CELL-FREE TRANSLATION OF HOMOGENATE, FREE AND MEMBRANE-BOUND RIBOSOMAL mRNA OF LIVER: USE OF GUANIDINIUM THIOCYANATE IN mRNA ISOLATION

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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For Professor E. Roger Boothroyd for many interesting and enlightening hours.

ABSTRACT

Total ribonucleic acid was isolated from rat liver homogenates and the free and membrane-bound polysomal subpopulations derived therefrom by a modification of the guanidinium thiocyanate/CsCl centrifugation technique of Ullrich et al. (Science, 196: 1313-1319, 1970). Messenger ribonucleic acid was isolated following affinity chromatography and purification of the polyadenylated ribonucleic acid species. The integrity of the messenger ribonucleic acid was assessed by cell-free translation in the heterologous micrococcal nuclease-treated reticulocyte lysate system. The results for the homogenate messenger ribonucleic acid revealed synthesis of peptides up to 145,000 in molecular weight. The newly synthesized radioactive peptide products correlated well with the major secretory product of the liver, the plasma peptides. Indeed, 30.8% of the radioactivity in the total translation products was found in a peptide of molecular weight similar to albumin and corresponded well to the 31% maximum calculated on theoretical grounds.

Synthesis of the plasma peptides (most notably albumin) was segregated to the messenger ribonucleic acid isolated from membrane-bound polysomes while peptides identified as cytosolic proteins were translated near exclusively by the messenger ribonucleic acid derived from free polysomes.

The guanidinium thiocyanate/CsCl centrifugation method revealed itself as a markedly superior method for the isolation of messenger ribonucleic acid from liver. The results disprove

the contention of translational control as responsible for the synthesis of albumin by the membrane-bound polysomal population of liver (Shafritz, D. (1974) J. Biol. Chem., 249: 81-88 and 89-93).

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Title of thesis: Cell-free translation of homogenate, free and membrane-bound ribosomal mRNA of liver: Use of guanidinium thiocyanate in mRNA isolation.

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RESUME

On a isolé l'acide ribonucléique total d'homogénates de foies de rats ainsi que les sous-populations polysomiques libres et liées à la membrane qu'on en a dérivées au moyen d'une modification de la technique de centrifugation au thiocyanate de guanidinium/CsCl d'Ullrich et coll. (Science 196: 1313-1319, 1979). On a isolé l'acide ribonucléique messager après la chromatographie d'affinité et la purification de l'espèce d'acide ribonucléique polyadénylé. On a vérifié l'intégrité de l'ARN-messager par traduction acellulaire dans le système de lysat de réticulocytes traités à la nucléase micrococcique. Les résultats obtenus dans le cas de l'ARN-messager font apparaître la synthèse de peptides dont le poids moléculaire atteint jusqu'à 145,000. On a noté une bonne corrélation entre les produits radioactifs de peptides nouvellement synthétisés et les principaux produits sécrétoires du foie, les peptides plasmatiques. En effet, on a observé 30.8% de la radioactivité des produits de traduction totaux dans un peptide de poids moléculaire semblable à celui de l'albumine et ce pourcentage correspondait bien aux 31% donnés par nos calculs théoriques.

La synthèse des peptides plasmatiques (et particulièrement de l'albumine) a été séparée de l'ARN-messager isolé de polysomes liés à la membrane tandis que les peptides identifiés comme protéines cytosoliques ont été traduits presque exclusivement par l'ARN-messager dérivé de polysomes libres. La

méthode de centrifugation au thiocyanate de guanidinium-CsCl s'est avérée nettement supérieure pour l'isolation de l'ARN-messager du foie. Ces résultats contredisent l'hypothèse selon laquelle la régulation de la traduction serait à l'origine de la synthèse de l'albumine par la population de polysomes liés à la membrane dans le foie (Shafritz, D., 1974, J. Biol. Chem., 249: 81-88 et 89-93.

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Titre de la thèse: Traduction acellulaire dans l'homogénat de foie d'ARN-messager ribosomique libre ou lié à la membrane: Utilisation du thiocyanate de guanidinium dans l'isolation de l'ARN-messager.

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TABLE OF CONTENTS

ABBREVIATIONS

APH	- 1-acetyl-2-phenylhydrazine
ATP	- adenosine-5'-triphosphate
AP	- ammonium persulfate
Bis	- N, N'-methylene bisacrylamide
BSA	- bovine serum albumin
BPB	- bromophenol blue
CB	- coomassie brilliant blue G
CB-Stain	- coomassie brilliant blue G-stained SDS-Page
CsCl	- cesium chloride
DNA	- deoxyribonucleic acid
DEP	- diethyl pyrocarbonate
DMSO	- dimethyl sulfoxide
DTT	- dithiothreitol
EGTA	- ethyleneglycol-bis(2-aminoethyl ether)-N,N'-tetraacetic acid
EDTA	- ethylenediamine tetraacetic acid
GTC	- guanidinium thiocyanate/CsCl density centrifugation
GTP	- guanosine-5'-triphosphate
HCl(acid)	- hydrochloric acid
H_2O_2	- hydrogen peroxide
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
mRNA	- messenger ribonucleic acid
poly(A)$^+$RNA	- polyadenylated ribonucleic acid

$poly(A)^{+}_{bd}$ RNA	- membrane-bound polysomal polyadenylated ribonucleic acid
$poly(A)^{+}_{fr}$ RNA	- free polysomal polyadenylated ribonucleic acid
$poly(A)^{+}_{hom}$ RNA	- homogenate polyadenylated ribonucleic acid
POPOP	- 1,4-bis-2-(5-phenyloxazolyl)-benzene
PPO	- 2,5-diphenyloxazone
R_c	- mobility of electrophoresed peptides relative to the mobility of the cytosolic peptide, C.
RNAse	- ribonuclease
RNA	- ribonucleic acid
rRNA	- ribosomal ribonucleic acid
SDS	- sodium dodecyl sulfate
SDS-PAGE	- SDS-polyacrylamide gel electrophoresis
tRNA	- transfer ribonucleic acid
TEMED	- N,N,N',N'-tetramethyl ethylenediamine
TCA	- trichloracetic acid
TRIS	- Tris (hydroxymethyl) aminomethane

INTRODUCTION

Cells are enveloped by a proteinaceous lipidic coat termed the cell membrane. Within each eukaryotic cell there is a functional and, indeed, physical compartmentalization. Structurally, the compartments are also enclosed by membranes e.g. the Golgi apparatus, lysosomes, endoplasmic reticulum, and the nucleus. Therefore, the elucidation of membrane biogenesis has been of immense interest to cell biology ever since its inception. One of the numerous procedures employed to investigate membrane biogenesis has been the use of in vitro translation systems (Bergeron et al., 1975; Blobel, 1980; Toneguzzo and Ghosh, 1978; Rachubinski et al., 1980).

Within eukaryotic cells, proteins are translated from mRNA either associated with polyribosomes free in the cytoplasm (free polysomes) or polyribosomes attached to the endoplasmic reticulum (membrane-bound polysomes) (Palade, 1975).

The majority of secretory proteins have been shown to be synthesized by membrane-bound polysomes in contrast to cytosolic proteins which are thought to be synthesized by free polysomes (as reviewed in Davis and Tai, 1980; Palade, 1975; Rolleston, 1974; Shore and Tata, 1977). Nascent secretory peptide chains of these secretory proteins are released into the cisternae of the endoplasmic reticulum

and then transported to the Golgi apparatus where they are modified and packaged to form secretory vesicles. The completed peptides are eventually released into the extracellular space after fusion of secretory vesicles with the plasma membrane during exocytosis (e.g. proteins: Caro and Palade, 1964; glycoproteins: Neutra and Leblond, 1966; Leblond and Bennett, 1977). Therefore, cell biologists initially assumed that all membrane proteins would be synthesized on membrane-bound polysomes. This has been suggested for 5'-nucleotidase (Bergeron et al., 1975), VSV G-protein (Toneguzzo and Ghosh, 1978; Lodish and Rothman, 1979), and cytochrome P450 (Bar-Nun et al., 1980). However, with the use of in vitro translation systems, it was also revealed that the synthesis of integral membrane proteins could be directed by free polysomal mRNA (e.g. cytochrome b_5: Rachubinski et al., 1980).

All the above findings are, however, based on the assumptions that the heterologous or homologous in vitro translation systems are valid and that the methods used to isolate RNA result in intact, valid and representative mRNA.

Cell-free translation systems such as the reticulocyte lysate system have been rigorously tested for the efficacy, verity and reproducibility of mRNA translation (Pelham and Jackson, 1976; Shields and Blobel, 1978; Shore et al., 1979). However, those peptides synthesized in vitro could only be considered bona fide if the mRNA translated were also bona fide.

Bona fide mRNA could be obtained from intact biologically active RNA free of protein, polysaccharides and DNA. However, when mRNA is liberated from its confines within ribosomal subunits through cellular disruption, the mRNA becomes vulnerable to enzymic degradation because RNAse is also released from lysosomes. Since mRNA is a large macromolecule, it is also accessible to mechanical fragmentation.

Traditionally, the mRNA used in cell-free translation systems has been isolated by means of an organic solvent extraction which employed the use of phenol-chloroform (Penman, 1966; Perry et al., 1972).

Is it possible that this procedure may be too harsh for mRNA isolation due to the nature of its materials e.g. phenol and the numerous sequences of vigorous shaking and transfer required to extract RNA free of contaminants?

In 1974, Glisin et al., presented an RNA isolation procedure which used CsCl equilibrium density centrifugation (Glisin et al., 1974). The feature of equilibrium density centrifugation which is advantageous for the isolation of RNA, is that macromolecules possess specific and characteristic buoyant densities. Therefore, in one step it was then possible to isolate RNA (buoyant density ca 1.9g/.cm^3) free of DNA (buoyant density ca 1.7g/.cm^3), polysaccharides and glycogen (buoyant density ca 1.67 g/.cm^3), and proteins (buoyant density ca 1.2-1.4 g/.cm^3) including RNAse (Anderson et al., 1966; Counts and Flamm, 1966; Flamm et al., 1972;

Piko et al., 1967).

In 1968, Cox showed that a chaotropic agent, guanidinium hydrochloride, could be used in RNA isolation for the inactivation of RNAse (Cox, 1968). However, a more powerful chaotropic agent than guanidinium hydrochloride exists. It is guanidinium thiocyanate. The cation and anion of guanidinium thiocyanate effectively dissolve, denature and dissociate proteins (Jencks, 1969; Sela, 1957).

Then in a 1977 paper on the cloning of rat insulin genes, Ullrich et al. introduced another method of RNA isolation which incorporated the CsCl equilibrium density centrifugation procedure of the aforementioned Glisin et al. Ullrich et al. were able to isolate mRNA from islets of Langerhans from the rat pancreas, an organ replete in RNAse -200µg RNAse A/g tissue wet weight (Beintema et al., 1973). In their bibliography and exclusively in a footnote, they briefly indicated that the pancreas was homogenized in a powerful deproteinizing solution, 4M guanidinium thiocyanate /1 M mercaptoethanol, pH 5.0 and then layered over 5.7M CsCl and sedimented according to Glisin et al. (Ullrich et al., 1977).

It was therefore decided to employ this method. First, it implied a guarantee of the demise of RNAse. Its homogenizing solution also contained besides the guanidinium thiocyanate, the powerful reductant, 2- mercaptoethanol. 2 - Mercaptoethanol reduces the disulfide bonds of RNAse

which are essential for its catalytic activity (Sela, 1957). Secondly, it appeared a priori that the RNA would be handled more gently than by the phenol-chloroform extraction.

Anatomically and physiologically, the liver has presented itself as a reasonable experimental model for protein characterization and therefore, membrane biogenesis. The liver is one of the few tissues where the technique of subcellular fractionation has been validated and employed as a useful tool for testing various theories concerning membrane biogenesis (e.g. Dallner et al., 1966; Dehlinger and Schimke, 1971; Posner et al., 1979; Bergeron et al., 1973).

Albumin is a liver protein which has been well characterized with respect to its structure, sequence and mode of secretion. Mature rat liver plasma albumin has been shown to bear a molecular weight of 65,000 (Judah et al., 1976; Peters, 1970) whereas the molecular weight of its precursor, preproalbumin has been shown to be 68,000-70,000 (Strauss et al., 1978; Rachubinski et al., 1980). The proportion of albumin synthezized by the liver compared to the total liver proteins synthesized in vivo determimed by several workers has varied from 3.5% (Schreiber et al., 1971) to 10.9% (Peters and Peters, 1972) to 12% (Peavy et al., 1978) to 13% (Keller and Taylor, 1976).

Though the techniques available to cell biologists over the past 25 years for determining peptide spectra e.g. SDS-PAGE had limited sensitivity, nevertheless, it was found

that most plasma proteins except gamma globulins were made almost exclusively by the liver (Bergeron et al., 1978; Crane and Miller, 1974; Miller et al., 1954; Glaumann and Ericsson, 1970; Glaumann et al., 1975; Jones et al., 1967; Miller and Bale, 1954; Miller et al., 1954; Morgan and Peters, 1971; Noel and Rubinstein, 1974; Peters et al., 1971; Redman et al., 1975).

Albumin represents the major peptide of plasma and the main secretory product of the liver.

Therefore, significant levels of mRNA from the liver should code for plasma proteins and more specifically, albumin. This prediction could be useful in assessing the integrity of mRNA isolated from rat liver homogenates i.e. the ability of this mRNA to direct the synthesis of albumin.

If it could be established that the isolation of homogenate mRNA by the guanidinium thiocyanate CsCl centrifugation procedure (henceforth considered as the GTC procedure) were superior to the phenol-chloroform extraction procedure, would it then be worthwhile and significant to further subfractionate the homogenate into the two main populations of ribosomes namely, free and membrane-bound polysomes for their subsequent isolation of mRNA?

Previous workers have described the synthesis of albumin mainly on membrane-bound polysomes (Hicks et al., 1969; Takagi and Ogata, 1968; Takagi et al., 1969; Takagi et al., 1970) and of a cytosolic protein, ferritin, on free polysomes

(Hicks et al., 1969; Redman, 1969). However, a controversy exists in the literature.

That is, exclusivity of the translation of albumin directed by mRNA on membrane-bound polysomes had been questioned by Shafritz in 1974. From his studies of 1974, he claimed that 'read-out' experiments showed (as expected) that the synthesis of albumin was on membrane-bound polysomes solely, whereas heterologous in vitro translation experiments showed an identical distribution of mRNA coding for albumin in both free and membrane-bound polysomes (Shafritz, 1974). Shafritz explained that translational control could account for the near-exclusive synthesis in vivo of albumin on membrane-bound polysomes.

One point of note is that most of the in vitro studies which used mRNA translation to assess albumin synthesis had employed the phenol-chloroform extraction procedure. As well, little attempt was made to correlate the proportion of albumin synthesized by the liver in vitro with the expected 13% (Keller and Taylor, 1976) in vivo.

OBJECTIVE

It was therefore considered worthwhile to attempt the isolation of liver mRNA following whatever could be gleamed from Ullrich et al.'s footnote by the GTC procedure:

> 'Islets were homogenized in 4M guanidinium thiocyanate (Tridom/Fluka), 1 M mercaptoethanol

buffered to pH 5.0 (J.M. Chirgwin, A.Pryzbyla, W.J. Rutter, in preparation). The homogenate was layered over 5.7M cesium chloride and the RNA was sedimented as described by V. Glisin, R. Crkvenjakou, C. Byus, [Biochem. 13:2633 (1974)].' Science (1977) 196:Reference No. 6, p. 1319.

Messenger RNA would be isolated from rat liver homogenates and translated in the well-established reticulocyte lysate system. The integrity of the mRNA would therefore be assessed by comparing the translation products through SDS-PAGE with the predicted major synthetic products of liver, namely, the plasma proteins and specifically, plasma albumin.

If homogenate mRNA was shown to be relatively unfragmented, then the GTC procedure could be used to study the biogenesis of liver membranes. For this latter purpose, however, the free and membrane-bound polysomes of liver would need to be effectively separated and their mRNA (isolated as above) assessed for integrity. The integrity of membrane-bound polysomal mRNA would be determined by comparing its translation products to plasma proteins (liver secretory products). As well, the integrity of free polysomal mRNA would be determined by comparing its translation products to liver sedentary proteins namely, the cytosolic proteins.

MATERIALS

1. All chemicals were of analytical grade.

Lactated Ringer's solution was obtained from Abbott Laboratories, Ltd., Montreal, Quebec.

PPO was purchased from Amersham, Arlington Heights, Illinois.

BPB, glacial acetic acid, hydrogen peroxide, absolute methanol, TCA and p-xylene were purchased from Anachemia Ltd., Lachine, Quebec.

DMSO was obtained from J.T. Baker Chemical Co., Phillipsburg, New Jersey.

SDS was obtained from Beckman Instruments Inc., Palo Alto, California.

Bio-Rad dye, high and low molecular weight protein standards and Econo-Columns (0.5 cm X10 cm) were purchased from Bio Rad Laboratories, Mississauga, Ontario.

Whatman No. 1 filter paper and No. 3 MM filter disks were purchased from Canlab, Montreal, Quebec.

Oligo(dT)-cellulose type T3, was obtained from Collaborative Research Inc., Waltham, Massachusetts.

Absolute ethanol was obtained from Consolidated Alcohols Ltd., Toronto, Ontario.

Bis was purchased from Eastman Kodak Co., Rochester, New York.

