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CANADIAN THESES ON MICROFICHE / *THÈSES CANADIENNES SUR MICROFICHE*

NAME OF AUTHOR/*NOM DE L'AUTEUR* David Wayne O'Keefe

TITLE OF THESIS/*TITRE DE LA THÈSE* Cell-free studies on the synthesis and regulation of alpha-fetoprotein

UNIVERSITY/*UNIVERSITÉ* University of Alberta

DEGREE FOR WHICH THESIS WAS PRESENTED/ *GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* M.Sc.

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE GRADE* 1975

NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* Dr. T. Tamaoki

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DATED/*DATÉ* June 3, 1975 SIGNED/*SIGNÉ* David W. O'Keefe

PERMANENT ADDRESS/*RÉSIDENCE FIXE* 3304 - 33rd Ave
Vernon, B.C.

NL-91 (3-74)

THE UNIVERSITY OF ALBERTA

CELL-FREE STUDIES ON THE SYNTHESIS AND REGULATION OF ALPHA-FETOPROTEIN

by

© DAVID WAYNE O'KEEFE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL, 1975

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ...CELL-FREE STUDIES.... .....ON THE SYNTHESIS AND REGULATION OF..... .....ALPHA-FETOPROTEIN..... submitted by .....DAVID WAYNE O'KEEFE..... in partial fulfilment of the requirements for the degree of Master of Science.

Tacke Tsuchi
Supervisor
A. R. Morgan
Wm Paranchych
2nd Signature

Date June 3, 1975

ABSTRACT

Alpha-fetoprotein (α-FP) is a serum protein that is synthesized by fetal liver but not by adult liver. In order to understand the control mechanism for α-FP production, cell-free synthesis of α-FP was attempted.

Polysomal RNA from fetal and adult mouse liver was added to a heterologous cell-free system prepared from Sarcoma 130 cells and the synthesis of α-FP was determined. The ability of the cell-free system to translate poly(U) was also examined.

Several parameters, such as pre-incubation time to eliminate endogenous protein synthesis and concentration of $MgCl_2$, KCl, ATP, GTP and tRNA, were studied. Significant differences were observed between poly(U) and polysomal RNA translation in this system: poly(U) translation required added tRNA but not high salt extract of ribosomes (ribosomal wash, source of initiation factors), whereas the translation of polysomal RNA required ribosomal wash, but not tRNA.

The synthesis of α-FP in the cell-free system was analysed by measuring radioactivity in material precipitated by antibody against α-FP. Albumin synthesis was also measured as an internal control. The synthesis of α-FP and albumin was obtained when fetal polysomal RNA was translated in the presence of ribosomal wash from either adult liver,

fetal liver or Sarcoma 130 cells. With any of these wash preparations, adult liver polysomal RNA failed to produce α-FP, although albumin synthesis was obtained. It was concluded that active mRNA for α-FP was not present in adult liver. This indicates that α-FP synthesis is controlled primarily at the level of transcription.

The protein precipitated by anti-α-FP or retained by a column containing anti-α-FP attached to Sepharose 4B was analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate. The results showed that 10-23% of the protein thus recovered was complete molecules of α-FP (MW 70,000). The remainder most likely represented nascent α-FP chains. This possibility was supported by the observation that fragments of α-FP (MW 12-53,000) produced by cyanogen bromide cleavage reacted with anti-α-FP.

ACKNOWLEDGEMENTS

I would like to thank Dr. Tamaoki for the guidance he has given me throughout the course of these studies. I am also grateful to Irma Schindler and Pat Banks for helpful discussion as well as for the antibody and ribosomal wash preparations that I used on many occasions. Also, I wish to thank Kelly Thomas and Amin Mirza for much useful discussion and almost all of the polysomal RNA preparations used.

I am grateful to have been a holder of a Medical Research Council Studentship.

Finally, I wish to thank my wife, Catherine, for the many hours she spent typing this thesis.

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LIST OF ABBREVIATIONS

mRNA	messenger ribonucleic acid
tRNA	transfer ribonucleic acid
rRNA	ribosomal ribonucleic acid
poly(U)	polyuridylic acid
Phe	phenylalanine
Leu	leucine
Glu	glutamic acid
Val	valine
ATP	adenosine-5'-triphosphate
GTP	guanosine-5'-triphosphate
TCA	trichloroacetic acid
SDS	sodium dodecyl sulphate
DTE	dithioerythritol
EDTA	ethylenediaminetetraacetic acid
β-ME	β-mercaptoethanol
DOC	deoxycholate
PBS	0.01 M phosphate buffered saline
BSA	bovine serum albumin
α-FP	alpha-fetoprotein
Hb	hemoglobin
IF1	initiation factor 1
IF2	initiation factor 2

EMC virus	Encephalomyocarditis virus
cpm	counts per minute
ma	milliampere
m, u	milli, micro
CP	creatine phosphate
CPK	creatine phosphokinase

INTRODUCTION

The developing fetal liver synthesizes alpha-fetoprotein (α-FP), while the adult liver synthesizes only trace amounts if any. Also, some primary liver carcinomas and some teratomas synthesize α-FP and detection of α-FP in serum is used in diagnosing these disorders. Fig. 1 illustrates the level of α-FP in the serum throughout the life cycle. High serum levels occur during embryonic and early post-natal life and can recur during liver regeneration, primary liver carcinomas and teratomas. For an excellent review on α-FP see Abelev (1971).

Alpha-fetoprotein is a glycoprotein of molecular weight of about 70,000 , of which about 4.5% is carbohydrate. The biological function of α-FP is unknown. However, α-FP has been shown to bind estrogen (Aussel et al., 1973). Also, α-FP appears to inhibit lymphocyte proliferation and could possibly have an immunosuppressive activity important during pregnancy (Parmely and Thompson, 1974). Sell et al. (1974) have shown that the production of α-FP is closely coupled to cellular division. Serum α-FP concentrations rise following rounds of hepatocellular proliferation. However, it is not understood how α-FP synthesis and mitotic activity of hepatocytes are

