lactoperoxidase (Krohn and Welch, 1974), electrolytic (Rosa et al, 1964), chloramine T (Greenwood et al, 1963), iodine monochloride (Bale et al, 1966), oxidative monochloride (Helmkamp et al, 1967), (all of which attach I predominantly to tyrosine residues), and conjugation of the iodinated Bolton-Hunter reagent to free amino groups (Bolton and Hunter, 1973).

The oxidative monochloride method has been used for most of the work here because of the high specific activities attainable without apparent denaturation.

The RIA can be performed in either of two basic forms- the equilibrium (as described above) and the sequential procedures. In the sequential procedure, instead of incubating standard or unknown and labelled antigen simultaneously with the antibody, the standad or unknown is preincubated with the antibody and labelled antigen is later added. Incubation is terminated

before equilibrium is reached. The sequential form of the assay has the advantage of having a lower optimal detection range, but the assay range is more limited (the maximum amount of antigen which can be measured is the amount which will saturate the antigen binding sites of the antibodies during the preincubation period).

C. SERUM ALBUMIN

1. FUNCTION

Albumin is a multifunctional protein. It serves three known general purposes in serum:

1) maintenance of colloid osmotic pressure of serum;
2) transport of many substances through the vasculature;
3) source of amino acids for tissues.

Isotope dilution studies in humans indicate that only 40% of total body albumin is found in the serum at any one time, 25% in the skin and muscle, and the remainder predominantly in the liver and gut (Peters, 1975). Katz et al (1970), using radial immunodiffusion to measure albumin, have found only 20-25% of rat albumin in serum, 35-40% in the carcass (mostly in muscle), 20-25% in skin, and less than 10% in the gut. These studies do not distinguish between undegraded RSA and that which may be partially degraded and still retain its antigenic activity. Albumin comprises 60% of the protein found in normal serum, yet it accounts for 80% of the colloid osmotic pressure of blood (Peters, 1975).

Amongst the substances which are bound by albumin in the serum are (Peters, 1975; Peters, 1970):

cations -	Cu^{++}, Ni^{++}, Mn^{++}, Co^{++}, Zn^{++}, Cd^{++}, Ca^{++}, Hg^{++}
anions -	fatty acids
bilirubin	
pyridoxal	
amino acids-	L-tryptophan, cysteine
hormones-	cortisol, testosterone, estrogen, thyroxin
drugs-	acetyl salicylate, digitoxin, barbiturates, sulfonamides, penicillin, strptomycin, warfarin, cyclamates, etc.
dyes	
soluble disulfides-	cysteine, glutathione

The sites of binding of many of these substances will be discussed in the section on structure.

Albumin is taken up by cells in peripheral tissues by pinocytosis. After degrading albumin, these cells can utilize the resultant amino acids (Peters, 1975).

As will be discussed in the section on synthesis, there is considerable evidence that the nutritional and hormonal status of the animal affect the rate of synthesis of albumin; however, reports indicating that osmotic pressure of serum affect the rate of RSA synthesis are of questionable validity. There has been little or no research reported on whether albumin synthesis is affected by any of the substances bound by albumin in the serum.

2. STRUCTURE

Much research has been done on the structure of albumin, especially on bovine and human serum albumin. Although there is some variation in the amino acid sequences amongst these species, the variations usually do not affect the functioning of the albumin molecule.

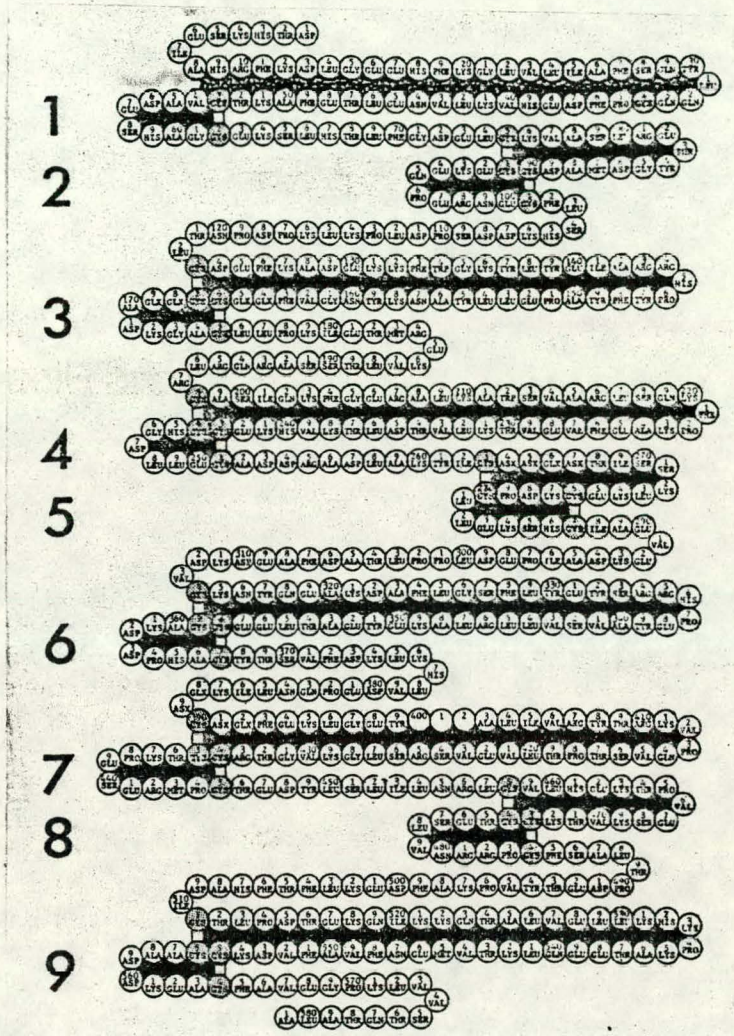
Rat serum albumin (RSA) is composed of 575 amino acids in a single polypeptide chain. The molecular weight, calculated from the amino acid composition, is 64,616 grams/mole. Albumin is the only known serum protein which contains no carbohydrate moieties (Peters, 1975).

Albumin contains 17 disulfide bonds and one free SH group. These disulfide bonds are totally inaccessible to reducing agents (mercaptoethanol, thioglycolate) between pH 5 and pH 7. The disulfide bonds gradually become more accesible outside of this pH range, but complete reduction requires the presence of denaturing agents (GuHCl, urea, or SDS) (Katchalski et al, 1957; Alexander and Hamilton, 1960; Goetzl and Peters, 1972). Brown (1975) has proposed a covalent structure for bovine serum albumin (Figure 1). The albumin molecule appears to be folded into three domains containing loops 1-3, 4-6, and 7-9. There is some homology in the amino acid sequences and immunochemical cross reactivity of the three domains, suggesting that albumin may have evolved from a molecule one third of its present size (Peters, 1977).

Isolated fragments of albumin (produced by limited proteolysis with pepsin or trypsin) usually maintain their native conformations. Some of these fragments, after denaturation in the presence of sulfhydryl reducing agents and 8M urea, can even regain their native structure (Teale and Benjamin, 1976). Ligands can be bound by some of the isolated fragments, thus allowing the determination of binding sites of these ligands (Figure 2).

FIGURE 1

Figure 1: Amino Acid Sequence of Bovine Serum Albumin

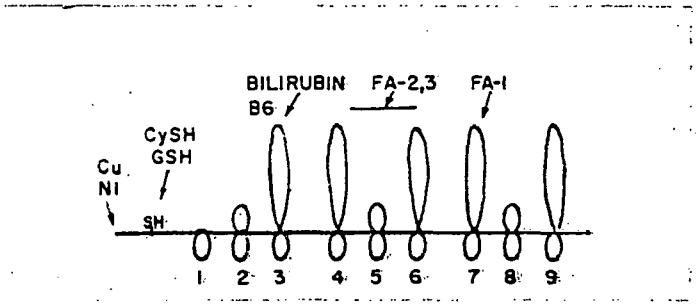


from Brown (1975).

Amino acids are indicated by their official three-letter abbreviations, with the amino-terminus at the top of the page. Disulfide bonds between Cys-Cys residues are designated by double bars.

FIGURE 2

Figure 2: Schematic of the Bovine Serum Albumin Molecule, Summarizing Ligand-Binding Sites



from Peters (1977).

CySH- cystine
GSH - glutathione
B6 - pyridoxal
FA - long-chain fatty acids

3. SYNTHESIS AND CATABOLISM

Serum albumin is synthesized exclusively by the liver (Miller et al, 1951; Miller and Bale, 1954; Miller et al, 1954). Normally it comprises 3-11% of total liver protein synthesis (Rotermund et al, 1970; Keller and Taylor, 1976; Peters and Peters, 1972; Taylor and Tse, 1976). Immunofluorescent (Hamashima et al, 1964) and enzyme conjugated antibody techniques (Feldmann et al, 1972) have been used to determine which cells and how many cells synthesize albumin. Both techniques indicate that parenchymal cells synthesize albumin, and that any particular cell contains either a considerable amount of albumin or no albumin at all. The immunofluorescent technique also shows the presence of albumin in Kupfer cells. These cells may be in the process of catabolizing albumin from the serum.

The intracellular site of albumin synthesis is almost exclusively on membrane-bound polysomes (Ikehara and Pitot, 1973; Redman and Cherian, 1972; Peters, 1962; Jamieson and Ashton, 1973). These polysomes normally consist of between 9 and 14 ribosomes per mRNA (Brown and Papaconstantinou, 1977) and a maximum of 19 ribosomes per mRNA (Peters, 1970). It takes

between 1 and 2 minutes for the polypeptide chain to be synthesized (Mathews et al, 1973). The albumin molecule passes directly to the cisternae of the rough endoplasmic reticulum, without attaching to the membrane itself. After intravenous injection of ^{14}C-leucine, peak radioactivity incorporated into material immunoprecipitable by anti-albumin antibodies is found in the rough endoplasmic reticulum at 5-10 minutes, in the smooth endoplasmic reticulum at 15-16 minutes and in the Golgi complex at 22-30 minutes. Radioactive albumin first appears in the serum at 15-20 minutes (Peters et al, 1971; Jamieson and Ashton, 1973). Estimates of the amount of albumin in the membranous channels listed above vary from .3 to .9 mg/g liver (Peters, 1962; Jamieson and Ashton, 1973; Marsh et al, 1958; Glaumann and Ericsson, 1970). Over a five-fold range of rate of albumin synthesis, the amount of intracellular albumin only doubles (Peters, 1977), indicating that there is little or no intracellular accumulation of albumin.

There are two lines of evidence indicating that albumin is first synthesized in a precursor form: 1) Urban et al (1974) have found a highly labelled protein to contaminate the mature intracellular albumin precipitated by anti-albumin antibodies. This contaminant was found to be identical to the pure RSA,

except for an additional pentapeptide (Urban et al, 1974) or hexapeptide (Russel and Geller, 1975) at the amino terminus. This oligopeptide is termed the pro-piece; 2) translation of rat liver poly (A) containing RNA in a cell-free protein synthesizing system derived from wheat germ gives a product immunoprecipitable by anti-RSA that is slightly heavier than mature RSA. Cleavage of this product with cyanogen bromide produces peptide fragments identical to those produced by similar treatment of mature albumin, except for the fragment from the amino terminus (Strauss et al, 1977; Yu and Redman, 1977). This fragment is approximately 2500 daltons heavier than the corresponding fragment from mature RSA. Strauss and coworkers have recently determined the complete amino acid sequence of this fragment (Figure 3):

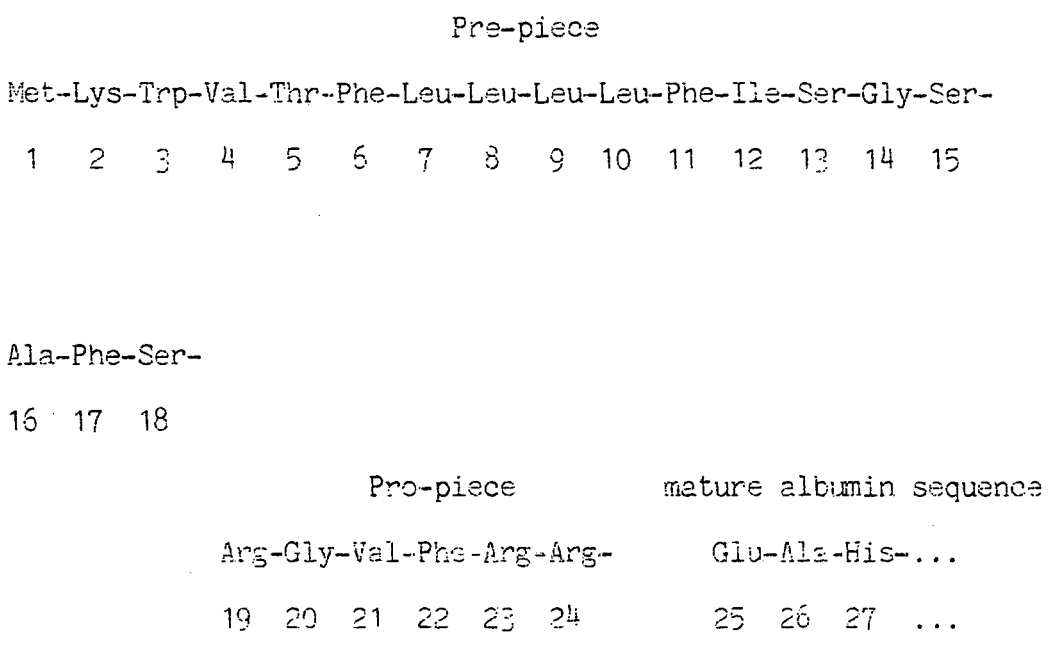


Figure 3: Amino acid sequence of the amino terminus of Pre-proalbumin

Blobel and coworkers (Blobel and Sabatini, 1971; Blobel and Dobberstein, 1975a,b) have postulated that the pre-piece serves as a signal for the interaction of the nascent peptide chain with the rough endoplasmic reticulum and to allow the peptide chain to pass through to the cisternum. The pre-piece of albumin and of many other proteins studied are all rich in hydrophobic amino acids. This supports their possible role in allowing the

protein to pass through the membrane. The pre-piece is most likely cleaved by proteases in the microsomal membrane while the remainder of the protein is still being synthesized. The function of the pro-piece is not known, but Schreiber et al (1976) have suggested that it might mask the binding sites for metal ions, that the cleaved oligopeptide may serve as a signal for increased albumin synthesis (the rate of cleavage being sensitive to changes in serum albumin concentration), and several other possibilities. Mature RSA does not accumulate within the cell; instead, the pro-piece is cleaved in the Golgi complex just prior to secretion of albumin from the cell. Recently it has been suggested that cleavage of the pro-piece is dependent upon fusion of protease and proalbumin containing Golgi vesicles (Judah et al, 1978). The protease, possibly Cathepsin B, then cleaves off the pro-piece, leaving mature RSA, which is then secreted.

The rough endoplasmic reticulum contains a disulfide interchange enzyme which may facilitate the formation of the disulfide bonds of RSA and other proteins (Anfinsen, 1972; Ansorge et al, 1973).

II. CONTROL OF SERUM LEVELS

The normal concentration of albumin in the serum of adult male rats is 2.97±.21 g% (Peters and Peters, 1972). This is approximately 60% of total serum protein. Only about 40% of total body albumin is in the vasculature at any one time (Peters, 1975). Serum albumin concentration is regulated by the rates of albumin synthesis and degradation, by transfer of albumin between intravascular and extravascular pools, and by changes in the plasma volume (Hoffenberg, 1970).

The rate of synthesis is responsive to changes in amino acid supply to the liver, hormone levels, and osmotic pressure. The rate of degradation is normaly proportional to the serum concentration (i.e., follows first order kinetics) (McFarlane, 1963) and is not related to the rate of synthesis. Serum albumin is synthesized exclusively in the liver (Miller et al, 1951; Miller and Bale, 1954; Miller et al, 1954), but there is no specific tissue or organ which accounts for the majority of albumin catabolism.

The adult male rat liver normally synthesizes 1.07±.08 mg RSA/100 g body weight/hour or 0.34 mg/g liver/hour (Peters and Peters, 1972). The half life ($T_{\frac{1}{2}}$) of albumin in the plasma is

2.5 days in the rat (Munro, 1969).

Albumin synthesis has been measured in rats fasted for short periods (18 to 48 hours) and for long periods (5 or 6 days) and in rats maintained on no or low protein diets with adequate calories for even longer periods of time. Measurement by in vivo incorporation of amino acids into albumin (Kirsch et al, 1968; Morgan and Peters, 1971), by net albumin production in liver perfusions (John and Miller, 1968; Rothschild et al, 1969; Kelman et al, 1972), by albumin synthesis directed by total liver mRNA in cell-free protein synthesizing systems, (Pain et al, 1978), and by cDNA-RNA hybridization studies (Yap et al, 1978a,b) all indicate that the rate of albumin synthesis under conditions of protein depletion is at most 50% of its normal value. Refeeding protein and/or amino acids to depleted animals causes a return to normal albumin synthesis in 24 to 48 hours and above normal rates for a limited time thereafter (Kirsch et al, 1968; Morgan and Peters, 1971). Under conditions of protein deprivation, serum albumin concentrations are maintained at near normal levels by the transfer of albumin from the extravascular to the intravascular pool (Hoffenberg et al, 1966) and by a decrease in plasma volume. The rate of catabolism does not fall until the 6^{th} or 7^{th} day of fasting, after which it drops off sharply. After commencement of

refeeding, the catabolic rate slowly increases along with the serum albumin concentration (Kirsch et al, 1968).

The effects of various hormones on albumin synthesis and catabolism are fairly controversial. In in vitro liver perfusion studies of livers from normal rats, John and Miller (1969) have shown that glucose, amino acids, cortisol and insulin are necessary for maximum albumin synthesis, and that growth hormone has little effect. However, in perfusion of livers from hypophysectomized rats, only 50% of the normal amount of RSA is synthesized. This cannot be reversed by addition of cortisol, insulin, thyronine and bovine growth hormone to the perfusate. Injection of these hormones into the rat 2 days prior to perfusion does bring RSA synthesis up to normal levels. Growth hormone therefore needs more than 12 hours to affect RSA synthesis or else it acts through a mediator on the liver (Griffin and Miller, 1974). Other investigators have also shown that growth hormone increases albumin synthesis and that hypophysectomy decreases albumin synthesis (Keller and Taylor, 1975; Feldhoff et al, 1977; Takeda, 1964). In perfusion studies of livers from rats injected with turpentine 24 hours prior to perfusion, (when glucocorticoid levels are high) albumin synthesis is significantly below normal. Livers from hyperthyroid rats produce more RSA than do livers from

euthyroid or hypothyroid rats in the perfusion system. However, addition of thyroxine to the perfusion of livers from hyper- or euthyroid rats brings RSA synthesis below control levels (Griffin and Miller, 1973). Clearly, other factors such as dosage of hormone, changes in amino acid supply, interaction of several hormones, and time after hormone treatment must be considered when interpreting these results.

Rothschild et al (1964) have claimed that albumin synthesis is increased under conditions of low osmotic pressure and decreased under conditions of high osmotic pressure. These changes in RSA synthesis, may, however, actually be due to other factors such as poisoning by substances used to increase osmotic pressure or changes in hormone and amino acid composition of serum. Rothschild et al (1964) suggest that the site of regulation may be in the interstitial fluid of the liver.

D. α_1-ACID GLYCOPROTEIN

There are several distinct proteins of rat serum known as α_1-acid glycoprotein (α_1-AGP). The one involved in the present study is the same as that of Darcy (1967), Gordon and Louis (1969), and John and Miller (1968).

1. FUNCTION

The function of α_1-AGP is not known. It is considered an acute phase protein because of its elevated concentration in the presence of tumors, as the result of tissue damage, and other types of stress (Darcy, 1967).

2. STRUCTURE

Gordon and Louis have partially characterized α_1-AGP (Table I):

Table I: Characterization of α_1-acid glycoprotein

Molecular Weight	45,000 - 46,000 daltons
Sedimentation Coefficient	3.3 - 3.6S
Isoelectric Point	pH4.4 - 4.8
Amino Acid Analysis	see Table II
Carbohydrate Content	15%
Carbohydrate Analysis	see Table III

Although the protein gives a single band on immunodiffusion, there is a fairly wide range of isoelectic points. This may be due to differnces in sialic acid content or to the existence of more than one protein with very similar properties.

3. SYNTHESIS AND CATABOLISM

John and Miller (1968) and Weimer et al (1965) have shown that α_1-AGP is synthesized in isolated rat liver perfusions and by rat liver tissue culture, respectively. There is no specific information on the pathway of biosynthesis of α_1-AGP. It can only be assumed, until such research is done, that the pathway is similar to that of other glycoproteins, particularly those of

TABLE II AND TABLE III

Table II: Amino Acid Composition of α_1-Acid Glycoprotein

	Amino acid composition (moles/100 moles of total amino acids)		
	Material after stage 2 (means ± s.d. of four analyses) (*a*)	Material after stage 3 (single analysis) (*b*)	*P*
Lys	8·10 ± 0·142	8·87	0·01
His	3·32 ± 0·450	4·18	—
Arg	3·02 ± 0·170	3·07	0·02
Asp	11·98 ± 0·684	10·74	—
Thr	7·57 ± 0·656	6·48	—
Ser	6·05 ± 0·640	5·54	—
Glu	14·50 ± 0·632	12·96	—
Pro	4·70 ± 1·000	3·50	—
Gly	6·65 ± 1·062	4·77	—
Ala	7·75 ± 1·506	5·46	—
CyS	6·00*	6·39	—
Val	6·39 ± 0·496	6·56	—
Met	1·08 ± 0·708	1·28	0·02
Ile	3·75 ± 0·208	3·07	0·05
Leu	7·92 ± 0·754	7·59	—
Tyr	3·02 ± 0·288	2·73	—
Phe	4·42 ± 0·428	6·14	0·02

* This value was assumed to be the same as in the material after stage 3. Actual recoveries were low owing to losses during hydrolysis.

from Gordon and Louis (1969).

Table III: Carbohydrate Composition of α_1-Acid Glycoprotein

Results are given as means with the numbers of preparations analysed in parenthesis. The stages are defined in Table I.

	Composition (% of dry wt.)	
	After stage 2	After stage 3
Sialic acid		
Before treatment with neuraminidase	3·7 (6)	3·3 (5)
After treatment with neuraminidase	1·3 (3)	1·3 (3)
Hexosamine	5·9 (10)	—
Hexose	4·05 (13)	—
Methylpentose	Trace (9)	—

from Gordon and Louis (1969).

rat liver. Jamieson and Ashton (1973) have investigated the biosynthesis of a different α_1-AGP (it has a higher carbohydrate content and lower isoelectric point) made by rat liver. This protein follows the same subcellular pathway as does albumin: after intravenous injection of H-leucine, peak radioactivity in α_1-AGP immunoprecipitable material is found in the rough endoplasmic reticulum at 5-10 minutes, in the smooth e.r. at 15 minutes, and in the Golgi complex at 30 minutes. Radioactivity first appears in the serum 15-20 minutes after ^{3}H-leucine injection. Several investigators (Molnar and Sy, 1967; Robinson, 1969) have shown that, in liver, initial glycosylation occurs while the polypeptide chain is still attached to the ribosome. However, Jamieson (1977), working with a specific protein, has found that addition of glucosamine (usually the first sugar to be added) occurs after the release of the polypeptide chain from the ribosome. Other investigators (Redman and Cherian, 1972; Pless and Lennarz, 1977) have shown that the core sugar residues (glucosamine and mannose) are added en masse from a lipid carrier of the oligosaccharide. Glycoproteins remain attached to the membrane of the rough e.r. while these core sugars are added (albumin, however, is released directly into the cisternum of the r.e.r.) (Redman and Cherian, 1972). Galactose is then added as the protein is released into

the cisternum and passes through the smooth e.r. Fucose and sialic acids are added in the Golgi complex just prior to secretion.

There is no specific information on the catabolism of α_1-AGP. It has been shown that removal of terminal sialic acid residues of most, but not all, other serum glycoproteins (Stockert et al, 1977; Scheinberg, 1976) studied allows these proteins to be bound by a hepatic binding protein located on the plasma membrane of hepatocytes, and then to be catabolized by the lysosomes (Ashwell and Morell, 1974; Bocci, 1976). The hepatic binding protein has been shown to specifically recognize the galactose residues which are exposed after desialylation. Exposure of two out of approximately ten penultimate galactosyl residues of ceruloplasmin is sufficient to mark the protein for catabolism (Ashwell and Morell, 1974). The serum half-lives of most desialylated glycoproteins (asialotransferrin is an exception) is considerably shorter than that of the native protein. It has not, however, been shown that this is the normal route of turnover of serum glycoproteins.

4. CONTROL OF SERUM LEVELS

The normal α_1-AGP serum concentration is 150 - 300 mg%. Values as high as 3.0 g% occur 24 hours after induction by turpentine injection. In vivo, after stress by turpentine injection, the serum levels of α_1-AGP begin to rise at about 7 or 8 hours and continue to rise until 24 to 48 hours. Darcy (1965) claims that adrenalectomy increases the response to turpentine, and that administration of cortisol prior to turpentine injection reduces the response normally seen. It must be noted, however, that pharmacological doses of cortisol were used. Weimer and Coggshall (1967) have shown that the effect of cortisol on seromucoid (the protein fraction to which α_1-AGP belongs) concentration is very dose dependent. Pharmacological doses of cortisol (3.0 mg/100 g body weight) given daily decrease the seromucoid concentration in normal rats and inhibit the normal response to turpentine injection. Administration of cortisol at smaller doses (less than .6 mg/100 g body weight) enhanced the response of adrenalectomized rats to turpentine injection, while pharmacological doses inhibited the response.

Gordon and Darcy (1967) originally reported that perfusion of livers from normal rats with blood from turpentine treated

rats caused an induction of α_1-AGP syntheis. These conclusions were, however, based on only 1 or 2 rats per group. It was also later shown by Gordon and Koj (1968) that there were inaccuracies in the measurement of α_1-AGP in these experiments. Gordon and Koj (1968) also showed that livers from rats injected with talc 4 1/2 hours prior to perfusion produce twice the normal amount of α_1-AGP during 5 hours of perfusion. This suggests that at least some of the mediator of the response to talc had reached the liver by 4 1/2 hours.

Neuhaus et al (1966) have shown that Actinomycin D, administered up to 2 hours after turpentine injection, can prevent the normal increase in concentration of seromucoids. Actinomycin D administered at 4 hours allows a partial response to the turpentine injection, indicating that some, but not all of the necessary RNA synthesis has occured at this time. Puromycin (Darcy, 1967) prevents the response to turpentine, showing that increased levels of α_1-AGP are due to increased synthesis and not just secretion of preformed protein.