AP, iso-butanol, ethylene glycol, ether, glycerin, HCl (acid), magnesium acetate, potassium acetate, potassium hydroxide (pellets), sodium acetate, sodium chloride, sodium hydroxide and sucrose were purchased from Fisher Scientific Co. Ltd., Montreal, Quebec.

Guanidinium thiocyanate was obtained from ICN-K & K Laboratories, Inc., Plainview, New York.

Royal X-O-Mat, X-Ray film, KLX developer and Rapid Fixer were from Kodak, Montreal, Quebec.

Nitrogen gas was from Liquid Carbonic, Montreal, Quebec.

GSWP filters of pore size 0.22 µm and diameters 13 mm and 25 mm were purchased from Millipore Ltd., Mississauga, Ontario.

Sodium pentobarbital, 65mg/ml, was obtained from M.T.C. Pharmaceuticals, Hamilton, Ontario.

1mCi/ml of ^{3}H-L-Leucine (L-[3,4,5-^{3}H(N)])with a specific activity of >110 Ci/mmol, Protosol and standard linear polyethylene LSC vials were purchased from New England Nuclear Canada, Lachine, Quebec.

POPOP was obtained from Packard Instruments Co., Inc., Downers Grove, Illinois.

APH, acrylamide, ATP, all 20 cold L-amino acids, brilliant cresyl blue, CaCl, CsCl, CB, creatine phosphate, creatine phosphokinase, cycloheximide, DEP, EDTA EGTA, GTP, hemin,

heparin, HEPES, 2-mercaptoethanol, micrococcal nuclease, TEMED, Tris, Triton X-100 and wheat germ tRNA were purchased from Sigma Chemical Co., St. Louis, Missouri.

2. ANIMALS

Male Sherman rats were obtained from the breeding colony of the Department of Anatomy, McGill University. They were maintained on Purina Rat Chow unless starved. The duration of starvations are indicated where applicable. As well, the rats received water ad libitum.

METHODS

I. Methods used in the Operation and Analysis of SDS-PAGE.

1 SDS-PAGE with a 5-15% resolving gel and discontinuous buffer system

The estimation of the molecular weights of proteins and their subunits by SDS-PAGE was introduced by Shapiro, Vinuela and Maizel (1967), the discontinuous buffer system by Davis (1964), and the acrylamide gradient by Blobel and Dobberstein (1975). Based on the work of the above authors and other considerations as outlined by Maizel (1971), the following protocols were designed for SDS-PAGE.

Throughout this work, doubly-distilled water was used. As well, all % solutions refer to W/V measurements unless otherwise specified.

a Stock solutions of the resolving and spacer gels

COMMON

i	ii
30% acrylamide	Tank buffer;
0.8% Bis	2.88% L-glycine
Water to volume	0.6% Tris, pH 8.3
Filtered with Whatman No. 1 paper and stored at 4°C for 2 months	0.1% SDS
	Water to volume
	Made freshly

iii

10% AP
Water to volume
Stored at 4°C for two weeks

iv

10% SDS
Water to volume
Stored at room temperature for two weeks

Resolving Gel	Spacer Gel
i	i
Buffer:	Buffer:
2.0M Tris-HCl, pH 8.85	0.5M Tris-HCl, pH 6.75
Water to volume	Water to volume
Stored at 4°C (for 1.5 weeks	Stored at 4°C for 1.5 weeks
ii	ii
85% sucrose	60% sucrose
Water to volume	Water to volume
Stored at 4°C	Stored at 4°C

B Preparation of the gel.

A 5-15% continuous gradient was formed for the resolving gel as follows:

% Acrylamide	5%	15%
Constituents /volume (ml)	for 60 ml	for 60 ml
30% acrylamide/0.8% Bis	10	30
Resolving gel buffer, pH 8.85	12	12
TEMED	0.05	0.05
85% sucrose	nil	8
10% SDS	0.6	0.6
Water	37.0	9
10% AP	0.3	0.3

The catalyst and accelerator for polymerization were AP and TEMED, respectively (Davis, 1964)

The 5% solution was made first and 19.5ml was placed in the gradient mixer chamber with a closed internal channel. Then the 15% solution was made and 17.5 ml added to the chamber with closed internal and external channels. The Buchler gradient mixer was immediately turned on, the channels opened and the continuous gradient then flowed through the external channel into the gel form to produce a slab gel after polymerization and hardening. Finally, four drops of iso-butanol were added to the upper edge of the resolving gel prior to polymerization so as to form a level surface. Since iso-butanol is immiscible, it was poured off after polymerization.

The spacer gel was made after the resolving gel. It must be of a concentration less than the starting concentration of the resolving gel gradient (Ornstein, 1964; and Davis, 1964).

Therefore, a 4% spacer gel was formed as follows:

% Acrylamide	4%
Constituents/volume (ml) for 50 ml	
30% acrylamide /0.8% Bis	6.6
Spacer gel buffer, pH 6.75	6.0
TEMED	0.05
10% SDS	0.5
60% sucrose	12.5
Water	24
10% AP	0.4

The spacer gel was added directly to the upper edge of the resolving gel. A comb was immediately placed in the spacer gel and removed after polymerization, thus forming pockets for sample loading.

c. Preparation of the sample:

A 'sample preparation' was made which contained the necessary chemicals to denature the protein sample (Reynolds and Tanford, 1970).

This solution was made as follows:

In a total volume of 5 ml there was:

1 ml of 10% SDS to coat the denatured hairpin-shaped protein with negative charges when heated at 100°C.

1 ml of spacer gel buffer, pH 6.75 to resuspend the protein in a pH identical to the spacer gel

1 ml of 0.1% BPB to be the marker for the gel front

1 ml of 2-mercaptoethanol to reduce the protein to prevent the formation of disulfide bonds. The latter result in aggregates and nonuniform conformations of the peptides.

1 ml of glycerin to make the sample solution more viscous and thereby allow it to be underlayed in a small volume into the gel.

50µl of the 'sample preparation' was added to the required volume of sample or standard. Usually, 40µg of

protein was sufficient.

The sample solution was heated at 100°C for two minutes and immediately loaded into the gel pockets of the spacer gel by Peterson pipettes.

The negative hairpin shape must be formed for two reasons.

When different proteins assume the form of a hairpin loop, the differences of tertiary structures are eliminated with respect to migration. As well, the obtention of a uniform negative charge in the proteins enables all proteins to now migrate to the anode strictly according to the length of the hairpin loop and thus directly proportional to molecular weight.

d. Operation of the gel

The gel box was constructed of plexiglas based on the design of Reid and Bieleski (1968) as modified by Studier (1972).

The rationale for the use of spacer and resolving gels was put forward by Ornstein (1964) in which advantage was taken of the Kohlrausch (1897) phenomenon. Namely, at the large pore size and low pH of the spacer gel, 6.7, (as well as the Tank buffer) chloride ions will migrate markedly faster than the protein molecules and the (very few) glycinate ions at these pH's. Thus a difference in potential will be set up between the rapidly migrating chloride ions (leading ions) and the much more slowly

migrating (and very few) glycinate ions (trailing ions). This sets up a considerable difference in potential and the peptides which are negatively charged are caught in between and accelerated— thereby stacked by the Kohlrausch phenomenon— toward the chloride front. However, upon reaching this, they follow the chloride ions in front, uniformly as a stack. Upon a change in pH (to 8.9) and pore size (Orstein, 1964) many more glycine molecules (in fact, a vast excess) become negatively charged. As these ions are of a much smaller size than the negatively charged peptides, the former rush past the peptides to follow the chloride front. The uniform distribution of glycine in the Tank buffer now eliminates the Kohlrausch phenomenon and the peptides are now subject to molecular sieving. Since all charge and shape differences have been removed by the SDS effect, the proteins then separate according to molecular weight with the gradient of acrylamide preventing diffusion of the lower molecular weight species (i.e., smaller pore size).

Gels were electrophoresed at an amperage setting of 17.5 mamps using a Heath-Schlumberger Regulated High Voltage Power Supply, Model SP-17A. To maintain this amperage, it was necessary to increase the initial voltage. The maximum allowable voltage was 240 volts beyond which the gel would overheat. After this voltage was reached, the amperage gradually decreased. Gels were electrophoresed for approximately 7 hours.

e. Development of the gel for analysis

After electrophoresis, the gel was stained overnight in the following solution using a modified method of Weber and Osborn (1969):

0.1% CB
50.0% absolute methanol (V/V)
10.0% glacial acetic acid (V/V)

The gel was then destained for one day in the following solution:

7.0% acetic acid (V/V)
30.0% absolute methanol (V/V)

The destaining solution was changed several times in order to obtain a sharp contrast of band migration and separation.

2. Gel Standards

Bio-Rad SDS-PAGE molecular weight standards were used. The approximate concentration of each protein was 2 mg/ml. 5 µl of the high and low molecular weight standards was used.

The protein composition of each set of standards was:

High Molecular Weight Standards.

Protein	Molecular Weight
Myosin	200,000
B-Galactosidase	130,000
Phosphorylase B	94,000
Bovine Serum Albumin	68,000
Ovalbumin	43,000

Low Molecular Weight Standards:

Protein	Molecular Weight
Phosphorylase B	94,000
Bovine Serum Albumin	68,000
Ovalbumin	43,000
Carbonic Anhydrase	30,000
Soybean trypsin inhibitor	21,000
Lysozyme	14,300

3. Estimation of the molecular weights of sample peptides

When a sample was electrophoresed in SDS-PAGE, the molecular weights of its peptide constituents were determined from a plot of Standard molecular weight vs. Mobility relative to the mobility of the cytosolic peptide, C (i.e., relative mobility, R_c) (Weber and Osborn, 1969).

The Bio-Rad high and low molecular weight standards were used for the Standard molecular weight vs R_c plots. R_c's were determined as follows:

After electrophoresis of the standards, gel staining and destaining, the distances of individual bands from the upper edge of the resolving gel were measured relative to the cytosolic peptide, C. This ratio represented R_c. Then on semi-logarithmic graph paper, the molecular weight values were plotted on the ordinate and the R_c values on the abscissa.

Thus, to estimate the molecular weights of the peptide constituents of a sample, the R_c's of the sample's peptide

bands were determined and the molecular weights read off a standard curve of Molecular weight vs. R_c (e.g. Fig. 2a). The samples were electrophoresed in the same gel as the standards, unless noted.

II Isolation of Rat Liver Cytosol, Serum and Plasma

1. Isolation of rat liver cytosol

Liver cytosol was prepared as described by Rachubinski et al., (1980).

A male Sherman rat starved for 12 hours was anaesthetized with 0.1% sodium pentobarbital and then perfused, using a perfusion height of 1.2 m, with Lactated Ringer's via the left ventricle. The liver was excised, washed once in ice-cold 0.25 M sucrose, homogenized in a Potter-Elvejham homogenizer (7-9 strokes), filtered with nylon boulting cloth and made to a 1:3 homogenate with 0.25 M sucrose.

The homogenate was centrifuged for 10 min. at 12,000g in the Sorvall RC-2 refrigerated centrifuge.

The 12K supernatant was centrifuged at 150,000g_{max} for 1 h in an International (IEC) A-237 rotor using the IEC B-60 ultracentrifuge. This final supernatant (i.e. I50K) was operationally defined as the cytosolic fraction.

The 150K supernatants were pooled, samples taken for protein determination and the remainder stood at -70^{o}C in a Revco upright freezer.

Approximately 110 µg-132 µg of cytosol was used for electrophoresis.

b. Isolation of rat liver serum

Liver serum was prepared according to Bergeron et al., 1978.

A male Sherman rat starved for 12 hours was anaesthetized with ether and decapitated. The blood was collected, left at room temperature for 1 h and then left at 0-4°C for 3 hours. The resulting blood clot was loosened at its periphery with a wooden applicator stick. The clot was then removed by centrifugation at 1,000 g_{av} for 15 min at 0-4°C in a IEC clinical centrifuge. The IK supernatant was operationally defined as serum. This supernatant was removed with a Pasteur pipette, samples taken for protein determination and the remainder stored at -70°C in the Revco upright freezer.

Approximately 60 µg of serum was used for electrophoresis.

c. Isolation of rat liver plasma

An unstarved male Sherman rat was decapitated. Its blood was collected into tubes rinsed with 1% heparin and centrifuged at 0-4°C and 1,000g_{av} for 10 min. The IK supernatant above a white buffy area was operationally defined as plasma. However, this supernatant was not used if it had a reddish tinge. The IK supernatant was gently removed with a Pasteur pipette, samples taken for protein determination and the remainder stored at -70°C in an upright Revco freezer.

Approximately 100 µg of plasma was used for electrophoresis.

III Bio-Rad Protein Assay

Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Technical Bulletin 1051: April, 1977).

1. Solutions

4.0 ml of doubly distilled water was added to 1.0 ml of the Bio-Rad dye reagent. This solution was filtered through Whatman No. 1 filter paper and stored at room temperature for up to 2 weeks. Then a standard BSA stock solution of 10mg/ml was made, from which 2mg/ml of BSA was prepared at the time of an assay.

A typical set of BSA standards were:

Desired µg Protein	Vol 2mg/ml BSA (in µl)	Vol of Water
20	10	90
40	20	80
60	30	70
80	40	60
100	50	50
140	70	30
160	80	20
200	100	Nil

2. Method

A total volume of 0.1 ml of each standard, blank and sample was prepared. 5.0 ml of the diluted Bio-Rad dye reagent was added to the 0.1 ml and mixed immediately. Between 5 min and 1 hr, optical absorption readings were taken at 595 nm in a Beckman Model Du Quartz spectrophotometer.

IV Methods used to carry out Cell-Free Protein Synthesis

1. Isolation of RNA from three subcellular fractions of rat liver

All glassware, metal instruments, polyallomer and polycarbonate tubes as well as the graduated cylinders used for RNA isolation were sterilized by dry-heating at 65°C for at least 6 hr, except the components for Millipore filtering which were sterilized in an Amsco autoclave for 1 hour at 15 psi and 120°C.

All solutions for RNA isolation were passed through 0.22 µm GSWP Millipore filters unless otherwise noted. After filtering they were tightly sealed. They were made at the most 3 days before the experiment. Cycloheximide and 2-mercaptoethanol were added to solutions on the day of an experiment. The rats used for RNA isolation were starved for 12 h but given water ad libitum.

a. Homogenate

100-150g starved male Sherman rats were anaesthetized with 0.1% sodium pentobarbital using a 1 ml sterile plastic syringe with a 25 5/8 gauge needle. The mediastinum of rats was entered in order to carry out cardiac perfusion. It was entered by cutting along the length of the linea alba in an inferior to superior direction, cutting the lower ribs from the superior end of the linea alba laterally, to expose the inferior surface of the diaphragm. Then the xiphoid process was raised and the heart exposed by incising the anterior margin of the diaphragm.

Perfusion was carried out using unfiltered (ice-cold) Lactated Ringer's containing 5µg/ml of cycloheximide, via the

left ventricle with a 21 gauge needle until the liver was clearly blanched.

This was done to flush blood and thus, the RNAse it contains from the liver. As well, cycloheximide prevents ribosomes from running off mRNA by inhibiting elongation (Ennis and Lubin, 1964; reviewed by Vazquez, 1979), thereby eliminating the problem of membrane-bound polysomes becoming distributed in the free polysomal population.

Livers were excised, trimmed of fat, connective tissue, and bile duct, blotted, and placed in a 4 M guanidinium thiocyanate / 1 M 2-mercaptoethanol, pH 5.0 / 5 µg/ml cycloheximide solution (a modification of the method of Chirgwin, Pryzbyla and Rutter as outlined by Ullrich et al. in 1977).

The guanidinium thiocyanate solution was prepared by heating the required amount of guanidinium thiocyanate on a Tek Pro Heat Stir 36 at a low heat, and stirring until the solution turned from a light brown cloudy solution to a clear solution with a brown precipitate. 2-mercaptoethanol was added to the guanidinium thiocyanate solution after it had cooled to room temperature. The solution was adjusted to pH 5.0 with glacial acetic acid, made up to volume, and filtered through Whatman No. 1 filter paper. It was then filtered through a 0.22 µm GSWP Millipore filter. This solution was filtered again on the day of the experiment through another 0.22 µm GSWP Millipore filter after the addition of cycloheximide.

5.7 M CsCl/0.1 M EDTA was treated with 0.2% DEP to inactivate any RNAse present in the CsCl (Taylor, 1976; Ehrenberg et al., 1976). After the addition of DEP, the solution's pH was adjusted to pH 6.5 and then left to stand for 20 min to inactivate RNAse. After 20 min, the solution was boiled for 2 min to inactivate DEP (Chirgwin, personal communication). The solution was then re-equilibrated to its original volume.

The rat livers were diced in a second change of the guanidinium thiocyanate solution and then homogenized at 0-4°C with a Potter-Elvehjem 40 ml homogenizer. The homogenate was filtered through a nylon boulting cloth, transferred to room temperature, and made up to the required volume with 4 M guanidinium thiocyanate / 1 M 2-mercaptoethanol, pH 5.0/5µg/ml cycloheximide to yield a 5% (W/V) homogenate.

Approximately 2.8 ml of the homogenate was layered onto 1.0 ml of 5.7 M CsCl/0.1 M EDTA, pH 6.5/6 mM 2-mercaptoethanol/ 5 µg/ml cyloheximide solution in each IEC SB405 polyallomer tube. About 16.8 ml of the homogenate was used. Six tubes were equilibrated on a Harvard Trip balance and centrifuged in an IEC SB405 rotor for 20-22 h at 192,000 g_{max} and 25°C using an IEC B-60 ultracentrifuge (Glisin et al., 1974; Martin and ter Meulen, 1976; Chirgwin, personal communication).