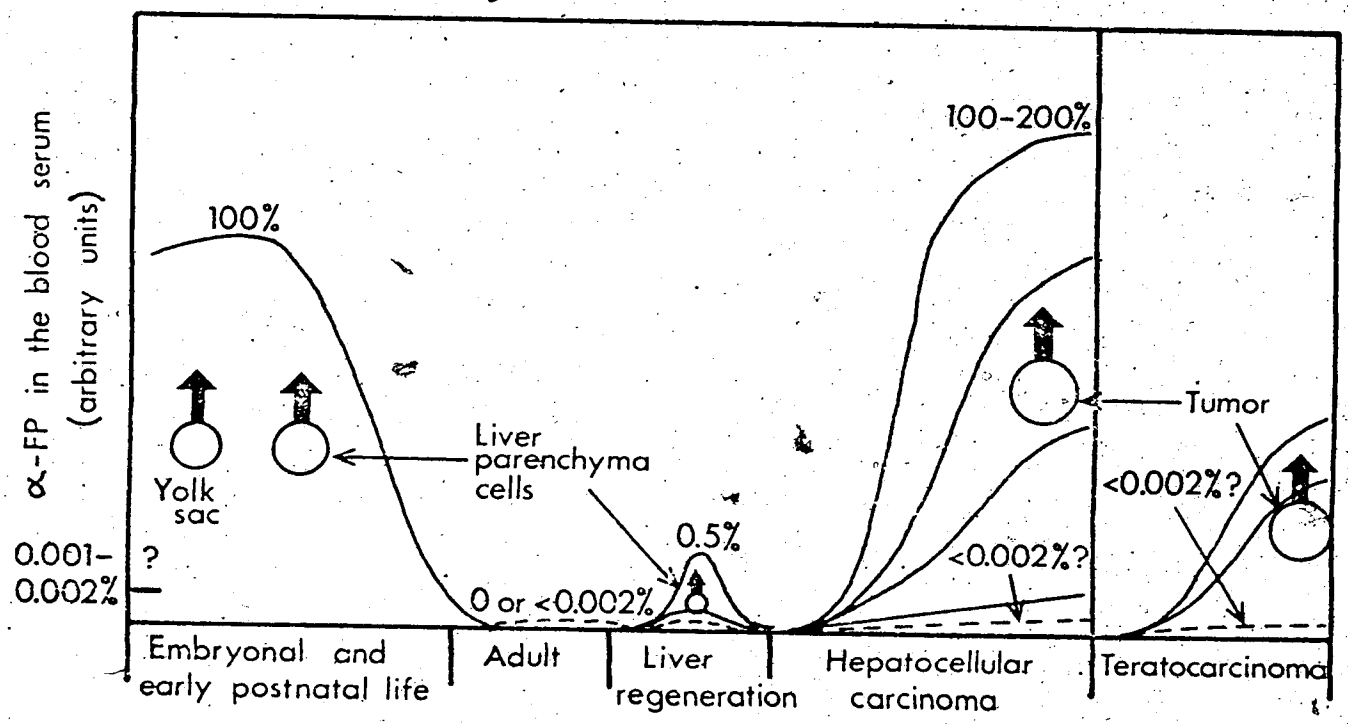


Figure 1. Schematic representation of α-FP synthesis in normal development and pathologic states. Solid line, serum α-FP level, in arbitrary units; broken line, expected α-FP level; ?, not known. (From Abelev, 1971)

interrelated.

Studying α-FP synthesis in a cell-free system may reveal some aspects of the control of α-FP synthesis. Using fetal liver lysates, Tamaoki et al. (1974) demonstrated the cell-free synthesis of mouse α-FP. Koga and Tamaoki (1974) showed that α-FP synthesis occurred on membrane-bound polysomes in fetal mouse liver. Also, Kanai et al. (1974) reported similar findings using rat ascites hepatoma cells. More recently, α-FP synthesis directed by exogenous mRNA was accomplished in an homologous cell-free system (Koga et al., 1974). The present work aims at establishing a heterologous cell-free system which is capable of translating α-FP and albumin mRNA extracted from fetal and adult mouse liver.

Initially a cell-free system from wheat germ (Roberts and Paterson, 1973) was tested for its ability to translate mouse liver polysomal RNA. The cell-free system contained ribosomes, tRNA and many other components required for the translation of exogenous mRNA. It was found that this system was active in phenylalanine incorporation directed by poly(U), but not capable of translating mouse liver polysomal RNA. A cell-free system from mouse Sarcoma 180 cells was therefore tried. These cells have been maintained in the McEachern Laboratory and were readily available. In addition, published results suggest that the Sarcoma system has several advantages over other cell-free systems, such as the Krebs II lysate. These advantages include a stronger stimulation by a wide range

of RNAs (viral and non-viral) and a longer time course of protein synthesis than the Krebs II system (Jenkins et al., 1973). The results described below indicate that this system was indeed capable of translating polysomal RNA from adult and fetal mouse liver. From the study of the requirements for the synthesis of α-FP and albumin in this system, the possible site of regulation of α-FP synthesis was determined.

METHODS AND MATERIALS

A. Chemicals and buffers

All chemicals used were of certified quality. Ultra-pure RNase-free sucrose was purchased from Swarz/Mann. ATP, GTP, creatine phosphate, creatine phosphokinase and SDS were purchased from Sigma Chemical Co.; puromycin dihydrochloride, 20 amino acids and rabbit anti-albumin, from Nutritional Biochemicals Corp.; poly(U) and mouse albumin, from Miles Laboratories Inc.; Sephadex G-25 and cyanogen bromide-activated Sepharose 4B, from Pharmacia; Scintiverse, from Fisher Scientific Co.; and Aquasol, from New England Nuclear. Radioactive amino acids purchased from Amersham/Searle were as follows: ^{14}C-leucine, 343 mCi/mmole and 311 mCi/mmole; ^{14}C-phenylalanine, 477 mCi/mmole; ^{3}H-leucine, 1 Ci/mmole and 190 mCi/mmole; ^{3}H-valine, 2 Ci/mmole and 17.7 Ci/mmole; ^{3}H-phenylalanine, 1 Ci/mmole and 20 Ci/mmole; and ^{3}H-glutamic acid, 1.4 Ci/mmole and 34 Ci/mmole.

The following buffers all contain 20 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 6 mM β-ME and 0.1 mM EDTA. The KCl and DTE concentrations for each are as follows:

Buffer A	100 mM KCl, 0.5 mM DTE
Buffer B	25 mM KCl, 0.5 mM DTE
Buffer C	100 mM KCl, 0.0 mM DTE

B. Preparation of adult mouse liver supernatant and ribosomal wash

ICR mice were decapitated and their livers were rinsed and homogenized in Buffer B containing 0.25 M sucrose. After centrifugation at 12,000 x g (av) for 30 minutes, the top two-thirds of the supernatant was removed (post-mitochondrial supernatant). This was centrifuged in a Beckman 60 Ti rotor at 214,000 x g (av) for 2 hours. The supernatant referred to as "S-100" was either used for the preparation of polysomes or used in the preparation of the supernatant enzyme fraction by collecting proteins precipitated by $(NH_4)_2SO_4$ between 35-75% saturation. The precipitated protein was dissolved in Buffer A containing 20% glycerol, dialysed against the same overnight and then stored in liquid N_2.

Ribosomal wash was prepared from adult and fetal mouse liver and also from Sarcoma 180 cells according to Prichard et al. (1971) with some modifications. The post-mitochondrial supernatant (see above) was centrifuged at 214,000 x g (av) for 2 hours. The pellet was washed 3 or 4 times with 0.25 M sucrose, 1 mM DTE and 0.1 mM EDTA (pH 7.0), and suspended in this same buffer. Four M KCl was added slowly to a final concentration of 0.5 M and the suspension was stirred for 30 minutes at 0°. The sample was then centrifuged in a Type 65 rotor at 176,000 x g (av) for 2 hours at 4°. The top three-quarters of the supernatant was dialysed overnight against Buffer A containing 20% glycerol and stored in liquid N_2.

C. Preparation of mouse liver tRNA

One-tenth volume of 2% Macoloid, one-twentieth volume of 20% potassium acetate (pH 5) and an equal volume of water-saturated phenol were added to adult mouse liver high speed supernatant ("S-100"). The mixture was shaken at room temperature for 15 minutes, centrifuged at 7,700 x g (av) for 15 minutes and the aqueous layer was removed. The phenol layer was re-extracted with one-half volume of water. The aqueous layers were combined and extracted 4 times with an equal volume of ether to remove phenol. RNA was precipitated by the addition of 2.5 times volume of 95% ethanol. The pellet was dissolved in one-third original volume of 0.05% potassium acetate (pH 5) and ethanol precipitation was repeated once more. The pellet was dissolved in 2 M Tris-HCl (pH 8.5) and incubated at 37° for 40 minutes. Again one-twentieth volume of 20% potassium acetate (pH 5) was added along with 2.5 times volume of ethanol and precipitation was allowed to continue for 4 hours at -20°. The resulting pellet was washed twice with ethanol and twice with ether. The tRNA pellet was then dried with a stream of N_2 and dissolved in water to approximately 20 A_{260} per ml and stored at -20°.