Miller and John (1970) have shown that 4 to 6 hours after addition of growth hormone, insulin, cortisol and amino acids to the perfusate of a normal liver, there is a 4 fold increase in the rate of α_1-AGP synthesis. Although livers from rats fasted

for 6 days synthesize α_1-AGP at the same rate as livers from normal rats, the former do not respond to the addition of amino acids, growth hormone, insulin and cortisol by the end of 12 hours of perfusion.

II. MATERIALS AND METHODS

A. MATERIALS

The following materials were obtained from the indicated company:

Bio-Gel A-5m, A-150m	Bio-Rad
Bio-Gel P-2	Bio-Rad
Bolton-Hunter Reagent[1]	Pierce
Bovine Serum Albumin-Fraction V	Miles Laboratories
Carbodiimide	
1-ethyl-3(3-dimethyl-aminopropyl)	Sigma
Cyanogen Bromide	Eastman
Cycloheximide	Sigma
Deoxycholic Acid	Sigma
Dithiothreitol (DTT)	Sigma
Divinyl Sulfone	Aldrich
Ethyl Chloroformate	Eastman Organic
Heparin-Na salt, Grade 1 from Porcine Intestinal Mucosa- 160.9 J-A units/mg	Sigma

HEPES[2]	Sigma
Indole	Sigma
I and I-high specific activity	New England Nuclear
	Amersham-Searle
MES[3]	Sigma
Orotate- C-14	New England Nuclear
Sephacryl S-200, superfine	Pharmacia Fine Chemicals
Sephadex G-25, G-50, G-100, etc.	Pharmacia Fine Chemicals
Sepharose 2B, 4B, and 6B	Pharmacia Fine Chemicals
Sucrose-ultrapure	Schwarz-Mann
TES[4]	Sigma
Tris (base)	Schwarz-Mann
Triton X-100	Beckman
Tween 20[5]	Sigma

1. Bolton-Hunter reagent- 3-(4-hydroxy phenyl)propionic acid N-hydroxysuccinimide ester
2. HEPES- N-2-Hydroxyethyl(piperazine-n'-2-ethanesulfonic acid
3. MES- 2(N-Morpholino)ethane sulfonic acid
4. TES- N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid
5. Tween 20- polyoxyethylene sorbitan monolaurate

B. RADIOIMMUNOASSAY

1. RADIOIMMUNOASSAY PROCEDURE

RIA Buffer	Wash Buffer
100 mM Tris HCl, pH 7.4	.85% NaCl
25 mM KCl	.025% NaN_3
10 mM $MgCl_2$	.5% Tween 20
.025% NaN_3	
.5% Tween 20	
.2% Bovine Serum Albumin	

1. .5 ml of standard at 0, 1, 2, 4, 8, and 16 ng/ml is pipetted into 12 x 75 mm polystyrene tubes.
2. .5 ml polysomes, at 3 different concentrations, is pipetted into tubes.
3. Sephadex immunoadsorbent, 30% (v/v) in RIA buffer, is stirred with a magnetic bar. .5 ml is pipetted into each of the above tubes.
4. Tubes are stoppered and continuously turned in the cold room for 24 hours.
5. Iodinated antigen (1 ng or 10,000 cpm) is added to each

tube and the tubes are turned for another 24 hours.

6. Total radioactivity in the tubes is measured. The immunoadsorbent is then washed (3 times with wash buffer so that in control tubes only .1 to .2% of the radioactivity remains) and radioactivity bound to the immunoadsorbent is measured.

7. $\frac{\text{Bound radioactivity}}{\text{Free radioactivity}}$ is calculated and plotted against ng antigen on semi-log paper. (see figure 4)

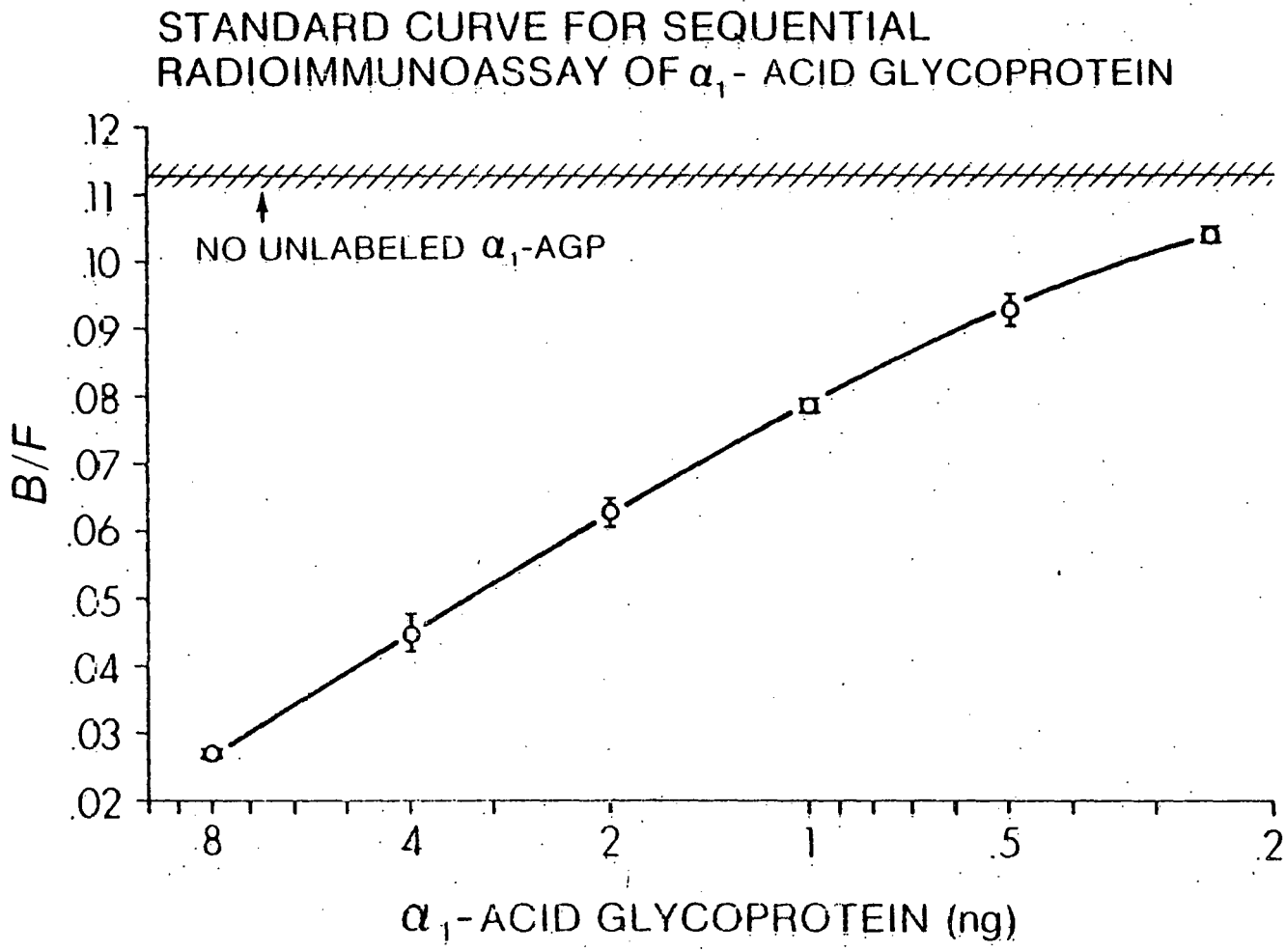
2. PURIFICATION OF ANTIGEN AND ANTIBODY

The procedures of John and Miller (1968) were used for the original purification of RSA and α_1-AGP and for the immunization of rabbits with these antigens to produce antisera. The α_1-AGP prepared in this way contains some RSA, which was removed by a Sepharose- anti-RSA immunoadsorbent (see affinity chromatography). Purified antigen was coupled to Sepharose 4B. This immunoadsorbent was then used to purify specific antibody from the antisera. The specific rabbit antibody was then coupled to Sepharose 4B, and this immunoadsorbent was used to purify the specific antigen from rat serum.

FIGURE 4

Figure 4: Typical Standard Curve for Radioimmunoassay

A sequential radioimmunoassay for α_1-acid glycoprotein was performed as described in Materials and Methods (pages 44-45). The mean and range of triplicate determinations are shown for each antigen concentration.


STANDARD CURVE FOR SEQUENTIAL
RADIOIMMUNOASSAY OF α1- ACID GLYCOPROTEIN
NO UNLABELED α1-AGP
B/F
.12
.11
.10
.09
.08
.07
.06
.05
.04
.03
.02
8
4
2
1
.5
.2
α1-ACID GLYCOPROTEIN (ng)

The affinity purified α_1-AGP was then used to immunize new rabbits. Several procedures were tried to obtain an antiserum with higher titer and antibodies with higher avidity for α_1-AGP: 1) Affinity purified α_1-AGP was conjugated to hemocyanin (Kagan and Glick, 1974), and this conjugate was injected into rabbits; 2) According to the procedure of Tung et al (1976), 8 week old BALB/c mice were injected intraperitoneally with .2 ml of the hemocyanin-α_1-AGP conjugate (mixed 1:9 with Complete Freund's Adjuvant), using a 24 G, 5/8" needle. Injections were repeated on days 15, 22 and 29. Between injections, the protein-CFA mixture was stored at -20°. It was mixed with a Virtis homogenizer before injection. Ascites fluid was obtained by draining the peritoneal cavity with an 18 G 1 1/2" needle between days 26 and 37.

The anti-α_1-AGP antibody preparation was further purified by adsorption of contaminants using affinity columns with α_2, β, and γ serum proteins and with α_1-Macroglobulin. The α_2, β, and γ proteins were obtained by Pevicon block electrophoresis (John et al, 1964) of normal rat serum. All fractions not containing α_1-AGP were pooled and coupled to Sepharose 4B. α_1-Macroglobulin was obtained by pooling the void volume fractions of normal rat serum passed through a Sephacryl S-200

column.

Glycoproteins remaining in the RSA preparation were removed by adsorbtion to Con A Sepharose. The α_1-AGP preparation was electrophoresed on Pevicon to remove contaminants with β and γ mobilities. It was also attempted to remove these contaminants by adsorbing an α_1-rich Pevicon fraction with anti-normal rat serum (crosslinked with ethyl chloroformate by the method of Ternynck and Avrameas, 1976). Dimers and other contaminants were removed by gel filtration of RSA and α_1-AGP on Sephadex G-100. Antigen is diluted to 32 ng/ml in RIA buffer with 5% sucrose (diluent buffer) and stored in aliquots at -70 C. The antigen is serially diluted for each assay in diluent buffer.

3. AFFINITY CHROMATOGRAPHY

Two types of immunoadsorbents were used for affinity chromatography. The first type consists of antigen or antibody coupled to Sepharose 4B by an adipic acid dihydrazide succinate sidearm. The second type consists of protein coupled to the Sepharose by divinyl sulfone. Both types of immunoadsorbent have very low nonspecific adsorbtion properties.

a. Adipic Acid Dihydrazide sidearm-

Adipic acid dihydrazide was coupled to Sepharose 4B activated with cyanogen bromide (CNBr) (March et al., 1974).

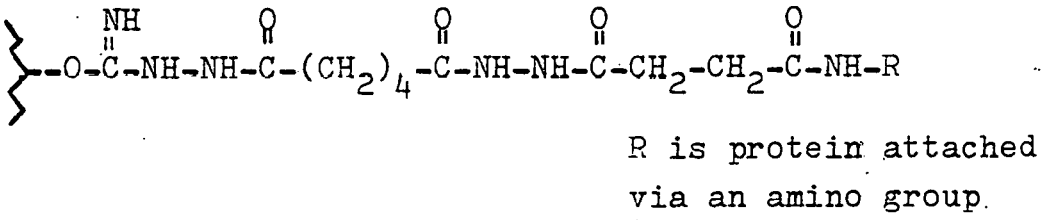


Figure 5: Sepharose 4B-adipic acid dihydrazide-succinate

For 100 mls of Sepharose, 30 g of CNBr and 10.5 g of adipic acid dihydrazide were used. The amount of adipic acid dihydrazide coupled to the agarose was determined by the trinitrobenzene-sulfonate (TNBS) test (Cuatrecasas, 1970; Failla and Santi, 1973; Wilchek and Miron, 1974). The free hydrazide groups were acylated with succinic anhydride (10 g per 100 mls Sepharose) in p-dioxane (Nishikawa and Bailon, 1975). The gel could be stored in this form in .02% NaN_3 at 4°C. Protein was coupled to the gel using 10 mg 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide HCl (EDC-CD) per ml Sepharose according to the Pharmacia instructions for CH-Sepharose. Typically, 10 µmoles adipic acid dihydrazide were coupled per ml Sepharose 4B. The

amount of protein coupled varied, but in all but one case was between 4.5 and 7.5 mg/ml Sepharose. The amount of protein coupled was determined on the basis of absorbtion at 280 nm. of supernatant. The Lowry procedure (1951) could not be used because the carbodiimide gives a very strong, positive reaction (unpublished observations).

The gel was poured into a Pharmacia column (K9/15 or K9/30) and washed free of unbound protein with 10 bed volumes of phosphate buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl) and two cycles of 10 volumes each of acetate buffer (.1M NaAcetate, pH 4.0, 1M NaCl) followed by bicarbonate buffer (.1M $NaHCO_3$, pH 9.0, 1M NaCl). The column was finally equilibrated with phosphate buffer.

For purification of specific antibody or antigen, the column was loaded with specific antiserum or normal rat serum, respectively. The serum was allowed to be in contact with the gel for at least 1 hour in the cold room. The column was then washed with phosphate buffer until the absorbance of the eluate at 280 nm had reached baseline. This was followed by 2 cycles of 5 bed volumes each of acetate and bicarbonate buffers. There was usually only a small amount of protein washed through in the first cycle. After reequilibration with phosphate buffer, the

specific protein was eluted with .2M glycine-HCl, pH 2.4 to 2.8. The eluate was collected into tubes containing sufficient borate buffer to raise the pH to 7.0. The specifically eluted fractions were tested for both specific and contaminating proteins by the method of Ouchterlony (1956), using whole rat serum or anti-whole rat serum. Meanwhile, the column was reequilibrated with phosphate buffer. The desired fractions were pooled and dialyzed against either water or dilute saline and then lyophilized.

b. Divinyl Sulfone sidearm-

The divinyl sulfone procedure for coupling proteins was much simpler.

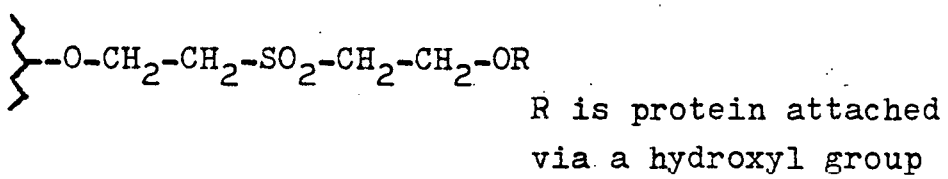


Figure 6: Sepharose 4B-Divinyl sulfone

100 mls of Sepharose 4B, in an equal volume of 1M Na_2CO_3, pH 11.0, were activated with 20 mls divinyl sulfone for 70

minutes at room temperature (Porath, 1974). The gel was washed with distilled water until the pH of the wash was 7.0. The gel was then stored in .02% NaN_3 at 4°C or used immediately. At least 2 mg of protein/ml Sepharose was added to the activated gel in .25M $NaHCO_3$, according to the procedure of Sairam and Porath (1976). After a 6 hour incubation at room temperature, either glycine or ethanolamine was added to block unreacted groups. The incubation was continued overnight. Based on absorbtion at 280 nm, approximately 2 mg protein were coupled per ml Sepharose.

Chromatography was carried out according to the procedure of Sairam and Porath (1976). The fractions eluted with NH_4OH were tested for the specific and contaminating proteins as for the adipic acid dihydrazide coupled columns. The desired fractions were pooled amd directly lyophilized.

4. IODINATION OF ANTIGEN AND ANTIBODY

a. Lactoperoxidase-

α_1-AGP was iodinated by the lactoperoxidase method as described by Krohn and Welch (1974). The iodinated protein was separated from lactoperoxidase and other reagents by affinity chromatography on a Sepharose-anti-α_1-AGP immunoadsorbent.

b. Bolton-Hunter-

α_1-AGP was reacted with the Bolton-Hunter reagent (3-4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester) (Bolton and Hunter, 1973) and was then iodinated by the iodine monochloride method (see below).

c. Iodine Monochloride-

Protein was iodinated with ^{125}I or ^{131}I to a low specific activity (usually less than 1 µCi/µg) by a modification of the iodine monochloride method of Bale et al (1966): .5 mg of purified antibody, dissolved in .5 ml of 2X Borate buffer, was put in a 13 x 100 mm borosilicate tube with a small magnetic bar. 10 µCi of ^{125}I or ^{131}I (.5 mCi/ml, in 1X Borate buffer),

as NaI, was added to the tube and stirring was started. The ICl reagent was diluted into 2M NaCl (1 to 1280) so that in .2 ml of this dilution there would be suficient I for 1I/molecule of antibody. .2 ml of this dilution was added to the tube with the protein and stirring was continued for 1 minute. Bovine serum albumin or normal rabbit serum was added as a protective protein. Free I was separated from the protein on a Sephadex G-25 column. The void volume fractions were pooled and frozen after the addition of more protective protein.

d. Oxidative Iodine Monochloride

Protein was iodinated to a high specific activity (greater than 5 μCi/μg) by a modification of the oxidative iodine monochloride method (Helmkamp et al., 1967):

1. 25 g of α_1-AGP or RSA are dissolved in .2 ml 1.5X Borate buffer, in a borosilicate tube. Let N = # moles protein.
2. KI is diluted, so that in 100 μl there are $\frac{2/3 \times N \times \#I/\text{molecule protein desired}}{\text{expected efficiency of iodination}}$ moles of KI.
3. KIO_3 is diluted so that its concentration is five times that of KI.

4. The following reagents are added to a tube in order:

   100 µl diluted KI

   1 to 10 µl NaI (^{125}I or ^{131}I) stock in 1X borate buffer or dilute NaOH

   100 µl 5M NaCl

   100 µl 2X distilled water

   100 µl diluted KIO_3

   100 µl 0.25N HCl

   The tube is shaken and allowed to incubate for 10 minutes.

5. After the 10 minute incubation, 10 µl of 3N NaOH are added to the protein.

6. The iodine mixture is immediately drawn up into a 1 ml tuberculin syringe with an 18 G, 1 1/2" needle, and is jetted into the protein.

7. After 1 minute, protective protein is added and free I is separated from protein on a Sephadex G-25 column, equilibrated with RIA buffer containing 4% BSA. Before being used for the RIA, the iodinated antigen is again passed through a Sephadex G-25 column to remove any protein which may have been lysed during storage at -20 C.

Specific activities of 10 to 20 µCi/µg were attainable with efficiencies between 33 and 60%.

5. DETERMINATION OF DESIRED ANTIBODY CONCENTRATION ON SEPHADEX

It is desired that functional antibody molecules be spaced far enough apart on the Sephadex so that the average inter-antibody distance is greater than the maximal specific polysome size.

Let r = radius of a ribosome = 1.15×10^{-6} cm.

l = interribosomal spacing = $.5 - 1.0 \times 10^{-6}$ cm.

n = maximum number of ribosomes per polysome

L = total length of polysome = $2rn + nl$

sa = surface area generated by polysome = πL^2

R = radius of swollen Sephadex G-25 superfine

= $1.0 - 4.0 \times 10^{-3}$ cm.

SA = surface area of Sephadex particle = $4\pi R^2$

For $n = 19$ and $l = 0.5 \times 10^{-6}$ cm. (calculations for RSA system; ribosomes are spaced as closely as possible):

$L = 5.32 \times 10^{-5}$ cm.

$sa = 8.89 \times 10^{-9}$ cm.^{2}

For $R = 2.5 \times 10^{-3}$ cm.:

$SA = 7.85 \times 10^{-5}$ cm.^{2}

There can then be $\frac{SA}{sa} = 8.8 \times 10^{3}$ antibody molecules per Sephadex

particle.
In 1 ml of swollen Sephadex there are $\frac{1 \text{ cm.}^3}{\frac{4 \pi R^3}{3}}$

$= 1.5 \times 10^7$ Sephadex particles

$= 2.3 \times 10^{-13}$ moles antibody

This is equivalent to 14.9 ng of RSA (M.W. = 66,000 daltons) binding capacity/ml Sephadex.

To determine the binding capacity of the immunoadsorbent once it is made, the following equations are used:

$$(Ag) + (Ab) \rightleftarrows (AgAb)$$

$$K = \frac{(AgAb)}{(Ag)(Ab)} = \frac{(Ag^*Ab)}{(Ag^*)(Ab)}$$

where Ag = antigen, not bound to antibody (free)

Ag* = labelled antigen (^{125}I), not bound to antibody (free)

Ab = antibody covalently bound to Sephadex, without antigen bound

AgAb = antigen-antibody complex

Rearranging the above equation,

$$\frac{\text{Bound antigen (B)}}{\text{Free antigen (F)}} = \frac{(Ag^*Ab)}{(Ag^*)} = \frac{(AgAb)}{(Ag)} = K(Ab)$$

Although an oversimplification, it has been assumed that all antibodies have equal avidity for the antigen, and that K is

therefore a constant. $\left(\frac{B}{F}\right)$ is maximum when only a tracer amount of antigen is incubated with the antibody. From the last equation, we see that $\left(\frac{B}{F}\right) = \frac{1}{2}\left(\frac{B}{F}\right)_{max}$ when half of the antibody molecules are bound with antigen.

Let $\frac{B}{T}$ = $\frac{\text{dpm bound to Sephadex}}{\text{Total dpm added to assay tube}}$

$\left(\frac{B}{T}\right)_{\frac{1}{2}}$ = $\frac{B}{T}$ value when $\frac{B}{F} = \frac{1}{2}\left(\frac{B}{F}\right)_{max}$

$(Ag)_{\frac{1}{2}}$ = concentration of antigen standard added to assay tube giving $\frac{B}{F} = \frac{1}{2}\left(\frac{B}{F}\right)_{max}$

Antigen binding capacity/ml = $2\ (Ag)_{\frac{1}{2}}(B/T)_{\frac{1}{2}}$/volume Sephadex.

For example, from the standard curves shown in Figures 7 and 8, the following parameters are obtained (Table IV):

FIGURE 7

Figure 7: Determination of Binding Capacity of Sephadex-Anti-RSA for RSA

0.1 ml Sephadex-anti-RSA is incubated with varying amounts of RSA (both labelled and unlabelled RSA concentrations are varied as shown in insert). Total reaction volume is 1.0 ml. Incubation is for 7 days in the cold room. RSA was iodinated by the oxidative iodine monochloride method.

DETERMINATION OF BINDING CAPACITY OF SEPHADEX-ANTI RSA FOR RSA

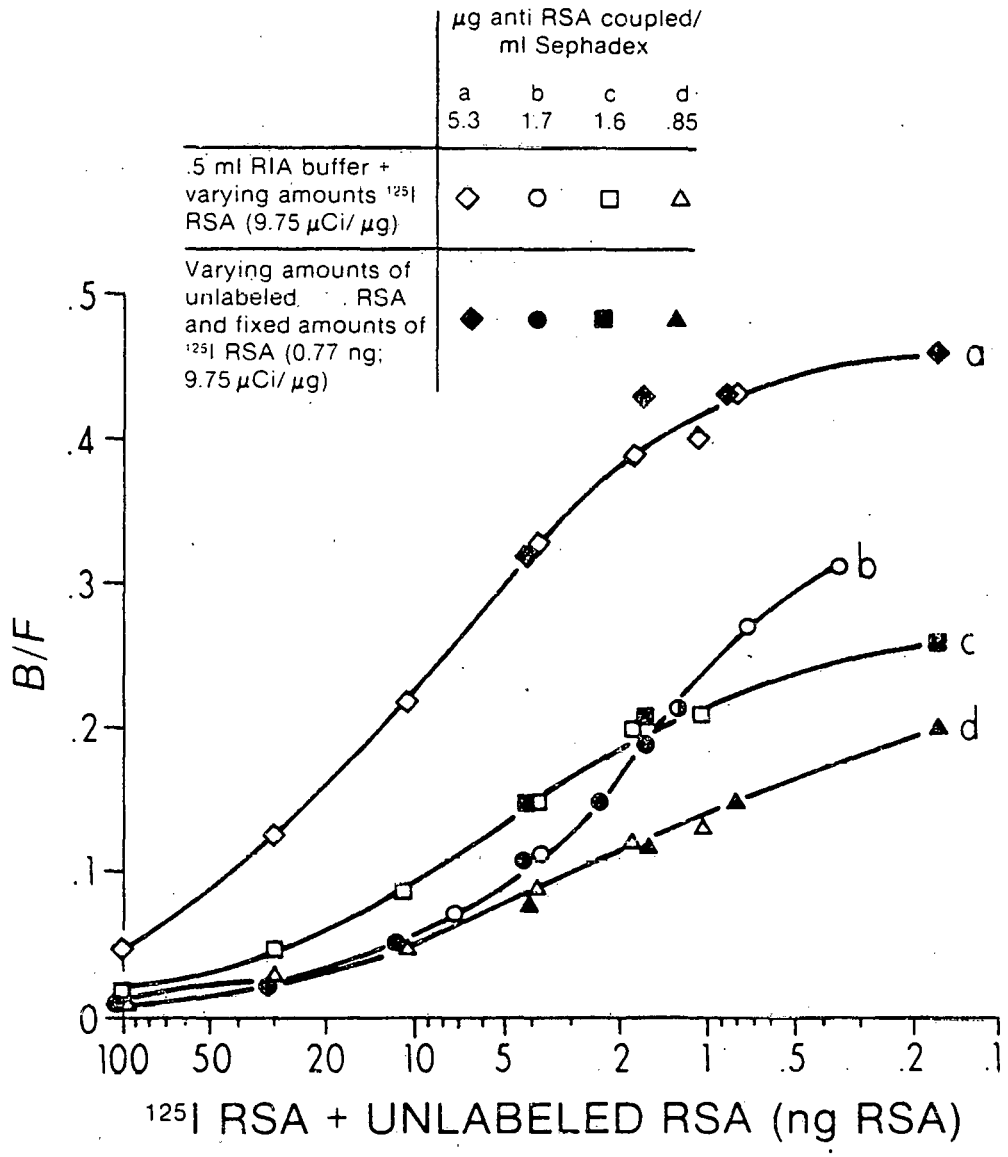


FIGURE 8

Figure 8: Determination of Binding Capacity of Sephadex-Anti-α_1-AGP for α_1-AGP

0.1 ml Sephadex-anti-α_1-AGP is incubated with varying amounts of α_1-AGP (both labelled and unlabelled α_1-AGP concentrations are varied as shown in insert). Total reaction volume is 1.0 ml. Incubation is for 7 days in the cold room. α_1-AGP was iodinated by the oxidative iodine monochloride method.