After centrifugation, all the supernatant, which included a DNA band at the guanidinium thiocyanate - CsCl interface, was aspirated with a Pasteur pipette leaving a clear glassy pellet. The tubes were inverted and the

walls of the tubes wiped with Kimwipes to remove any traces of DNA. The RNA in the clear pellet was resuspended in 1 volume of 100mM Tris acetate/100 mM sodium acetate, pH 9.0/ 6mM 2-mercaptoethanol at room temperature using a Teflon pestle. A 0.1 volume of 4°C 2.0M potassium acetate, pH 5.5/ 6 mM 2-mercaptoethanol solution was added in the cold and then 2.5 volumes of -20°C cold absolute ethanol was added. The resulting solution was kept at -20°C at least 12 h to precipitate the RNA. (Bergeron et al., 1975). Further processing will be described later.

b. Membrane-bound polysomal fraction

Membrane-bound polysomes were isolated following the procedure of Ramsey and Steele (1976) as modified by Rachubinski et al. (1980).

The rat livers were perfused and excised in the same manner outlined for homogenate RNA isolation. However, the livers were placed in ice-cold Polysomal Buffer-0.25M sucrose, which consisted of 0.2M Tris acetate, pH 8.5 at 2°C/75mM potassium acetate/5mM magnesium acetate/6mM 2-mercaptoethanol/5µg/ml cycloheximide/150 units /ml heparin and 0.25 M sucrose, diced in a second change of the same buffer and homogenized at 0.4°C with a Potter-Elvehjem 4.0 ml homogenizer. The homogenate was filtered through nylon boulting cloth and diluted with the buffer to give a 1:8-1:10 (W/V) homogenate.

The homogenate was centrifuged at 740 g_{av} for 2 min,

then at 131,000 g_{av} for 12 min in a Beckman L5-65 ultracentrifuge at 0-4°C, acceleration rate 10 using SW27 polyallomer tubes and the SW27 rotor. Most of the 131K supernatant (contained free polysomes) was poured into a graduated cylinder measured, and kept in the cold, 0-4°C, until further use. The supernatant closest to the pellet, was discarded.

The SW27 tubes were inverted at 0-4°C to drain the supernatant and the walls were wiped with Kimwipes. The upright tubes were placed on ice and transferred to room temperature. The 131 K pellets (containing bound polysomes) were scooped from the tubes with a spatula and placed in a 20 ml Dounce homogenizer at room temperature containing 4 M guanidinium thiocyanate/1 M 2-mercaptoethanol, pH 5.0/5 µg//ml cycloheximide. The pellet was then homogenized using the 'A' pestle and either diluted with the guanidinium thiocyanate solution to give a final concentration of 9-11% (equivalent starting liver wet wt./V) or left undiluted. (Dilution yielded a smaller DNA band).

Half the suspension was used for RNA isolation (ca. 16.8 ml). The RNA was isolated via CsCl density contrifugation and precipitated with absolute ethanol following the same procedures described for the isolation of homogenate RNA.

c. Free polysomal fraction

Free polysomes were isolated following the procedure of Ramsey and Steele (1976) as modified by Rachubinski et al., (1980)

After approximately 3 h at 0-4°C, the 131,000 g_{av} supernatant was added to Beckman 60 Ti polycarbonate tubes half-way (13.0-13.5 ml). When necessary, Polysome Buffer-0.25 M sucrose was added to the supernatant to make up the appropriate volume. Each tube was then underlaid first with 7 ml of Polysome Buffer-1.38 M sucrose, pH 8.5 at 2°C, using a 20 or 30 ml sterile plastic syringe with a 15 or 17 gauge needle with 2-3 cm of tubing attached at its tip and secondly, with Polysome Buffer-2.0 M sucrose, pH 8.5 at 2°C, to capacity in the same manner.

The tubes were centrifuged in a Beckman 60 Ti rotor for 15-17 h, at 169,000 g_{max} using a Beckman L5-65 ultracentrifuge at 0-4°C and at an acceleration rate of 10.

The supernatant and sucrose cushions were aspirated with a Pasteur pipette, the walls of the tubes wiped with Kimwipes and a few drops of 4 M guanidinium thiocyanate/1 M 2-mercaptoethanol, pH 5.0/5µg/ml cycloheximide were added to the free polysomal pellet. The pellets were resuspended using a glass rod with a large round tip and the suspensions removed with a Pasteur pipette to a 20 ml Dounce homogenizer containing guanidinium thiocyanate solution. The suspension was homogenized using the 'A' pestle and diluted with the guanidinium thiocyanate solution to approximately 16.8 mls which was used for RNA isolation. The RNA was obtained via CsCl density centrifugation and

precipitated with absolute ethanol following the same procedures described for the isolation of homogenate RNA.

2. Isolation of Poly $(A)^+$ RNA

The RNA precipitates of the rat liver homogenate, free, and membrane-bound polysomal preparations were collected by centrifugation. 45 ml of the absolute ethanol-RNA solution was added to 50 ml conical pyrex polycarbonate tubes and centrifuged at 0-4°C for 10 min at 1,500 g_{max} in an IEC Model V Size 2 centrifuge using its large swinging buckets. The 1.5 K supernatant was discarded, the remainder of the absolute ethanol-RNA solution added to the pellet while resuspending the pellet with a glass rod, and the volume made up to 45 ml with ice-cold absolute ethanol and centrifuged for another 10 min. The resultant 1.5 K supernatant was discarded, the pellet resuspended in 45 ml of absolute ethanol to wash it and centrifuged for 10 min. This 1.5 K supernatant was discarded, the pellets were dried under nitrogen gas and the tubes kept on ice until denaturation.

Affinity chromatography was carried out according to a modified version of Aviv and Leder's procedure (1972). 200 mg of Type 3 Oligo (dT)-cellulose (contains oligomers of an average chain length of 14-16 nucleotides which is suitable for quantitative binding—Collaborative Research, Inc., 1977: Catalog 3) was suspended in a small volume of the Salt buffer, i.e., 10 mM Tris acetate, pH 7.6/0.4 M sodium chloride/0.5% SDS, and added to a 0.5 x 10.0 cm glass Bio-Rad Econo column. The cellulose was washed with

10 ml of 0.1 M potassium hydroxide, 20 ml of doubly-distilled water and finally with 20 ml of the Salt buffer. The flow rate of the wash and affinity chromatography was maintained between 1 and 1.5 ml per minute.

The dried RNA pellets were resuspended in 2.5 ml of 10 mM Tris acetate, pH 7.6/1% SDS and heat denatured at 65°C for 5 min. Then 2.5 ml of ice-cold 10 mM Tris acetate, pH 7.6/ 0.8 M sodium chloride was added and 10 µl taken for RNA analysis. The RNA was added to the column and after it had flowed into the cellulose, was allowed to bind for 5 min to enable interaction between mRNA polyadenylic acid and column oligothymidylic acid.

The nonabsorbed material, non-adenylated mRNA, rRNA, tRNA, and DNA, was eluted by washing with 15 to 20 ml of the Salt buffer with 0.5% SDS and 2.0 ml of the Salt buffer without SDS to prevent inhibition of translation by SDS (Shore, personal communication). 100 µl of the salt eluant was taken for RNA analysis.

The material retained by the column was eluted with 5 to 8 ml of Non-salt buffer, 10 mM Tris acetate, pH 7.6. 1.0 ml of the poly(A)$^+$ fraction was taken for RNA analysis and added back to the remaining poly(A)$^+$ fraction. The volume was adjusted to fill polycarbonate A321 tubes which were then centrifuged at 0-4°C and 210,000 g_{max} for 12 to 14 h in an IEC A321 rotor using an IEC B-60 ultracentrifuge to pellet the poly(A)$^+$ RNA.

3. RNA Analysis

The concentration of RNA extracted from rat liver homogenates, free and membrane-bound polysomes was determined as described by Munro and Fleck (1966) using $E^{1\%}_{1\ cm}$ = 312 where an O.D. of 1.000 at 260 nm indicates a concentration of 32 µg/ml of RNA.

A total volume of 1 ml of sample was read in a 1 ml quartz cuvette of 1 cm light path distance at 260 nm using a Beckman DU Quartz Spectrophotometer.

For Oligo (dT)-cellulose affinity chromatography, samples were taken before elution (10 µl), after salt elution (100 µl), and lastly, after non-salt elution (1 ml). Readings at 280 nm were taken additional to those at 260 nm since the ideal 260 nm:280 nm ratio of 2.05:1 (Warburg and Christian, 1941) indicated the sufficient removal of protein from RNA. This is desired for translation.

4. Preparation of the reticulocyte lysate cell-free translation system

a. Reticulocyte lysate preparation

Reticulocyte lysates were prepared from 3 kg male New Zealand white rabbits as outlined by Hunt et al. (1972).

Rabbits were injected subcutaneously with 5 mls of 10 mg/ml of APH in saline (0.9% NaCl). This was repeated for four to five days decreasing the dosage 1 ml a day after

two days until the eyes became a pale pink.

The rabbits were then left untreated for two or three days while the eye colour began to return. Then the reticulocyte content of the blood was determined by staining the blood with 0.3% brilliant cresyl blue in ethanol. If more than 75% of the cells were observed to contain a dark blue reticular network, hence reticulocytes, then the rabbits were bled.

Before bleeding, the animals were injected with 1.0 to 1.5 ml of sodium pentobarbitol intravenously, in one ear. After the animals fell asleep, 0.1 ml of 10 mg/ml heparin in saline was injected intravenously to prevent blood clotting.

Blood was then collected by cardiac puncture using a heparinized needle and tubing leading to a bottle containing 0.1 to 0.2 ml of heparin three feet below the rabbit. This enabled the blood to flow into the container by gravity.

The blood was placed on ice and then red blood cells were collected by centrifugation at 0-4^{o}C for 5 min at 2,500 g_{max} using the large swinging buckets of the IEC Model V Size 2 centrifuge.

The 2.5 K supernatant containing plasma and a buffy layer of white blood cells was removed by aspiration and the upper half of the pellets washed three times with a 0.14 M sodium chloride/5 mM potassium chloride/5 mM magnesium acetate. The lower half of the pellets contained erythrocytes.

The cells were then lysed by adding 1 or 2 volumes of doubly-distilled water to the final 2.5 K pellet. The cells

were left on ice for 10 min, then centrifuged at 0-4°C for 10 min at 20,000g_{max} in the SS34 fixed angle rotor and the Sorvall RC-2 centrifuge. The 20 K supernatant was dispensed in small aliquots e.g. 0.4 to 1.0 ml, frozen in liquid nitrogen, and stored at -70°C.

b. Nuclease treatment of reticulocyte lysates

Reticulocyte lysates were rid of endogenous mRNA by the method of Pelham and Jackson (1976).

Endogenous lysate mRNA competes with exogenous mRNA for translation. A system which reduces endogenous protein synthesis to a negligible level is provided by digesting endogenous lysate mRNA by micrococcal nuclease. This nuclease requires calcium ions for its activity which are then removed by chelation with EGTA thereby inactivating the nuclease.

0.4 to 1.0 ml of lysate was treated with 4 to 10 µl of 7.5 K units/ml micrococcal nuclease and 4 to 10 µl 0.1 M calcium chloride at 20°C for 10-20 min.

Further hydrolysis was terminated by the addition of 8-20 µl 0.1 M EGTA, pH 7.5, and transfer to ice.

This was done immediately before protein synthesis.

c. Reticulocyte lysate cell-free translation system

Translation by reticulocyte lysates was carried out following the procedure of Shore and Tata (1977).

A cocktail was prepared which contained 10 mM ATP

neutralized with Tris crystals; 2 mM GTP, 0.3 M of 18 amino acids (minus cysteine and leucine) and 0.1 M HEPES, pH 7.5, in doubly-distilled water. The cocktail was stored at -70°C along with the other components unless otherwise noted.

The other components were: 50 mM magnesium acetate, 2.0 M potassium chloride, 9.3% creatine phosphate, creatine phosphokinase, hemin (see below), $65A_{260}$/ml wheat germ tRNA, freshly prepared 0.088% cysteine, lysate, mRNA and water.

To 6.5 mg hemin the following materials were added in the order: 0.25 ml 1.0 M potassium hydroxide, 0.5 ml 0.2 M Tris acetate, pH 7.6, 8.89 ml ethylene glycol, 0.19 ml 1.0 M HCl and 0.05 ml H_2O. This was stored at -20°C. Aliquots of hemin were removed at -20°C.

Translation of exogenous polyadenylated RNA was carried out as follows:

A reaction mixture save the lysate was prepared either immediately before or during the treatment of lysate with nuclease. The reaction mixture contained in a total volume of 42.7 µl:3µl of cocktail, 5 µl of 9.3% creatine phosphate, 2 µl of 50 mM magnesium acetate, 2 µl 2 M potassium chloride, 2.5 µl wheat germ tRNA, 0.2 µl fresh 0.088% cysteine, 1 µl hemin and 2 µl 10 µCi ^{3}H-leucine. After digestion, 25 µl of lysate was added. Volumes were increased proportional to the number of reactions carried out plus one. Therefore, to each plastic Eppendorf microtube on ice containing the appropriate amount of RNA and water in 7.3 µl, 42.7 µl of

reaction mixture was added yielding a total reaction volume of 50 µl. This was then mixed gently and incubated for 60 min at 29°C. The reaction was terminated by placing the tubes on ice.

Usually, five concentrations of poly(A)+RNA were translated so that the mRNA dependency of the reticulocyte lysate translation system, i.e., for rat liver homogenate, free and membrane-bound polysomal mRNA, could be determined.

V Analyses of the Peptide Products of the Reticulocyte Lysate Cell-free Translation System

1. Initial determination of the presence and amount of peptide product

The measurement of radioactive amino acid incorporation in the reticulocyte lysate cell-free translation system was carried out by TCA precipitation of the peptide products on filter-paper disks as described by Mans and Novelli (1961).

Duplicate disks of Whatman No. 3 MM chromatography paper, 2.3 cm in diameter, were spotted with 5 µl samples of translation products. The disks were air dried and then added to a beaker containing ice-cold 10% TCA/10 mM leucine (there should be at least 3 ml per disk) for at least 60 min. The TCA precipitates protein into the matrix of the disks and the nonlabeled leucine dilutes the specific activity of the radioactive leucine to inhibit nonspecific binding.

The 10% TCA solution was poured off and the disks washed

with 5% TCA/10 mM leucine for 15 min.

The disks were then resuspended in 5% TCA/10 mM leucine and incubated at 90°C for 30 min to hydrolyze and extract RNA and amino acid-charged tRNA. The 5% TCA solution was decanted and the disks washed with 5% TCA/10 mM leucine.

The disks were then resuspended in ether/ethanol (V/V) and incubated at 37°C for at least 30 min to extract TCA and lipids, the ether/ethanol solution decanted, and the disks washed with ether/ethanol.

The disks were resuspended in ethyl ether for 15 min at room temperature to remove the water and alcohol, the ethyl ether decanted, and the disks air-dried.

Each disk was then added to a plastic counting vial.

Usually, 10 mls of scintillation solution, namely, 70% p-xylene/30% Triton X-100/0.7% PPO/0.03% POPOP, would have been added next. However, it was found that the lysate caused color quenching. This was found by the presence of a large discrepancy between the efficiencies as determined by the channels-ratio and the external standard methods. (Wang and Willis, 1965). Therefore, the disks were treated with Protosol, a tissue solubilizer, and 30% H_2O_2. After this treatment, it was found that the isotope counting efficiencies determined by the channels-ratio and external standard methods agreed.

The disks were treated as described by NEN (NEN Protosol Tissue and Gel Solubilizer: General Instructions).

Disks were thoroughly wet with 0.3 ml of water. 1.0 ml

of Protosol was added to the vial and the vial cap secured tightly to avoid loss of the solvent. The vials were incubated at 65°C for 30 min. Decolorization was carried out by the addition of 0.1 ml of 30% H_2O_2 and incubation at 65°C for another 30 min. The samples were cooled to room temperature, scintillation solution added and the samples counted for 10 min in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3003.

The radioactivity values obtained from the counter are given in cpm. Conversion to dpm requires the determination of the % (counting) efficiency of an isotope, e.g., tritium is the isotope of ^{3}H-leucine. The % efficiency is determined from the counts-ratio and external standard values (given by the spectrometer) as outlined by Wang and Willis (1965). Therefore, dpm = $\frac{\text{cpm}}{\text{\% efficiency}} \times 100$, where the cpm values were corrected for background. Background was determined by counting a vial only containing 10 mls scintillation fluid and a radioactivity control, e.g. unspotted disks treated as experimental disks.

The % efficiency of ^{3}H in this work was found to be ca 34%.

2. Resolution of the amount, molecular weight and origin of synthesis of labeled peptide products

The molecular weights of the protein products were estimated after electrophoresis through 5-15% SDS-PAGE.