D. Preparation of total polysomes, polysomal RNA and rRNA

Autoclaved glassware and solutions were used for all procedures. Livers from adult ICR mice or 14-15 day old fetal mice were used. The mice were killed by decapitation

and the livers were removed, rinsed in Buffer B containing 0.25 M sucrose and homogenized in the same buffer. The homogenate was centrifuged at 7,700 x g (av) for 10 minutes at 4°. The top two-thirds of the supernatant was removed and adjusted to a final concentration of 1% Triton X-100 and 1% DOC, and left on ice for 30 minutes. The homogenate was then layered on a discontinuous sucrose gradient of 1 ml of 1.5 M sucrose and 1 ml of 1 M sucrose. Both sucrose solutions were in a 1:1 mixture of Buffer B and adult mouse liver supernatant ("S-100"). "S-100" was used as a source of ribonuclease inhibitor. The sample was centrifuged at 149,000 x g (av) for 3 hours at 4° in an SW 50.1 rotor. The pellet containing the polysomes was suspended in Buffer A.

The RNA was extracted from the polysomes according to the method of Perry et al. (1972). SDS was added to the polysome preparation to a final concentration of 1%, and the solution was left at room temperature for 15 minutes. An equal volume of phenol/chloroform (1:1) was added and the mixture was shaken for 5 minutes at room temperature. (The phenol had been equilibrated overnight with 0.1 M acetate (pH 6), 0.1 M NaCl and 1 mM EDTA.) The sample was centrifuged at 17,000 x g (av) for 15 minutes at 4°, and the upper aqueous phase was removed. The lower phenol/chloroform phase was extracted twice with an equal volume of 0.1 M acetate (pH 6), 0.1 M NaCl and 1 mM EDTA. All the aqueous layers were combined and NaCl was added to a final concen-

tration of 0.2 M. After 2.5 times volume of 95% ethanol was added the sample was left at -20° overnight. The pellet was collected by centrifugation at 5,900 x g (av) for 20 minutes and was washed 3 times with ethanol. The remaining ethanol was removed with a stream of N_2 and the RNA was dissolved in water, freeze dried and stored at -20°.

Ribosomal RNA was prepared from mouse liver run-off 80S ribosomes (A. Faber, Ph. D. Thesis, 1974; Falvey and Staehelin, 1970) in a similar fashion. The run-off 80S ribosomes showed little endogenous activity and therefore contamination with mRNA was small.

E. Preparation of Sarcoma S-30

S-30 was prepared from Sarcoma 180 cells according to Jenkins et al. (1973). The cells were grown intraperitoneally in BDF mice and harvested 7 days after transplantation. They were washed 3 or 4 times with 20 mM Tris-HCl (pH 7.8) and 150 mM NaCl using low speed centrifugation in an International centrifuge (Model PR-6). Packed cells were suspended in 20 mM Tris-HCl (pH 7.8), 1.5 mM $MgCl_2$ and 10 mM KCl (1.75 times their volume); Nonidet P-40 was added to a final concentration of 0.5% and left at 4° for 10 minutes. The resulting lysate was adjusted to 5 mM $MgCl_2$ and 110 mM KCl by the addition of one-ninth volume of 20 mM Tris-HCl (pH 7.8), 37 mM $MgCl_2$, 1.01 M KCl and 60 mM β-ME. The lysate was then centrifuged at 30,000 x g (av) for 10 minutes and the top two-thirds of

the supernatant was carefully removed. This supernatant was incubated at 37° for 60 minutes with the following components per ml: 1 umole ATP, 0.3 umole GTP, 50 uM each of the 20 amino acids, 10 umole creatine phosphate and 50 ug creatine phosphokinase. The reaction mixture was cooled and then passed through a Sephadex G-25 column equilibrated with Buffer C. The protein fraction in the void volume was collected, adjusted to 20% glycerol and frozen quickly in small aliquots in liquid N_2. This preparation is referred to as S-30 and had a protein concentration of 10-14 mg/ml.

F. Protein and RNA determination

Protein concentration was estimated using the method of Lowrey et al. (1951) or by spectrophotometric determinations of A_{260} and A_{280}. A standard concentration curve was constructed using BSA. RNA concentration was determined by measuring A_{260} and assuming 1 mg of RNA equals 20 A_{260} units.

G. Sucrose density-gradient analysis

Exponential sucrose gradients (10-35%) were made up in 20 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 100 mM KCl and 0.1 mM EDTA. The sample was layered on top and centrifugation was for 30 minutes at 149,000 x g (av). The gradients were analysed by measuring the A_{260} using a flow cell attached to a Gilford Model 2000 spectrophotometer.

H. Liquid scintillation counting

All liquid scintillation counting was done with a Beckman counter using either toluene-based liquid scintillation fluid, Aquasol (New England Nuclear) or Scintiverse (Fisher Scientific Co.). Counting efficiency for ^{14}C and ^{3}H was approximately 80% and 25%, respectively.

I. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was done essentially according to Davis (1964). In the present work, 7% gels at pH 8.9 were run at 4 ma per tube. The gels were stained with amido black (1% in 7% acetic acid), destained electrophoretically and scanned at 650 nm. If the sample was radioactive the gel was sliced into 2mm fractions which were dissolved in 30% H_2O_2 at 60° overnight. Ten ml of Aquasol (New England Nuclear) or Scintiverse (Fisher Scientific Co.) was added and the radioactivity was measured in a liquid scintillation counter. The gels will be referred to as polyacrylamide gels, pH 8.9 .

Gel analysis was also performed in the presence of 0.1% SDS and 6 M urea (Dunker and Rueckert, 1969; Weber and Osborn, 1969). These were stained with Coomassie Blue (0.63% in 2.5% acetic acid and 50% methanol), destained electrophoretically and scanned at 650 nm. If the sample was radioactive the gel was sliced and counted in the same manner as described above. These gels will be referred to as SDS gels.

Polyacrylamide gel electrophoresis at pH 8.9 will resolve α-FP and albumin as illustrated in Fig. 2. However, SDS polyacrylamide gel electrophoresis does not separate α-FP and albumin as both proteins have molecular weights of about 70,000 .

J. Preparation of mouse α-FP and albumin

Mouse α-FP was prepared from mouse fetuses that were 14-16 days old. The whole fetal bodies were homogenized in Buffer B and 0.25 M sucrose and centrifuged at 5,000 x g (av) for 10 minutes. The supernatant was centrifuged at 214,000 x g (av) for 90 minutes and the resulting supernatant was adjusted to pH 4.8 with acetic acid. The precipitate that formed was removed by centrifugation and the supernatant was adjusted to 40% $(NH_4)_2SO_4$ and stirred at 0° for 20 minutes. The precipitate formed was recovered by centrifugation and dissolved in 20 mM Tris-HCl (pH 7.8) and 0.15 M NaCl. This was adjusted to pH 4.8 with acetic acid and the precipitate was discarded. The supernatant was heated to 60° for 10 minutes and the precipitate was discarded. The supernatant was run on polyacrylamide gels at pH 8.9 and stained with 8-Anilo-1-naphthalenesulfonic acid (0.3 mg/ml). Proteins react with this reagent, becoming fluorescent and visible under ultra-violet light. α-FP bands were homogenized in 50 mM Tris-HCl (pH 7.0). Following centrifugation at 12,000 x g (av) for 10 minutes,

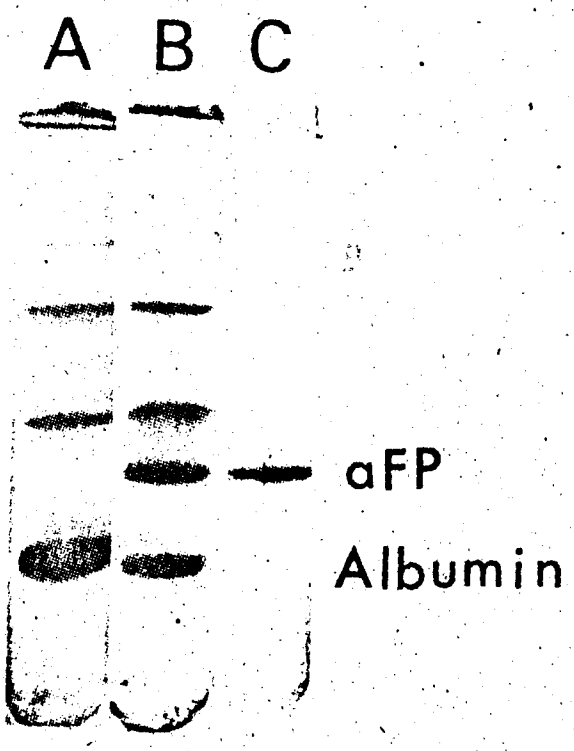


Figure 2. Polyacrylamide gel electrophoresis at pH 3.9 of: A) adult mouse serum; B) fetal mouse serum; C) α-FP.