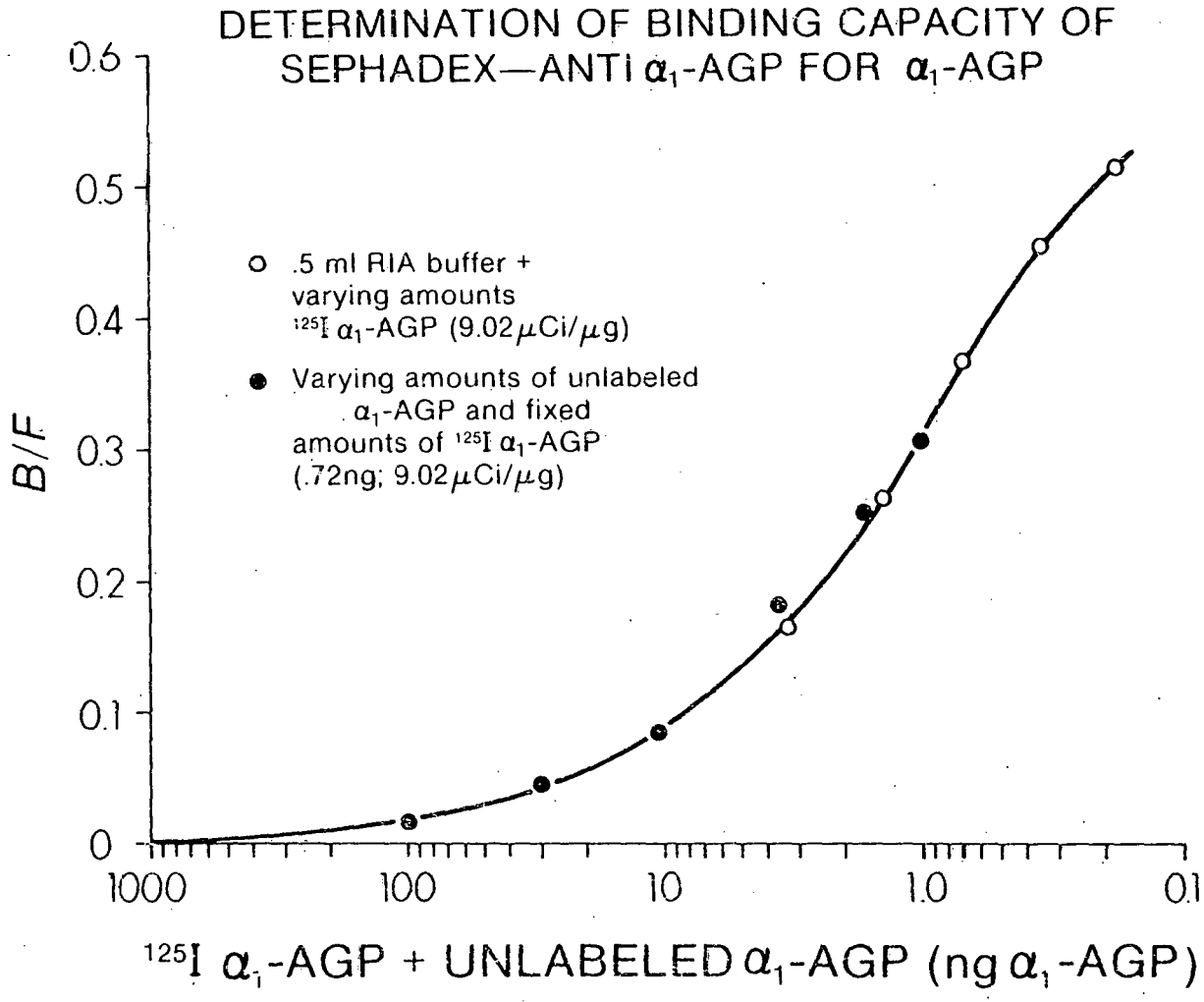

DETERMINATION OF BINDING CAPACITY OF
SEPHADEX—ANTI α1-AGP FOR α1-AGP
B/F
0.6
0.5
0.4
0.3
0.2
0.1
0
○ .5 ml RIA buffer + varying amounts 125I α1-AGP (9.02μCi/μg)
● Varying amounts of unlabeled α1-AGP and fixed amounts of 125I α1-AGP (.72ng; 9.02μCi/μg)
1000
100
10
1.0
0.1
125I α1-AGP + UNLABELED α1-AGP (ng α1-AGP)

Table IV: Binding Capacities of Sephadex Immunoadsorbents

0.1 ml Sephadex-anti-RSA or Sephadex-anti-α_1-AGP is incubated with varying amounts of RSA or α_1-AGP, respectively for 7 days in cold room. Total volume = 1.0 ml.

amount antibody coupled/ml Sephadex	$\left(\frac{B}{F}\right)_{max}$	$(Ag)_{\frac{1}{2}}$	$\left(\frac{B}{T}\right)_{\frac{1}{2}}$	capacity/ml Sephadex
5.3 µg	.46	10.5 ng	.187	39.2 ng RSA
1.7 µg	.34	1.95 ng	.145	5.7 ng RSA
1.6 µg	.27	5.2 ng	.119	12.4 ng RSA
0.85 µg	.22	2.3 ng	.099	4.6 ng RSA
----	.6	1.0 ng	.23	4.3 ng α_1-AGP

6. PREPARATION OF IMMUNOADSORBENT

a. Sephadex G-25 with adipic acid dihydrazide sidearm-

Antibody was coupled to Sephadex G-25, superfine with an adipic acid dihydrazide sidearm essentially as described in Affininty Chromatography section, except that protein was coupled directly to the hydrazido-Sephadex, without the

succinate extension (Wilchek and Miron, 1974). The quantities of reagents were, however, reduced as follows:

	Protein Purification	RIA
matrix	100 ml Sepharose	100 ml Sephadex
CNBr	30 g	168 µg
Adipic Acid Dihydrazide	10.5 g	188 µg
Succinic Anhydride	10 g	
EDC Carbodiimide	1 g	20 µg

b. Sephadex G-25 with divinyl sulfone sidearm-

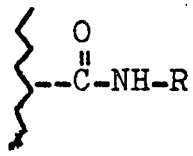
Anti-RSA or anti-α_1-AGP, purified by affinity chromatography, is coupled to Sephadex G-25 superfine by a divinyl sulfone linkage (see Affinity chromatography). 10 µl of DVS is dispersed into 10 mls of 1M Na_2CO_3 (1:1000 dilution) and part of this is added to the Sephadex. The extent of mixing of the DVS in the carbonate is probably extremely critical- 0.02 to 10 µl DVS/100 ml Sephadex have given similar binding capacities of the resultant immunoadsorbant. It might be worthwhile to homogenize (with Virtis homogenizer) the DVS in the carbonate to ensure a homogeneous solution.

Excess protein (2 mg/100 ml Sephadex) was added to the activated gel in order to drive the reaction to completion.

Glycine is added to block activated sidearms to which protein is not coupled. The extent of coupling was measured by inclusion of a tracer amount of iodinated antibody with the cold antibody. The immunoadsorbent is washed with bicarbonate and then acetate buffers. It is sonicated while still in the acetate buffer. The sonication helps to release free DVS and protein. After turning end over end overnight, (at about 60 rpm) the Sephadex is suspended in the RIA buffer at a concentration of 30% (v/v).

c. Bio-Gel P-2

Bio-Gel P-2 was tried as an alternative to Sephadex as an insoluble matrix.



R is protein attached via a carbon

Figure 9: Bio-Gel P-2

Normal rabbit IgG was coupled to Bio-Gel P-2 according to the procedure of Inman and Dintzis (1969), except that concentrations of all reagents were reduced. The procedure is

as follows: 1 g P-2 (-400 mesh) is swelled in 4.5 ml distilled water. 0.3 ml of a 1:10 dilution of hydrazine is added. This is incubated at 50°C for 10 minutes. The gel is washed with .10M NaCl on a Buchner funnel with Whatman #1 filter paper (it would be better to use a paper with a smaller pore size). The gel is washed with .3N HCl and then resuspended (only 1.5 ml gel remained) in 15 ml .3N HCl and cooled in an ice bath. 1 ml 1M $NaNO_2$ is added to the gel in a 30 ml beaker. After 20 minutes at 3 C, the gel is washed on a funnel with 200 ml .3N HCl, 100 ml .1M sulfamic acid, and then 150 ml cold water. The gel is resuspended in 10 ml of 4.6 mg/ml 1,7 hexane diamine (in .1M Borax) and stirred for 1 hour in an ice bath. 2 ml of 1M NH_4OH, 1M NH_4Cl are added and stirring is continued for another 2 hours. The gel is then washed with 200 ml .2N NaCl and then H_2O. It was left in the cold room overnight. 0.6 ml of P-2-hexane diamine is adjusted to pH 4.2 with .4M HAc. 4.7 mg carbodiimide is added and mixing continued for 2 minutes. Normal rabbit IgG (.25 mg) is added and mixing continued overnight. Glycine is then added to block any active groups remaining. Incubation is continued for another day. The gel is then washed as for other immunoadsorbents. 12% of the protein (48μg/ml gel) was bound to the P-2.

7. POLYSOME PREPARATION

Polysomes are prepared as described below and then thawed in the cold room and diluted with 3 volumes of RIA buffer, but with 4/3 the indicated concentrations of NaN_3, Tween 20, and BSA, giving final concentrations equal to that of the diluent buffer. Further dilutions are made with the diluent buffer.

C. POLYSOME ISOLATION PROCEDURES

1. Mg^{++} PRECIPITATION PROCEDURES

Two different procedures were used to isolate polysomes by Mg^{++} precipitation. The procedure of Palmiter (1974) was followed according to instructions. The procedure of Ilan (1976) is given in more detail because parts of it are used extensively in this research:

Buffer A	50 mM	Tris-HCl	pH 7.4
	25 mM	KCl	
	5 mM	MgCl	
	250 mM	sucrose	
	1 mM	DTT	
	5 µg/ml	cycloheximide	

Buffer B	100 mM	MES	pH 6.0
	300 mM	KCl	
	20 mM	MgCl	
	1 mM	DTT	
	5 µg/ml	cycloheximide	

Buffer C	100 mM	TES	pH 7.5
	25 mM	KCl	
	1 M	sucrose	
	1 mM	DTT	
	5 µg/ml	cycloheximide	

Buffer D	100 mM	Tris-HCl	pH 7.4
	25 mM	KCl	
	10 mM	MgCl	
	1 mM	DTT	
	5 µg/ml	cycloheximide	

Buffer E	100 mM	TES	pH 7.5
	300 mM	KCl	
	1 mM	DTT	
	5 µg/ml	cycloheximide	

1. Rinse liver in ice cold buffer A
2. Homogenize diced liver in 3 volumes Buffer B using 5 strokes of the loose pestle of a Dounce homogenizer.
3. Add 1/9 volume 10% Triton X-100, homogenize with 3 more strokes and let sit in cold room for 5 minutes
4. Centrifuge for 5 minutes at 2000 g and 10 minutes at 12,000 g
5. Add 1/9 volume 1M $MgCl_2$, adjust pH to 5.5 with 1M acetic acid, let sit at 4 C for 1 hour
6. Layer supernatant over Buffer C, centrifuge at 30,000 g for 10 minutes
7. Resuspend polysomal pellet in Buffer E using gentle homogenization
8. Clarify polysomal suspension by centrifugation at 12,000 g for 10 minutes

2. GEL FILTRATION PROCEDURES

K 26/40 and K 26/100 Pharmacia columns with adapters were used for gel filtration of post-mitochondrial supernatants and polysomes. Samples were applied from the barrel of a syringe through an 18 g needle and capillary tubing. Elution was in an upwards direction. Columns were run in the cold room. Unless otherwise noted, PMS was prepared according to the procedure of Ilan.

3. FINAL POLYSOME ISOLATION PROCEDURE

a. Treatment of rats

1. Untreated rats are allowed water and food ad libitum
2. Stressed rats are injected with 1.0 ml turpentine subcutaneously at the designated time
3. Fasted rats are allowed water, but food is removed for 144 hours (6 days) prior to liver excision
4. Fasted and then refed rats are fasted as in #3 above and are then refed for the designated time prior to liver excision
5. Livers of experimental animals were excised between 7:30 and 9:00 p.m. No time schedule was kept for excision of

livers used only to set up the isolation procedure.

6. Male Sprague-Dawley rats weighed 350 to 450 g at the time treatment was started

b. Procedure

All buffers refer to corresponding ones of Ilan procedure.

1. Excise 5 to 6 g liver, rinse in Buffer A, wrap in aluminum foil, and freeze between 2 blocks of dry ice.
2. Mince liver in 3 volumes of Buffer B; homogenize with 5 strokes in a size C Potter-Elvehjem homogenizer, clearance = .15 to .33 mm.
3. Add 1/9 vol. 20% (w/v) Triton X-100 to the homogenate to free endoplasmic reticulum from the nucleus. Homogenize with 3 more strokes.
4. After waiting 5 minutes, centrifuge for 5 minutes at 2000g and 10 minutes at 12000 g (HB-4 rotor, Sorvall RC2-B centrifuge, 0°C.
5. Add 1/9 volume of sodium deoxycholate (DOC) to the supernatant (PMS) to free the bound polysomes from the rough endoplasmic reticulum. Homogenize as in #2, above..
6. Centrifuge as above for 5 minutes at 5,000 g to remove small amount of insoluble material.
7. Layer 8.1 ml of supernatant over sucrose gradients made

in Buffer D with 80 units/ml heparin, as in Figure 10; Centrifuge for 12 hours at 174,500 g (SW-41 rotor, Beckman L2-65B ultracentrifuge, 0°C).

8. Freeze and store tubes in an upright position in a -70 freezer.
9. Cut tubes with a hacksaw 2 11/16" from the top.
10. Thaw bottom portions of the tubes in the cold room and dilute to 10 ml with Buffer D. Homogenize polysomes with a Ten Broeck homogenizer (clearance = .1 to .18 mm); centrifuge to remove any insoluble material.
11. Centrifuge as in #7, above, but for 20 hours.
12. Freeze, store, and cut tubes as in #8 and #9, above. Dilute to 8.5 ml, as above, but with Buffer D containing no DTT or cycloheximide.
13. After centrifugation to remove insoluble material, store polysomes in -70°C freezer in polypropylene tubes.

4. POLYSOME PROFILES

20%-50% (w/w) sucrose gradients in Buffer D (Ilan, 1976) were formed with a Beckman gradient former into 9/16" x 3 1/2" cellulose nitrate tubes. 0.5 ml samples were layered over 12 ml gradients and centrifuged for 90 to 120 minutes at 240,000 g in

Figure 10: Sucrose Gradients for Isolation of Polysomes

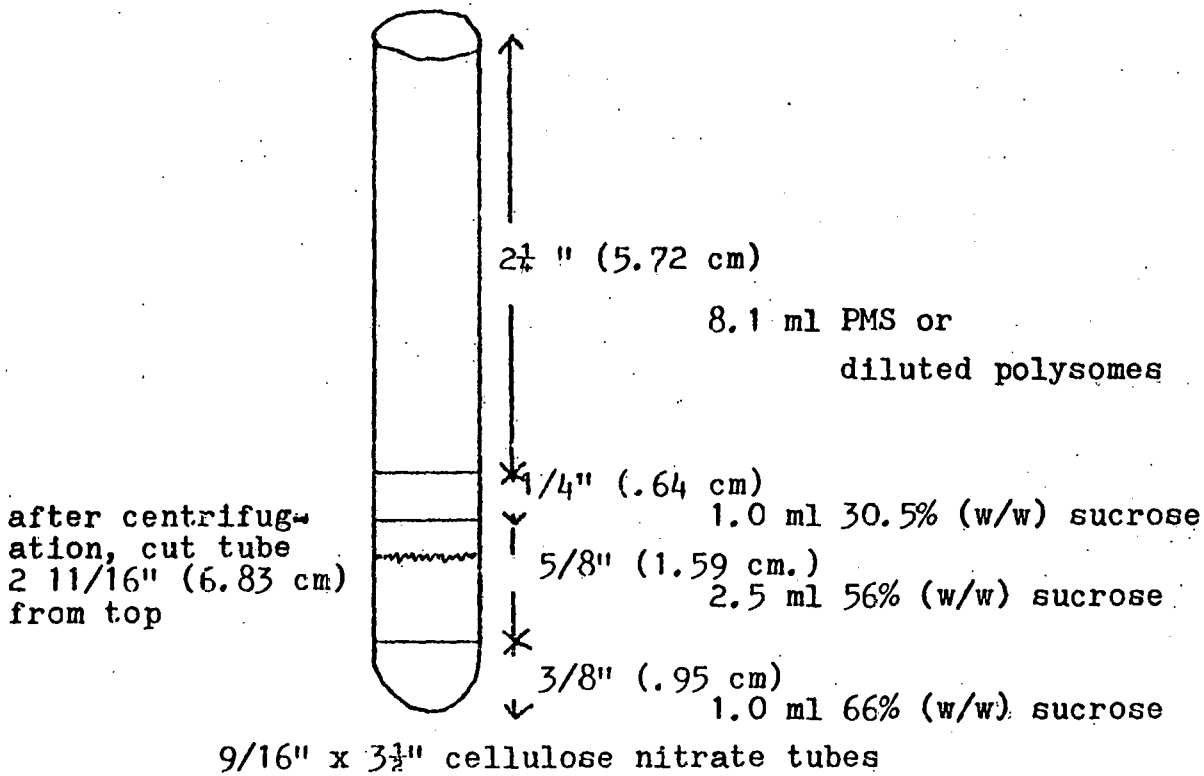


FIGURE 10

an SW 41 rotor. Gradients were eluted using a Beckman eluter. Cesium chloride was pumped into the bottom of the tube and fractions were collected from the top of the tube. Absorbtion at 254 nm was monitored with an ISCO UV monitor.

D. CALCULATION OF RESULTS

The amount of polysomes synthesizing either RSA or a_1-AGP have been expressed in three ways:

$$A = \frac{\mu g \text{ antigen}}{mg \text{ polysomal RNA}} = \left(\frac{\mu g \text{ antigen - background}}{OD_{260} \text{ unit}}\right)\left(\frac{OD_{260} \text{ units}}{mg \text{ polysomal RNA}}\right)$$

This expresses, on a relative scale, the fraction of RNA which is engaged in the synthesis of a particular protein.

$$B = \frac{\mu g \text{ antigen}}{mg \text{ homogenate DNA}} = \frac{A \times mg \text{ polysomal RNA}}{mg \text{ homogenate DNA}}$$

This expresses the number of polysomes/mg DNA engaged in the synthesis of the specific protein. It is based on the actual amount of RNA recovered in the polysome region after the first ultracentrifugation step.

$$C = \frac{\mu g \text{ antigen}}{mg \text{ homogenate DNA}} = \frac{A \times 0.8 \times mg \text{ homogenate RNA}}{mg \text{ homogenate DNA}}$$

This expresses the number of polysomes/mg DNA engaged in the synthesis of the specific protein. However, in contrast to B, C assumes that 80% of homogenate RNA is polysomal RNA, even though

actual recoveries of RNA in the polysome region may have been much lower. For RSA synthesizing polysomes, all three terms (A, B, and C) have been set = 1.0 for polysomes from normal rat liver. Values for other samples are based on normal rat liver = 1.0. The actual values obtained for normal polysomes are:

1) A = .071 ± .015 µg RSA/mg RNA

2) B = .111 ± .018 µg antigen/mg DNA

3) C = .164 ± .033 µg RSA/mg DNA

E. MISCELLANEOUS PROCEDURES

1. RNA DETERMINATION

RNA was extracted by the method of Munro and Fleck (1966) from homogenates, post-mitochondrial supernatants and polysome samples. The amount of RNA was determined by measurement of optical density at 260 nm. (1 $O.D._{260}$ unit = 32 µg RNA; light path length = 1 cm.). One time, polysomes were labelled by i.p. injection of 6-^{14}C-orotate (15 µCi, 35 µCi/mg) into 400 g rats 24 and 48 hours prior to liver excision. Relative amounts of RNA were then determined as dpm ^{14}C. Polysomes were labelled to .02 µCi/mg polysomal RNA.

2. DNA DETERMINATION

DNA was determined on the final PCA precipitates of the RNA extractions (Munro and Fleck, 1966) by the Ceriotti procedure (Ceriotti, 1952), as modified by Short et al (1968).

3. PROTEIN DETERMINATION

Protein was determined by the procedure of Lowry et al (1951), using BSA as a standard.

4. SPECIFIC SERUM PROTEIN DETERMINATION

Serum concentrations of RSA and α_1-AGP were determined by the Mancini (1965) single radial immunodiffusion method, as modified by Fahey and McKelvey (1965).

5. BASIC IMMUNOCHEMICAL TECHNIQUES

Ouchterlony was performed in 1.2% agar in either a phosphate or veronal acetate buffer. Immunoelectrophoresis was performed according to directions in the LKB manual. Slides were washed and stained with either Coomassie Blue or Amido Black. For autoradiographs, the indicated iodinated protein (1500 to 150,000 dpm) was used for immunoelectrophoresis or double radial immunodiffusion. After washing and staining, slides were incubated with Kodak no-screen X-ray film. Incubation was for a few hours to 10 days, depending upon

how much radioactivity was used. Film was developed according to directions.

6. GAMMA COUNTING

A Nuclear Chicago (model 4230 or 4233) automatic gamma counter with the following settings was used:

	^{125}I	^{131}I
high voltage	630 volts	630 volts
attenuator		
coarse	1	4
fine	27.5	24.5
differential	wide	wide
base	0.96	3.0
window	2.40	1.40
efficiency	67%	40%

7. LIQUID SCINTILLATION COUNTING

A Packard Tri-Carb liquid scintillation counter (Model 2002) was used to count ^{14}C labelled samples (aqueous) in Aquasol. The following settings were used:

gain-50%

window-0.50 to ∞

efficiency-84% for 1 ml aqueous sample in 10 ml Aquasol

III. RESULTS AND DISCUSSION

A. RADIOIMMUNOASSAY

1. AFFINITY CHROMATOGRAPHY - ISOLATION OF ANTIGENS AND ANTIBODIES

a. Immunoadsorbents

Using the adipic acid dihydrazide succinate sidearm for coupling protein to Sepharose reduced the problem of nonspecific binding which is associated with simple alkane sidearms (Nishikawa and Bailon, 1975). Contamination in specifically eluted proteins was not detected by Ouchterlony. However, after 2 or 3 cycles, the capacities of the immunoadsorbents were greatly reduced so that they were no longer useful for purifying protein. This may be due to denaturation of the coupled protein or to its leakage from the Sepharose. Wilchek and Miron (1974) recommend using a polyhydrazide instead of the adipic acid dihydrazide to overcome the latter problem. The multiple attachment points of the polyhydrazide make it improbable that

the sidearm will become dissociated from the Sepharose.

The adipic acid dihydrazide sidearm worked extremely well for the RSA - anti-RSA system. The yield of purified protein was very high for the first two cycles of the column. No contaminants were detected in the RSA and anti-RSA purified in this manner. There were, however, two problems associated with the α_1-AGP - anti-α_1-AGP system:

1) The α_1-AGP did not couple to the Sepharose as well as RSA did (this procedure was only tried once, so a general conclusion is not valid); and

2) Anti-α_1-AGP was eluted from the Sepharose-α_1-AGP adsorbent to only a very small extent by glycine buffers of pH as low as 2.2. As the pH was raised with the phosphate buffer, a large amount of specific antibody was eluted from the column. The reason for this is not known, but it is possible that antibody is eluted at the low pH but is insoluble until the pH is raised.

The divinyl sulfone (DVS) sidearm has several advantages over the adipic acid dihydrazide succinate sidearm:

1) the coupling procedure was both simpler and less time consuming;

2) The gel did not lose its capacity to specifically bind protein even after repeated usage;

3) The eluted protein could be lyophilized without prior dialysis. However, not all of the lyophilized material eluted from the column could be solubilized in either distilled water or normal saline. This insoluble material is not soluble in dilute base (.1 N NaOH) and is therefore probably not protein. Insoluble material could be eluted from Sepharose containing no coupled protein. This material is therefore probably merely a breakdown product of the Sepharose.

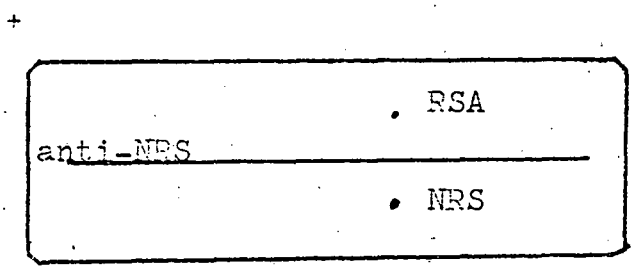
b. Purification of RSA

RSA, purified by affinity chromatography, showed no contamination by immunoelectrophoresis (Figure 11). It was filtered through Sephadex G-100 to remove any aggregates (see Figure 17).

c. Purification of α_1-AGP

α_1-AGP, purified from acute phase serum by affinity chromatography, contains several contaminants (Figures 12a,b). Despite these contaminants, the autoradiographs (Figures 13a-d) show that α_1-AGP is the most heavily iodinated. Figures 13c and d do not reveal any cross contamination between the iodinated RSA and α_1-AGP preparations.