The cellular levels and origin of synthesis of the

peptide products were determined first by radioactivity profile determinations and then by fluorography of 5-15% SDS-PAGE's. Molecular weights of sample and standard proteins were estimated from both gel sets. Electrophoresis was carried out as outlined in Methods I.

a. Radioactivity profiles

Approximately 2 x 10^5 cpm of each sample of peptide translation products (unless otherwise noted) directed by homogenate, free, and membrane-bound polysomal mRNA fractions were electrophoresed. The amount of counts of the blank (i.e., no exogenous mRNA translated) that were loaded on the gels was limited to the largest volume of experimental (i.e., exogenous mRNA translated) sample loaded. Electrophoresis was carried out and the gels prepared for radioactivity profile determination.

After destaining, the spacer gel was excised at the spacer-resolving gel boundary and 2 mm slices were cut from the upper edge of the resolving gel towards (and including) the bottom of the gel. The slices were placed in plastic counting vials, 0.5 ml 30% H_2O_2 added and the vials incubated at 37°C overnight (Bergeron et al., 1975; Strauss et al., 1975). The vials were cooled to room temperature, 10 ml of scintillation solution added, and the vials counted for 10 min in the Model 3003 Packard Scintillation Spectrometer.

Using 18 x 25 cm K and E No. 461521 graphpaper (10 x 10 to the cm),

the cpm values of each sample were plotted on the ordinate as a function of the number of gel slices which was plotted on the abscissa. The radioactivity profiles of the protein products translated from the three subcellular fractions could then be compared.

The percentage of a particular protein of each fraction relative to the total amount of proteins synthesized in that fraction could then be estimated. In this work, as indicated above, the cpm values of ^{3}H-leucine incorporation were used to plot radioactivity profiles. However, for dpm values, the cpm values can be considered as:

$$dpm = \frac{cpm}{\%\ \text{efficiency of}\ ^{3}H} \times 100 = \frac{cpm}{34} \times 100 = \frac{cpm}{.34}$$

b. Fluorography:

Fluorography was carried out as outlined by Laskey and Mills (1975).

After destaining, gels were placed in 500 ml once-used DMSO for 30 min, then in a new solution of 500 ml DMSO for another 30 min. Next, the gel was put into a solution of 200 ml 22.5% PPO/0.05% POPOP in DMSO for 3 h. Finally the gel was placed in 2 to 3 l of 2% glycerol for 60 min.

After the glycerol wash, the gel was prepared for drying. The gel was gently placed on a wet piece of Whatman No. 1 filter paper (lying over 4 thick paper sheets) covered with Saran wrap and then a plastic sheet. A rubber mat was placed over the gel and the drier turned on. The gel was dried for

90 min at 85°C on a Hoefer Scientific Instruments (HSI) Model SE-540 gel drier.

The dried gel was then placed in immediate contact with a sheet of pre-flashed Royal X-O-Mat X-Ray film in the dark. The film was exposed for 10 days in a light-free 14 x 17 in^2 Westinghouse Int X-Ray holder at -70°C in a Revco upright freezer.

The film was developed by immersion into KLX Developer for 4 min, washed in running tap water for 1 min, fixed in Rapid fixer for 10 min, and, lastly, washed in 20°C running tap water for 3 min. The film was air dried.

It should be noted that those samples containing the highest amount of radioactive ^3H-leucine were chosen for analysis by gel slicing and fluorography since the sample volume was small (50 µL) thereby permitting limited analyzing volumes.

Table 1

Recovery of homogenate RNA fractions from Oligo(dt)-cellulose columns

Sample	Wet wt. liver (g)	mg RNA/ g liver wet wt.	mg poly(A)$^-$/ g liver wet wt.	mg poly(A)$^+$/ g liver wet wt.	Recovery of RNA	Poly(A)$^+$
Experiment 1	3.4	4.57	3.87	9.36×10^{-2}	87%	2.0%
Experiment 2	3.7	5.00	3.43	9.90×10^{-2}	71%	2.0%

RNA was extracted as described in Methods (IV, 1a) from rat liver homogenates. The results from two separate isolation procedures are indicated.

Fig. 1. mRNA dependency of cell-free translation as directed by homogenate mRNA (poly(A)$^{+}_{hom}$ RNA)

Increasing amounts of poly (A)$^{+}_{hom}$ RNA were incubated with micrococcal nuclease-treated lysate for 60 min at 29°C. Radioactivity incorporation (^{3}H-leucine) into TCA precipitates was assessed by the method of Mans and Novelli as described in Methods (V, 1). Cell-free protein synthesis was a linear function of added poly(A)$^{+}_{hom}$ RNA until ca 1 µg added per 50 µg incubation mixture. Two separate poly(A)$^{+}_{hom}$ RNA isolations from 107 g (●——●) and 88 g (o- - -o) rats were carried out two weeks apart, to test the reproducibility of the isolation procedure.

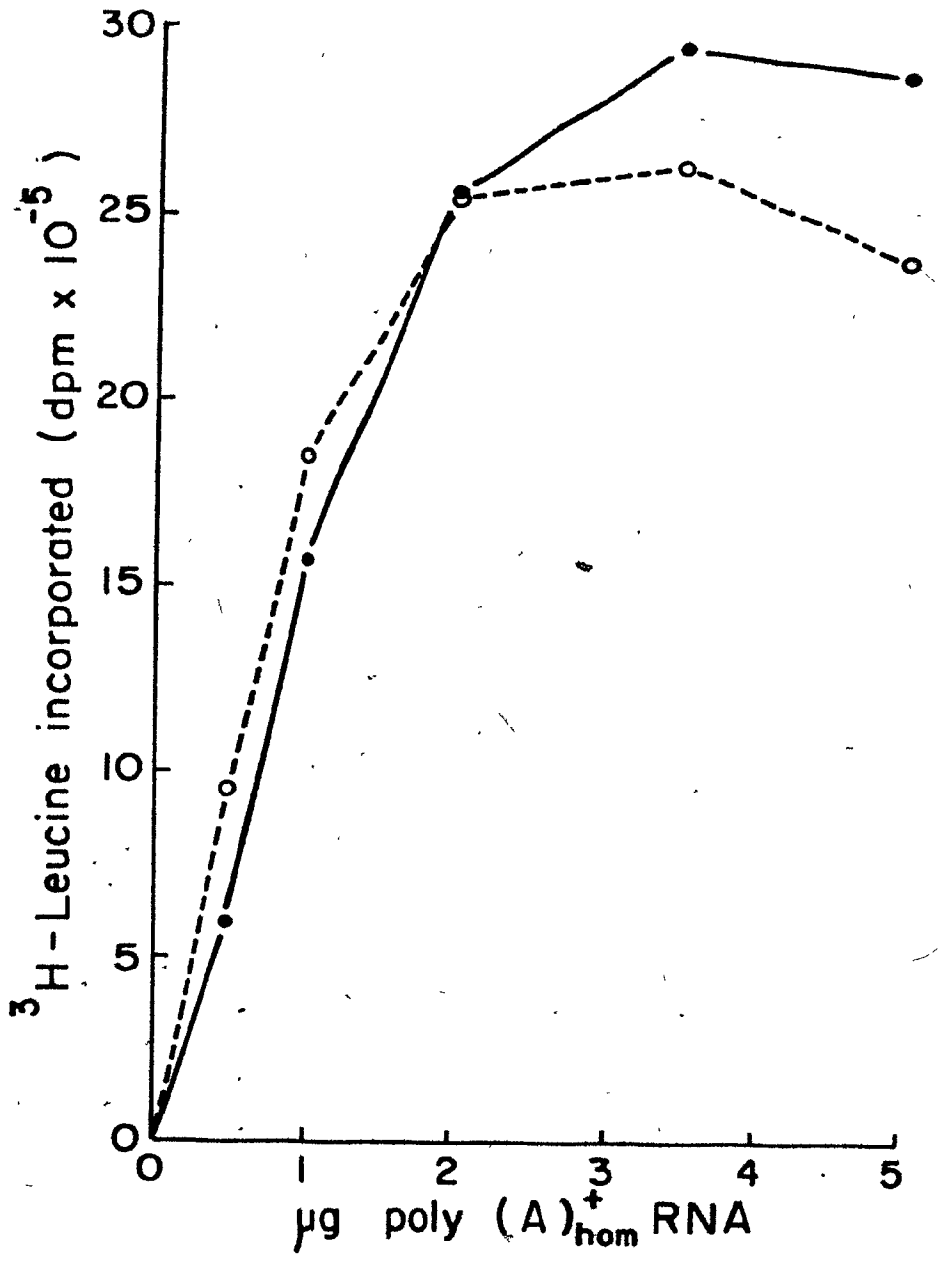
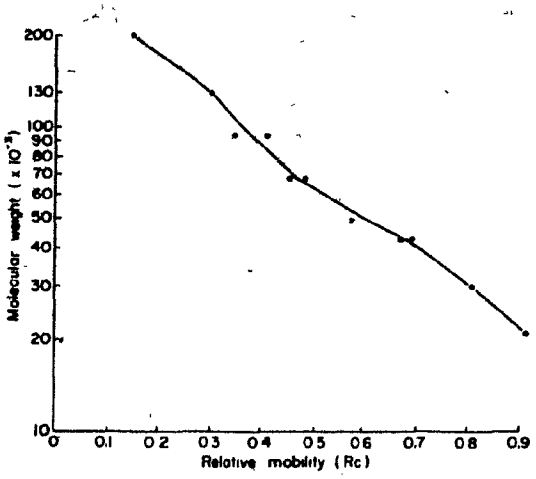

30
25
20
15
10
5
0
0
1
2
3
4
5
^{3}H-Leucine incorporated (dpm x 10^{-5})
μg poly $(A)^+_{hom}$ RNA

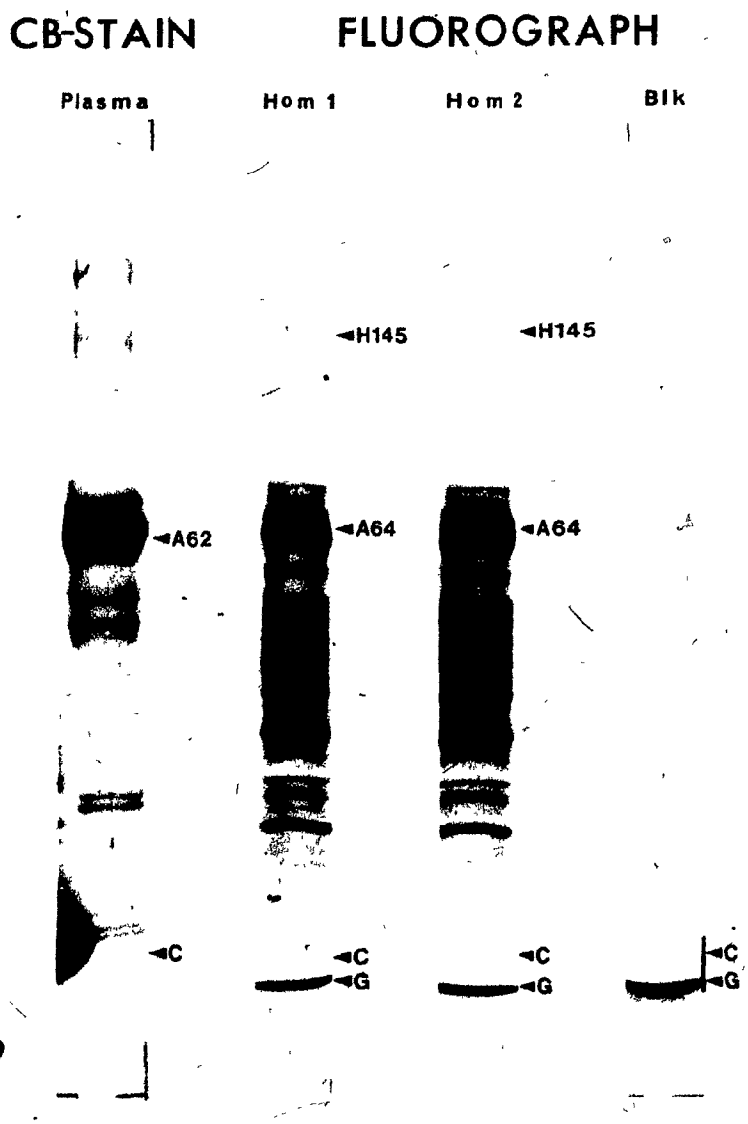
Fig. 2. Fluorographic analysis of peptides synthesized by homogenate mRNA (poly(A)$^{+}_{hom}$ RNA)

a. Calibration curve of the SDS-PAGE gels of Fig. 2b. The molecular weight standards were as described in Materials and Methods.

b. The spectrum of CB-stained plasma peptides (Plasma) was compared to the fluorograph of cell-free translation products (2 x 10^5 cpm applied) directed by poly(A)$^{+}_{hom}$ RNA from two separate experiments (Hom 1 and Hom 2). The fluorographs were exposed for 10 days. The radioactive products (3 x 10^4 cpm) from a comparable volume of endogenous translation products (12 µl) is also indicated (Blk). Both Hom 1 and Hom 2 showed identical translation products with a major band (Mr 64,000, labeled as A64) of slightly decreased mobility to that of liver plasma albumin (Mr 62,000 labeled as A62). Of note, is a band of molecular weight 145,000 (H 145) which was the highest observed molecular weight peptide synthesized by the system. G refers to the major translation product, globin, of the endogenous reticulocyte m-RNA. C represents the cytosolic peptide whose mobility was used to determine the relative mobility of electrophoresed peptides while in samples other than cytosol, the equivalent mobility was used.


200
130
100
90
80
70
60
50
40
30
20
10
Molecular weight (x 10^{-3})
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Relative mobility (Rc)

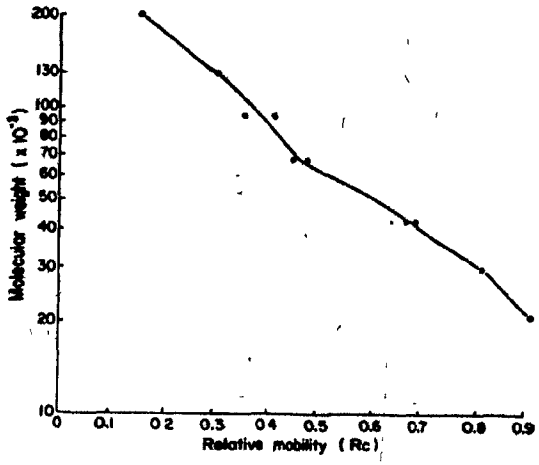
a


CB-STAIN
FLUOROGRAPH
Plasma
Hom 1
Hom 2
Blk
◄H145
◄H145
◄A62
◄A64
◄A64
◄C
◄C
◄G
◄C
◄G
◄C
◄G

b

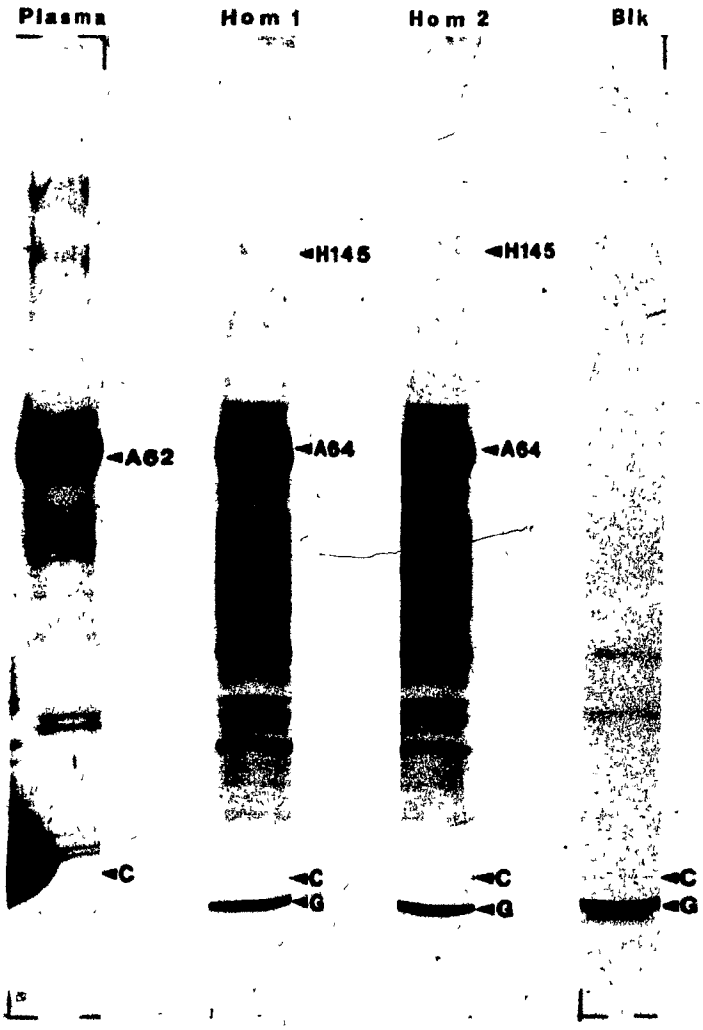
Fig. 2. Fluorographic analysis of peptides synthesized by homogenate mRNA ($poly(A)^{+}_{hom}$ RNA)

Same as outlined in 43a. However, the facing fluorograph (Hom 1, Hom 2, Blk) was overexposed to highlight H145.