4 volumes of 95% ethanol were added to the supernatant. The precipitate (α-FP) was dissolved in 50 mM sodium acetate (pH 5).

Commercial albumin (Miles Laboratories) was further purified by polyacrylamide gel electrophoresis at pH 8.9 as described above for α-FP.

K. Preparation of antibodies

Both commercial anti-albumin (Nutritional Biochemicals Corp.) and anti-albumin prepared in Dr. Tamaoki's laboratory were used. For the preparation of anti-albumin, purified albumin (2 mg) was mixed with an equal amount of complete Freund's adjuvant and was injected into a goat intramuscularly. This was followed by four additional weekly injections using incomplete Freund's adjuvant. Two weeks after the final injection blood was collected from the animal and left at 37° for 60 minutes and then at 0° overnight. The serum was removed and Na_2SO_4 was added to 18% and the mixture was stirred for 60 minutes at room temperature. The precipitate formed was collected by centrifugation at 12,000 x g (av) for 10 minutes at room temperature and washed with 18% Na_2SO_4. The pellet was dissolved in 20 mM Tris-HCl (pH 7.8) and 0.15 M NaCl. Na_2SO_4 precipitation was repeated once more and the pellet was dissolved in the Tris-saline solution and dialyzed against the same at room temperature for 2 hours and then at 0° overnight. Any precipitate was removed by centri-

fugation and the supernatant containing the antibody was frozen.

For the preparation of anti-α-FP, purified α-FP was injected into a rabbit or goat as described for the injection of albumin. The animal was bled and the serum was incubated with adult mouse serum and high speed supernatant from adult mouse liver at 37° for 60 minutes and then left at 0° overnight. The precipitate that formed was removed by centrifugation at 17,000 x g (av) for 20 minutes. The supernatant was incubated with half volumes of adult mouse serum and liver supernatant as above. This process was repeated until there was no precipitation. The absorbed serum was then fractionated by Na_2SO_4 precipitation as described above for anti-albumin.

The antibody preparations were tested using Ouchterlony double immunodiffusion plates and immunoelectrophoresis. For double immunodiffusion anti-α-FP developed a single precipitin line with fetal serum which fused with the precipitin line formed with purified α-FP (Fig. 3A). The anti-α-FP did not react with albumin, adult mouse serum or adult liver high speed supernatant (Fig. 3A). The specificity of the antibody preparations were further checked by immunoelectrophoresis (Fig. 3B). Anti-α-FP gave a single precipitin line with a preparation containing both α-FP and albumin. The line was at a site corresponding to α-FP.

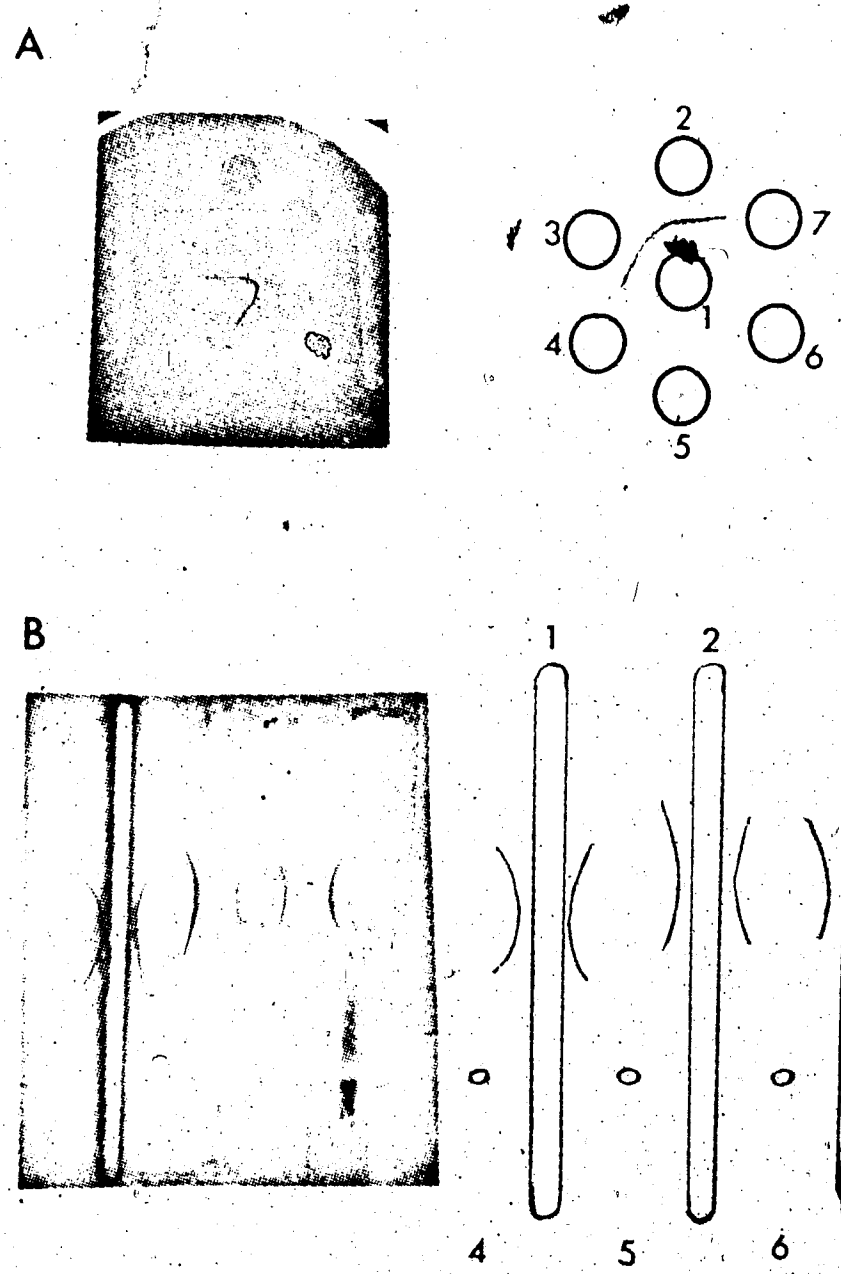


Figure 3. Double immunodiffusion plate and immunoelectrophoresis.

A. 1) anti-α-FP, 2) α-FP, 3) fetal serum, 4) adult serum, 5) adult liver high speed supernatant, 6) albumin, 7) adult serum.

B. 1) anti-α-FP, 2) anti-albumin, 3) anti-albumin, 4) α-FP, 5) α-FP and albumin, 6) albumin, 7) adult liver high speed supernatant.