FIGURE 11: IMMUNOELECTROPHORESIS OF RAT SERUM ALBUMIN PURIFIED BY AFFINITY CHROMATOGRAPHY



RSA- 2 µl @ 0.8 mg/ml rat serum albumin purified by affinity chromatography

NRS- 2 µl undiluted normal rat serum

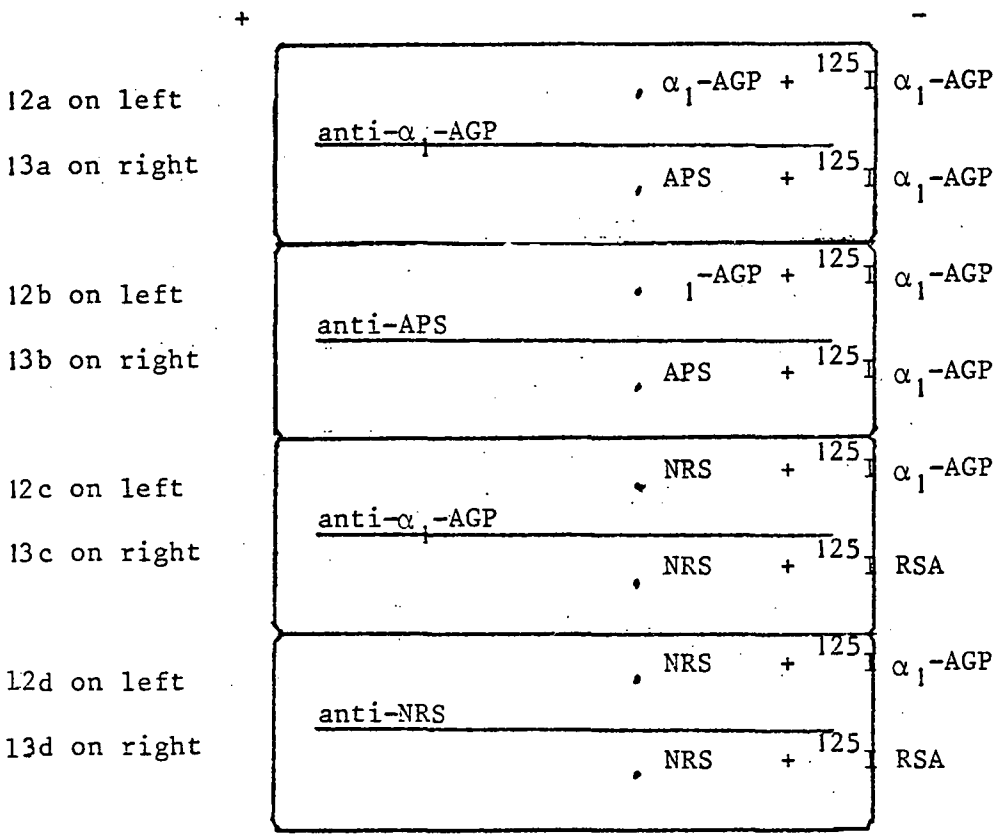
anti-NRS- 0.1 ml undiluted rabbit anti-NRS antiserum

Immunoelectrophoresis was as described in Materials and Methods

FIGURE 12 AND FIGURE 13

Figure 12: Immunoelectrophoresis of α_1-Acid Glycoprotein

Figure 13: Autoradiography of Figure 12



Immunoelectrophoresis was as described in Materials and Methods.

1 to 2 µl antigen and 0.1 ml antiserum were used.

NRS - normal rat serum

APS - acute phase serum

α_1-AGP - α_1-acid glycoprotein, purified by affinity chromatography @ 7mg/ml

antisera were raised in rabbits and used undiluted

$^{125}\alpha_1$-AGP - α_1-AGP iodinated by the oxidative ICl method; 150,000 dpm

^{125}RSA - RSA iodinated by the oxidative ICl method; 150,000 dpm

13a,b,c were exposed to X-ray film for 62 hours

13d was exposed for 14 hours

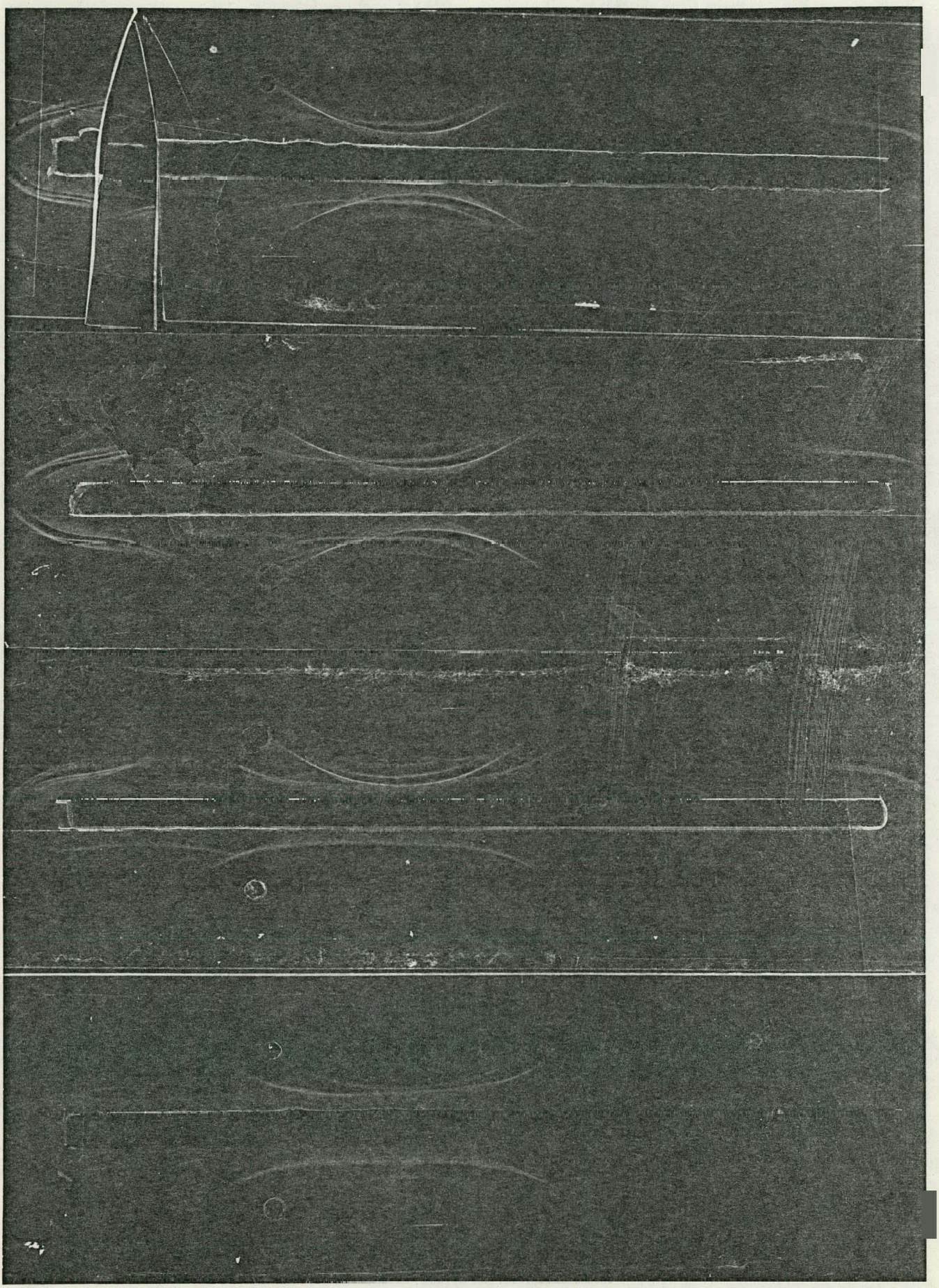
Figures 12 and 13, continued

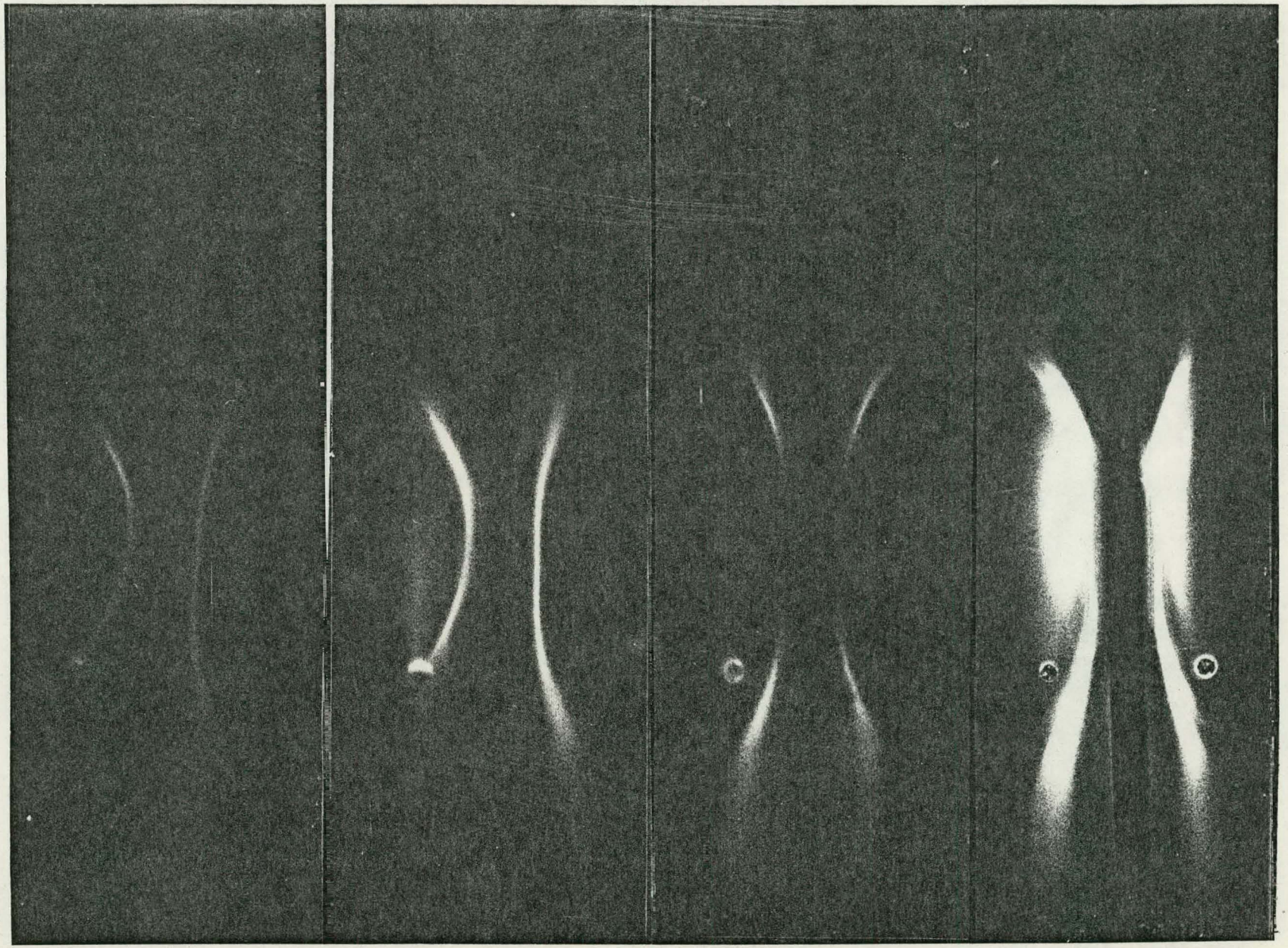
12a and 13a: Anti-α_1-AGP antiserum is used to demonstrate the contaminants in the affinity purified α_1-AGP. The autoradiograph is purposely overexposed to show the contaminants in the iodinated preparation.

12b and 13b: Same as 12a and 13a, but anti-APS is used instead of anti-α_1-AGP. Note that the autoradiography does not show any contaminants. Comparison of 12b with 12a shows that anti-APS contains antibodies to many proteins in APS that are not in anti-α_1-AGP. However, anti-APS contains fewer antibodies to proteins in the α_1-AGP preparation than does anti-α_1-AGP.

12c and 13c: 12c shows all of the proteins in NRS with which anti-APS reacts. Although the X-ray film is overexposed, the $^{125}I\alpha_1$-AGP and ^{125}I RSA each appear pure. The arcs formed by the two proteins are quite distinct from each other.

12d and 13d: Same as 12c and 13c, but using anti-NRS. The film is properly exposed.





Two methods were tried to remove the contamination from the α_1-AGP:

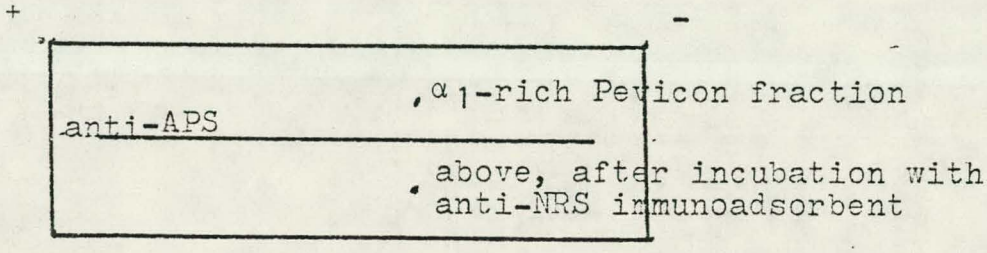
1) Since anti-normal rat serum does not react strongly with α_1-AGP, it was thought that an immunoadsorbent made from anti-NRS might be capable of removing the contaminants. The antiserum was cross-linked with ethylchloroformate. As a trial run, an α_1-rich Pevicon fraction (from the electrophoresis of acute phase serum) was adsorbed with the immunoadsorbent. As shown by immunoelectrophoresis, (Figure 14), some of the contaminants in the β and γ regions were removed. It did not appear, however, that all contaminants could be removed by this method. It was therefore rejected.

2) The affinity chromatography purified α_1-AGP was electrophoresed by Pevicon block electrophoresis (Figures 15a-f, 16). All fractions were dialyzed, lyophilized and dissolved to 3.5 mg/ml for immunoelectrophoresis. As can be seen, fractions 20-24 appear to be pure by staining, but autoradiography reveals a slight heterogeneity. All autoradiographs appeared similar, so only that corresponding to Figure 15c is shown. Using anti-α_1-AGP antiserum, instead of anti-APS antiserum, revealed more impurities in the fractions. They were all minor impurities. Later fractions do contain contaminants. It should be noted that higher numbered fractions (those migrating further

FIGURE 14

Figure 14: Immunoelectrophoreseis of α_1-rich Pevicon Fraction Adsorbed by Anti-Normal Rat Serum Immunoadsorbent

An α_1-rich pevicon fraction (containing 40 mg protein) was incubated with 20 ml anti-NRS, polymerized with ethyl chloroformate. The supernatant after incubation was dialyzed against distilled H_2O, lyophilized, and reconstituted to the original volume.



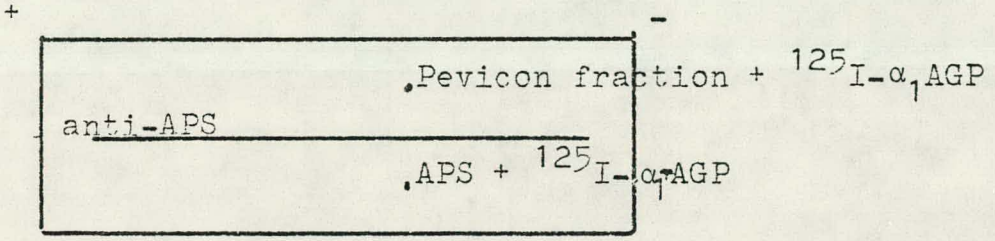
APS- acute phase serum

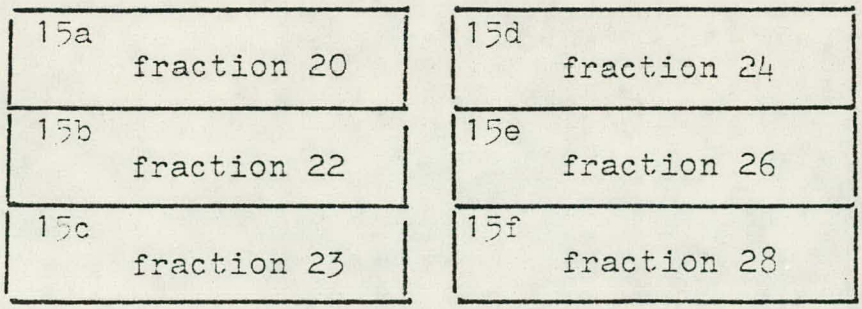
FIGURE 15 AND FIGURE 16

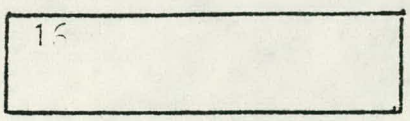
Figure 15: Immunoelectrophoresis of Affinity Chromatography Purified α_1-Acid Glycoprotein, Separated by Pevicon Block Electrophoresis

Figure 16: Autoradiography of Figure 15c

α_1-AGP was purified by affinity chromatography. This preparation was electrophoresed through Pevicon. The Pevicon fractions were dialyzed, lyophilized, and reconstituted to 3.5 mg/ml. Anti-APS was used to show the proteins in each of these fractions by immunoelectrophoresis.





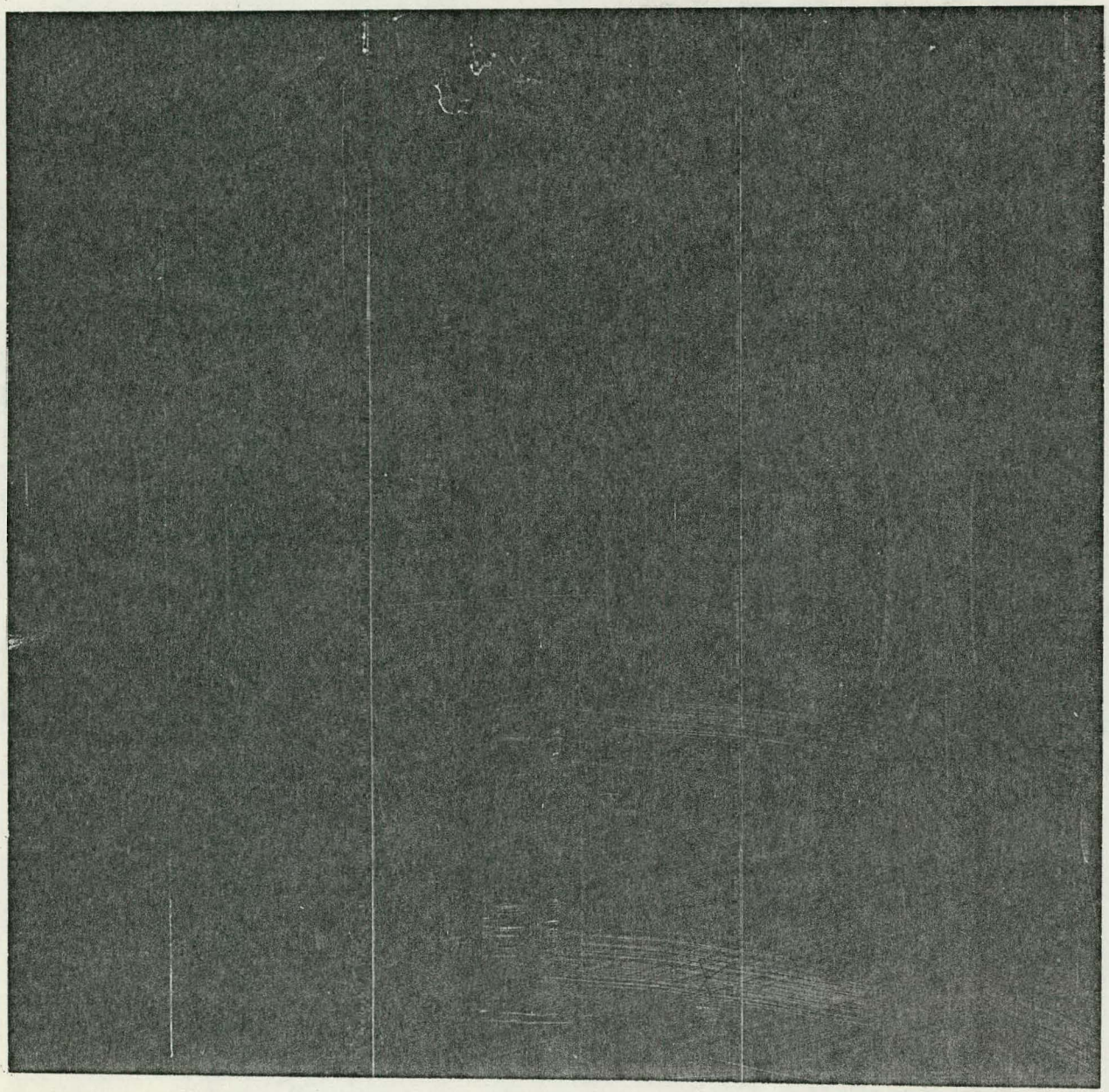


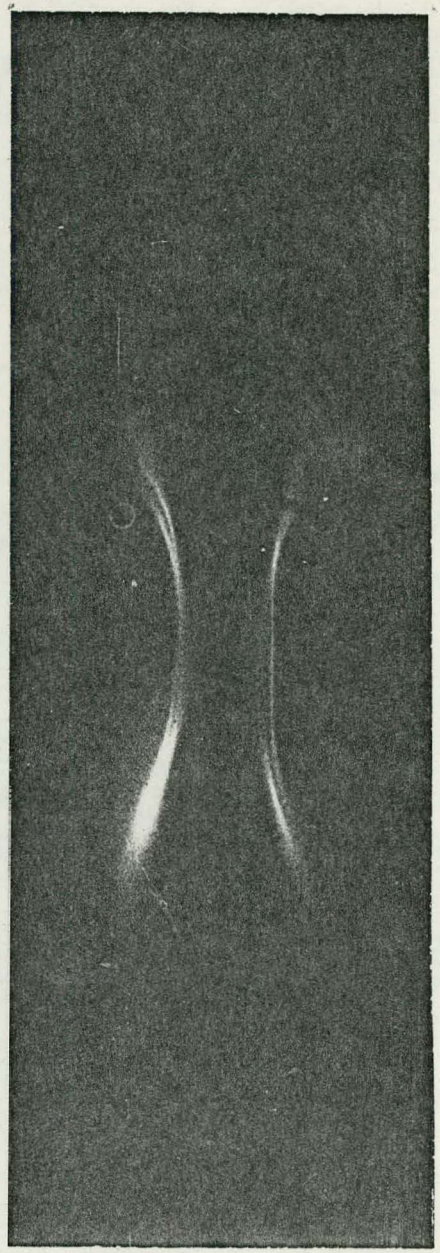
Pevicon fraction- number of fraction indicated in each slide

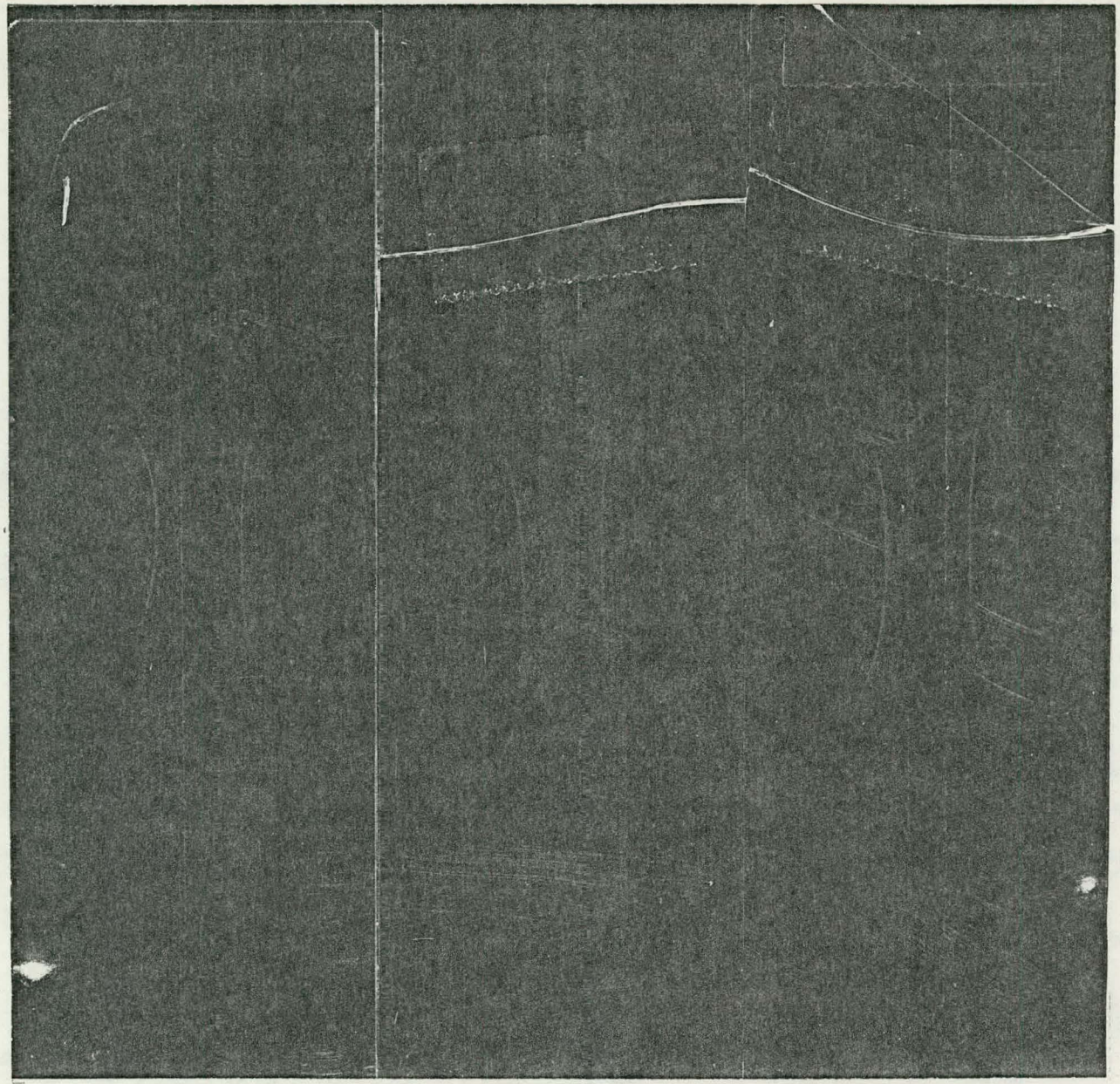
APS- 1 to 3 dilution of APS

anti-APS- .075 ml, undiluted

^{125}I-α_1AGP- 150,000 dpm. affinity purified α_1-ACP was iodinated by the oxidative monochloride method







toward the anode during Pevicon electrophoresis) again migrated further during immunoelectrophoresis. This indicates a heterogeneity in the α_1-AGP preparation. This may be due to either heterogeneity in the serum or reduction in mobility caused by removal of some charged groups (carbohydrates) during purification.

Aliquots of fractions 23 and 24 were iodinated, to be used in the RIA. The remainder of fractions 19 to 29, although containing some impurity, was pooled. Contaminants with β and γ mobility had been removed. Immunoelectrophoresis and autoradiography of the iodinated protein did not show any impurities.