a

CB-STAIN FLUOROGRAPH

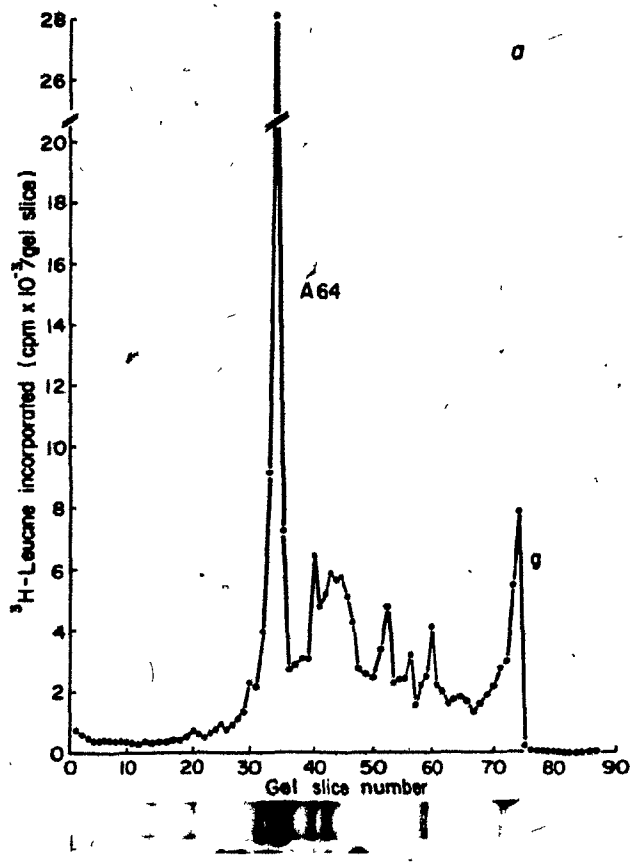


b

overexposed

Fig. 3. Radioactivity profile of peptides synthesized by homogenate mRNA (poly $(A)^{+}_{hom}$ RNA): Experiment 1 & 2

2×10^{5} cpm of translation products directed by poly$(A)^{+}_{hom\,1}$ RNA (a) and poly$(A)^{+}_{hom\,2}$ RNA (b) loaded on 5-15% linear gradient SDS-PAGE gels and cut into 2 mm slices for radioactivity profile determinations. The radioactivity profiles were compared to the peptides of CB-stained plasma of which the major peak (A64) corresponded to plasma albumin (A) but with a slightly decreased mobility. Peak G refers to the globin zone of the gel profiles.


a
A64
g
^{3}H-Leucine incorporated (cpm x 10^{-3}/gel slice)
Gel slice number

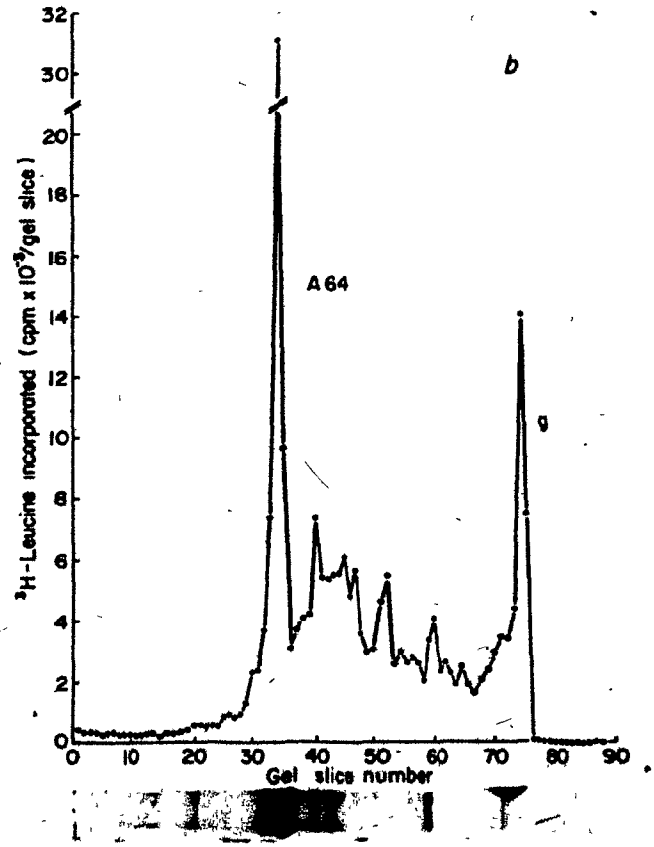

b
A64
g
^{3}H-Leucine incorporated (cpm x 10^{-3}/gel slice)
Gel slice number

Table 2

Recovery of free and membrane-bound polysomal RNA fractions from Oligo (dT)-cellulose columns

Sample	Wet wt. liver (g)	mg RNA/g liver wet wt.	mg poly(A)$^-$/g liver wet wt.	mg poly(A)$^+$/g liver wet wt.	Recovery of RNA	Poly(A)$^+$
Experiment 1	3.79					
Bound Polysomes		3.26	1.84	3.91×10^{-2}	58%	1.2%
Free Polysomes		1.32	7.49×10^{-1}	2.27×10^{-2}	58%	1.7%
Experiment 2	3.96					
Bound Polysomes		2.25	1.72	3.78×10^{-2}	78%	1.7%
Free Polysomes		1.05	7.37×10^{-1}	2.26×10^{-2}	72%	2.2%
*Experiment 3	3.74					
Bound Polysomes		1.15	.95	2.02×10^{-2}	84%	1.8%
Free Polysomes		.88	.72	2.34×10^{-2}	84%	2.7%

RNA was extracted as outlined in Materials and Methods. The results from three separate isolation procedures are indicated.

*The results of Fig. 4 were obtained from translation of the poly(A)$^+_{bd}$ RNA and poly(A)$^+_{fr}$ RNA of Experiment 3.

Fig. 4. mRNA dependency of cell-free translation as directed by free and membrane-bound polysomal mRNA (poly $(A)^+_{fr}$ RNA and poly $(A)^+_{bd}$ RNA, respectively)

Increasing amounts of poly $(A)^+_{fr}$ RNA and poly $(A)^+_{bd}$ RNA were incubated with micrococcal nuclease-treated lysate for 60 min at 29°C. Radioactivity incorporation (^{3}H-leucine) into TCA precipitates was assessed by the method of Mans and Novelli as described in Methods (V,1). Cell-free protein synthesis was a linear function of added poly $(A)^+_{fr}$ RNA (Δ—Δ) and poly $(A)^+_{bd}$ RNA (▲—▲) until ca 1 µg and ca 3.5 µg added per 50 µl of incubation mixture, respectively. The data is compared to that obtained for poly $(a)^+_{hom}$ RNA (●—○; see Fig. 1 also).

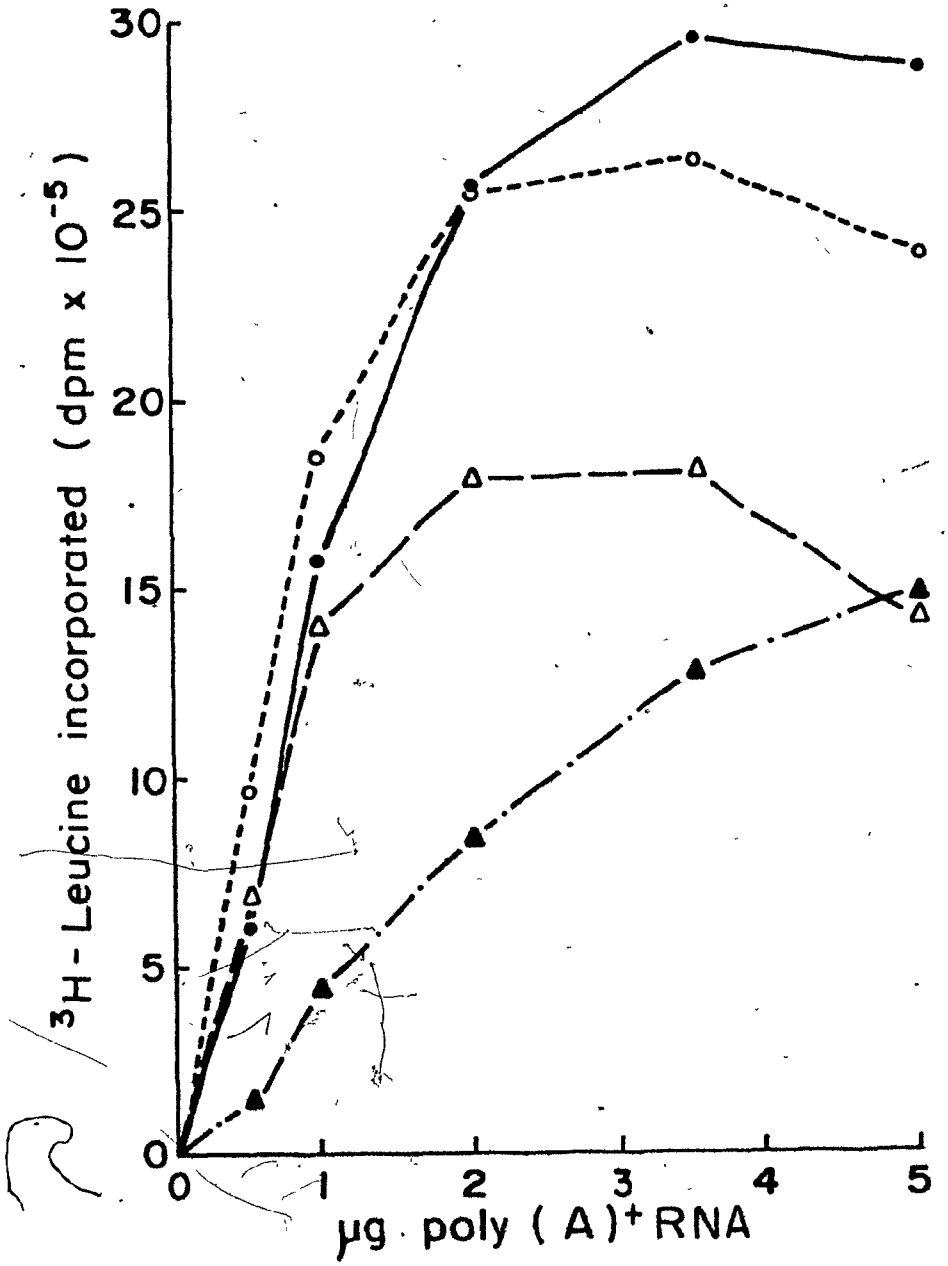
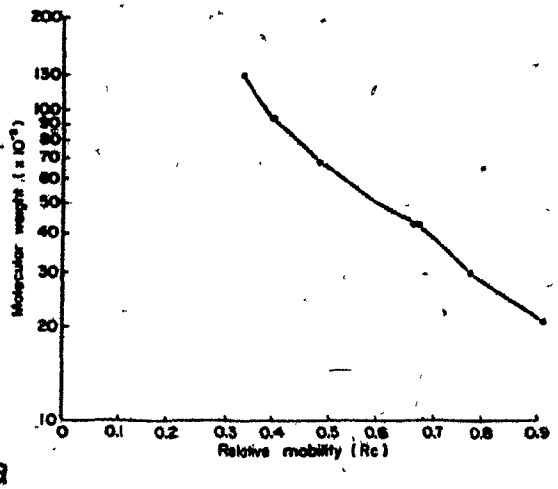

30
25
20
15
10
5
0
0
1
2
3
4
5
^{3}H- Leucine incorporated (dpm x 10^{-5})
µg poly (A)$^+$ RNA

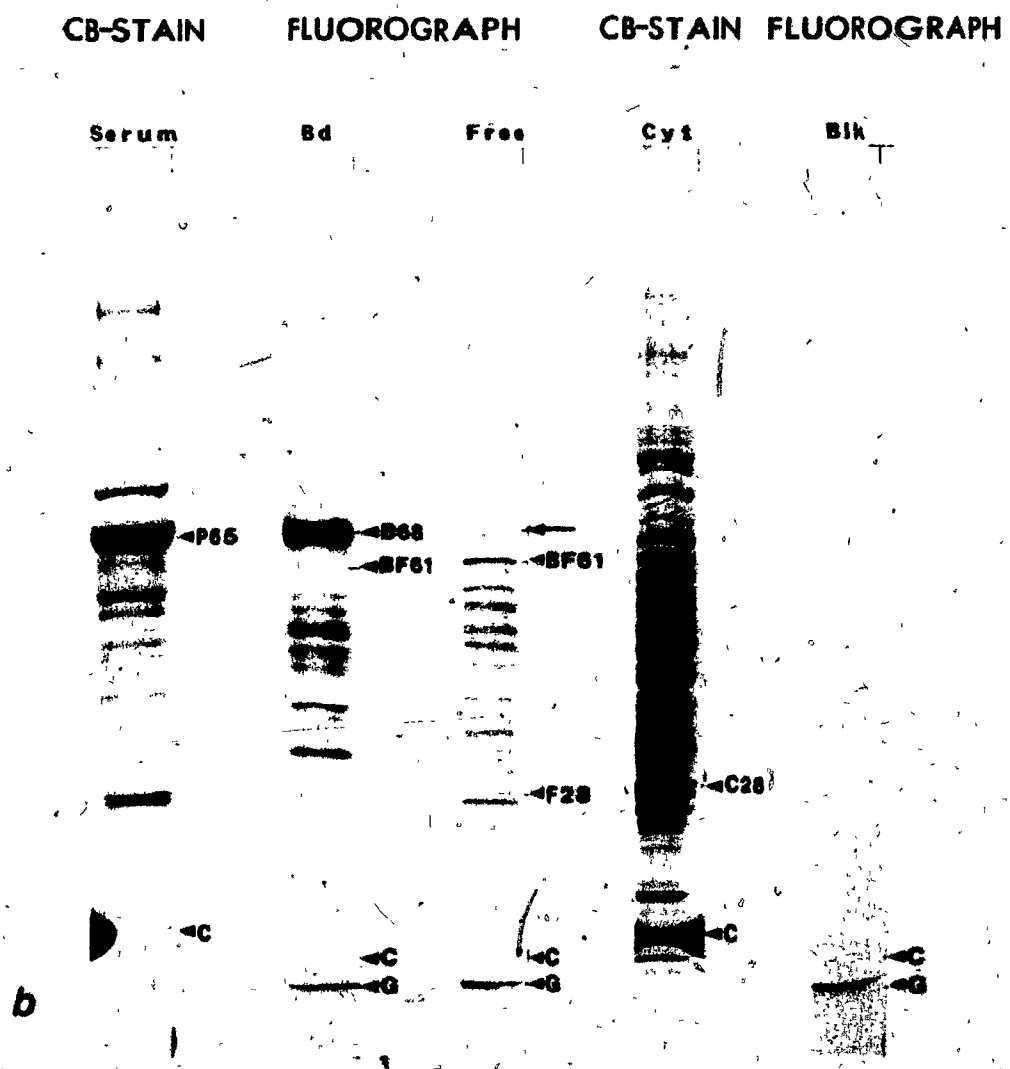
Fig. 5. Fluorographic analysis of peptides synthesized by free and membrane-bound polysomal mRNA (poly(A)$^+_{fr}$ RNA and poly (A)$^+_{bd}$ RNA, respectively): Experiment 1

a. Calibration curve of the SDS-PAGE gels of Fig. 6a. The molecular weight standards were as described in Materials and Methods.

b. The spectrum of CB-stained serum peptides (Serum) and cytosolic peptides (Cyt) is compared to the fluorograph of cell-free translation products (2 x 10^5 cpm applied) directed by either poly(A)$^+_{fr}$ RNA (Free) or poly(A)$^+_{bd}$ RNA (Bd). The radioactive products (3 x 10^4 cpm) from a comparable volume of endogenous translation product (7.5 µl) is also indicated (Blk). The fluorographs were exposed for 10 days. The major translated product of poly(A)$^+_{bd}$ RNA (B68) had a decreased mobility compared to that of CB-stained serum albumin (P65). Only small amounts of this peptide were found in the translation products of poly(A)$^+_{fr}$ RNA (labeled by the arrow). In contrast, peptide F28 was found in the translation products of poly(A)$^+_{fr}$ RNA but not poly(A)$^+_{bd}$ RNA and corresponded in mobility to CB-stained C28. A peptide which appeared shared between the translation products of poly(A)$^+_{fr}$ RNA and poly(A)$^+_{bd}$ RNA is designated BF61. For explanations of labels G and C, see legend of Fig. 2.


200
150
100
90
80
70
60
50
40
30
20
10
Molecular weight (x 10⁻³)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Relative mobility (Rc)

a

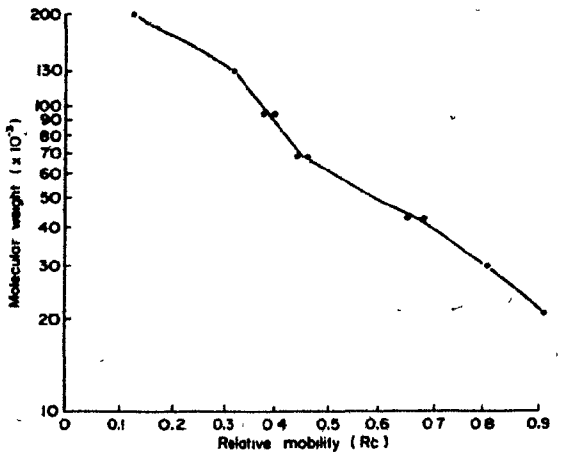

CB-STAIN
FLUOROGRAPH
CB-STAIN
FLUOROGRAPH
Serum
Bd
Free
Cyt
Blk
P65
B68
BF61
BF61
F28
C28
C
C
G
C
G
C
C
G

b

Fig. 6. Flurographic analysis of peptides synthesized by free and membrane-bound polysomal mRNA (poly(A)$^{+}_{fr}$ RNA and poly(A)$^{+}_{bd}$ RNA, respectively): Experiment 2

a. Calibration curve of the SDS-PAGE gels of Fig. 6b. The molecular weight standards were as described in Materials and Methods.