Anti-albumin showed a single precipitin line at a site corresponding to albumin when tested with albumin, adult liver high speed supernatant and a preparation containing α-FP and albumin. By the criteria of double immunodiffusion and immunoelectrophoresis the antibody preparations employed were monospecific.

L. Immunoprecipitation

Immunoprecipitation of α-FP and albumin was done in the following manner. The cell-free reaction mixture was centrifuged at 125,000 x g (av) for 2 hours in a Type 50 Rotor pelleting the ribosomes which otherwise cause non-specific precipitation. To a 300-600 ul portion of the supernatant, carrier antigen (α-FP or albumin) and an excess amount of antibody (as determined by Ouchterlony plates) were added. Also a solution of 10% Triton X-100 and 10% DOC in PBS (pH 7) was added to a final concentration of 1-1.1% Triton X-100 and DOC. This was to help prevent non-specific precipitation (Rhoads et al., 1973). The mixture was incubated at 30° for 60 minutes and then at 4° overnight. The antigen-antibody complex was precipitated by centrifugation at 58,000 x g (av) for 60 minutes in an SW 50.1 rotor. A 0.9 M sucrose cushion containing 10 mM leucine, valine and phenylalanine, 1% Triton X-100 and 1% DOC in PBS (pH 7) was used. The supernatant was removed carefully and the pellet was washed twice with 20 mM Tris-HCl (pH 7.8) and 150 mM NaCl. The pellet was

then resuspended in this Tris-saline solution and centrifuged through a sucrose cushion as iescribed above. The pellet was rinsed with the Tris-saline solution and dissolved in 4 M urea, 1% SDS and 1% β-ME by incubating at 45° for 30 minutes. The sample was then either counted with Aquasol (New England Nuclear) or Scintiverse (Fisher Scientific Co.), or run on an SDS gel.

M. Affinity column chromatography

Two grams of cyanogen bromide-activated Sepharose 4B (Pharmacia) were swollen with water and washed with a total of 1 litre of water by repeated suspension and filtration through a scintered glass funnel. This was followed by further washing with 200 ml PBS (pH 7.2), 200 ml 0.1 M NH_4HCO_3 and 500 ml PBS (pH 7.2). The washed Sepharose was mixed with anti-α-FP and stirred for 60 minutes at 4° and then left overnight at 4° for the coupling reaction to continue. The non-bound antibody was removed by 500 ml PBS (pH 7.2), 200 ml 1 M NH_4OH and 500 ml PBS (pH 7.2) The A_{280} of the effluent was checked to determine that no more protein was being washed off the Sepharose.

After the Sepharose 4B with antibody attached was further washed with 1 litre of 0.1 M NH_4HCO_3 and 0.5 litre of PBS (pH 7.2), it was added to the sample; this sample containing α-FP was in PBS (pH 7.2). The mixture was stirred in a beaker for 60 minutes at 4° and then left without stirring overnight at 4°. The mixture was filtered and

washed with 1 litre of PBS (pH 7.2) and 0.5 litres of 0.1 M NH_4HCO_3, and packed into a small column where it was washed further with 0.25 litres of 0.1 M NH_4HCO_3. The column was then eluted with 1 M NH_4OH and 1 ml fractions were collected. The samples were either measured for A_{230} or if radioactive, an aliquot was tested for radioactivity with Aquasol (New England Nuclear) or Scintiverse (Fisher Scientific Co.).

The column was found to be re-usable 3 or 4 times.

N. Cyanogen bromide cleavage

The procedure followed was very similar to that described by Gross (1967). The protein to be digested was dissolved in 70% formic acid, and cyanogen bromide was added to give a ratio of cyanogen bromide to methionine residues of about 100:1 . This ensured an excess of cyanogen bromide for cleavage. The mixture was incubated at room temperature for 24 hours, diluted tenfold with water and freeze dried. The resulting material was dissolved in 4 M urea, 1% SDS and 1% β-ME and analysed by SDS gel electrophoresis.

O. Amino acid incorporation directed by poly(U) and natural mRNA

The reaction mixture using poly(U) as messenger usually had the following components in a final volume of 0.1 ml: 0.1 umole ATP, 0.03 umole GTP, 1 umole creatine phosphate, 5 ug creatine phosphokinase, 50 ug poly(U), 20 mM Tris-HCl (pH 7.8), 6 mM β-ME, 0.1 mM EDTA and

0.083 uCi of ^{14}C-phenylalanine (477 mCi/mmole). Other components included were $MgCl_2$, KCl, mouse liver tRNA and S-30 with the concentration of each depending on the experiment. The reaction mixture was incubated at 30° for 10, 15 or 30 minutes. These variables will be defined with the data for each experiment.

After incubation the procedure for hot TCA precipitation was followed. Cold 10% TCA (2 ml) was added to the sample. After 10 minutes at 4° the sample was placed in boiling water for 15 minutes and then kept at 4° for 15 minutes. The sample was filtered onto a glass fibre or millipore filter, washed 4 times with cold 5% TCA and dried under a heat lamp. Toluene-based scintillation fluid was added and the sample was counted in a Beckman liquid scintillation counter.

In the assay of amino acid incorporation directed by natural mRNA, the following components were included in a final volume of 0.1 ml: 0.1 umole ATP, 0.03 umole GTP, 1 umole creatine phosphate, 5 ug creatine phosphokinase, 20 mM Tris-HCl (pH 7.8), 6 mM β-ME, 0.1 mM EDTA and 5 nmoles each of 19 amino acids (excluding leucine). The $MgCl_2$ and KCl concentrations were 3.5 mM and 70 mM, respectively, unless otherwise stated. The concentrations of mouse liver tRNA, ^{14}C-leucine, ribosomal wash protein, S-30 protein and polysomal RNA will be given for each experiment. The reaction mixture was incubated for 30 minutes at 30° followed by hot TCA precipitation and liquid scintillation counting as described above.

For product analysis the volume of reaction mixtures was increased to 1-5 ml. The concentration of the components was similar to the small scale reactions with the following per ml: 3.5 mM $MgCl_2$, 70 mM KCl, 20 mM Tris-HCl (pH 7.8), 6 mM β-ME, 0.1 mM EDTA, 1 umole ATP, 0.3 umole GTP, 10 umoles creatine phosphate, 50 ug creatine phosphokinase, 0.05 umole each of 16 amino acids (The amount of tritiated leucine, valine, phenylalanine and glutamic acid will be given for each experiment.), approximately 50 ug mouse liver tRNA, 2.25 mg S-30 protein, 1.25 mg ribosomal wash protein and 150-200 ug of polysomal RNA. The reaction mixture was incubated at 30° for 2 hours. The sample was then centrifuged for 2 hours at 125,000 x g (av) in a Type 50 rotor or at 149,000 x g (av) in a SW 50.1 rotor through a 1 M sucrose cushion. The supernatant was removed and radioactive α-FP or albumin was analysed by immunoprecipitation as described above.