The pooled Pevicon fractions were applied to a G-100 column. This separated the α_1-AGP into high (HMW) and low (LMW) peaks (Figure 17). The LMW α_1-AGP peak elutes in approximately the same volume as RSA. However, the reported molecular weights of RSA and α_1-AGP are 66,000 and 45,000, respectively. This discrepancy may be due to either the aberrant behavior of glycoproteins on gel filtration or to differences in the amount of carbohydrate, depending upon purification procedure. No carbohydrate analysis has been performed to compare it with that by

FIGURE 17

Figure 17: Gel Filtration of Rat Serum Albumin and α_1-Acid Glycoprotein through Sephadex G-100

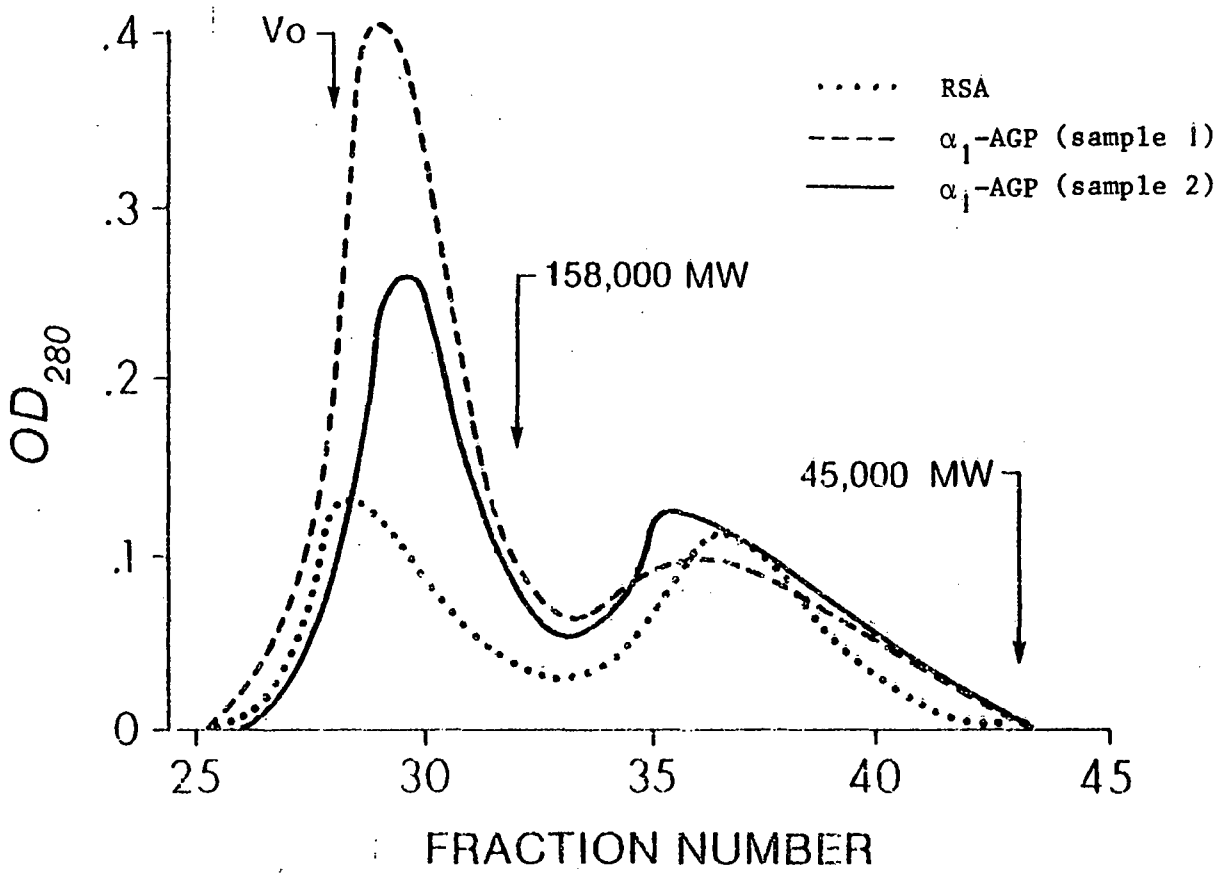
A 2.5 cm x 80 cm Pharmacia column, packed with Sephadex G-100 was equilibrated with .04M $(NH_4)_2CO_3$. For molecular weight determinations, the column was calibrated with blue dextran, aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease A. RSA (purified by affinity chromatography on an anti-RSA immunoadsorbent followed by passage through a Conconavalin A-Sepharose column to remove glycoproteins) and α_1-AGP (purified by affinity chromatography on an anti-α_1-AGP immunoadsorbent followed by Pevicon block electrophoresis) were separately filtered through the column to separate monomers from aggregated protein.

4.5 ml fractions were collected.

RSA fractions 34 to 43 were pooled and lyophilized. This is the RSA monomer used in the radioimmunoassay.

α_1-AGP fractions 33 to 44 were pooled and lyophilized. This is the α_1-AGP monomer used in the radioimmunoassay.

α_1-AGP fractions 26 to 32 (high molecular weight peak) were pooled and treated with .01M Tris, pH 5.5, .1M β-mercaptoethanol at 37° for 1 hour. The protein was reapplied to the G-100 column, which had been reequilibrated with this buffer. The protein eluted in the same fractions as before (26 to 32).

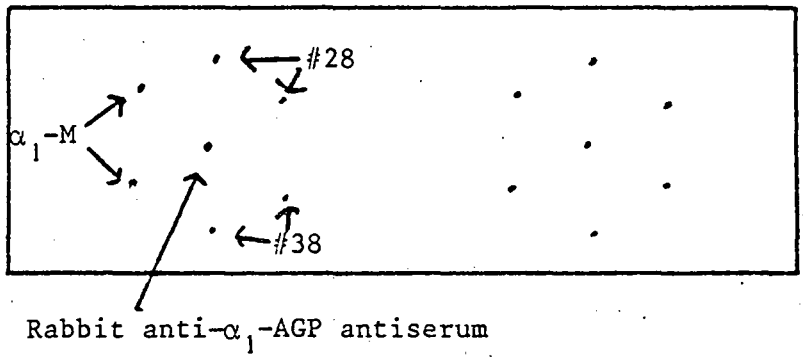

Vo
158,000 MW
45,000 MW
RSA
α1-AGP (sample 1)
α1-AGP (sample 2)
OD280
.4
.3
.2
.1
0
25
30
35
40
45
FRACTION NUMBER

Gordon and Louis (1969). Ouchterlony (Figure 18) shows at least 2 precipitin lines between anti-α_1-AGP and the HMW peak (fraction #28). The outer, sharp line is continuous with the line with α_1-Macroglobulin (α_1-M). The inner, diffuse line(s) is continuous with those of the LMW peak (fraction #38). There is a lack of cross reactivity between the LMW peak and α_1-M. Filtration of normal rat serum through Sephacryl S-200 confirmed these results. The G-100 fractions were immunoelectrophoresed against anti-α_1-AGP (Figure 19). α_1-M was included with the G-100 fraction in the bottom well of each immunoelectrophoresis slide. It was hoped that this would show the α_1-M line in comparison with the α_1-AGP line. The α_1-M line, however, could not be distinguished. More of this protein should have been applied to the slides. It can be seen that immunoelectrophoresis of fractions 28-32 of the G-100 column gives longer lines and greater mobility than that of the later fractions. Fractions 33-35 appear to be pure and fractions 36-38 have a contaminant with slightly greater mobility. The monomer peak (fractions 33 to 44) was pooled and iodinated. This iodinated α_1-AGP was used in the RIA. The pooled monomer peak is seen to contain one contaminant with α_1 mobility (Figure 20).

FIGURE 18

Figure 18: Ouchterlony to Show the Degree of Cross Reactivity Amongst α_1-Macroglobulin and the High and Low Molecular Weight α_1-Acid Glycoprotein Fractions of G-100 Column

For procedure, see legend to Figure 17.



α_1-M - α_1-Macroglobulin

#28 - high molecular weight α_1-AGP peak

#38 - low molecular weight α_1-AGP peak

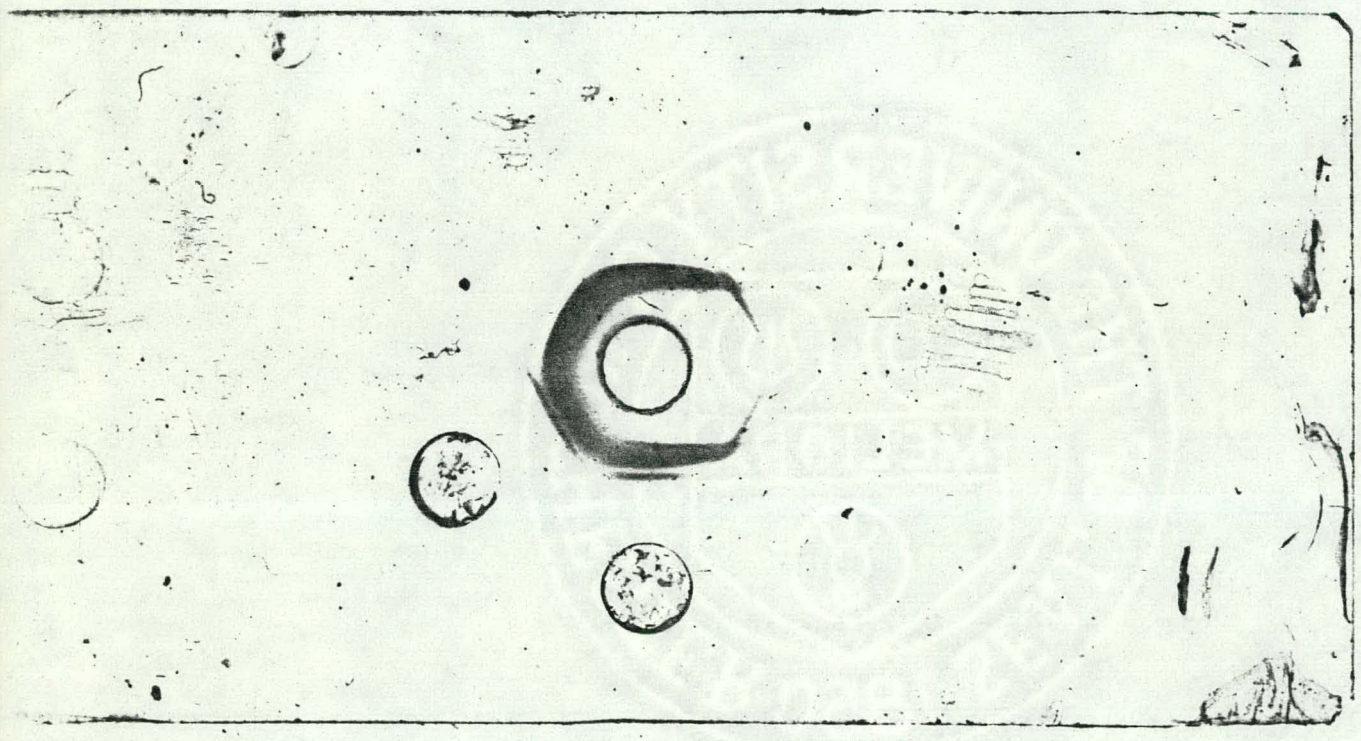


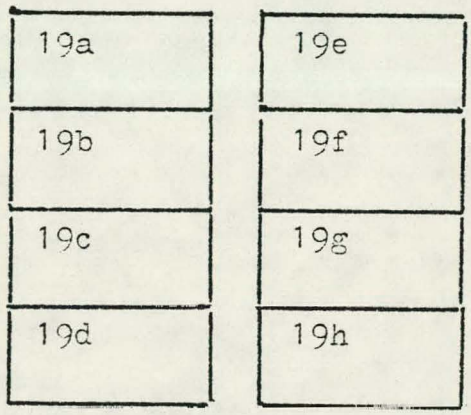
FIGURE 19

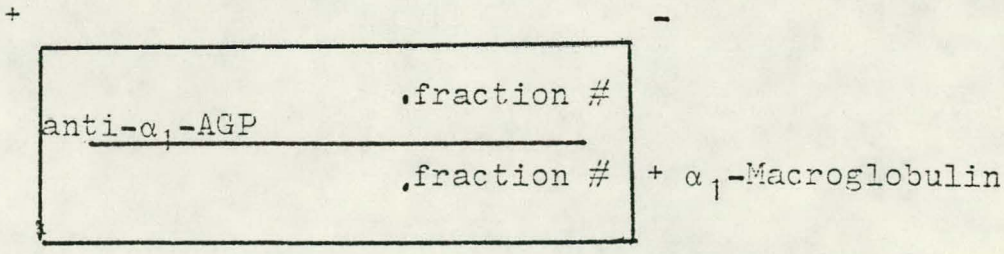
Figure 19: Immunoelectrophoresis of Sephadex G-100 Fractions of α_1-Acid Glycoprotein

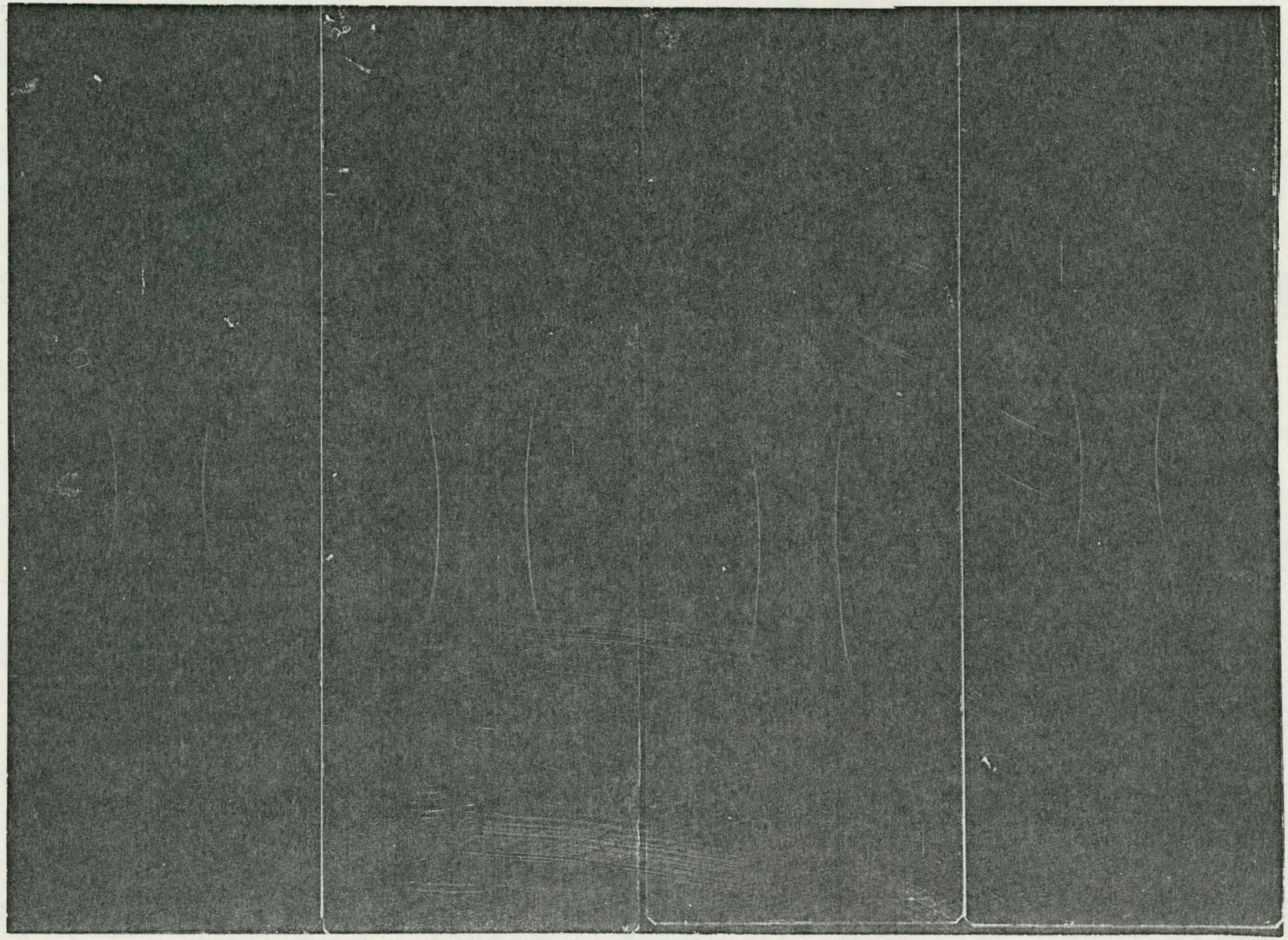
For procedure, see legend to Figure 17.

In addition to the indicated G-100 fraction, α_1-Macroglobulin was added to the bottom antigen well of each slide. This was done to help differentiate the α_1-Acid Glycoprotein line from the α_1-Macroglobulin line. However, the procedure did not help to resolve the two lines.

slide	fraction #
a	28
b	31
c	32
d	33
e	34
f	35
g	36
h	38







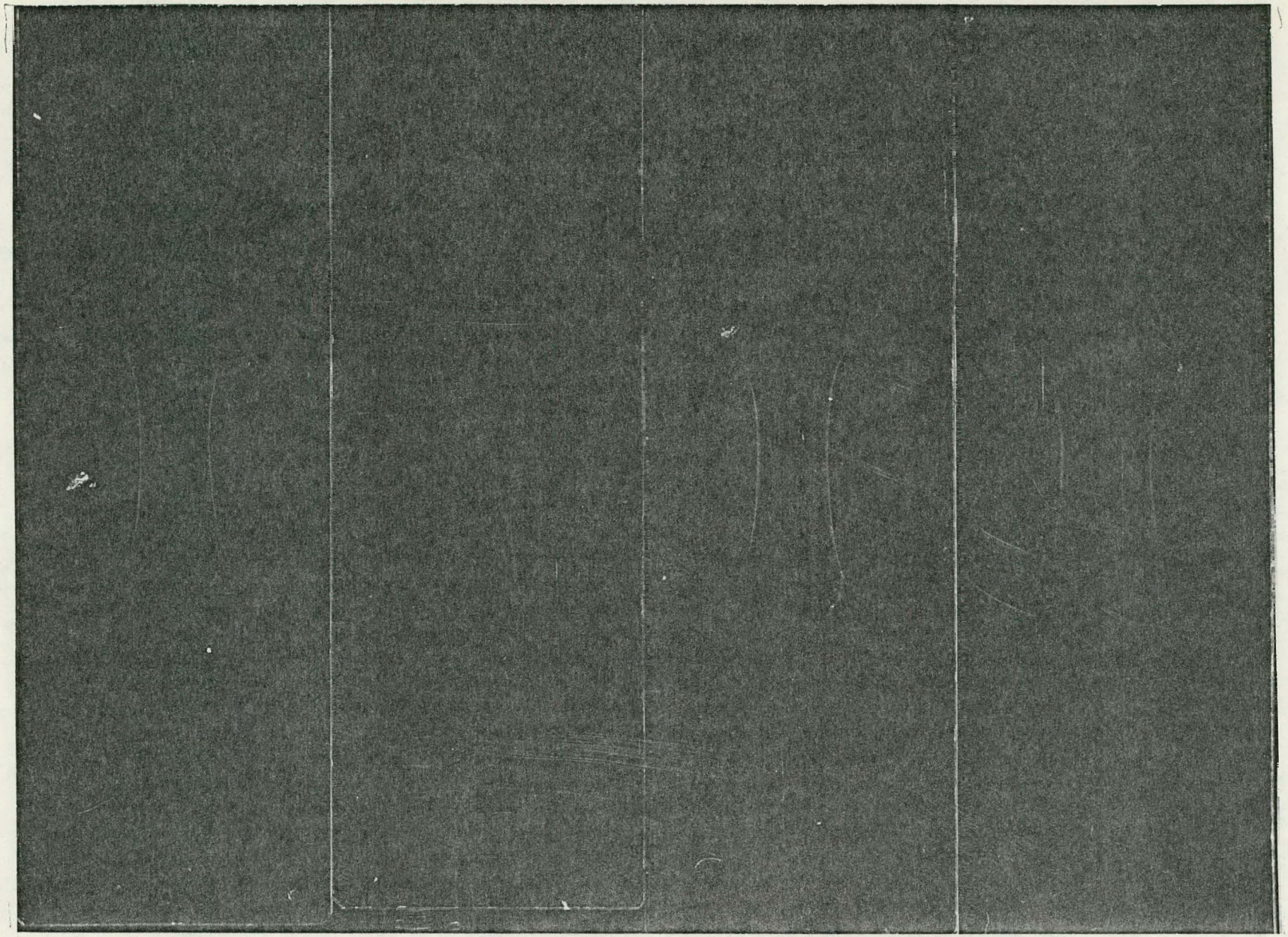
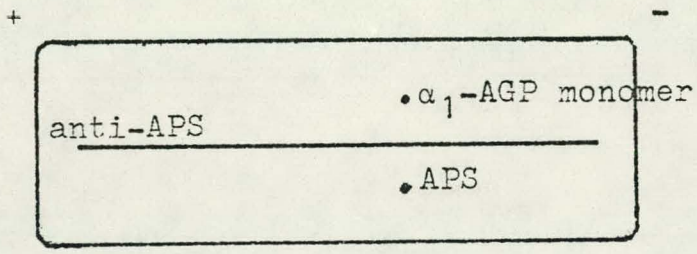


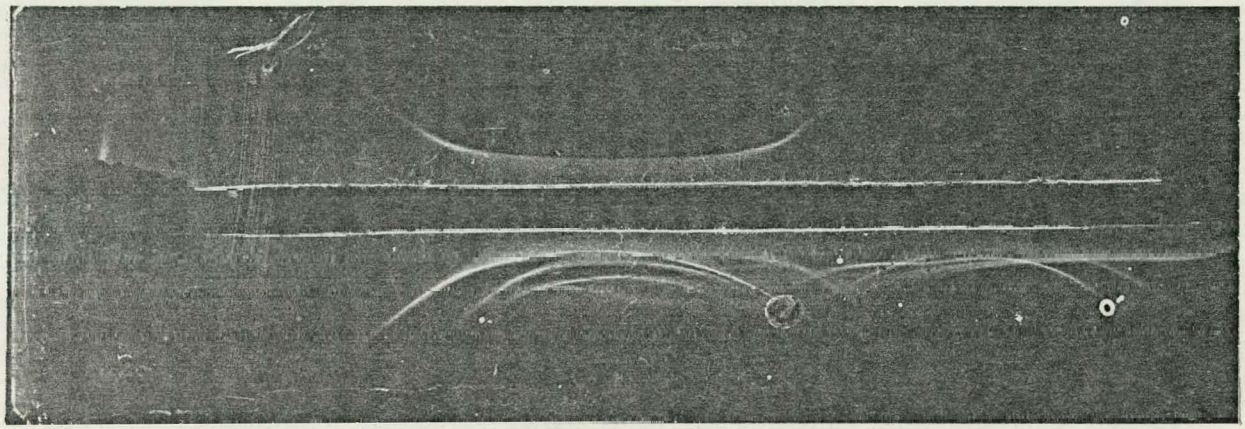
FIGURE 20

Figure 20: Immunoelectrophoresis of α_1-Acid Glycoprotein Monomer

α_1-AGP monomer was obtained as described in the legend to Figure 17. The antigen wells are purposely overloaded with protein to reveal any contaminants.



APS- acute phase serum



d. Purification of anti-RSA

Anti-RSA of sufficient avidity for the detection of 1 ng RSA was obtained with no trouble by the methods described.

e. Purification of anti-α_1-AGP

The anti-α_1-AGP immunoadsorbents continuously bound less labelled antigen than the anti-RSA immunoadsorbent. Part of the problem was due to contamination in the anti-α_1-AGP. These contaminants were almost all removed in the immunoadsorbent prepared for use in the RIA of polysomes. Several methods were tried to improve the quality of the antiserum:

1) Affinity Chromatography- antibody was adsorbed to an antigen column. It was hoped that antibody of weaker avidity would elute with .1M NH_4OH and that antibody of higher avidity would elute with .5M NH_4OH. However, immunoadsorbent prepared with antibody eluted with .5M NH_4OH was no better than that prepared with antibody eluted at the lower concentration.

2) Time of bleeding after immunization- antibody avidity is supposed to increase with time after immunization. Rabbits were therefore bled weekly for several weeks and the antisera were tested by Ouchterlony. Antibody was purified from those showing

high titers and fairly sharp lines; a small increase in binding was seen when antibody from later bleedings was used.

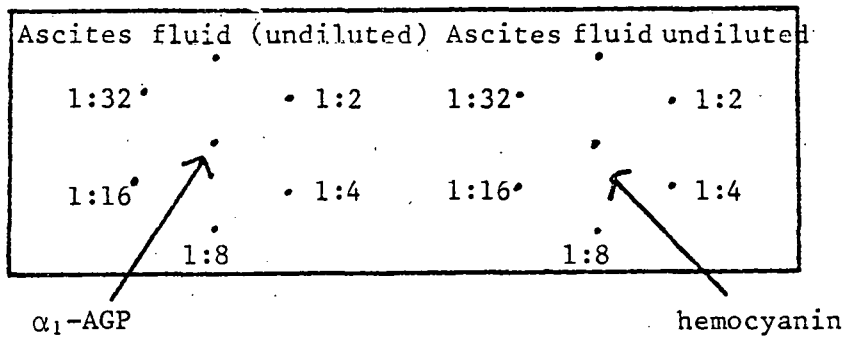
3) Conjugation of antigen with hemocyanin- α_1-AGP was conjugated with hemocyanin. Hemocyanin is known to be a potent elicitor of the immune response. It was hoped that the hemocyanin-α_1-AGP complex would be a more potent antigen than α_1-AGP alone. This complex was used to raise antibodies in both rabbits and mice (see below). Testing of the rabbit antiserum showed that the reaction to the hemocyanin was much more potent than that to the α_1-AGP. It is impossible to determine whether this method improved the quality of the antiserum because controls with sufficient rabbits were not run.

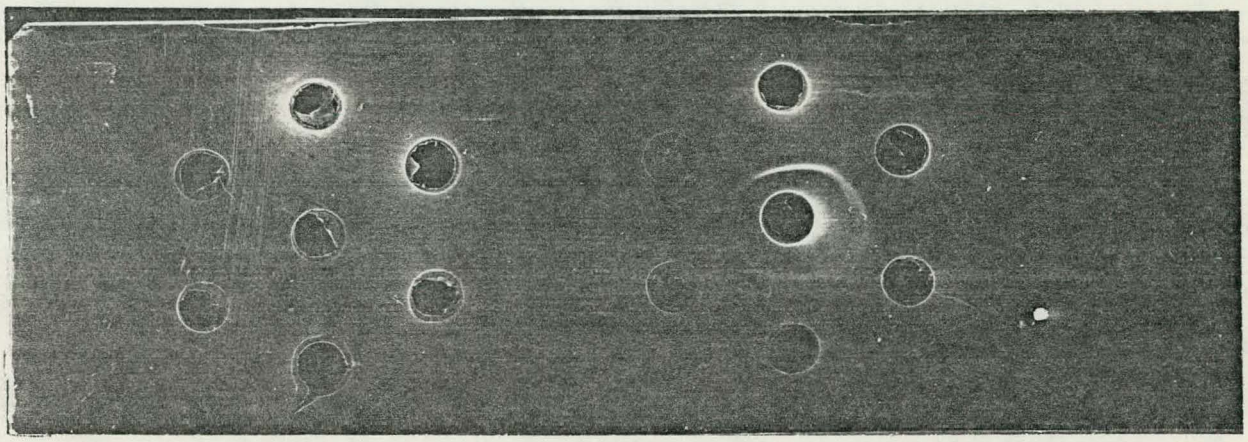
4) Raising antibodies in mice- Intraperitoneal injection of antigen in complete Freund's adjuvant causes the accumulation of ascites fluid containing antibodies in mice. It was attempted to raise anti-α_1-AGP in mice, even though there is a good deal of cross reactivity between rat and mouse α_1-AGP (seen using rabbit anti-rat α_1-AGP). Ouchterlony of the ascites fluid did not show any precipitin lines with rat α_1-AGP. However, there was a strong reaction with hemocyanin (Figure 21). The ascites fluid was passed through a Sepharose-α_1-AGP column. The column was eluted. The eluted material did react with rat α_1-AGP by Ouchterlony. A total of 2 to 3 mg of specific antibody were

FIGURE 21

Figure 21: Ouchterlonys of Ascites Fluid of Mice, Injected with α_1-Acid Glycoprotein-Hemocyanin Conjugate, Against α_1-Acid Glycoprotein and Against Hemocyanin

Mice were injected, intraperitoneally, with an α_1-AGP - hemocyanin conjugate in complete Freund's Adjuvant. Ascites fluid containing antibodies was obtained as described in Materials and Methods.