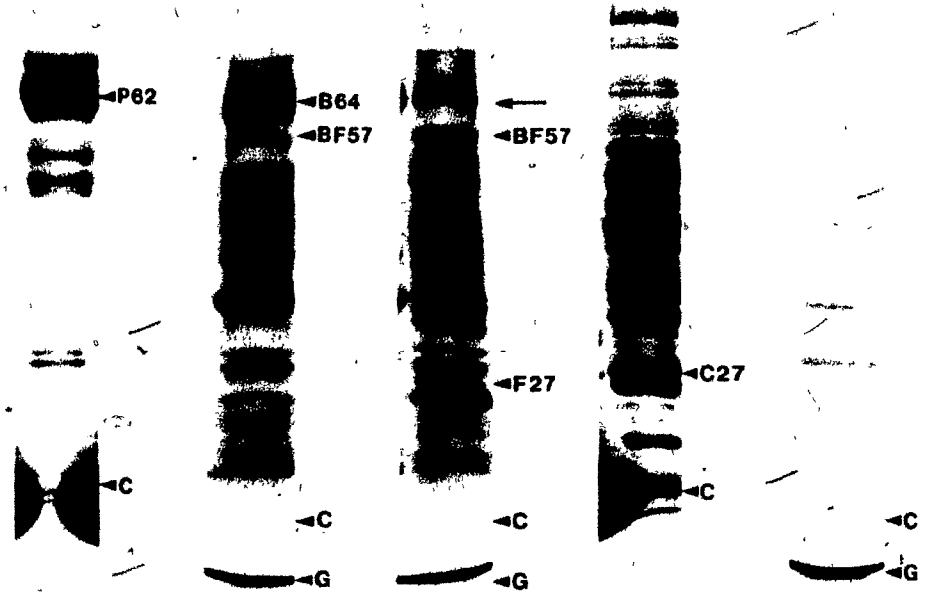
b. Identical experiment to that described in Fig. 5 except that different mRNA preparations were used. As for Fig. 5, the radioactive products (6×10^4 cpm) from a comparable volume of endogenous translation products (21µl) are also indicated. The notations of indicated peptides were the same but their molecular weights appeared slightly different (e.g. P62, Fig. 6, as compared to P65, Fig. 5). The spectrum of CB-stained plasma peptides (Plasma) is also shown.



a

CB-STAIN FLUOROGRAPH CB-STAIN FLUOROGRAPH

Plasma Bd Free Cyt Blk



b

Fig. 7. Radioactivity profiles of peptides synthesized by free and membrane-bound polysomal mRNA ($poly(A)^+_{bd}$ RNA and $poly(A)^+_{fr}$ RNA, respectively): Experiment 1

5×10^5 cpm of translation products directed by $poly(A)^+_{bd}$ RNA (a) and 2×10^5 cpm of translation products directed by $poly(A)^+_{fr}$ RNA (b) were loaded on 5-15% linear gradient SDS-PAGE gels and cut into 2 mm slices for radioactivity profile determinations (●——●).

The radioactivity profiles of the labeled products (5×10^4 cpm) from a comparable volume of endogenous translation products (20 µl) are also shown (o——o).

The radioactivity profile of the translation products synthesized by $poly(A)^+_{bd}$ RNA was compared to the peptides of CB-stained serum of which the major peak (A') corresponded to rat serum albumin (A) with a slightly decreased mobility. The radioactivity profile of polypeptides translated from $poly(A)^+_{fr}$ RNA was compared to the peptides of CB-stained cytosol.

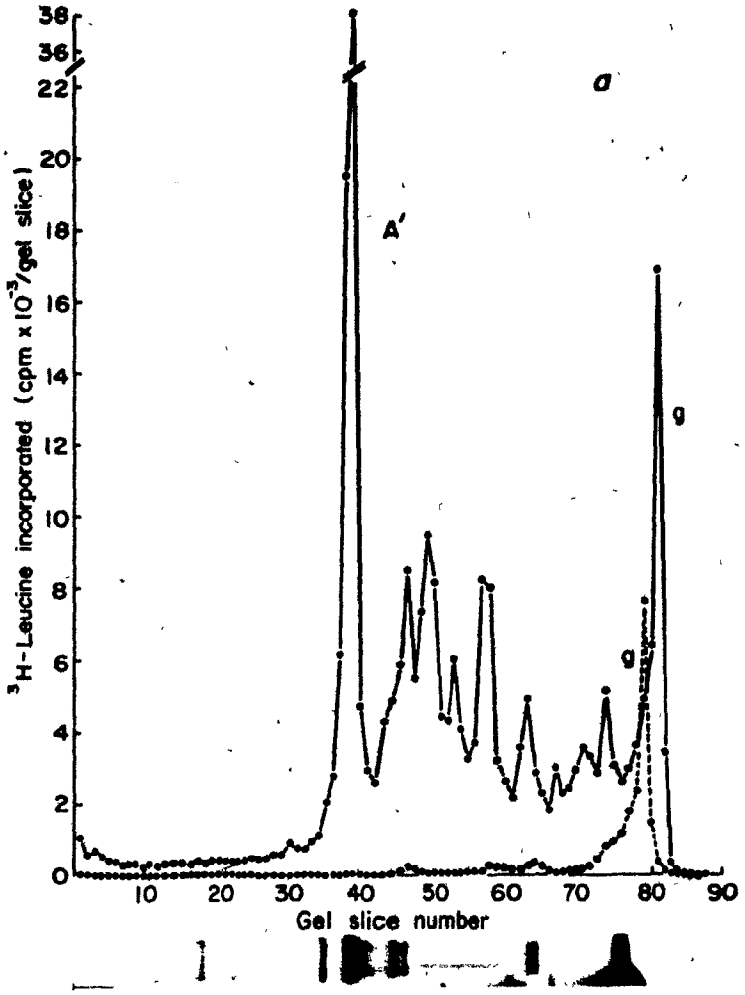
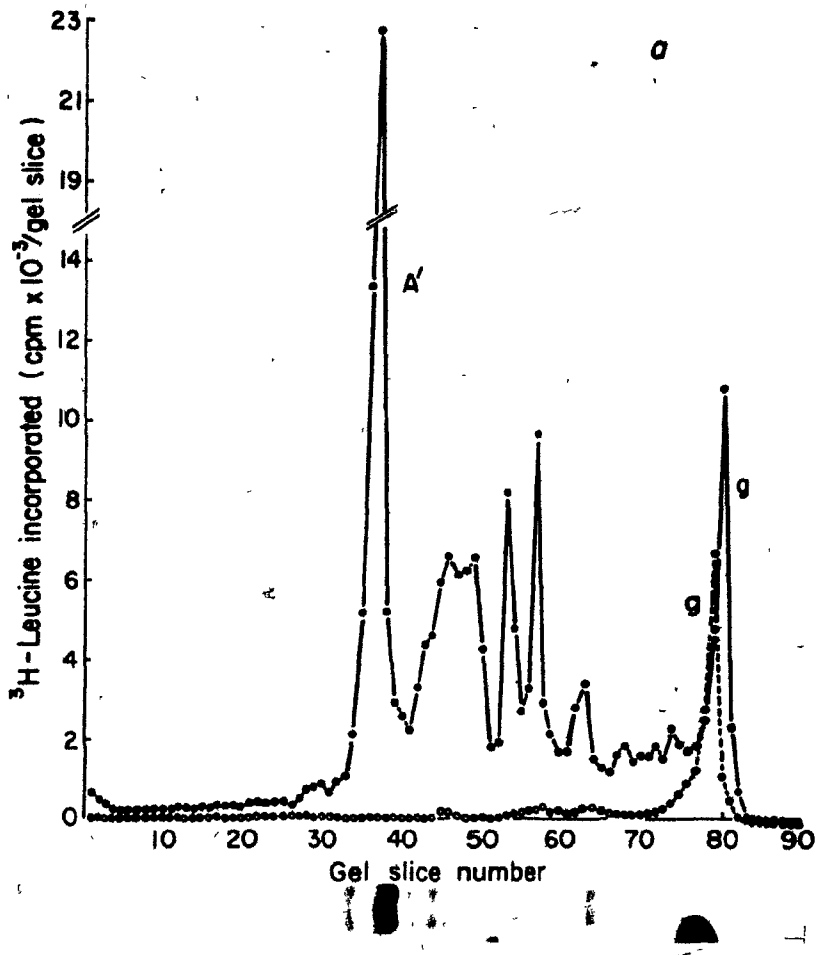

a
A'
g
g
^{3}H-Leucine incorporated (cpm x 10^{-3}/gel slice)
Gel slice number
b
g
g
^{3}H-Leucine incorporated (cpm x 10^{-3}/gel slice)
Gel slice number

Fig. 8. Radioactivity profiles of peptides synthesized by free and membrane-bound polysomal mRNA (poly(A)$^+_{bd}$ RNA and poly(a)$^+_{fr}$ RNA, respectively): Experiment 2

Identical to those experiments described in Fig. 7 except that equal amounts (2 x 10^5 cpm) of translation products from poly(A)$^+_{bd}$ RNA (a) and poly(A)$^+_{fr}$ RNA (b) were applied to the gel (●—●). Different mRNA preparations were used from that described in Fig. 7 to indicate the reproducibility of the method. As for Fig. 7, the radioactivity profile from comparable volumes of endogenous-translation products (3 x 10^4 cpm) are indicated (o—o). All other notations are otherwise identical to Fig. 7.


a
A'
g
g
^{3}H-Leucine incorporated (cpm x 10^{-3}/gel slice)
Gel slice number
0
2
4
6
8
10
12
14
19
21
23
10
20
30
40
50
60
70
80
90

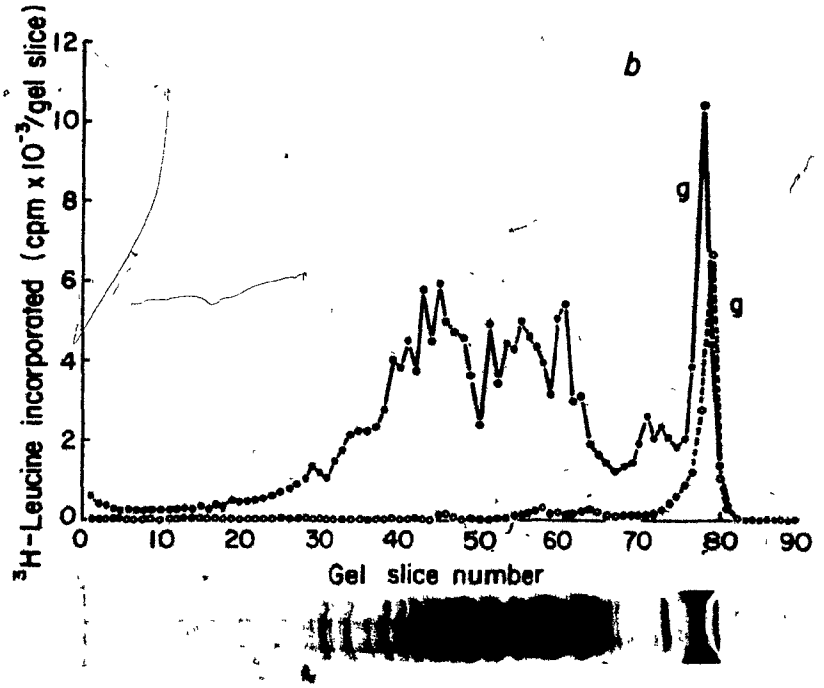
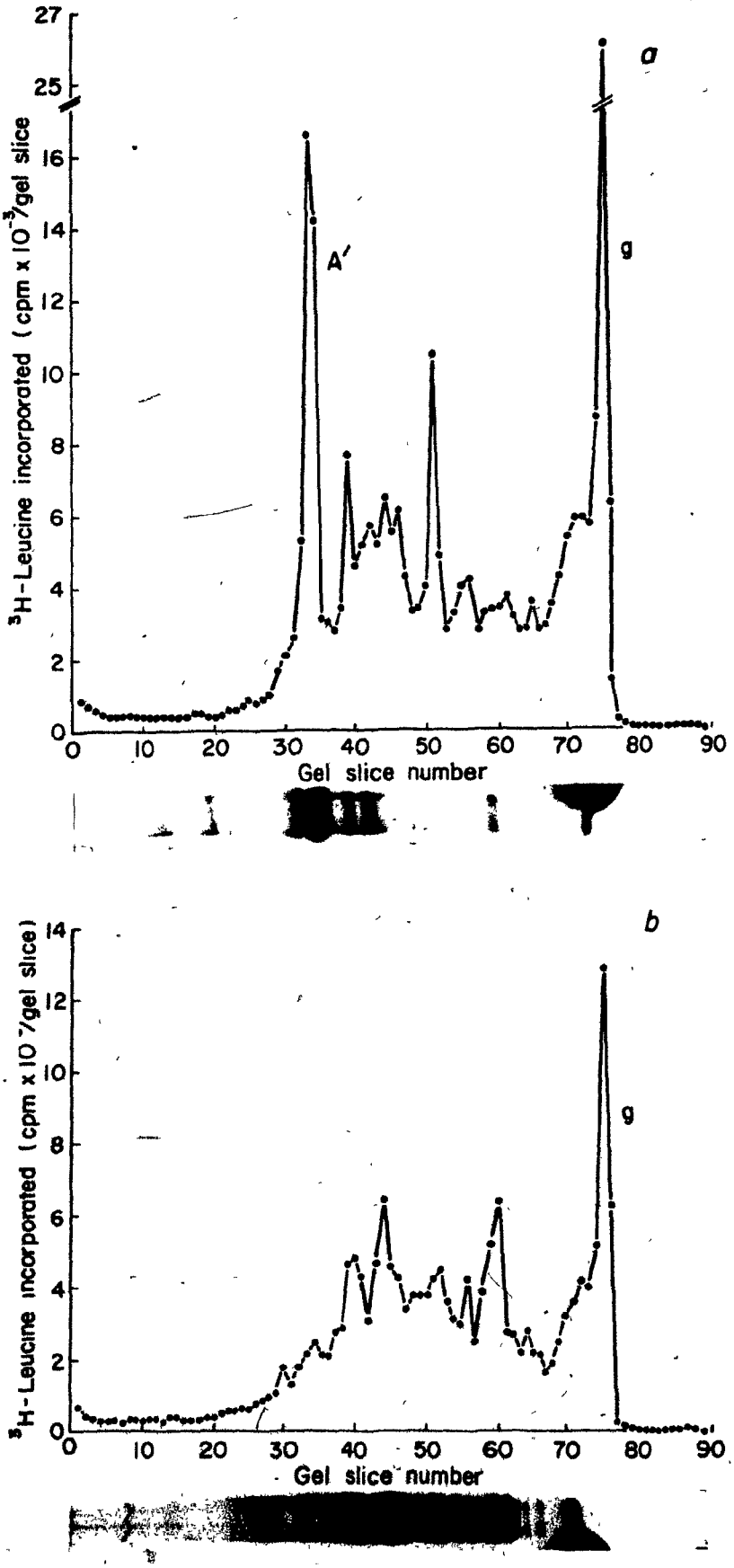

b
g
g
^{3}H-Leucine incorporated (cpm x 10^{-3}/gel slice)
Gel slice number
0
2
4
6
8
10
12
0
10
20
30
40
50
60
70
80
90

Fig. 9. Radioactivity profiles of peptides synthesized by free and membrane-bound polysomal mRNA (poly$(A)^+_{fr}$ RNA and poly $(A)^+_{bd}$ RNA, respectively): Experiment 3.

Identical to those experiments described in Fig. 7 except that equal amounts (2×10^5 cpm) of translation products from poly$(A)^+_{bd}$ RNA (a) and poly$(A)^+_{fr}$ RNA (b) were applied to the gel (•——•). Different mRNA preparations were used from that described in Figs. 7 and 8 to indicate the reproducibility of the method. In Fig. 9a, CB-stained plasma replaced CB-stained serum where its major peak (A') corresponded to rat plasma albumin (A). As well, no radioactivity profile of endogenous polypeptides is indicated. All other notations are otherwise identical to Fig. 7.


a
b
A'
g
g
^{3}H-Leucine incorporated (cpm x 10^{-3}/gel slice
^{3}H-Leucine incorporated (cpm x 10^{-3}/gel slice)
Gel slice number
Gel slice number

RESULTS

I Rat Liver Homogenate

The use of guanidinium thiocyanate for the isolation of rat liver homogenate RNA resulted in an average of 4.8 mg RNA/g liver of which an average of 2.0% was poly(A)$^+$ RNA (Table 1, Experiments 1 and 2). Cell-free translation of increasing amounts of this poly(A)$^+_{hom}$ RNA by micrococcal nuclease-treated reticulocyte lysate was linear and reproducible until ca 1 µg poly(A)$^+_{hom}$ RNA was added per 50 µl incubation mixture (Fig. 1).