RESULTS

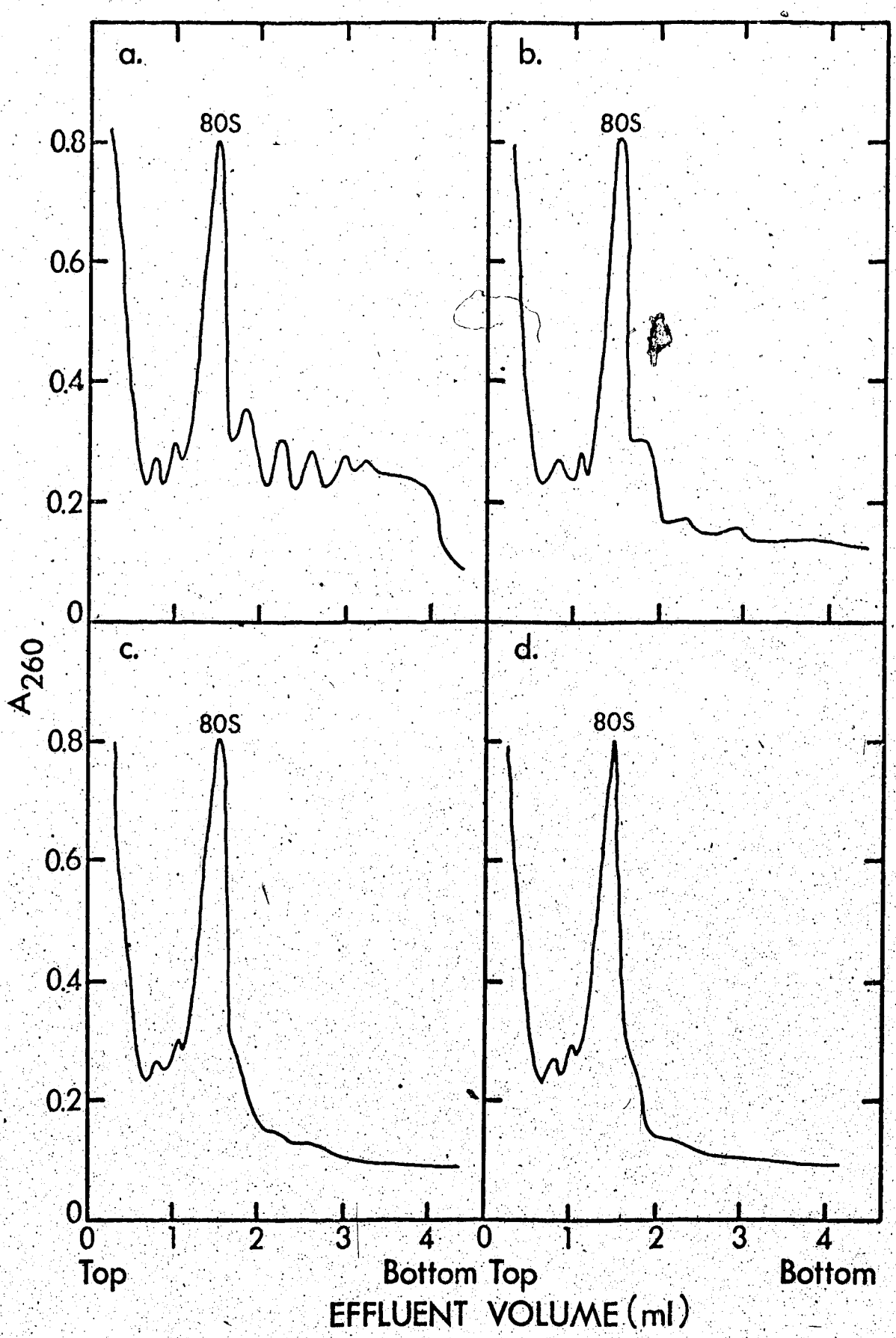
A. Preparation of S-30

The procedure for the preparation of S-30 was essentially the same as that of Jenkins et al. (1973). The details are described in the Methods and Materials section. Briefly, the cells were lysed with Nonidet P-40 and the lysate was centrifuged at 30,000 x g (av) for 10 minutes. The supernatant was pre-incubated for one hour at 37° to lower the endogenous protein synthetic activity. The preparation was then passed through a Sephadex G-25 column to remove free amino acids and other small molecular weight material. Some aspects of this procedure were studied in detail and the results obtained are described below.

Firstly, the effect of the incubation of the 30,000 x g supernatant at 37° was studied. The 30,000 x g supernatant was incubated at 37° for 0, 5, 10 and 15 minutes, and the patterns of polysomes present in each preparation were analysed by centrifugation through sucrose gradients (Fig. 4). The supernatant without incubation showed a large 80S peak and considerable absorbance in the polysome region of the gradient. After 5 minutes of incubation at 37° the large polysomes disappeared with small polysomes containing two to four ribosomes remaining. The pattern was essentially unchanged after 10 minutes of incubation and it appeared that the run-off of polysomes was completed within 15 minutes of incubation.

Figure 4. The effect of pre-incubation on the polysome pattern of S-30. Sarcoma 180 cells were lysed in hypotonic buffer(20 mM Tris-HCl (pH 7.8), 1.5 mM $MgCl_2$ and 10 mM KCl) and 0.5% Nonidet P-40 at 0° for 10 minutes. The salt conditions were adjusted to 20 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$ and 110 mM KCl. The lysate was centifuged at 30,000 xg (av) for 10 minutes at 4°. The supernatant was removed and the following were added per ml: 1 umole ATP, 0.3 umole GTP, 10 umole creatine phosphate, 50 ug creatine phosphokinase and 50 nmoles each of 20 amino acids. The sample was divided into 4 portions and incubated for 0, 5, 10 or 15 minutes at 37°. These samples were layered on pre-formed 10-35% sucrose gradients(see Methods and Materials) and centrifuged at 149,000 xg (av) for 30 minutes in an SW 50.1 rotor. The gradients were pumped out and scanned at 260 nm using a Gilford model 2000 spectrophotometer.

A, pre-incubated 0 minutes; B, 5 minutes; C, 10 minutes; D, 15 minutes.


a.
b.
c.
d.
80S
80S
80S
80S
0.8
0.6
0.4
0.2
0
A_{260}
0 1 2 3 4 0 1 2 3 4
Top
Bottom Top
Bottom
EFFLUENT VOLUME (ml)

The data in Table I show leucine incorporation, in response to added polysomal RNA, by S-30 preparations pre-incubated at 37 ^{o} for 0, 15, 30 and 60 minutes. Ribosomal wash was present in each reaction mixture, since in its absence, leucine incorporation was low (see below and Fig. 14A). The sample without pre-incubation showed little increase in incorporation in the presence of added polysomal RNA, giving a ratio of incorporation with polysomal RNA to without polysomal RNA of 1.01 . This ratio increased to 1.47 and 1.79 after pre-incubation for 15 and 60 minutes, respectively. This shows that the endogenous activity continued to decrease after 15 minutes incubation although there was no detectable change in the polysome pattern as analysed in sucrose gradients (Fig. 4). This may be due to continued degradation of endogenous mRNA which had been released from polysomes. The S-30 preparations used in the following experiments were, therefore, routinely pre-incubated for 60 minutes.

Secondly, the effect of the salt conditions during pre-incubation on the protein-synthesizing activity of the S-30 was studied. Table 2 shows that S-30 prepared with pre-incubation at 5 mM Mg^{2+} and 110 mM K^{+} ($S\text{-}30_{5\text{-}110}$) gave a higher stimulation with exogenous polysomal RNA than S-30 prepared at 3.5 mM Mg^{2+} and 70 mM K^{+} ($S\text{-}30_{3.5\text{-}70}$). The kinetics of leucine incorporation, in response to added polysomal RNA, by these two S-30 preparations showed that

Table 1. The effect of pre-incubation time on the stimulation of S-30 with exogenous polysomal RNA

Length of pre-incubation	Hot TCA precipitable ^{14}C-leucine			
	endogenous	with ribosomal wash	with RNA and ribosomal wash	with RNA / without RNA
min.	cpm	cpm	cpm	
0	7,940	8,498	8,610	1.01
15	1,240	2,041	2,996	1.47
30	684	1,363	2,067	1.52
60	623	1,278	2,290	1.79

The S-30 preparations were all prepared as described in Methods and Materials except that the pre-incubation time was 0, 15, 30 or 60 minutes. Each preparation was assayed with exogenous polysomal RNA. The following components were in a final volume of 120 ul: 0.1 umole ATP, 0.03 umole GTP, 1 umol[illegible] phosphate, 5 ug creatine phosphokinase, 20 mM [illegible]H 7.8), 6 mM β-ME, 0.1 mM EDTA, 3.5 mM $MgCl_2$, 70 mM [illegible]es each of 19 amino acids (excluding leucin[illegible]ouse liver tRNA, 0.083 uCi ^{14}C-leucine (343 mC[illegible], 150 ug ribosomal wash protein, 550 ug S-30 protein [illegible]ug of polysomal RNA from adult liver. The reaction[illegible]ncubated at 30° for 30 minutes and the hot TCA preci[illegible]ble counts are shown for the endogenous reaction, [illegible]h just ribosomal wash added and with both polysomal [illegible] and ribosomal wash present. Also, the ratio of incorpora[illegible] with RNA to incorporation without RNA is shown.