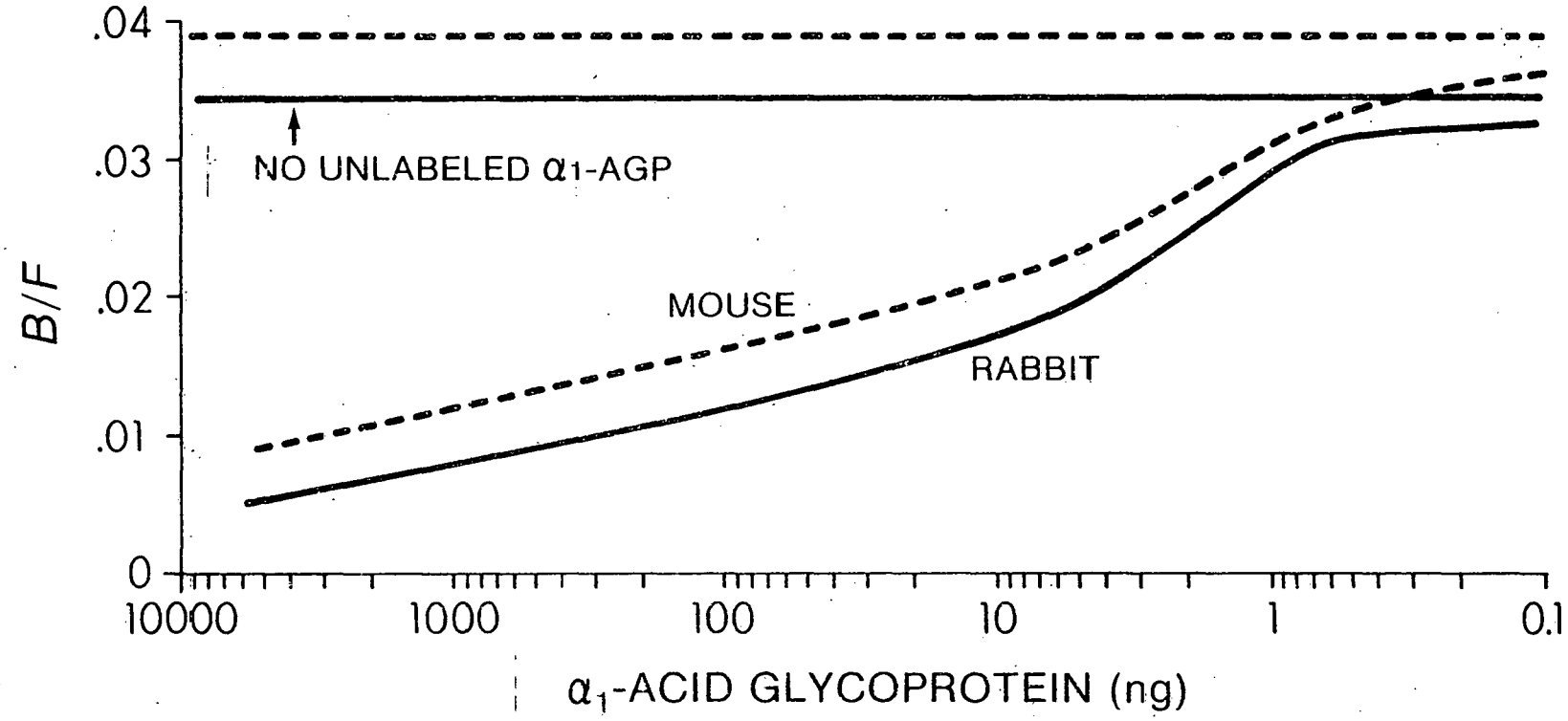
obtained from 4 mice. This antibody was coupled to Sephadex via a DVS sidearm and a sequential radioimmunoassay was performed. Amounts of immunoadsorbent were chosen so that the mouse and rabbit immunoadsorbents would bind equal amounts of labelled antigen in 24 hours when no unlabelled antigen was present. The two standard curves were almost identical (Figure 22). The mouse immunoadsorbent has not been tested with polysomes (it was made after all assays were already completed). One might expect the mouse antibodies to be less heterogenous than the rabbit antibodies (since the mouse and rat α_1-AGPs cross react). One might speculate that if the determinants with which the mouse antibody reacts were located near the COOH terminus of the protein, this would provide a very simple means for assuring that a polysome (mRNA) would react with only one antibody.

Anti-α_1-AGP, purified by affinity chromatography, still contained some contaminating antibodies, as evidenced by immunoelectrophoresis. Although no contaminants were detected (by polyacrylamide gel electrophoresis) in the α_1-AGP preparation, animals sometimes produce significant amounts of antibody to a protein present in only minute amounts. These antibodies might then be selected for during affinity chromatography. Since most of the contaminating antibodies were directed against proteins

FIGURE 22

Figure 22: Comparison of Immunoadsorbents Made with Mouse and Rabbit Anti-α_1-Acid Glycoprotein

Mouse ascites fluid and rabbit antisera were obtained after immunization with an α_1-acid glycoprotein - hemocyanin conjugate. Mouse and rabbit antibodies were separately purified by affinity chromatography on Sepharose-α_1-AGP, followed by removal of contaminants with immunoadsorbents containing α_1-macroglobulin and proteins with α_2, β, and γ mobility. The purified antibodies were coupled to Sephadex G-25 via a divinyl sulfone sidearm. A sequential radioimmunoassay was performed as described in Materials and Methods, except that 15,000 dpm (3 ng) ^{125}I- α_1-AGP was used. The total reaction volume was 1.5 ml. Either .36 ml packed mouse immunoadsorbent or .09 ml packed rabbit immunoadsorbent was used. This was done to equalize the capacities of the two immunoadsorbents. The apparent high non-specific binding to the mouse immunoadsorbent is probably due to the less efficient washing procedure (since a large Sephadex volume was used).


.04
.03
.02
.01
0
B/F
NO UNLABELED α1-AGP
MOUSE
RABBIT
10000
1000
100
10
1
0.1
α1-ACID GLYCOPROTEIN (ng)

with mobilities different than that of α_1-AGP, two new immunoadsorbents were made to remove these contaminants. Normal rat serum was fractionated by Pevicon block electrophoresis. All fractions containing no α_1-AGP and containing proteins with mobilities slower than that of α_1-AGP were pooled and used for the immunoadsorbent. Contaminating antibodies were removed by adsorbing the anti-α_1-AGP with the above immunoadsorbent, with a Sepharose-α_1-Macroglobulin and a Sepharose-RSA adsorbent. Immunoelectrophoresis showed that only one minor contaminant remained (Figure 23). It reacted with a protein with the same mobility as α_1-AGP.

2. RADIOACTIVE LABELLING OF PROTEIN

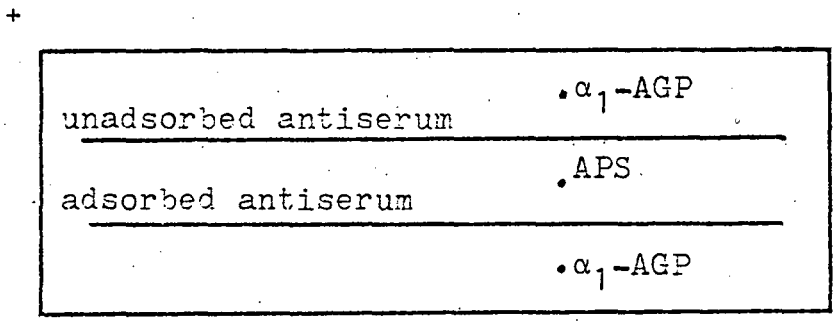
a. Choice of label

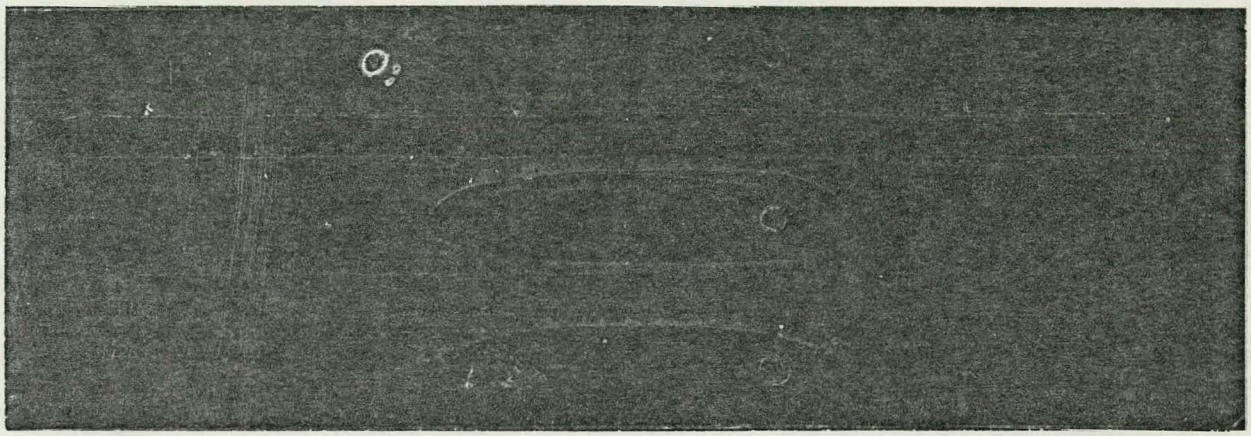
For a sensitive radioimmunoassay, it is essential that the antigen be labelled to a high specific activity, without loss of immunological activity. ^{125}I and ^{131}I are the labels of choice for proteins because of the high specific activities attainable. One ^{131}I atom per insulin molecule provides the same number of

FIGURE 23

Figure 23: Immunoelectrophoresis Showing Purification of Anti-α_1-Acid Glycoprotein Antibody by Adsorption of Antiserum with α_1-Macroglobulin and α_2, β, and γ Mobility Proteins

Anti-α_1-AGP antiserum was passed through Sepharose -α_1-macroglobulin and Sepharose -α_2, β, γ (normal rat serum was separated by Pevicon block electrophoresis- proteins with α_2, β, and γ mobility were pooled and coupled to Sepharose).




O₂

dpm as 586 tritium atoms and 200 times the number of dpm as if all 263 carbon atoms were ^{14}C (Hunter, 1971). The half-lives of ^{131}I and ^{125}I are 8 days and 60 days, respectively. However, ^{131}I is provided at a maximum specific activity of 25 Ci/mg and ^{125}I is provided at a maximum specific activity of 17 Ci/mg. Therefore, within 1 week of labelling, protein labelled with ^{125}I would have a higher activity than that labelled with ^{131}I. Unless a double-lable experiment is to be done or a rapid decay is required, ^{125}I is the label of choice.

b. Choice of method of iodination

In Table V are tabulated various parameters and results for iodinations by various methods. The lactoperoxidase procedure was only tried once. After iodination of α_1-AGP by this method, the α_1-AGP was repurified by affinity chromatography (in order to separate it from the lactoperoxidase). Only 4% of the radioactivity bound to the immunoadsorbent. This may be due either to a poor efficiency of iodination or to denaturation of the protein. The former possibility is favored because the lactoperoxidase method is reputed to be one of the gentlest iodination procedures. The affinity purified, iodinated α_1-AGP bound very well to the Sephadex immunoadsorbent and was of

TABLE V

Table V: Iodination of Proteins

Iodination of proteins was performed as described in Materials and Methods. The following abbreviations are used:

Lactoper.	Lactoperoxidase
ICl	Iodine monochloride
Oxid. ICl	Oxidative iodine monochloride
Bolton-Hunter, ICl	see Results and Discussion, page 104
Aff. Chr.	Protein was purified by affinity chromatography on a Sepharose-antigen or antibody column
Pevicon	Proteins with β and γ mobility were removed from the affinity purified α_1-AGP by Pevicon block electrophoresis
PCA	refers to the original method (John and Miller, 1968) used to purify α_1-AGP, which included a perchloric acid extraction
Monomer	α_1-AGP, after Pevicon block electrophoresis, was filtered through a Sephadex G-100 column to obtain the monomer peak
	Affinity chromatography purified RSA, after removal of glycoproteins with ConA-Sepharose, was filtered through a G-100 column to obtain the monomer peak
α_1-M, α_2, β, γ	Affinity purified anti-α_1-AGP was passed through immunoadsorbents containing α_1-macroglobulin and serum proteins with α_2, β, and γ mobilities
$(NH_4)_2SO_4$	IgG from normal rabbit serum was precipitated with 1/3 saturated ammonium sulfate

METHOD	PROTEIN TYPE	PROTEIN PURIFIC.	PROTEIN AMT. (μG)	#I/MOL.	EFFIC.	SP. ACT. μCi/μG	COMMENTS
Lactoper.	α_1-AGP	Aff. Chr.	30		5%	8.5	
Bolton-Hunter, ICl	"	"	50	3	60%	0.95	
"	"	"	50	3	25%	2.70	
ICl	"	PCA	50	3	52%	0.66	
"	"	"	50	6	56%	0.78	
"	"	Aff. Chr.	63	4	42%	0.43	I-125 and ICl mixed, and then protein added
"	"	"	63	4	18%	0.88	
"	"	"	63	4	35%	1.69	
"	"	"	63	4	21%	2.14	
"	"	"	63	6	22%	2.35	
Oxid. ICl	"	"	25	12	59%	12.8	
"	"	"	25	6	5%	5.2	
"	"	"	26	12	54%	6.0	
"	"	"	26	12	44%	13.8	
"	"	"	26	8	40%	2.5	
"	"	"	26	10	36%	44.6	
"	"	Pevicon	25	3	69%	66.0	
"	"	"	59	3	22	9.8	
"	"	Monomer	47	8	14%	9.0	Larger reaction volume
ICl	RSA	Aff. Chr.	100		7%	2.3	
Oxid. ICl	"	"	25	4	36%	19.0	87% immunoprecipitable
"	"	"	26	4	26	0.32	
"	"	"	26	4	22%	16.5	
"	"	Monomer	25	6	43%	1.79	
"	"	"	25	6	18%	15.3	>76% immunoprecipitable

METHOD	PROTEIN TYPE	PURIFIC.	AMT. (µG)	#I/MOL.	EFFIC.	SP. ACT. (µCi/µG)	COMMENTS
ICl	anti-RSA	Aff. Chr.	1000	4	50%	0.78	
"	"	"	540	1	46%	0.009	I-131
Oxid. ICl	"	"	28	10	37%	0.54	I-131
"	"	"	108	4	61%	1.76	82 % immunoprecipitable with goat anti-rabbit IgG
ICl	anti-α_1-AGP	Aff. Chr.	900	4	40%	0.9	68% of labelled antibody would bind to immuno-adsorbent (antigen)
ICl	"	Aff. Chr.	500	1	8%	0.002	I-131
Oxid. ICl	"	α_1-M, α_2, β, γ	25	15	21%	0.32	
ICl	Normal IgG	$(NH_4)_2SO_4$	1000	4	68%	0.007	I-131

sufficient specific activity. However, a 4% efficiency is very wasteful of the ^{125}I, so this method was not used.

Instead of iodinating the Bolton-Hunter reagent and then reacting this with the protein, the reagent was reacted with the protein and the protein was then iodinated. This did not improve the iodination of α_1-AGP. However, it was not determined how well the first reaction proceeded. It may be that the Bolton-Hunter reagent reacts better in the iodinated form. The electrolytic method for iodinating protein was not tried because it is difficult to scale this procedure down to g quantities of protein.

As can be seen from Table V, the maximum specific activity for α_1-AGP was 2.5 μCi/μg, except when the oxidative ICl method was used. Using the latter method, specific activities up to 66 μCi/μg were attained. Other investigators have labelled bovine serum albumin to 300-600 μCi/μg by the ICl method (but immunological activity was not tested) (Hunter, 1971). However, since it was desirable to maintain as much consistency as possible between the RSA and α_1-AGP systems, RSA was also labelled by the oxidative ICl method. Specific activities up to 19 μCi/μg were attained. No methodical system was used to

optimize parameters (#I/molecule protein, # ^{125}I/^{127}I, amount protein, etc.) for the iodination of either protein and the procedures were never exactly repeated to determine the reproducibility. The oxidative method does seem to consistently give efficiencies of 30 to 60% and specific activities of over 10 μci/μg when 25 to 100 μg of RSA or α_1-AGP are iodinated. As can be seen in figures 7 and 8, binding of α_1-AGP or RSA to their respective immunoadsorbents is not impaired by iodination of the proteins. At least one preparation of iodinated α_1-AGP had high (1 to 2%) non-specific binding to Sephadex, but not to P-2 (polyacrylamide beads supplied by Bio-Rad). It is therefore advisable to test each newly labelled preparation of iodinated protein. 75% ± 9% of the ^{125}I-RSA (iodinated by the oxidative ICl method) is immunoreactive with anti-RSA (anti-RSA is either coupled to Sephadex or free antibody is precipitated with goat anti-Rabbit IgG). It might be possible to increase the amount of ^{125}I-RSA immunoprecipitable with anti-RSA by selecting the immunoreactive protein by affinity chromatography. However, it is possible that the protein would be denatured by the eluting agent. It is also possible that the source of the non-immunoreactive ^{125}I RSA is the purification procedure and not the iodination procedure.

c. Choice of protective protein

The ^{125}I-RSA is stored in buffer containing 4% BSA. Although no precipitin lines were seen between BSA and anti-RSA by double radial immunodiffusion, there have been reports of approximately 13% cross reactivity between RSA and BSA using anti-BSA (Weigle, 1961) (note that this does not necessarily mean that anti-RSA would react with BSA). Binding of iodinated BSA to anti-RSA immunoadsorbent would indicate cross-reactivity. BSA was used as a protective protein because it is relatively inexpensive, and the preparation used contained no ribonuclease activity (normal rabbit serum, which would otherwise be a good protective protein, rapidly degraded polysomes). Even if anti-RSA does bind to BSA, BSA should not interfere with the RIA because it would merely block all antibodies directed against it. There should still be abundant antibodies to react with the RSA. Chicken ovalbumin would probably have been a more appropriate protective protein because it is both inexpensive and does not cross-react with RSA. It would only be necessary to check that it does not contain ribonuclease activity.

d. Antibody iodination

It was not necessary to label antibody to high specific activities. Either the ICl or the oxidative ICL method was used for the labelling. Labelled antibody was used as a tracer to determine the amount of antibody coupled to Sephadex for the radioimmunoassay.

3. ASSAY CONDITIONS

a. Immunoadsorbents

Immunoadsorbent for the RIA was originally prepared using the adipic acid dihydrazide succinate coupled to Sephadex. The specific binding of the labelled RSA was high and the non-specific binding was less than 1%. Since it was found that this sidearm was less stable than the DVS sidearm in affinity chromatography, the DVS was used in later experiments.

As mentioned above, one preparation of iodinated α_1-AGP had high non-specific binding properties to Sephadex, but not to

P-2. Experiments were continued using the Sephadex immunoadsorbent and a good preparation of α_1-AGP. It was decided to prepare an immunoadsorbent using P-2 as an alternative to the Sephadex. The P-2 could then be used if the frequency of non-specific adsorbtion increased. P-2 was activated with a hexane diamine sidearm and normal rabbit IgG was coupled to it. The non-specific binding of iodinated RSA and iodinated α_1-AGP was only .3%. Non-specific binding of polysomes and specific binding of antigen to specific antibody were not determined. It was noted that the P-2 packed better than Sephadex upon centrifugation. This would make the washing procedure easier.

All RIA experiments discussed below utilized the DVS sidearm.

As stated in the methods section, it is necessary for the inter-antibody spacing to be greater than the length of a specific polysome. Immunoadsorbents were, however, initially prepared with approximately a 5-fold excess of antibody. Many parameters had to be checked before the immunoadsorbent could be used to assay polysomes and the greater antibody concentration allowed shorter incubation times.

b. Choice of buffer

First, a suitable buffer system had to be chosen. The buffer needed to be good for antigen-antibody binding, to inhibit non-specific binding to the immunoadsorbent of both labelled antigen and non-specific polysomes, and to be free of ribonuclease activity. The original RIA method followed used a phosphate buffer containing .2% BSA and .5% Tween 20. This buffer caused extensive degradation of polysomes (as seen by polysome profiles). In addition, 5 mM Mg^{++} had to be added to the buffer to prevent dissociation of ribosomes into subunits. The Mg^{++} complexed with the PO_4^{-2} and formed a precipitate. While this did not seem to interfere with the binding of antigen to the immunoadsorbent, it did lower Mg^{++} concentrations.

Buffer D, used in the polysome isolation procedure, was modified to be used as the radioimmunoassay buffer: .5% Tween 20 and .2% BSA were added to reduce non-specific adsorbtion and .02% NaN_3 was added as a bacteriostatic agent. Fortunately, this buffer did not cause degradation of polysomes during a 24 hour incubation in the cold room.

Binding of labelled antigen to the immunoadsorbent in this buffer was as good as with the original phosphate buffer. However, rabbit liver polysomes, obtained by ultracentrifugation through sucrose gradients, significantly inhibited the binding of labelled antigen to the immunoadsorbent. Dialysis of these polysomes against Buffer D removed the inhibitory substance. One of the components of the sample removed by dialysis was sucrose. Experiments were performed to determine the effect of sucrose concentration on the binding of antigen to immunoadsorbent. It was found that the maximum final sucrose concentration which did not inhibit binding was 2 1/2% (w/w). Polysomes were thereafter diluted to 5.0% sucrose and equal volumes of sample and immunoadsorbent were used.

The next problem encountered was that the immunoadsorbent seemed to lose its binding capacity after 2 months in Buffer D. A sample of the immunoadsorbent stored in the phosphate buffer for 8 months did not lose its binding capacity. Although it is possible that this is due to a positive action of the phosphate buffer, it was thought that the DTT in Buffer D probably denatured the antibody during prolonged exposure. No effect of DTT could be seen in a 24 hour incubation. However, to be safe, immunoadsorbents were stored in Buffer D containing neither DTT

nor cycloheximide. Polysome and standard samples did contain both DTT and cycloheximide.

With most of the problems associated with the RIA already worked out, the antibody concentration in the immunoadsorbent was reduced. Again, problems were encountered with reduced binding capacity when week long incubations were performed. It was thought that the DTT may denature the antibody at dilute (µg/ml) antibody concentrations (i.e. that the DTT to antibody ratio was important; Sears et al 1977) in a shorter period of time. Binding was significantly higher when immunoadsorbent was diluted into fresh buffer containing no DTT. Since polysomes were prepared in buffers containing DTT, it was necessary to determine how much of the DTT had to be removed. In the meantime, polysome samples were being prepared in the usual manner, but DTT was not included in any buffers after the first ultracentrifugation step. Several experiments were performed to determine the effects of DTT and cycloheximide on antigen binding. No effects were seen up to 1 mM DTT and 5 µg/ml cycloheximide in the sample (these are the concentrations used in polysome buffers). It is thought that perhaps there was some antigen contamination introduced into the immunoadsorbent which caused the reduced binding. Care was therefore taken to always use the same magnetic bar and forceps (for removing the magnetic

bar from the flask containing the immunoadsorbent), neither of which were allowed to come in contact with serum or other source of antigen. No further major problems were encountered with reduced binding. Since many polysome samples had already been prepared using buffers (in final steps) containing no DTT, this procedure was continued. However, as mentioned in the introduction, the reducing agent may decrease RNAse activity, so if the experiments were to be repeated, it would probably be advantageous to include the DTT.

c. Washing immunoadsorbent

It was determined (see below) that only 1 to 2% of the antibody bound to immunoadsorbent is effective in antigen binding. Therefore, a serious problem could arise if any of this antibody were released from the solid phase into solution. Presumably, the antibody in solution would regain its effectiveness in binding antigen and would compete with the solid phase. It would not, however, precipitate the antigen because of the low concentration of antigen and antibody. This leakage could be a very serious problem. For example, if there were 1 µg antibody bound per ml Sephadex, and the bound antibody had a binding capacity only 2% of the same amount of free antibody (i.e. 1 µg bound antibody ≡ 20 ng free antibody)

release of 1% of the coupled antibody (10 ng) although not significantly reducing the immunoadsorbent capacity, would increase the effective antibody concentration in the supernatant to 50% of the solid phase! To determine whether this was a problem, immunoadsorbent was again washed just prior to being used in the assay and compared with the immunoadsorbent not washed the additional time. The rewashed immunoadsorbent had a 6% higher binding capacity. This does not represent very much leakage, but the immunoadsorbent was rewashed not more than one week prior to its use in a radioimmunoassay.

d. Filtration of iodinated antigen

An experiment was performed to determine whether the iodinated antigen underwent a significant amount of lysis during storage at -20°. A sample was filtered through a PD-10 column (Sephadex G-25 M, packed in a syringe barrel). 84% of the material eluted in the void volume. Binding of this material to the immunoadsorbent was compared with the binding of the original sample applied to the PD-10 column. Binding in 24 hours was 16% greater in the refiltered sample. Labelled antigen was therefore routinely filtered through a PD-10 column before being used in the RIA.

4. REPRODUCIBILITY OF RADIOIMMUNOASSAYS

Figures 24 and 25 show the intraassay reproducibility of the radioimmunoassays. The mean and range of triplicate determinations at each antigen concentration are shown. Table VI demonstrates the reproducibility of the two radioimmunoassays. One polysome sample was aliquotted into several tubes and frozen. One tube was used in each assay as a measure of the reproducibility of the assay of polysomes. Some of the variation can be accounted for by small variations (up to 1 hr) in incubation times and by differences in the amount of labelled antigen added. Both of these parameters should have been kept more constant.

FIGURE 24

Figure 24: Standard Curve for Radioimmunoassay of RSA

A sequential radioimmunoassay for RSA was performed as described in Materials and Methods (pages 44-45). The mean and range of triplicate determinations are shown for each antigen concentration.