Analysis of the translation products from 3.5 µg poly(A)$^+_{hom}$ RNA is illustrated in Figs. 2 and 3 in which two different methods of assay were carried out. By the method of fluorography (Fig. 2b), a major peptide of ca 64,000 molecular weight (A64) was observed. Comparison to the peptides of CB-stained rat liver plasma (Fig. 2b) showed a major peptide of similar but distinctly greater mobility (A62) which was plasma albumin. Also of note in the fluorograph, was a peptide with an apparent molecular weight of 145,000 (H145) which attested to the marked gentleness of the GTC system. The endogenous translation products of the micrococcal nuclease-treated reticulocyte lysate system demonstrated globin as the major product (G, Fig. 2b:Blk) and did not affect the interpretation of the fluorographs of the translation products of poly(A)$^+_{hom}$ RNA. The identical, fluorographs from two independent populations of poly(A)$^+_{hom}$ RNA (Fig. 2b, Hom 1 and Hom 2) emphasized the reproducibility

of the RNA isolation procedure.

The second method of analysis, namely, gel slicing (Fig. 3) confirmed that the major peptide translated by poly(A)$^+_{hom}$ RNA was similar in mobility to plasma albumin (compare the major peak of poly(A)$^+_{hom}$ RNA, A64, to the mobility of plasma albumin, A, shown on a CB-stained plasma sample beneath each graph). From gel slicing, it was additionally possible to determine that in the two experiments, 31.6% (Fig. 3a) and 30% (Fig. 3b) of the total translation product corresponded to this major peptide A64.[1]

II Rat Liver Free and Membrane-Bound Polysomes

The use of guanidinium thiocyanate for the isolation of rat liver free and membrane-bound polysomal RNA resulted in an average of 1.1 mg RNA_{fr}/g liver and 2.2 mg RNA_{bd}/g liver of which an average of 2.2% was poly(A)$^+_{fr}$ RNA and 1.6% was

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[1] Sample Calculation of the proportion of poly(A)$^+_{hom}$ RNA translation product in peptide A64 for the experiment described in Fig. 3a. That is,

$$\text{Proportion of albumin synthesized by poly(A)}^+_{hom}\text{ RNA} = \frac{\text{cpm in gel slices 31-36}}{\text{cpm in total translation products (gel slices 1-87)} - \text{cpm in endogenous translation products}} \times 100$$

$$= \frac{53{,}392.5 \text{ cpm}}{198{,}243 \text{ cpm} - 29{,}596 \text{ cpm}} \times 100$$

$$= 31.6\%$$

poly(A)$^+_{bd}$ RNA, respectively (Table 2, Experiments 1, 2 and 3). Cell-free translation of increasing amounts of poly(A)$^+_{fr}$ RNA and poly(A)$^+_{bd}$ RNA by micrococcal nuclease-treated reticulocyte lysate was linear until ca 1 µg poly(A)$^+_{fr}$ RNA and ca 3.5 µg poly(A)$^+_{bd}$ RNA added per 50 µl incubation mixture (Fig. 4).

Analysis of the translation products from 3.5 µg poly(A)$^+_{bd}$ RNA and 1 µg poly(A)$^+_{fr}$ RNA (Figs. 5 and 8: Free and Bound), and 5 µg poly(A)$^+_{bd}$ RNA and 3.5 µg poly(A)$^+_{fr}$ RNA (Figs. 6 and 9: Free & Bound) was determined by two methods of assay (Fluorography:Figs. 5 and 6; Gel slicing:Fig. 8 and 9).

By the method of fluorography, the major translated peptide of poly(A)$^+_{bd}$ RNA of ca 68,000 or 64,000 molecular weight (B68, Fig. 5b and B64, Fig. 6b, respectively) was observed to be similar but of a distinctly lesser mobility when compared to that of CB-stained serum albumin (P65, Fig. 5b) or CB-stained plasma albumin (P62, Fig. 6b). Only small amounts of this peptide were found in the translation products of poly(A)$^+_{fr}$ RNA (labeled by the arrow, Figs. 5b and 6b). In contrast, peptide F28 (Fig. 5b) or F27 (Fig. 6b) was found in the translation products of poly(A)$^+_{fr}$ RNA but not poly(A)$^+_{bd}$ RNA and corresponded in mobility to CB-stained C28 (Fig. 5b) or C27 (Fig. 6b). A peptide observed to be shared between the translation products of poly(A)$^+_{fr}$ RNA and poly(A)$^+_{bd}$ RNA was indicated as BF 61 (Fig. 5) or BF 57 (Fig.6).

The endogenous translation products of the micrococcal nuclease-treated reticulocyte system demonstrated globin as the major product (G, Figs. 5b and 6b:Blk) and did not affect the interpretation of the results of fluorographs of either poly(A)$^+_{fr}$ RNA or poly(A)$^+_{bd}$ RNA. Therefore, within the limits of the methods of analysis, the similar fluorographs of peptides directed by independent populations of poly(A)$^+_{fr}$ RNA or poly(A)$^+_{bd}$ RNA reflected the reproducibility of the RNA isolation procedure.

Though the molecular weights of analagous peptide products indicated that the peptides of Figs. 5b and 6b differed slightly, this difference was at most 6.6% (i.e., % difference between BF61 of Fig. 5b and BF57 of Fig. 6b) can be accounted for on the basis of differing gel conditions.

The translation products from a third population of poly(A)$^+_{fr}$ RNA and poly(A)$^+_{bd}$ RNA were also analyzed by the second method of analysis, namely, gel slicing where the third set of translation products were directed by 3.5 µg poly(A)$^+_{bd}$ RNA and 2 µg poly(A)$^+_{fr}$ RNA (Figs. 7a and b, respectively).

Radioactivity profile determinations (Figs. 7, 8 and 9) confirmed that the major peptide translated by poly(A)$^+_{bd}$ RNA (peak A' in Figs. 7a, 8a and 9a) was similar in mobility to serum (Figs. 7a, 8a) or plasma (Fig. 9a) albumin. From gel slicing, it was additionally possible to determine that in the three experiments, 30.3% (Fig. 7a), 30.7% (Fig. 8a) and 23.5% (Fig. 9a) of the total translation product corresponded

to this major peptide, A'.[2]

From both fluorography (Figs. 5b and 6b) and gel slicing (Figs. 7b, 8b and 9B), it was observed that little radioactivity was associated with the major peptide (albumin) as synthesized by poly(A)$^+_{fr}$ RNA. Indeed, strict accounting of the radioactivity which appeared in gel slices of the albumin zone as directed by poly(A)$^+_{fr}$ RNA, confirmed the overwhelming synthesis of albumin by poly(A)$^+_{bd}$ RNA. Thus in the experiment of the total radioactivity in the albumin zone (Fig. 7) 77% was directed by poly(A)$^+_{bd}$ RNA and 23% by poly(A)$^+_{fr}$ RNA; in Fig. 8, 74% by poly(A)$^+_{bd}$ RNA and 26% by poly(A)$^+_{fr}$ RNA; and in Fig. 9, 77% by poly(A)$^+_{bd}$ RNA and 23% by poly(A)$^+_{fr}$ RNA.[3]

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[2]Sample Calculation of the proportion of poly(A)$_{bd}$ RNA translation product in peptide A'. Example: Fig. 7a.

$$\text{Proportion of albumin synthesized by poly(A)}^+_{bd}\text{ RNA} = \frac{\text{cpm in gel slices 120-128}}{\text{cpm in total translation products (gel slices 1-88)} - \text{cpm in endogenous translation products (gel slices 1-87)}} \times 100$$

$$= \frac{79{,}920.4 \text{ cpm}}{285{,}807 \text{ cpm} - 22{,}319.9 \text{ cpm}} \times 100$$

$$= 30.3\%$$

[3]Sample calculation for the radioactivity present in the albumin zone. Example: Fig. 9.

Fig. 9a cpm in albumin zone as directed by poly(A)$^+_{bd}$ RNA = gel slices 28-35=46,559.8 cpm

Fig. 9b cpm in albumin zone as directed by poly(A)$^+_{fr}$ RNA = gel slices 28-35=13,675,8 cpm

$$\%\text{ made by poly(A)}^+_{bd}\text{ RNA} = \frac{46{,}559.8 \text{ cpm}}{46.559.8 \text{ cpm} + 13{,}675.8 \text{ cpm}} \times 100 = 77\%$$

$$\%\text{ made by poly(A)}^+_{fr}\text{ RNA} = \frac{13{,}675 \text{ cpm}}{13{,}675.8 \text{ cpm} + 46{,}559.8 \text{ cpm}} \times 100 = 23\%$$

DISCUSSION

The experimental protocol consisted of the isolation of rat liver homogenate, free and membrane-bound polysomal RNA following a detailed comprehensive modification and application of the GTC procedure of Chirgwin et al. to rat liver (Ullrich et al., 1977).

The RNA was ethanol precipitated and subsequently chromatographed through Oligo (dT)-cellulose to selectively isolate poly(A)$^+$ RNA, a well-known marker of mRNA. mRNA is the only class of RNA which possesses a 3'-tail of polyadenylic acid residues (Brawerman, 1976;Darnell et al., 1973).

The freshly prepared poly(A)$^+$ RNA was translated in the micrococcal nuclease-treated rabbit reticulocyte lysate cell-free translation system containing one labeled amino acid, ^{3}H-leucine, and the other remaining nineteen cold amino acids.

The cellular levels and origin of synthesis of the peptide products were determined quantitatively by radioactivity profiles of SDS-PAGE's and qualitatively by fluorography of gels. Gel profiles of the translation products were always compared to the electrophoretic spectrum of either the major classes of secreted proteins (rat liver plasma) or sedentary proteins (liver cytosolic peptide).[4]

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[4]This is a simplification since lysosomal content proteins may be considered as a special class of secretory proteins (Palade, 1975). They have not been analyzed by this author. As well, transmembrane proteins might also be considered to be another special class of secretory proteins (Bergeron et al., 1975; Rogers et al., 1980). Nevertheless, the peptides chosen for comparison, namely, plasma (secretory) and cytosol (sedentary) seemed reasonable and experimentally feasible.

One of the purposes of this work was to determine whether or not the mRNA isolated by GTC was superior to that extracted by phenol-chloroform. If so, the use of GTC could prove to be a valuable tool in the investigation and elucidation of membrane biogenesis.

I. Homogenate mRNA

The homogenate RNA's which eluted from Oligo d(T)-cellulose columns with a wash of a non-salt buffer designated as poly(A)$^+_{hom}$ RNA's (Table 1, Results) were chosen to be translated since their OD260nm:OD280 nm ratios, i.e., 2.24 and 2.08, respectively, indicated the sufficient removal of peptides from the RNA (Warburg and Christian, 1941). Therefore, these poly(A)$^+_{hom}$ RNA samples were translated.

The RNA yield of 4.8 mg RNA_{hom} /g rat liver wet wt. was lower than that of other investigators who had used the phenol-chloroform method (Pain et al., 1974; Blobel and Potter, 1967; Keller and Taylor, 1976). However, the 2.0% recovery of poly(A)$^+_{hom}$ RNA was comparable to other investigators who have used the phenol-chloroform procedure (e.g., Rachubinski et al., 1980).

Two criteria which attested to the validity of mRNA translation by the in vitro translation system were the exogenous) mRNA dependency of the system as well as the reproducibility of translation.

Thus, it was shown that the incorporation of ^{3}H-leucine into hot TCA precipitates was dependent on the addition of poly(A)$^+_{hom}$ RNA and a linear response was noted in the range of 0-1 µg RNA with saturation occurring at ca 2 µg of

poly(A)$^{+}_{hom}$ RNA added. These results were identical over two separate experiments (Fig. 1 of Results).

The translation products were visualized by fluorography (method of Laskey and Mills) and compared to CB-stained profiles of the major synthetic products of the liver, namely the plasma proteins (Bergeron et al., 1978; Crane and Miller, 1974; Miller et al., 1954; Glaumann and Ericsson, 1970; Glaumann et al., 1975; Jones et al., 1967; Miller and Bale, 1954; Miller et al., 1954; Morgan and Peters, 1971; Noel and Rubenstein, 1974; Peters et al., 1971; Redman et al., 1975). Therefore, the integrity of the rat liver mRNA was established by the ability of the mRNA to direct the synthesis of the marker proteins of the liver as well as large peptides.

Fluorographic analysis of cell-free translation products directed by rat liver poly(A)$^{+}_{hom}$ RNA from two separate experiments showed an identical spectrum of radioactive products with a major band running close to the mobility of serum or plasma albumin. Close analysis revealed that this major radiolabeled band had a mobility slightly less than that of plasma albumin equivalent to a difference in molecular weight of ca 2,000 (Fig. 2, Results). Such a difference is equivalent to the prepro sequence of albumin which is comprised of ca 24 amino acids (Strauss, 1977; Raehubinski et al., 1980) and would not be processed by the reticulocyte lysate system (Shields and Blobel, 1977; Shore et al., 1979). Of note in the fluorographic analysis, were bands up to 145,000 in molecular weight which were easily visualized. This is

clearly superior to translation products using phenol-chloroform to isolate mRNA (e.g., compare Fig. 3 of Rachubinski et al., 1980 or Fig. 2 of Goldman and Blobel, 1978 to Fig. 2 of present results).

Radioactivity gel profile determinations enabled direct quantitative analysis of translation products. The major peak, A64, of Figs. 3a and b was judged to be 30.8% (average) of the total translation products. This value, 30.8%, was markedly superior to that found by those investigators who used phenol-chloroform to isolate liver mRNA (Table 3). Indeed, the results even exceeded the highest *in vivo* value[5] of % albumin synthesized as determined by Keller and Taylor (1976) as 13% (Table 3).

In Table 3, all the *in vitro* values of % albumin synthesized with the exception of the author's value, were obtained through the use of the phenol-chloroform mRNA extraction procedure.

The obvious discrepancy between the author's *in vitro* value of % albumin synthesized, 30.8%, and the other *in vitro* values, e.g., maximum 13% (see Table 3) implied that the GTC

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[5]All estimates made thus far by investigators on the amount of albumin synthesized by liver as a proportion of total protein synthetic products of liver must be considered highly suspect. These values (see Table 3) are usually based on immunoprecipitates. They have not, however, satisfied even the most elementary of criteria to ensure that the immunoprecipitation was quantitative and complete. The more correct way to assess % albumin in liver synthetic products should have been by competitive radioimmunoassay as described by Yalow (1978).

Table 3

In vivo and in vitro experimental comparisons of the proportion of albumin synthesized by liver homogenate mRNA relative to total synthesized peptides

Authors	Tissue	mRNA fraction	mRNA isolation procedure	in vitro Experiment Estimating % Albumin/ Total Liver Peptides	in vitro % Alb/ $Prot_T$	in vivo % Alb/ $Prot_T$
1. Schreiber et al., 1971	rat liver	----------	----------	in vivo ----------		3.5%
2. Rachubinski, 1978	rat liver	homogenate	phenol-chloroform	in vitro translation: wheat germ extract	3.6%	
3. Brown and Papaconstantinou, 1979	mouse liver	homogenate	phenol-chloroform/CsCl density centrifugation	in vitro translation: wheat germ extract	4.5%	
4. Peters and Peters, 1972	rat liver	----------	----------	in vivo ----------		10.9%
5. Peavy et al., 1978	rat liver	homogenate	phenol-chloroform	in vitro translation: reticulocyte lysates and cDNA hybridization	9.5%	
	rat liver	----------	----------	in vivo ----------		12%
6. Tse and Taylor, 1977	rat liver	homogenate	phenol-chloroform	in vitro translation: wheat germ extract	9.6%	
7. Keller and Taylor, 1976	rat liver	----------	----------	in vivo ----------		13%
8. (author) Power, 1980	rat liver	homogenate	guanidinium thiocyanate/ CsCl density centrifugation	in vitro translation: reticulocyte lysates	30.8%	

method was superior to the phenol-chloroform extraction procedure for the isolation of mRNA. However, this conclusion could only be made definite upon comparison of the GTC method to a method employing the CsCl density centrifugation along with phenol extraction thereby enabling a direct comparison between the efficacy of mRNA isolation by guanidinium thiocyanate to that of phenol-chloroform.

In 1979, Brown and Papaconstantinou isolated total liver mRNA by phenol-chloroform extraction with immediate centrifugation of the final aqueous phase using CsCl (5.7M). Their results showed the in vitro % albumin synthesized value to be 4.5% in contrast to the author's value of 30.8%.

Since the CsCl density centrifugation technique provides a system wherein RNA can be isolated free of contaminants under a singly gentle condition, it is therefore hightly probable to surmise that the great discrepancy in the in vitro % albumin synthesized values between the author and the cited investigators was indeed due to the harsh conditions of the phenol-chloroform technique as well as its inability to inactivate RNAse activity.

It is therefore concluded that the GTC procedure for the isolation of rat liver homogenate mRNA is vastly superior to the traditional phenol-chloroform method. It is furthermore predicted that when assessment of total protein synthesis by liver is carried out with a reasonable degree of sophistication, i.e., by careful radioimmunoassay assay (see

Yalow, 1978) then at least 31% of the total protein synthesis by liver could be attributed to albumin.[6]

For any future studies on membrane biogenesis, however, the validity of GTC method would have to be determined for the free and membrane-bound polysomal mRNA.

II Free and Membrane-bound Polysomal mRNA

Before determining the validity of the GTC method for the isolation of free and membrane-bound polysomal mRNA, it was necessary to choose a method which could isolate free and membrane-bound polysomes in proportions representative of their in vivo levels.