Table 2. The effect of salt conditions during pre-incubation of S-30

S-30 preparation	Hot TCA precipitable ^{14}C-leucine		
	endogenous	with ribosomal wash	with RNA and ribosomal wash
	cpm	cpm	cpm
$S\text{-}30_{3.5\text{-}70}$	597	1,349	2,994
$S\text{-}30_{5\text{-}110}$	367	1,467	4,332

S-30 was prepared as in Methods and Materials but with $S\text{-}30_{3.5\text{-}70}$ pre-incubated at 3.5 mM $MgCl_2$ and 70 mM KCl, while $S\text{-}30_{5\text{-}110}$ was pre-incubated at 5 mM $MgCl_2$ and 110 mM KCl. The two S-30 preparations were then assayed with exogenous polysomal RNA and the components of the reaction were the same as in Table 1 except that the volume was only 0.1 ml and 300 ug of S-30 protein were used. The hot TCA precipitable counts are shown for endogenous synthesis, with ribosomal wash present and with both polysomal RNA and ribosomal wash present.

$S\text{-}30_{5\text{-}110}$ was more active than $S\text{-}30_{3.5\text{-}70}$, throughout the time period studied (Fig.). This could be explained by the fact, that the run-off of ribosomes is favoured at high Mg^{2+} concentrations, while at low Mg^{2+} concentrations initiation is favoured (Mathews, 1972). Therefore, S-30 was routinely pre-incubated at 5 mM Mg^{2+} and 110 mM K^{+}.

For the S-30 preparations used in the above experiment, Sephadex G-25 column chromatography was used to remove low molecular weight material. Another technique for removing low molecular weight material, dialysis, was also tested. Table 3 compares S-30 which was dialysed to S-30 passed through a Sephadex G-25 column. Dialysed S-30 shows very low activity for translation of exogenous polysomal RNA as compared to S-30 prepared by Sephadex column chromatography. However, the dialysed preparation was active in poly(U)-directed phenylalanine incorporation, indicating a selective loss of the ability to translate natural polysomal RNA. This experiment was performed only once, so the validity of the results must await further experiments. However, for the purpose of preparation of S-30, dialysis did not offer a great advantage over Sephadex G-25 chromatography. In the following experiments, therefore, the Sephadex G-25 procedure was always used.

B. Studies using poly(U) as template

The ability of S-30 to synthesize proteins, in response

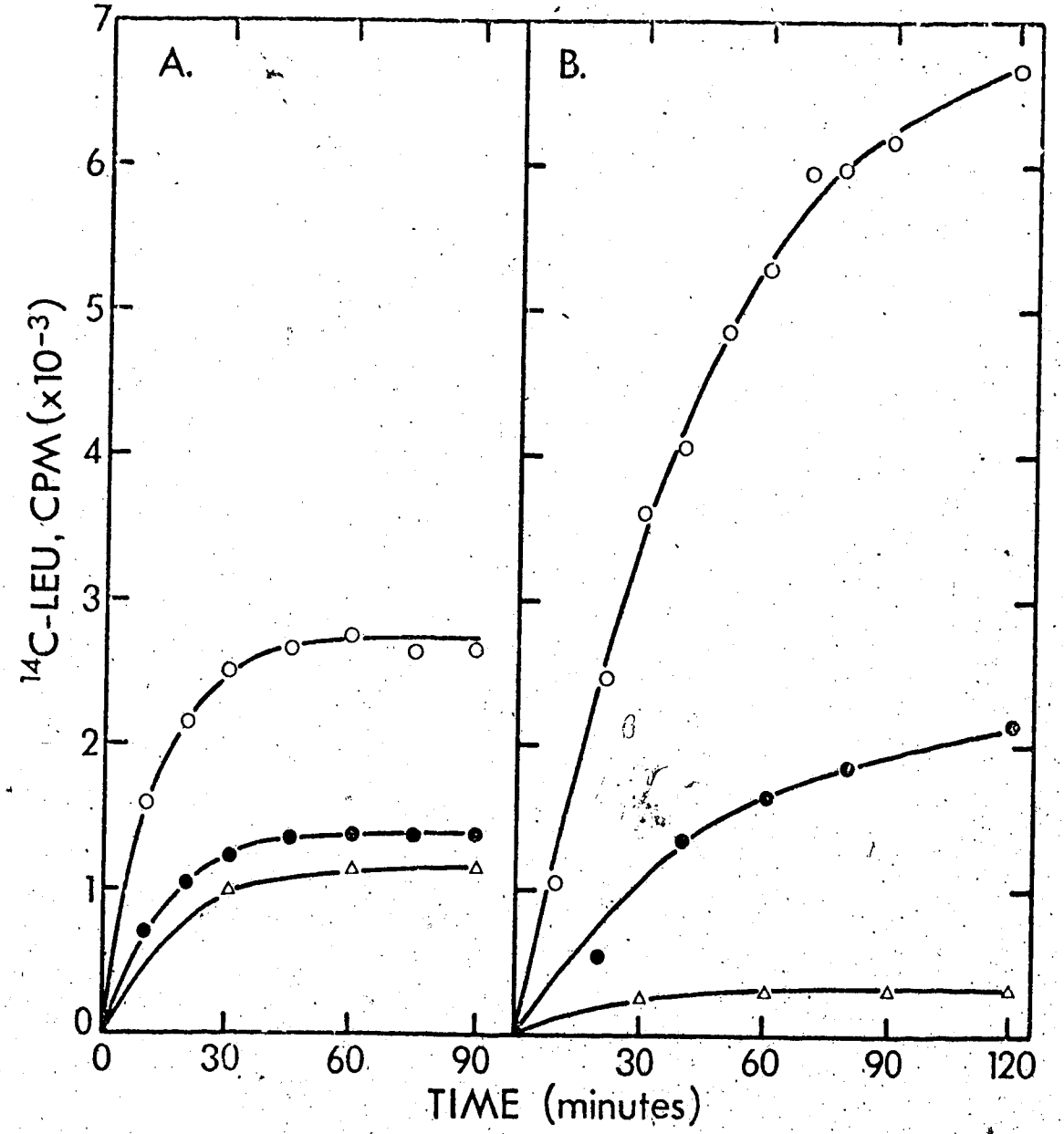


Figure 5. Time courses for pre-incubated S-30 preparations.

A. S-30 pre-incubated at 3.5 mM Mg^{2+} and 70 mM K^{+}. Conditions of assay are those described in Methods and Materials with the following differences: volume of 0.12 ml, 0.033 uCi ^{14}C-leucine (343 mCi/mmole), 8 ug mouse liver tRNA, 100 ug ribosomal wash protein, 600 ug S-30 protein and 16 ug adult polysomal RNA.