STANDARD CURVE FOR SEQUENTIAL
RADIOIMMUNOASSAY OF RSA

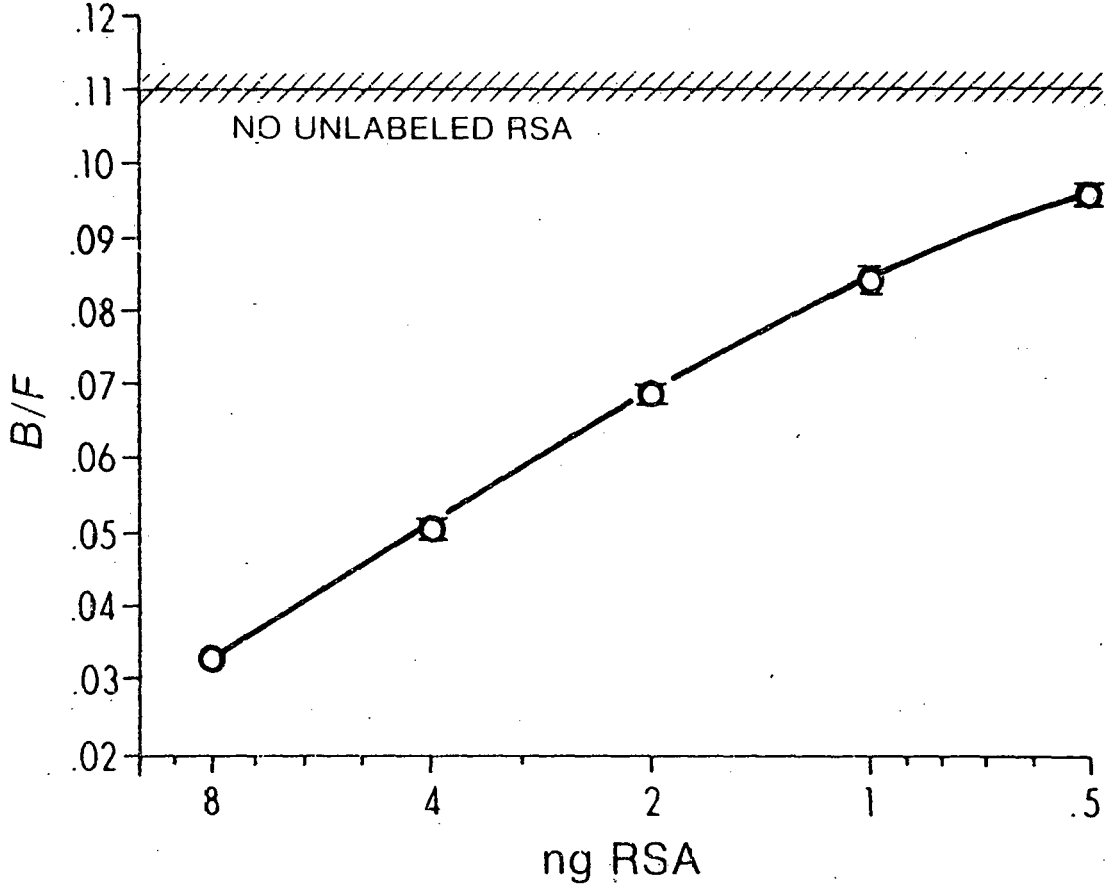


FIGURE 25

Figure 25: Standard Curve for Radicimmunoassay of α_1-AGP

A sequential radioimmunoassay for α_1-AGP was performed as described in Materials and Methods (pages 44-45). The mean and range of triplicate determinations are shown for each antigen concentration.

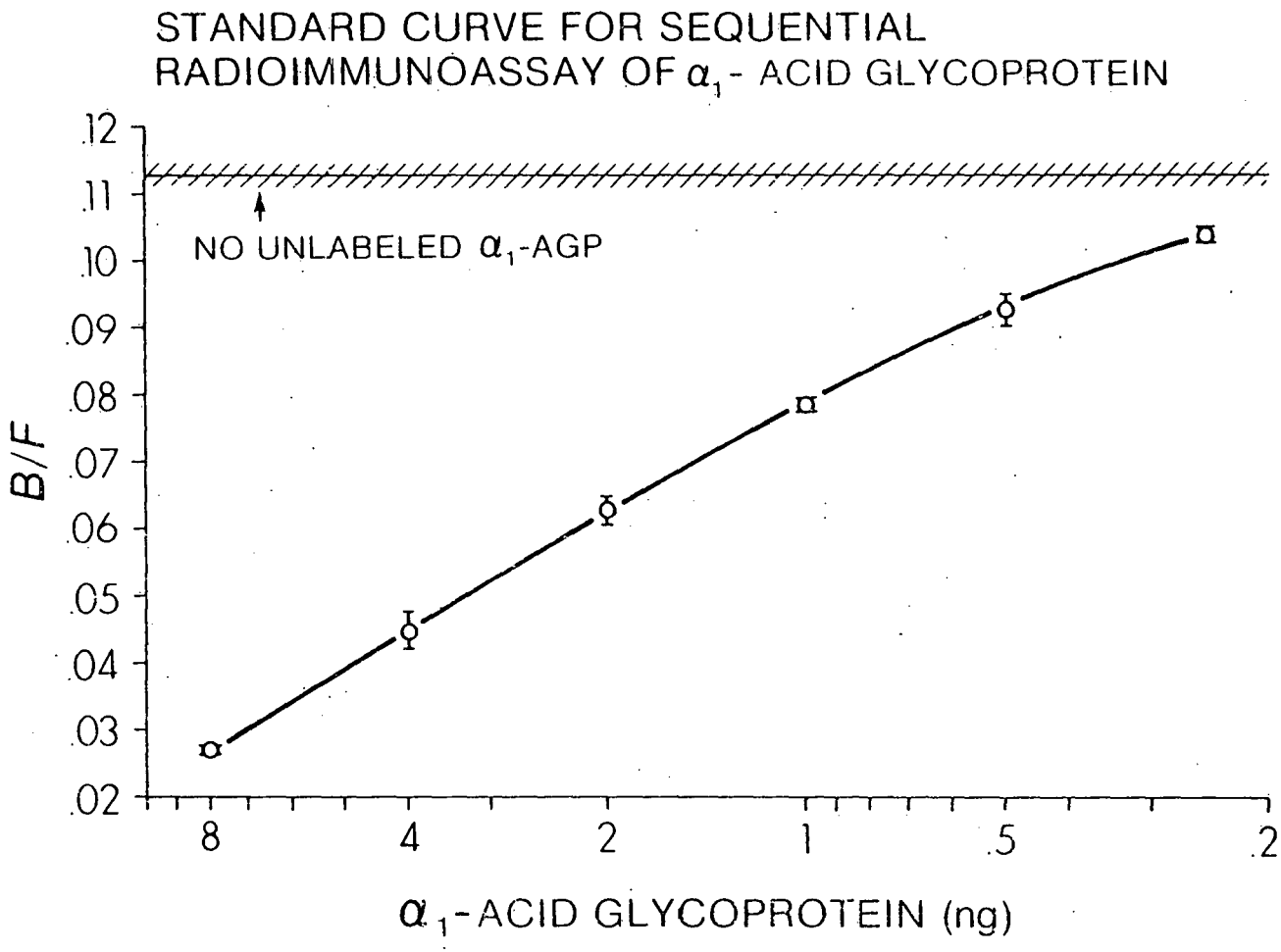

STANDARD CURVE FOR SEQUENTIAL
RADIOIMMUNOASSAY OF α1- ACID GLYCOPROTEIN
NO UNLABELED α1-AGP
B/F
.12
.11
.10
.09
.08
.07
.06
.05
.04
.03
.02
8
4
2
1
.5
.2
α1-ACID GLYCOPROTEIN (ng)

TABLE VI

Table VI: Reproducibility of Radioimmunoassays

Radioimmunoassays were performed as described in Materials and Methods section. The $\frac{B}{F}$ max values are the mean $\pm$ S.D. of four determinations. For determination of the inter-assay reproducibility of the assays for RSA polysomes, a sample of normal rat liver polysomes was prepared and aliquotted into separate tubes which were stored in a -70^{o} freezer until time for use. For determination of the interassay reproducibility of the assays for α_1-AGP polysomes, polysomes were prepared from livers of rats treated with turpentine 72 hours previous to liver excision.

PROTEIN	I-125 PROTEIN DPM	NG	$\frac{B}{F}$ max ± S.D.	NG PROTEIN / MG POLYSOMAL RNA
RSA	18,000	1.3	.1085 ± .0051	----
RSA	15,000	1.1	.1119 ± .0062	----
RSA	15,300	1.2	.1089 ± .0015	58.9
RSA	14,100	1.1	.1073 ± .0030	51.3
RSA	13,800	1.1	.1046 ± .0037	----
RSA	14,000	1.2	.1152 ± .0062	----
RSA	14,100	1.3	.1050 ± .0067	55.2
RSA	13.800	1.4	.0953 ± .0026	61.9
RSA	13,500	1.4	.0969 ± .0022	51.3
			.1060 ± .0065	55.7 ± 4.7
α_1-AGP	12,900	1.2	.1127 ± .0018	78.6
α_1-AGP	13,211	1.3	.1151 ± .0038	74.2
α_1-AGP	16,000	1.7	.1066 ± .0029	66.8
α_1-AGP	15,500	1.7	.0975 ± .0023	61.7
			.1080 ± .0078	70.3 ± 7.5

B. POLYSOME ISOLATIONS

Several methods were tried to isolate polysomes in a quantitative yield, without degradation by ribonucleases, and without contamination with serum proteins. The methods tried were Mg^{++} precipitation, ultracentrifugation through discontinuous sucrose gradients, and gel filtration. As will be shown, none of these methods worked alone to remove serum proteins, but by combining two methods or doing one method twice, satisfactory purification was achieved.

1. GEL FILTRATION

a. Isolation of polysomes

In order to avoid extensive use of the ultracentrifuge, I first attempted to isolate polysomes from a post-mitochondrial supernatant by gel filtration. Theoretically, all polysomes composed of at least two ribosomes per mRNA should elute in the void volume of either Sepharose 6B (exclusion limit = 4,000,000 daltons) or Bio-Gel A-5m (exclusion limit = 5,000,000 daltons). Almost all serum proteins (including RSA and α_1-AGP) should be

significantly retarded in their filtration through these two gels.

In order to determine whether the polysomes would quantitatively elute in the void volume of these two gels, polysomes labelled with ^{14}C orotate were first purified by Mg^{++} precipitation (Palmiter, 1974). These polysomes were filtered through a 20 ml Sepharose 6B column, equilibrated with 25 mM Tris, pH 7.4, 5 mM $MgCl_2$, 150 mM KCl. Only 70% of the ^{14}C label and 260 nm absorbing material eluted in the void volume. The rest of the material was not eluted with several volumes of buffer. The peak fractions in the void volume were pooled and applied to a fresh Sepharose 6B column, equilibrated with the same buffer. This time, only 60% of the material eluted in the void volume. This indicates that the column is non-specifically retaining a substantial fraction of the polysomes. A 48% recovery of ^{14}C-orotate labelled polysomes occured when a low salt buffer was used. The remainder of the label was eluted with .3N KOH at room temperature. Although the recovery using high salt buffer is better than that obtained by following most procedures in the literature, the non-specific retention might make stringent washing (with KOH) of the gel necessary before reuse of the column. Similar results were obtained with Bio-Gel A-5m as with Sepharose 6B.

It was determined that polysomes would pass through a Pharmacia column with no gel in it, or packed with Sephadex G-25. G-200, Sephacryl S-200, or Bio-Gel A-150m with at least an 85% recovery of the 260 nm absorbing material. The Sephadex would be unsuitable for purification of polysomes because serum proteins of high molecular weight would elute in the void volume. Sephadex G-25 might, however, prove to be useful in removing low molecular weight substances from a polysome preparation and G-200 would separate free IgG from IgG bound to polysomes. Bio-Gel A-150m has a fractionation range of 1 million to 150 million daltons for proteins. Polysomes should therefore be fractionated according to the number of ribosomes per mRNA. This gel is therefore not suitable for the isolation of unfractionated polysomes. If adequate resolution were obtained, it could be used as an alternative to the continuous sucrose gradients used to obtain polysome profiles. The fact that polysomes eluted from Bio-Gel A-150m in an 85% yield indicates that it is not agarose per se which retains the polysomes, but that the retention seen with Sepharose 6B and Bio-Gel A-5m may be something peculiar to that fractionation range.

^{125}I-RSA was added to a rat liver post-mitochondrial supernatant before filtration of the PMS on Bio-Gel A-150m to

determine whether RSA would completely separate from the polysomes. Only 1.5% of the label coeluted with the polysomes. It was determined that 0.8% of the label would elute in the fractions near the void volume even in the absence of any polysomal material. This label may represent either aggregated RSA or a contaminant in the RSA preparation used for iodination. It might also represent RSA which has been phagocytized by cells incompletely removed during serum preparation. Rat kidney polysomes were isolated from a PMS by gel filtration through Bio-Gel A-150m. The fractions were tested in a radioimmunoassay for RSA. Even the first polysome fractions contained RSA, although considerably less RSA than the retarded fractions. It is suggested that further experiments be performed to try to improve this method for polysome isolation: 1) Other filtration media, such as Ultra-Gel (cross-linked agarose and polyacrylamide, supplied by Bio-Rad), may give better results than agarose; 2) Further purification of the agarose (for example, to remove sulfate groups) may be helpful in reducing non-specific retention of polysomes; 3) A second passage of polysomes through one of the filtration media may sufficiently remove protein which origially eluted in the void volume; 4) Bio-Gel A-150m may be suitable for running polysome profiles and for demonstrating the specific binding of labelled (iodinated) antibody to polysomes.

b. Demonstration of binding of antibodies to polysomes

Since IgG does not elute in the void volume of Sephadex G-200, this gel can be used to show that antibody can specifically bind to polysomes. As is shown in Table VII and Figure 26, incubation of ^{125}I anti-RSA with polysomes causes a substantial fraction (32%) of the antibody to elute in the void volume with the polysomes. If the polysomes are preincubated with cold anti-RSA before being incubated with the labelled anti-RSA, the amount of ^{125}I anti-RSA eluting in the void volume is the same as the control value. Preincubation of the polysomes with anti-ferritin does not prevent the ^{125}I anti-RSA from eluting in the void volume. The 8% of the anti-RSA which elutes in the void volume under control conditions is probably aggregated IgG and/or IgM.

Table VII: BINDING OF ANTI-RAT SERUM ALBUMIN TO POLYSOMES

Polysomes were preincubated with unlabelled antibody or buffer for 1 hour and were then incubated with labelled anti-RSA for 4 hours. The total incubation mixture was then filtered thorough a G-200 column.

10 O.D. UNITS OF POLYSOMES	PREINCUBATION	INCUBATION (ANTI-RSA)	% OF ACTIVITY IN POLYSOME REGION
no	no	0.7 μCi (1.4 mg)	8
yes	no	1.3 μCi (0.03 mg)	32
yes	1 mg anti-RSA	0.9 μCi	8
yes	1 mg anti-ferritin	2.3 μCi (0.03 mg)	44

If ^{125}I anti-RSA is fractionated on Sepharose 6B, there are two distinct peaks of activity. When the lower molecular weight peak is rechromatographed, it elutes in the same volume as before. Sepharose 6B can be used in the same manner as G-200 to show that ^{125}I anti-RSA specifically binds to polysomes. The resolution of the polysome and antibody fractions is better, especially when the low molecular weight antibody fraction is used. When polysomes and ^{125}I anti-RSA are incubated for

FIGURE 26

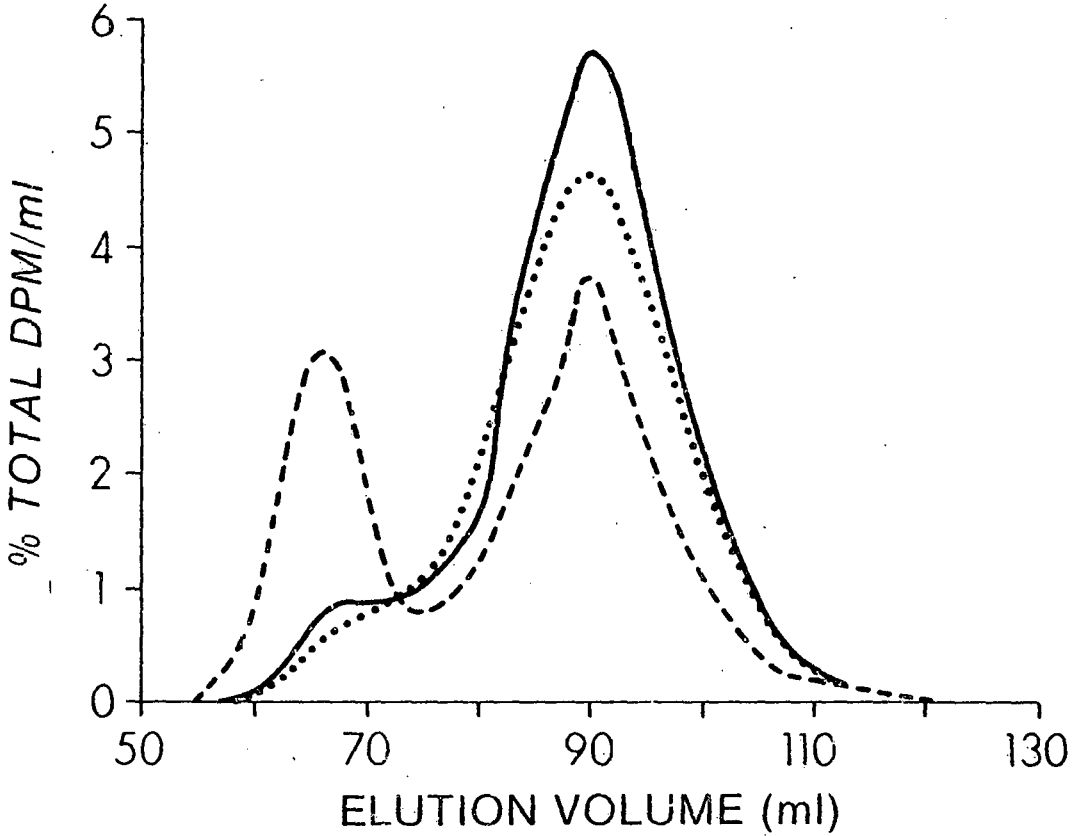
Figure 26: Binding of Anti-RSA to Polysomes

Polysomes were preincubated with unlabelled antibody or buffer for 1 hour and were then incubated with iodinated anti-RSA for 4 hours. The total incubation mixture was then filtered through a Sephadex G-200 column.
Although not shown in this figure, when polysomes were preincubated with anti-ferritin, the elution profile of the labelled anti-RSA was almost identical to that when polysomes were preincubated with buffer only. For details on amounts of antibody and polysomes used in the incubations, see Table VII (page 123).

..... ^{125}I anti-RSA only

- - - - ^{125}I anti-RSA and polysomes

——— ^{125}I-anti-RSA and unlabelled anti-RSA and polysomes


6
5
4
3
2
1
0
% TOTAL DPM/ml
50
70
90
110
130
ELUTION VOLUME (ml)

increasing times, an increasing amount of radioactivity appears in the void volume. Before gel filtration could be routinely used, it would be necessary to demonstrate by RNA analysis, and not by optical densisty measurements, that the polysomes quantitatively eluted in the void volume. As long as the antibody consists of only IgG, Sephacryl S-200 would probably be an ideal medium- it is fast and polysomes and IgG should be clearly separated.

2. Mg^{++} PRECIPITATION

Two methods of Mg^{++} precipitation were used to isolate polysomes (Palmiter, 1974; Ilan, 1976). In both cases the polysome pellet was obviously contaminated by other material, as evidenced by the reddish color of the polysome pellet. Radioimmunoassay of rat kidney and rat liver polysomes showed .281 µg and .344 µg RSA per mg RNA, respectively. Rabbit liver, rat kidney, and rat liver polysomes, first isolated by the Mg^{++} precipitation procedure of Ilan were repurified by ultracentrifugation through a 1M sucrose pad. Radioimmunoassay of samples taken from the top and bottom of the centrifuge tube showed that there was a definite separation of the polysomes and the contaminating free RSA. Polysome samples were then

dialyzed. RSA could be detected in rat liver polysomes, but not in polysomes from rabbit liver or rat kidney (sensitivity of assay was 1 ng).

3. ULTRACENTRIFUGATION THROUGH SUCROSE GRADIENT

Since ultracentrifugation sufficiently improved the purity of Mg^{++} precipitated polysomes, experiments were performed to determine whether ultracentrifugation alone of the PMS would remove free RSA from the polysomes.

The procedure of Ilan was used to obtain a PMS because it had been determined that 88-95% of the homogenate RNA was recovered in the PMS. The PMS was treated with deoxycholate to 1.3% to solubilize the endoplasmic reticulum, thus allowing all polysomes (not just free polysomes) to sediment through 2.0M sucrose.

In order to obtain a quantitative yield of polysomal RNA in the polysome fraction (80% of homogenate RNA is normally polysomal RNA), (Blobel and Potter, 1967) and at the same time to remove all free RSA and α_1-AGP, several parameters of the ultracentrifugation were varied. These included sucrose

concentration, buffer composition, and centrifugation time. The polysome fraction was defined as the material in the bottom 2.1 cm of the ultracentrifuge tube (9/16" x 3 1/2" tube). Aspiration of the top layers of the gradients left too much contaminating cell sap, so centrifuge tubes were frozen at -76°C and then cut with a hacksaw 2.1 cm from the bottom. A 2.5M sucrose pad was used so that the polysomes would not pellet to the bottom of the tube (it is dificult to resuspend a polysome pellet). This layer was overlaid with 2.5 ml layers of decreasing sucrose concentrations (2.0 M, 1.5M, and 1.0M) and then PMS. After a 90 minute centrifugation at 280,000 g, it was seen that both the 1.0M and 1.5M sucrose layers were pigmented, while the 2.0M layer was fairly clear. The 2.0M-2.5M interface was cloudy, indicating the probable presence of polysomes. In future experiments, a 1 ml 2.5M, 2.5 ml 2.0M and 1 ml 1.0M sucrose gradient was used. The 1.0M layer prevents the mixing of the PMS with the 2.0M layer when the PMS is layered over the gradient.

The gradients were centrifuged for 6 hr., 12 hr., and 18 hr. at 180,000 g to determine the time necessary to sediment all of the polysmes. 65% of the homogenate RNA (81% of polysomal RNA) was in the polysome fraction after either 12 hr. or 18 hr. of centrifugation. RIA of rat kidney polysomes showed that there

was contamination with RSA. Inclusion of BSA (.2%) in the gradient buffer, or using Buffer B (high salt, pH 6.0), or Buffer D with high salt as the gradient buffer did not eliminate this contamination. To determine whether the contamination was due to adsorption of RSA onto polysomes, normal rat serum was diluted into buffer or rabbit liver PMS and ultracentrifuged. In both cases, .25 to .33% of the RSA sedimented into the polysome region. This indicated that a second purification step would be necessary. (One would not expect that RSA monomer would sediment into the polysome region. This may represent denatured and/or aggregated RSA, possibly being ingested by phagocytes, etc. which were not adequately removed during preparation of the serum).

Polysomes from the first centrifugation step were diluted and layered over identical, but fresh sucrose gradients. It was found that 12 hours of centrifugation was not sufficient to recover all of the polysomes (63% recovery). After 20 hours of centrifugation, 77% of the polysomal RNA was recovered. RIA did not detect RSA contamination in rat kidney polysomes or rabbit liver polysomes isolated from a homogenate with normal rat serum.

Polysomes were centrifuged through continuous sucrose gradients to obtain polysome profiles and to determine whether any of the conditions used caused more or less polysome degradation than any of the other conditions. Buffer D was slightly (probably not significantly) better than Buffer B. Inclusion of heparin (80 units/ml) in Buffer D for the first ultracentrifugation step helped to preserve the polysome integrity.

The procedure now consisted of a first centrifugation of the PMS for 12 hours at 180,000 g through a sucrose gradient in Buffer D with heparin. The polysome layer was diluted, clarified by low speed centrifugation, and then centrifuged for 20 hours through a sucrose gradient made in Buffer D without heparin.

Polysome profiles showed that storage of polysomes at -76°C for over a month did not cause degradation of the polysomes. Incubation of the polysomes for 24 hours in the cold room (4°C), either in Buffer D, in RIA buffer (Buffer D with 0.2% BSA and .5% Tween 20) or in RIA buffer with Sephadex-anti RSA resulted in only a small amount of degradation of the polysomes. (The peak abosrbtion in the polysome profiles shifted from 6 ribosomes to 5 ribosomes per polysome). Incubation with normal

rabbit serum caused extensive degradation of the polysomes to predominantly disomes.

4. FINAL ISOLATION PROCEDURE

In developing the polysome isolation procedure, livers from fed rats were routinely used. The recovery of RNA in the polysome fraction after the first ultracentrifugation was 55% of homogenate RNA or 70% of polysomal RNA. This was considered to be an adequate recovery. Analysis of the RNA content of polysome fraction and homogenate of experimental animals showed that recoveries from fasted, turpentine treated and normal rats were all fairly consistently between 69 and 75%. However, only approximately 40% of homogenate RNA was recovered in the polysome fraction when livers from rats refed for 24 to 48 hours were used. It was observed that the post mitochondrial supernatants from these livers were cloudy, even after treatment with deoxycholate. The polysome samples were also cloudy, and could not be cleared by treatment with deoxycholate. It is thought that this may be due to contamination with glycogen, but this has not yet been investigated. Many investigators have used the optical density of a polysome sample at 260 nanometers as a measure of the amount of polysomes. To determine whether

this was valid for the polysomes isolated by the above procedure, RNA was extracted from the polysome samples. RNA was then determined by the conversion formula: 1 $O.D._{260}$ unit ≡ 32 µg RNA. It was found that polysomes samples from rats refed for 12 to 48 hours contained 20 to 25 µg RNA per $O.D._{260}$ unit; samples from all other rats contained 33 to 38 µg RNA per $O.D._{260}$ unit.

C. EFFECTS OF STRESS BY TURPENTINE INJECTION

Subcutaneous injection of turpentine causes an acute phase reaction in rats. Glucocorticoids (mainly corticosterone in rats) are thought to be one of the prime mediators of the response to this stress. Serum levels of many proteins (those termed acute phase proteins) increase, while levels of other proteins, including RSA, decrease. The experiments described here were designed to show some of the changes at the sub-cellular level which affect rates of protein synthesis.

1. DNA AND RNA LEVELS

DNA has been used in these experiments as a means of normalizing results. Liver weight is quite responsive to some of the experimental conditions (see section on fasting) and is therefore not a good parameter to use for normalization. In adult rats, even during fasting, total DNA levels (per whole liver) do not vary significantly.