The preparation of polysomes by isopynic centrifugation of a postmitochondrial supernatant fraction (Bergeron et al., 1975; Blobel and Potter, 1967; Shafritz, 1973) had been favored by investigators for several years since this fraction was relatively free of nuclease-containing organelles (Roth, 1967) and possessed a nuclease inhibitor (Roth, 1956;

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[6] A simple calculation reveals that such an estimate is not unreasonable. The author's proportion of membrane-bound to free polysomes in the liver was ca 2:1 (see Table 2 of Results and footnote 7; see also Ramsey and Steele, 1977). Most of the membrane-bound polysomes are involved in the synthesis of secretory proteins—most of which are the plasma peptides (see fig. 4 of Bergeron et al., 1978). Albumin represents ca 47% of the total protein in plasma (Sheving et al., 1968). Therefore, on these grounds alone it can be speculated that the amount of albumin synthesized by the liver is approximately the product of the proportion of membrane-bound polysomes multiplied by the proportion of albumin in plasma, i.e., .67 x .47 = .31 x 100 = 31%. Therefore, more than a third of the total proteins synthesized by liver could be albumin. Clearly, a careful in vivo study of the proportion of albumin made by the liver is required.

Bond et al., 1965). However, one problem which confronted investigators who wished to obtain a quantitative yield of membrane-bound polysomes was that the conventional preparation of polysomes from a postmitochondrial supernatant fraction (Bergeron et al., 1975; Blobel and Potter, 1967; Shafritz, 1973) resulted in recoveries 90 and 30 % of the total free and membrane-bound polysomes, respectively (Blobel and Potter, 1967) thereby yielding unrepresentative membrane-bound polysomal fractions.

In 1976, Ramsey and Steele outlined a method for the isolation of polysomes from whole rat liver homogenates by means of differential sedimentation as the key step for the separation of free and membrane-bound polysomes. This technique yielded a ca 95% recovery of total polysomes from the sum of free and membrane-bound polysomes (Ramsey and Steele, 1976). One minor problem as found by Rachubinski (1978) in the method of Ramsey and Steele consisted of the addition of detergent where this step increased RNAse activity, presumably through the liberation of lysosome-bound enzymes.

Therefore, it was decided to employ Rachubinski et al.'s modified version (i.e., no detergent treatment) of Ramsey and Steele's technique of polysomal isolation (Rachubinski et al., 1980).

Thus following the separation of free and membrane-bound polysomes by Rachubinski et al's modification, RNA was prepared from the respective polysomal populations (Methods IV,

1b and c) by the GTC procedure. Using the average values of mg RNA (free or membrane-bound)/g liver wet weight (Table 2), it was calculated that the free and membrane-bound polysomes comprised 33 and 67%, respectively, of the total polysomes.[7] These proportions are comparable to the estimates of other workers who also determined the proportion of these polysomal classes in liver. That is, the proportions of free and membrane-bound polysomes, respectively, were shown by Bergeron et al. (1975) to be 22 and 78%, by Blobel and Potter (1967) and Ramsey and Steele (1976) to be 25 and 75%, by Ramsey and Steele (1977) to be 33 and 67%, by Venkatsen and Steele (1972) to be 34 and 66% and Pain et al. (1974) to be 41 and 59%.

The use of GTC for the isolation of rat liver free and membrane-bound polysomal RNA, resulted in an average recovery of 2.2% poly(A)$^+_{fr}$ RNA and 1.6% poly(A)$^+_{bd}$ RNA (Table 2) after passing each polysomal RNA population through an Oligo d(T)-cellulose column. These values agreed with the aforementioned homogenate values (Table 1) as well as the polysomal estimates

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[7] Calculations from Table 2 of Results:

Membrane-bound polysomes: 2.2 mg RNA/g liver
Free polysomes: 1.1 mg RNA/g liver
3.3 mg RNA/g liver

% 'Bound' polysomes: $\frac{2.2 \text{ mg RNA/g liver}}{3.3 \text{ mg RNA/g liver}} \times 100 = 67\%$

% free polysomes: $\frac{1.1 \text{ mg RNA/g liver}}{3.3 \text{ mg RNA/g liver}} \times 100 = 33\%$

of other workers for the recovery of poly(A)$^+$ RNA (Rachubinski et al., 1980; Keller and Taylor, 1976).

Translation of poly(A)$^+_{fr}$ RNA and poly(A)$^+_{bd}$ RNA revealed that protein synthesis was a linear function of the addition of poly(A)$^+$ RNA from either polysomal class in the range 0-1 µg for poly(A)$^+_{fr}$ RNA and 0-3.5 µg for poly(A)$^+_{bd}$ RNA with saturation occurring at ca 2 µg poly(A)$^+_{fr}$ RNA and $\geq$ ca 5 µg poly(A)$^+_{bd}$ RNA added.

However, the slope of the various mRNA-dependency curves (Fig. 4) markedly differed for poly(A)$^+_{bd}$ RNA and poly(A)$^+_{fr}$ RNA. This could be explained by the presence of a significant amount of poly(A)$^+$ RNA in poly(A)$^+_{bd}$ RNA which had not participated in protein synthesis, i.e., evidence for some degraded mRNA in poly(A)$^+_{bd}$ RNA.[8] The similar slopes of the mRNA dependency curves for poly(A)$^+_{fr}$ RNA and poly(A)$^+_{hom}$ RNA (Fig. 4) indicated, in contrast, the lack of significantly degraded free polysomal mRNA.

It is difficult to compare these results with other investigators. That is, although Rachubinski et al, (1980) compared the translational ability of the free and membrane-bound polysomal mRNA of rat liver by comparing the

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[8]Alternatively, a less likely situation is that either a factor which copurified with poly(A)$^+_{bd}$ RNA had markedly inhibited protein synthesis or some property of the secondary structure of all poly(A)$^+_{bd}$ RNA's as opposed to that of poly(A)$^+_{fr}$ RNA's. However, as poly(A)$^+_{hom}$ RNA is mainly composed of poly(A)$^+_{bd}$ RNA (footnote 7) this would seem to make these explanations extremely unlikely.

respective values of ^{3}H-leucine incorporation as dpm/µg poly(A)$^{+}$ RNA they, as well as other workers (e.g. Bergeron et al., 1975; Faber et al., 1974; Shafritz, 1973; Shore and Harris, 1977), did not determine the mRNA dependency of their translation systems, so that it was not possible to clearly estimate the integrity of their mRNA populations. However, it is noteworthy that Taylor and Schimke (1973) found non-mRNA dependency of their reticulocyte lysate translational system (untreated with micrococcal nuclease) following the addition of exogenous rat liver (total) polysomal mRNA (see Table 1, Taylor and Schimke, 1973). Nevertheless, a comparison (see Figs. 7, 8 and 9) to the representative results of Rachubinski et al. (1980) (see Table 1) indicates the vast superiority of the poly(A)$^{+}_{bd}$ RNA as isolated by the GTC method to poly(A)$^{+}_{bd}$ RNA as isolated by the phenol-chloroform method.

Fluorographic analysis of cell-free translation products directed by rat liver poly(A)$^{+}_{fr}$ RNA and poly(A)$^{+}_{bd}$ RNA as isolated by GTC from two different experiments showed relatively identical spectrums of radioactive products, respectively (Figs. 5 and 6). Close analysis of the translation productions of poly(A)$^{+}_{bd}$ RNA revealed the near-exclusive synthesis of a major radiolabeled band with a slightly slower mobility than that of plasma (or serum) albumin equivalent to a difference in molecular weight of ca 2,000 (Figs. 5 and 6). This difference would be accounted for by the prepro leader sequence of albumin, i.e., 24 amino acids (Strauss,

1978) which would not be expected to be processed by the reticulocyte lysate translation system (Shields and Blobel, 1977; Shore et al., 1979). In contrast, analysis of the fluorographs of peptides translated by poly(A)$^+_{fr}$ RNA showed the presence of only a minor amount of this peptide.

Fluorography revealed the exclusive synthesis of a peptide directed by poly(A)$^+_{fr}$ RNA (ca 27,500 molecular weight) which corresponded exactly in mobility with a peptide found in the cytosolic fraction of rat liver (Figs. 5 and 6). As well, a labeled peptide such as that of mobility ca 59,000 molecular weight appeared to be directed by both poly(A)$^+_{fr}$ RNA and poly(A)$^+_{bd}$ RNA.[9] It is significant that such synthesis might be expected for peptides possessing an

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[9] The molecular weight of the peptide exclusively synthesized by poly(A)$^+_{fr}$ RNA was determined by two sets of experiments (see Figs. 5 and 6). In one set, the molecular weight of the labeled peptide, F28, was found to be 28,000 which corresponded to the CB-stained cytosolic peptide, C28, also of 28,000 molecular weight, (Fig. 5). Though Fig. 6 showed the equivalent peptide, the molecular weights of the labeled and cytosolic peptide were found to be 27,000 (F27 and C27, respectively). The % difference of the two molecular weight determinations was 3.6% which can be accounted for by the limitations of SDS-PAGE.

This consideration could also be applied to the peptide directed by both poly(A)$^+_{fr}$ RNA and poly(A)$^+_{bd}$ RNA where one experiment found the labeled peptide BF61 to be 61,000 in molecular weight (Fig. 5) and in another experiment the peptide, BF57, to be 57,000 in molecular weight (Fig. 6) which yielded a 6.6% molecular weight difference.

internal signal sequence e.g. ovalbumin (Lingappa et al., 1979).[10]

The radioactivity of gel profile determinations enabled direct quantitative analysis of translation products. Examination of the radioactivity gel profile determinations of rat liver poly(A)$^{+}_{bd}$ RNA translation products revealed that the major peak, A' (Figures 7a, 8a and 9a), was judged to be 30.3, 30.7 and 23.5%, respectively, of the total translation products. Therefore, the average value of % albumin synthesized by GTC isolated rat liver poly(A)$^{+}_{bd}$ RNA, 28.2%, was markedly higher than that as determined by other workers (see Table 4) where the highest previous value was obtained

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[10] Lingappa et al. (1979) found that chicken ovalbumin possessed an internal sequence as determined by testing the ability of tryptic ovalbumin fragments to inhibit the translocation of preprolactin. From their work, this author predicted that the first 65.7% of the ovalbumin molecule is made on free polysomes and that the remaining 34.3% on membrane-bound polysomes. That is, since ovalbumin consists of amino acid residues, 1-385, and the signal sequence bears amino acids residues, 234-253,

$\therefore$ % ovalbumin made on free polysomes: $\frac{253 \text{ amino acids}}{385 \text{ amino acids}} \times 100 = 65.7\%$

and % ovalbumin made on 'bound' polysomes: $\frac{132 \text{ amino acids}}{385 \text{ amino acids}} \times 100 = 34.3\%$

The first portion included the signal sequence since the free polysomes are only directed to the endoplasmic reticulum after the synthesis of the signal sequence (Blobel and Dobberstein, 1975).

Note that a testing of this prediction could be made, following the methods outlined in this thesis. That is, to determine the polysomal origin of ovalbumin by translation of oviduct poly(A)$^{+}_{fr}$ RNA and poly(A)$^{+}_{bd}$ RNA. This then, would resolve the controversy between Palmiter (Meek et al., 1980) and Blobel (Blobel and Dobberstein, 1975).

Table 4

In vitro experimental comparisons of the proportion of albumin synthesized by free and membrane-bound polysomal mRNA relative to total, synthesized peptides

Authors	Tissue	mRNA Fraction	mRNA isolation procedure	Experiment estimating % Albumin/Total Liver Peptides	% $Alb/Prot_T$
1. Shafritz, 1973	rabbit liver	bound polysomes: (postmitochondrial supernatant)	phenol-M-cresol	in vitro translation: KCl-treated reticulocyte ribosomes	3%
2. Shore and Harris, 1977	rat liver	rough microsomes (postnuclear supernatant)	phenol-chloroform	in vitro translation: reticulocyte lysates	3.1%
3. Faber et al., 1974	mouse liver	bound polysomes (postmitochondrial supernatant)	phenol-SDS	in vitro-translation: reticulocyte lysates	5-8%
4. Rachubinski et al., 1980	rat liver	bound polysomes (Ramsey and Steele, 1976)	phenol-chloroform	in vitro translation: wheat germ extract	8.6-8.8%
5. (author) Power, 1980	rat liver	bound polysomes (Ramsey and Steele, 1976)	guanidinium thiocyanate/CsCl density centrifugation	in vitro translation: reticulocyte lysates	28.2%

by Rachubinski et al., (1980). Most of these workers isolated membrane-bound polysomal mRNA via phenol-chloroform extraction (Shafritz used phenol-cresol extraction and Faber, 1974, used phenol-SDS). Therefore, Table 4 clearly illustrated the ability of GTC to isolate more intact polysomal mRNA than phenol-chloroform extraction.

Radioactivity gel profile determinations of rat liver poly(A)$^+_{fr}$ RNA translation products revealed little association of radioactivity in the albumin zone (see footnote 3 of Results). In fact, the amount of radioactivity associated with the albumin zone relative to a total radioactivity gel profile, i.e., total synthesized peptides, was found to be an average of 76% as synthesized by poly(A)$^+_{bd}$ RNA and 24% as synthesized by poly(A)$^+_{fr}$ RNA. Rachubinski et al. (1980) determined these values more specifically by the use of monospecific albumin antibodies. Anti-albumin immunoprecipitates of the translation products directed either by phenol-chloroform extracted rat liver poly(A)$^+_{fr}$ RNA or poly(A)$^+_{bd}$ RNA were analyzed by radioactivity gel profile determinations. Rachubinski et al. (1980) found that >90% of the radioactivity of the immunoprecipitates was synthesized by poly(A)$^+_{bd}$ RNA and 10% by poly(A)$^+_{fr}$ RNA. If monospecific albumin antibodies had been used to immunoprecipitate the translation products of GTC isolated rat liver poly(A)$^+_{fr}$ RNA or poly(A)$^+_{bd}$ RNA, the amount of radioactivity associated with albumin would probably increase dramatically in the radioactivity gel profiles of immunoprecipitates directed by poly(A)$^+_{bd}$ RNA. This assumption

is based upon close observation and comparison of the fluorographs of peptides directed by poly(A)$^+_{fr}$ RNA and poly(A)$^+_{bd}$ RNA.[11]

The values of % albumin synthesized either by GTC isolated rat liver poly(A)$^+_{hom}$ RNA (30.8%) or poly(A)$^+_{bd}$ RNA (28.2%) were shown to be approximately three times higher than the highest values previously obtained by investigators who had prepared these liver mRNA populations by phenol-chloroform extraction (Tables 3 and 4). As well, the values closely corresponded to the 31% maximum calculated on theoretical grounds (see footnote 6). A similar study could be carried out with the insulin receptor. Indeed, the high degree of integrity of liver mRNA as isolated by the GTC method should permit the eventual isolation of the insulin receptor gene(s). These results, as well as the fact that GTC isolated homogenate mRNA translated a very high molecular weight peptide, H 145, strongly suggests the superiority of the GTC method over phenol-chloroform for the isolation of mRNA. The extension of the studies to poly(A)$^+_{bd}$ RNA and poly(A)$^+_{fr}$ RNA could also serve to demolish the notion

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[11] Several minor bands were found in the albumin zone of fluorographs of poly(A)$^+_{fr}$ RNA peptide products (Figs. 5b and 6b, lane: Free) of which only one likely represented albumin. The remainder are probably peptides truly directed by poly(A)$^+_{fr}$ RNA. Current experiments are aimed at establishing this more specifically by the use of antialbumin antibodies.

of Shafritz that translational control could explain exclusive *in vivo* synthesis of albumin on membrane-bound polysomes (Shafritz, 1974). Clearly, the RNA of Shafritz must have been extensively degraded.

Further verification of albumin synthesis by rat liver membrane-bound polysomes as determined by the GTC mRNA isolation procedure and the modified Ramsey and Steele polysomal extraction technique could be elicited from the use of monospecific anti-albumin. The techniques outlined in this thesis could enable investigation of the polysomal origin of two membrane proteins, 5'-nucleotidase and the insulin receptor, again with the aid of *in vitro* translation concomitant with monospecific antibodies. It would also be interesting to determine whether the peptide of *ca* 59,000 molecular weight (i.e., BF 61, Fig. 5; BF 57, Fig. 6) has a dual polysomal origin as suggested in this thesis.

Since 5'-nucleotidase has never been sequenced and since it is an important membrane protein, it would be considered worthwhile to eventually prepare cDNA copies of the 5'-nucleotidase message in order to determine the sequence and number of its translatable genes which would enable an exact determination of its amino acid composition and molecular weight. Its composition and molecular weight. Its composition of sugar residues could be studied via radioautography.

In conclusion, the GTC method has been applied to the isolation of mRNA from homogenates, and free and membrane-bound polysomes of rat liver. This work demonstrated that

the GTC method resulted in a proportion of mRNA coding for (presumed) albumin by homogenate and membrane-bound polysomal mRNA higher than the proportions determined from the phenol-chloroform method. The cut clean differences in the translation products directed by free and membrane-bound polysomal mRNA has provided better evidence to date for the dichotomy of function of these two polysomal classes. Their distinct roles were shown to be due to specific mRNA's associated with each polysomal class and not to any translational control.

It should be noted that Chirgwin et al. finally published their paper on mRNA isolation by GTC as this author's work was being completed (Chirgwin et al., 1979). Their method was virtually similar to the author's extension of Uldrich et al.'s GTC procedure (1977). However, Chirgwin et al. did not determine the efficacy of the GTC method for the isolation of rat liver homogenate, free, and membrane-bound polysomal mRNA nor attempt analysis of liver translation products.

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