Table VIII shows that the DNA content per gram of liver does not change significantly following turpentine treatment. RNA/DNA levels, however, increase by almost 50% 48 hours following turpentine treatment. Synthesis of all species of RNA (rRNA, tRNA, hnRNA, mRNA) is said to increase under conditions of elevated glucocorticoid levels (Sekeris and Schmid, 1973; Earp, 1974; Munck, 1968).

Following stress, a constant percentage (55-57%) of total homogenate RNA has been recovered in the polysome fraction. Assuming that polysomal RNA accounts for 80% of total homogenate RNA (Blobel and Potter, 1967), this recovery represents 69 to 71% of the polysomal RNA. While 100% recoveries would have been ideal, one would expect that a 70% recovery is fairly representative of the total polysome population. It does not suggest that a substantial fraction of the membrane-bound polysomes were pelleted with the nuclei and mitochondria (preliminary experiments with normal rat livers showed that 90 to 95% of homogenate RNA was found in the PMS). Longer ultra-centrifugation time did not improve recovery of normal rat liver polysomes. Perhaps some of the polysomes have associated with lower density material.

TABLE VIII

Table VIII: Nucleic Acid Content of Livers of Turpentine Injected Rats

The number of experimental animals in each group is in parentheses. The figures for the recovery of polysomal RNA are based on 80% of homogenate RNA being polysomal RNA. DNA and RNA extractions and polysome purifications are as described in Materials and Methods. For RNA determinations, the optical density of the RNA extract was measured and the conversion formula: 1 $O.D._{260}$ unit ≡ .032 mg RNA (Munro and Fleck, 1966), was used.

Hours after Turpentine Injection		mg DNA / g liver	mg RNA / g liver	RNA / DNA	µg RNA / OD_{260}	OD_{260} / g liver	polysomal RNA / homogenate DNA	polysomal RNA / homogenate RNA	Yield of polysomal (%) RNA
0	(7)	2.3±.3	6.5±.5	2.9±.5	33.4±2.5	107±10	1.59±.27	.55±.10	69±12
8	(3)	(1.8-2.3) 2.0	(7.1-7.8) 7.4	(3.2-4.2) 3.7	35.0-364 35.7	108-126 115	1.66-2.47 2.08	(.53-.59) .55	(66-74) 69
12	(4)	2.2±.5	8.1±.5	3.8±.8	38.0±1.2	121±16	2.14±.31	.57±05	71±6
24	(6)	2.2±.3	8.0±.6	3.6±.5	36.2±2.6	127.3±10	2.11±.41	.58±.05	72±6
36	(3)	(2.4-2.6) 2.5	(8.3-8.8) 8.5	3.1-3.7 3.4	(34.1-35.0) 34.5	127-148 136	1.82-1.91 1.89	(.51-.64) .55	(63-76) 69
48	(3)	(2.3-2.3) 2.3	(9.0-9.8) 9.5	3.8-4.4 4.1	34.9-35.4 35.1	(146-155) 152	(2.27-2.33) 2.31	(.53-.61) .56	(66-76) 70
72	(4)	2.7+.3	8.9±.8	3.4±.4	33.8±1.9	145±12	1.86±.23	.56±.08	69±10

2. RSA SYNTHESIZING POLYSOMES

24 to 48 hours following turpentine treatment, the percentage of polysomes synthesizing RSA has fallen to 60% of normal levels. When the number of RSA synthesizing polysomes is expressed in terms of DNA levels, the mRNA level has decreased by only 20%. If protein synthesis were strictly proportional to specific mRNA content, one would expect that the rate of RSA synthesis in the in vitro perfusion system would be only 20% lower in livers from turpentine treated rats as in livers from normal rats. Instead, it is found that both the absolute rate of RSA synthesis and its rate relative to total serum protein synthesis have decreased by 70%! (Miller, unpublished results) Clearly, there are other regulatory factors than mRNA levels. **The efficiency of translation of RSA mRNA must have decreased, possibly due to changes in rates of initiation or elongation.**

3. α_1-AGP SYNTHESIZING POLYSOMES

α_1-AGP synthesizing polysomes were almost undetectable in livers of normal rats. This is due to the background level which must be subtracted from all measurements- both rabbit liver and rat kidney polysomes inhibit the binding of iodinated

FIGURE 27

Figure 27: RSA Polysomes in Livers of Stressed (Turpentine Treated) Rats

Polysomes were isolated from livers of rats stressed by subcutaneous injection of turpentine. Polysomes were assayed at three different concentrations in the RSA radioimmunoassay. In parentheses, is shown the number of rats in each group. See pages 74 to 75 for method of calculating results.

RSA POLYSOMES IN LIVERS OF STRESSED (TURPENTINE TREATED) RATS

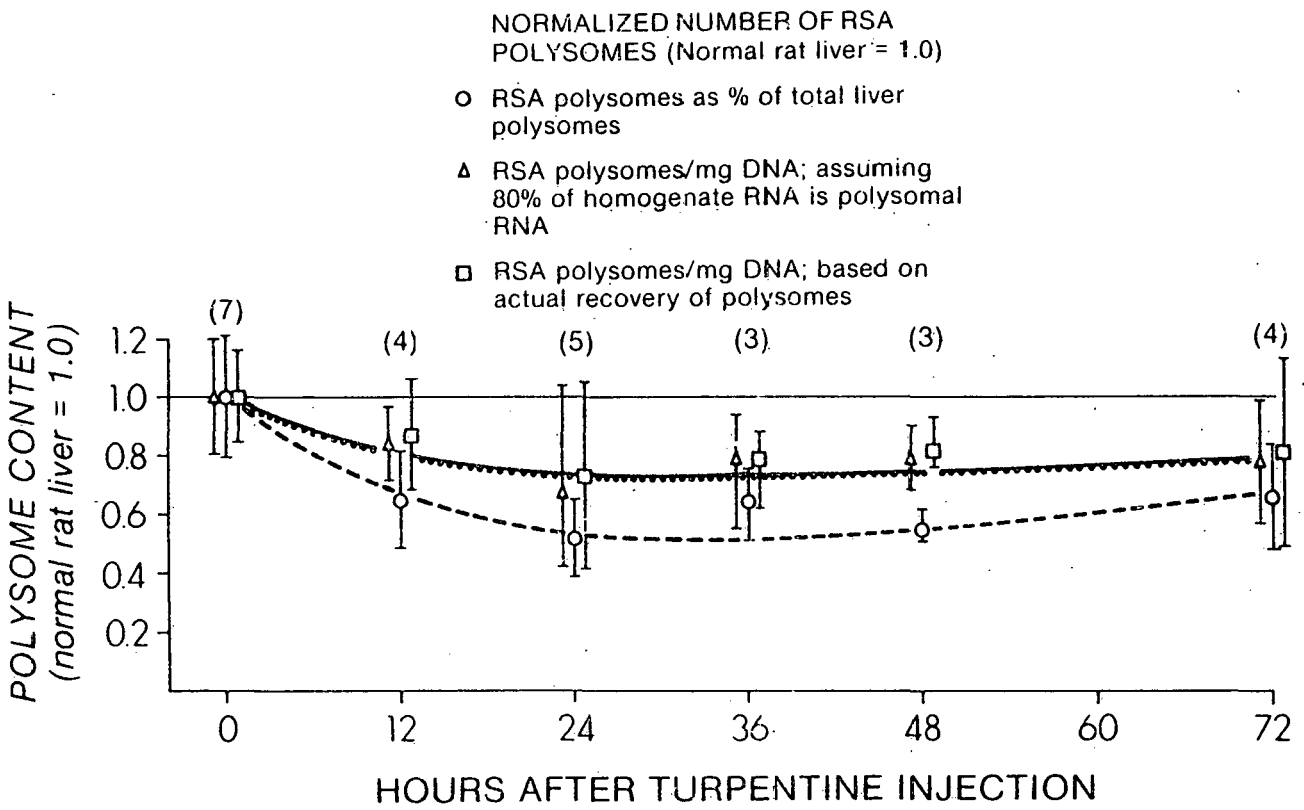
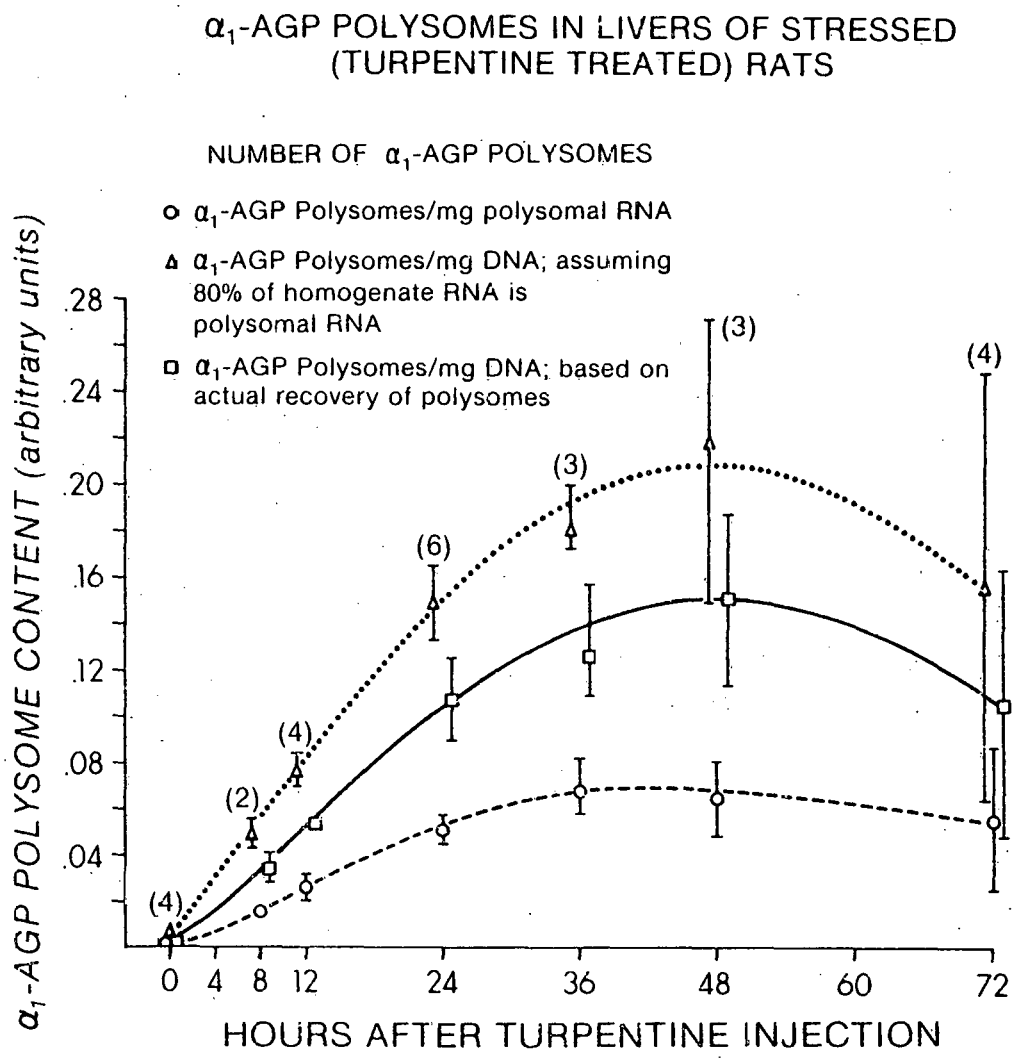


FIGURE 28

Figure 28: α_1-AGP Polysomes in Livers of Stressed (Turpentine Treated) Rats

Polysomes were isolated from livers of rats stressed by subcutaneous injection of turpentine. Polysomes were assayed at three different concentrations by the α_1-AGP radioimmunoassay. The background level found in rat kidney and rabbit liver polysomes was subtracted. In parentheses, is shown the number of rats in each group. See pages 74 to 75 for method of calculating results.


α1-AGP POLYSOMES IN LIVERS OF STRESSED (TURPENTINE TREATED) RATS
NUMBER OF α1-AGP POLYSOMES
o α1-AGP Polysomes/mg polysomal RNA
Δ α1-AGP Polysomes/mg DNA; assuming 80% of homogenate RNA is polysomal RNA
□ α1-AGP Polysomes/mg DNA; based on actual recovery of polysomes
α1-AGP POLYSOME CONTENT (arbitrary units)
.28
.24
.20
.16
.12
.08
.04
(4)
(2)
(4)
(6)
(3)
(3)
(4)
0 4 8 12 24 36 48 60 72
HOURS AFTER TURPENTINE INJECTION

RSA and α_1-AGP to their respective immunoadsorbents. There are two possible explanations for this: 1) The polysomes non-specifically bind to the immunoadsorbent. There have been reports of IgG binding non-specifically to polysomes, although neither the isolated F_c or F_{ab} fragment does (Holme et al, 1971). F_{ab} fragments could be used instead of the whole IgG molecule in preparation of the immunoadsorbents. 2) The iodinated antigen binds to polysomes in the supernatant rather than to the Sephadex. This is unlikely because the affinity of the antigen for the antibody should be much greater than its affinity for polysomes. This background level is approximately 1 ng antigen per 0.1 mg polysomal RNA. When α_1-AGP synthesis is induced, the mRNA levels found are 6 times this background level. However, in the uninduced state, the mRNA levels are less than twice the background, making precise measurements difficult. This background level needs to be reduced in order to measure low levels of α_1-AGP polysomes and to apply the system to proteins which comprise oly a small percentage of total protein synthesis.

At the present time, it is better to compare the 48 hour time point with the 8 hour time point, and just keep in mind that the mRNA has already been induced at 8 hours. There is seen to be at least a 4 fold increase in α_1-AGP polysomes per mg

DNA. By 72 hours, there is a small return toward normal levels. Perhaps the large standard deviations at 48 and 72 hours are indicative of the fact that some animals have begun to decrease the α_1-AGP polysome levels, while other animals are still increasing or maintaining the levels. The standard deviations for serum levels at these two times are equally as large.

The rate of α_1-AGP synthesis in perfusions of normal livers with cortisol, insulin, amino acids, and glucose (full supplementation) increases after 4 to 8 hours to 3.5 times normal levels (only amino acids and glucose in the perfusate; John and Miller, 1969). The fact that mRNA levels are already elevated at 8 hours, although the serum concentration of α_1-AGP has hardly increased, supports the fact that the induction by cortisol in the perfusion is first seen at 4 to 8 hours. Livers from rats treated with turpentine 24 hours prior to perfusion synthesize at least 6 times the normal amount of α_1-AGP during the first 12 hours of perfusion.

D. EFFECTS OF FASTING AND REFEEDING ON SPECIFIC mRNA LEVELS

1. DNA AND RNA LEVELS

The weight of the liver is extremely responsive to changes in nutritional status of the rat. For example, Yap et al (1978a) have shown that after 24 to 30 hours of fasting, there is a 30% decrease in liver weight. Morgan and Peters (1971) have shown a 50% decrease in liver weight after 10 days on a protein free diet. At the same time, the DNA content (per whole liver) decreased by less than 20% compared to controls. Part of this decrease is accounted for by the fact that young, growing animals were used, and therefore the DNA content of the livers of the control rats actually increased. Therefore, for comparison of results, expressing results on a per mg DNA basis is the best means of normalization.

After a 6 day fast, I have found that the RNA/DNA levels have dropped to 55% of the normal value (Table IX). The recovery of the RNA in the polysome fraction is 61 ± 12% (compare with 55 ± 10% in normal rats). The RNA/DNA ratio does not increase until 24 hours after commencement of refeeding. By 48 hours, it has reached normal levels.

TABLE IX

Table IX: Nucleic Acid Content of Livers of Rats Fasted for 6 Days and Refed for up to 3 Days

The number of experimental animals in each group is shown in parentheses. The figures for the recovery of polysomal RNA are based on 80% of homogenate RNA being polysomal RNA. DNA and RNA extractions and polysome purifications are as described in Materials and Methods. For RNA determinations, the optical density of the RNA extract was measured, and the following conversion formula was applied:

1 $O.D._{260}$ unit $\equiv$ .032 mg RNA, (Munro and Fleck, 1966).

		mg DNA / g liver	mg RNA / g liver	RNA / DNA	µg RNA / OD_{260}	OD_{260} / g liver	polysomal RNA / homogenate DNA	polysomal RNA / homogenate RNA	Yield of polysomal (%) RNA
Normal Rat	(7)	2.3±.3	6.5±.5	2.9±.5	33.4±2.5	107±10	1.59±.27	.55±.10	69±12
Hours of refeeding after 6 day fast									
0	(6)	4.0±.9	6.3±1.0	1.6±.2	35.4±2.6	107±11	.98±.21	.61±.12	76±15
4	(3)	(2.3-3.7) 3.0	(5.4-6.4) 6.0	(1.5-2.8) 2.1	(26.1-29.9) 28.1	(83-98) 90	(.64-1.03) .87	(.37-.47) .43	(46-58) 53
8	(2)	(3.1-3.3) 3.2	(5.3-58) 5.5	(1.6-1.9) 1.7	(20.8-28.2) 24.5	(93-119) 106	(.78-.85) .81	(.44-.50) .47	(55-62) 57
12	(3)	(3.2-3.7) 3.5	(5.8-6.9) 6.3	1.5-2.1 1.8	19.8-23.0 21.4	(112-135) 121	(.72-.75) .74	(.42-.46) .44	(53-58) 55
24	(5)	2.8±.3	6.0±.3	2.1±.1	21.4±6.1	110±17	.81±.25	.38±.11	47±14
36	(4)	2.2±.1	5.5±.6	2.5±.2	20.4±2.0	102±13	.94±.14	.38±.04	47±4
48	(3)	(2.3-2.5) 2.4	(7.3-7.5) 7.4	3.1±.2	24.4-29.7 27.1	98-121 112	(1.16-1.39) 1.28	(.40-.43) .41	(50-54) 51
72	(3)	(2.2-2.7) 2.5	(6.2-7.3) 6.7	(2.7-2.7) 2.7	(30.5-34.0) 31.8	(116-123) 117	(1.31-1.69) 1.52	(.48-.62) .56	(60-77) 70

2. LEVELS OF RSA SYNTHESIZING POLYSOMES

In rats fasted for 6 days, the number of RSA synthesizing polysomes per mg RNA is only slightly below normal, but the number of RSA polysomes per mg DNA has decreased by 40%. Liver perfusion studies show that the rate of synthesis of almost all (not α_1-AGP) serum proteins is 20 to 25% of normal levels. Thus there is a much sharper decrease in synthesis of most serum proteins than would be expected by the specific mRNA content (per whole liver). Yap et al (1978a) have found that even after only a 24 to 30 hour fast, 60% of RSA mRNA is found in the post-ribosomal supernatant as ribonucleoprotein complexes. These measurements were made using cDNA-RNA hybridization. These complexes do not contain ribosomal subunits, so they may be a storage form for the mRNA. These investigators (Yap et al, 1978b) have found that these mRNP complexes are rapidly mobilized into polysomes once refeeding (by gavage) has commenced.

Quantitation of RSA synthesizing polysomes in refed rats is made difficult by the fact that the recovery of homogenate RNA in the polysome fraction is only 40% between 24 and 48 hours

after commencement of refeeding. The amount of RNA recovered in the polysome fraction (per mg DNA in the homogenate) is actually less than in the fasted state! This suggests that a substantial fraction of the polysomes are not sedimenting into the polysome region. Further work is needed to determine the cause for this.

Results have been corrected to compensate for these low recoveries. Unfortunately, two assumptions had to be made: 1) a representative sample of polysomes was recovered and 2) 80% of homogenate RNA is polysomal RNA.

Twelve hours after commencement of refeeding, there is a definite increase in the amount of RSA polysomes (on both a per mg polysomal RNA and per mg DNA basis). At 48 hours, the amount of RSA mRNA/mg DNA is 60% higher than normal levels. By 72 hours, levels have returned to normal. Peters (1975) has found a similar response in the rate of RSA synthesis when rats maintaind on a protein free diet for 10 days are refed.

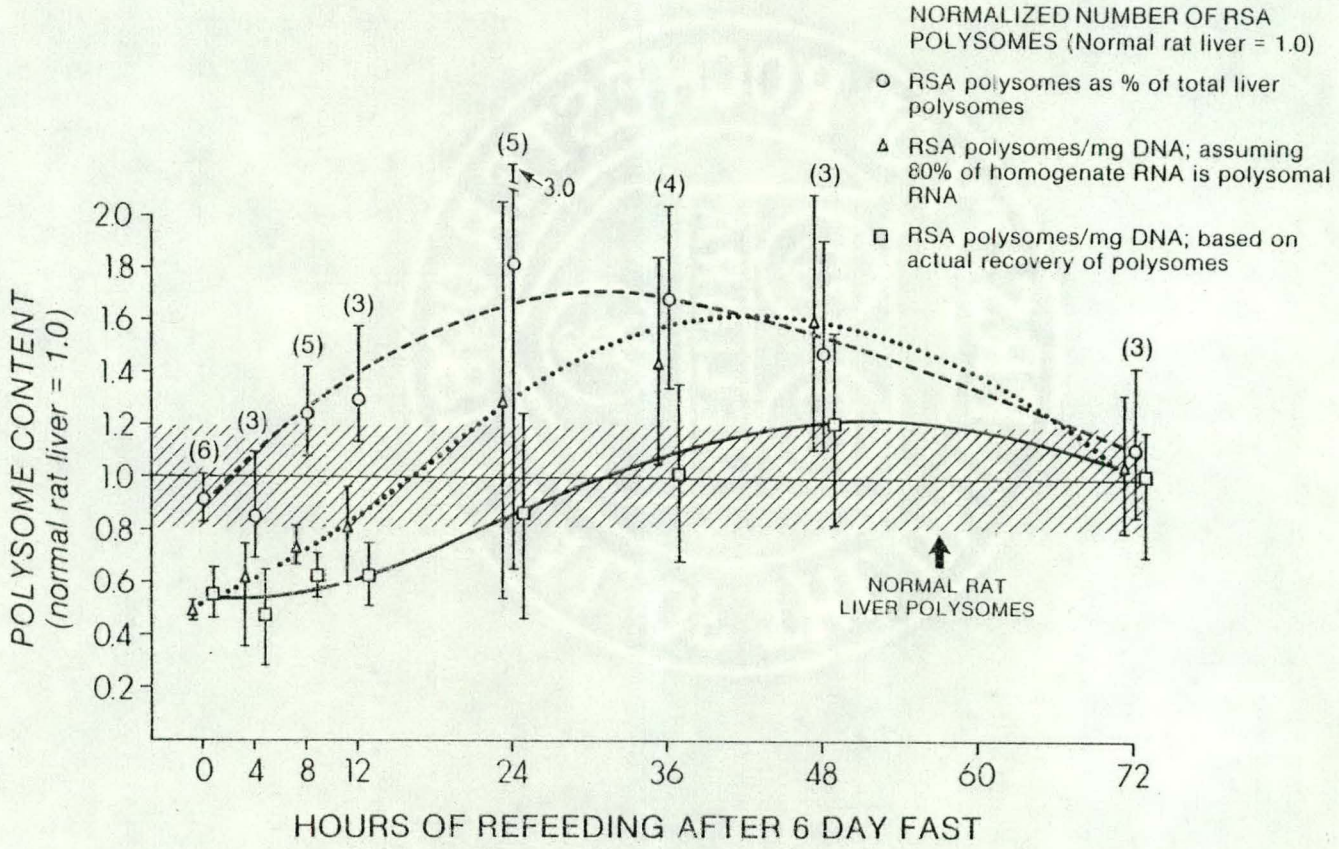
3. LEVELS OF α_1-AGP SYNTHESIZING POLYSOMES

Levels of α_1-AGP synthesizing polysomes were not significantly above background levels in either fasted or refed

FIGURE 29

Figure 29: RSA Polysomes in Rats Fasted for 6 Days and Refed for up to 3 Days

Polysomes were isolated from livers of rats fasted for 6 days and then refed for up to 3 days. Polysomes were assayed at three different concentrations by the RSA radioimmunoassay. In parentheses is shown the number of rats in each group. See pages 74 to 75 for method of calculating results.


NORMALIZED NUMBER OF RSA POLYSOMES (Normal rat liver = 1.0)
RSA polysomes as % of total liver polysomes
RSA polysomes/mg DNA; assuming 80% of homogenate RNA is polysomal RNA
RSA polysomes/mg DNA; based on actual recovery of polysomes
POLYSOME CONTENT (normal rat liver = 1.0)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
(6)
(3)
(5)
(3)
(5)
3.0
(4)
(3)
(3)
NORMAL RAT LIVER POLYSOMES
0
4
8
12
24
36
48
60
72
HOURS OF REFEEDING AFTER 6 DAY FAST

rats. Testing of these polysomes must therefore await the elimination of this background level.

IV. CONCLUSIONS

The radioimmunoassays for RSA and α_1-AGP are each sensitive to less than 1 ng of pure protein. There are several advantages to the polysome isolation procedure described:

1) No serum protein has been detected adsorbed to the polysomes;

2) In most cases, a 70% recovery of polysomal RNA has been attained.

However, there are several points in the procedure which need improvement:

1) There should be better control of ribonuclease activity- polysome profiles showed that the peak size was 6 ribosomes per mRNA;

2) The recovery of polysomes from livers of rats refed after a 6 day fast needs to be improved;

3) The nature of the substance in the purified polysomes which non-specifically inhibits the binding of labelled antigen to immunoadsorbent needs to be determined and this substance needs to be eliminated.

The experiments described here have shown that the method outlined, although needing some improvement, may be a very useful addition to the other methods already used to quantitate

specific mRNA. The method requires very little purified antigen and antibody, it does not require the purification of the specific mRNA, the immunoprecipitation of polysomes or the use of liquid scintillation counting. It measures only mRNA which is in the form of polysomes. The method is not sensitive to small changes, such as salt concentration, in the reaction mixture. The inter and intra- assay reproducibilities have been very good.

If one assumes (obviously not a valid assumption) that each RSA polysome is antigenically equivalent (both in association and dissociation rates to antibody) to an RSA molecule, then under normal conditions approximately 0.5% of liver mRNA would be RSA mRNA. However, RSA normally represents at least 5% of total liver protein synthesis. This suggests that the polysomes are actually very poor competitors for antibodies. Similar results were obtained with α_1-AGP. At the peak of the response to turpentine injection, with the assumptions made above, α_1-AGP mRNA would be only 0.6% ot total liver mRNA.

The experimental results show that during fasting and after stress by turpentine treatment, the synthesis of RSA decreases much more than does the mRNA level. These results support the hypothesis of Miller and Griffin (1975) that the synthesis of other

proteins, including acute phase proteins, takes precedence over the synthesis of RSA. Therefore under limiting conditions (possibly supply of amino acids, initiation and/or elongation factors) the translation of RSA mRNA may be severely compromised.

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