B. S-30 pre-incubated at 5 mM Mg^{2+} and 110 mM K^{+}. Conditions of assay are those of Methods and Materials with the following differences: volume of 0.1 ml, 0.033 uCi ^{14}C-leucine (343 mCi/mmole), 125 ug ribosomal wash protein, 240 ug S-30 protein and 10 ug adult polysomal RNA.

The time of incubation is shown along with the hot TCA precipitable counts.

○—○, complete system
●—●, without polysomal RNA
△—△, without polysomal RNA, without ribosomal wash (endogenous)

Table 3. Comparison of S-30 prepared using dialysis and Sephadex G-25 column chromatography

S-30 preparation	Hot TCA precipitable ^{14}C-leucine			^{14}C-phenylalanine
	endogenous	with ribosomal wash	with RNA and ribosomal wash	with poly(U)
	cpm	cpm	cpm	cpm
Sephadex G-25	127	201	1,026	32,371
Dialysis	173	151	216	51,494

The S-30's prepared by Sephadex G-25 column chromatography and dialysis were assayed with exogenous natural polysomal RNA and poly(U). The conditions of assay with exogenous polysomal RNA were those stated in Methods and Materials with the following differences: a final volume of 0.1 ml, 0.093 uCi ^{14}C-leucine (311 mCi/mmole), 8.3 ug mouse liver tRNA, 100 ug ribosomal wash protein, 9 ug polysomal RNA and 360 ug S-30 protein (Sephadex G-25) or 720 ug S-30 protein (dialysed). For poly(U), the conditions were those described in Methods and Materials with 10 mM $MgCl_2$, 94 mM KCl, 8.3 ug mouse liver tRNA and 240 ug S-30 protein (Sephadex G-25) or 480 ug protein for S-30 (dialysed). The reactions were incubated for 30 minutes at 30°. The hot TCA precipitable counts are shown for endogenous synthesis, with ribosomal wash, with polysomal RNA and ribosomal wash and for the poly(U) assay.

to exogenous RNA, was examined initially using poly(U) as template (Nirenberg and Matthaei, 1961).

The phenylalanine incorporation at various concentrations of Mg^{2+} and K^{+} is shown in Fig. 6. The K^{+} concentration curve had an optimum of approximately 100 mM while the Mg^{2+} concentration curve showed a sharp optimum in the range of 7-8 mM. The optimum Mg^{2+} concentration varied somewhat from one preparation of S-30 to another.

It has been reported for other cell-free systems that the addition of ribosomal wash results in stimulation of phenylalanine incorporation at low Mg^{2+} concentration, whereas, inhibition occurs at high Mg^{2+} concentration (Shafritz and Anderson, 1970; Murty et al., 1974). The Mg^{2+} optimum thus shifted to a lower concentration and this effect is attributed to initiation factors present in the ribosomal wash. In the present system, however, a "Mg^{2+} shift" was not observed but rather a general inhibition occurred (Fig. 7).

The concentration of S-30 and phenylalanine incorporation showed a linear relationship up to the maximum level of S-30 assayed, which was 600 ug of S-30 protein per 100 ul reaction mixture (Fig. 8).

The addition of mouse liver tRNA stimulated phenylalanine incorporation but the optimum tRNA concentration varied depending on the concentration of S-30 used. Fig. 9 shows the requirement for tRNA at three levels of S-30. The requirement for tRNA was lower at lower concentrations of S-30.

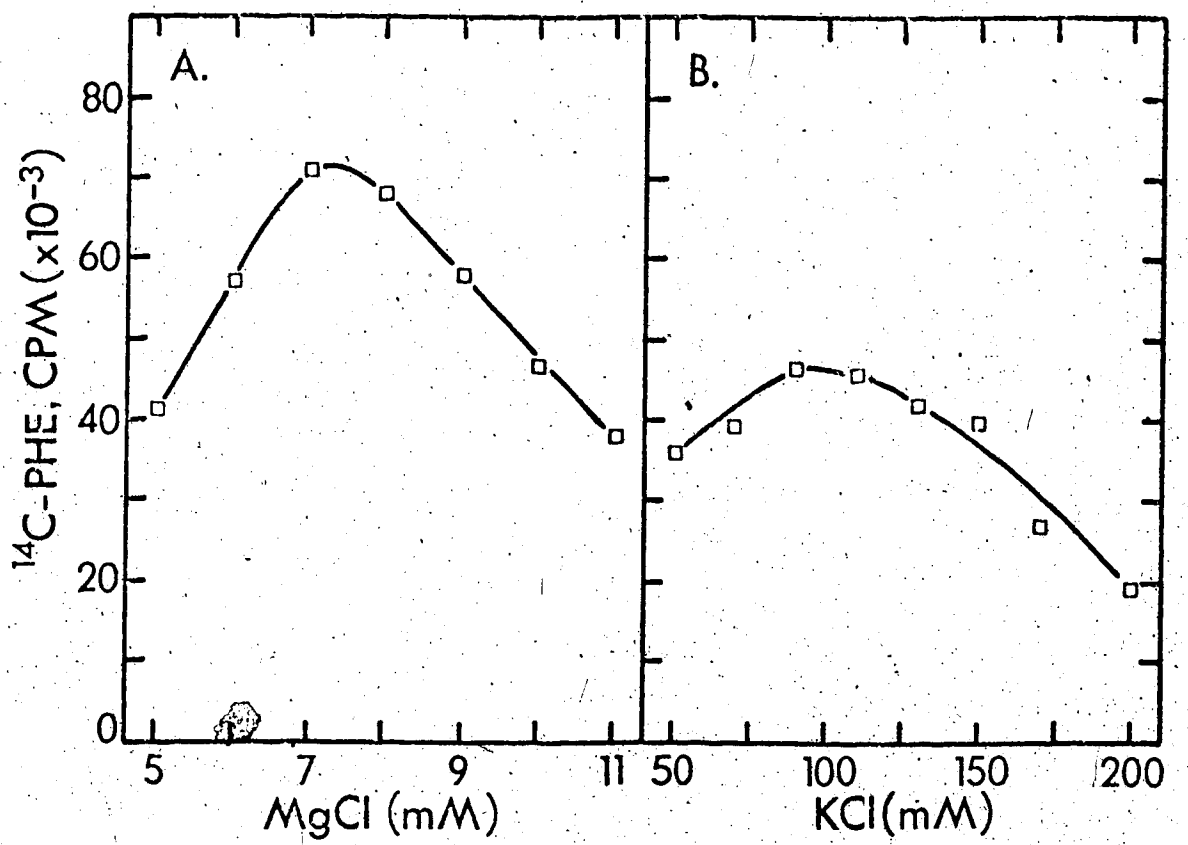


Figure 6. The effect of Mg^{2+} and K^{+} concentrations on poly(U)-directed phenylalanine incorporation.

A. The components in a final volume of 0.1 ml were: 0.1 umole ATP, 0.03 umole GTP, 1 umole creatine phosphate, 5 ug creatine phosphokinase, 50 ug poly(U), 20 mM Tris-HCl (pH 7.8), 6 mM β-ME 0.1 mM EDTA, 0.083 uCi ^{14}C-phenylalanine (477 mCi/mmole), 100 mM KCl, 5 ug mouse liver tRNA and 270 ug S-30 protein. The $MgCl_2$ concentration was as indicated and the reaction was incubated at 30° for 30 minutes. The hot TCA precipitable counts are shown.

B. The components were the same as in (A) except that 8 ug mouse liver tRNA, 240 ug S-30 protein and 10 mM $MgCl_2$ were used. The KCl concentration was as indicated and the reaction was incubated at 30° for 15 minutes. The hot TCA precipitable counts are shown.

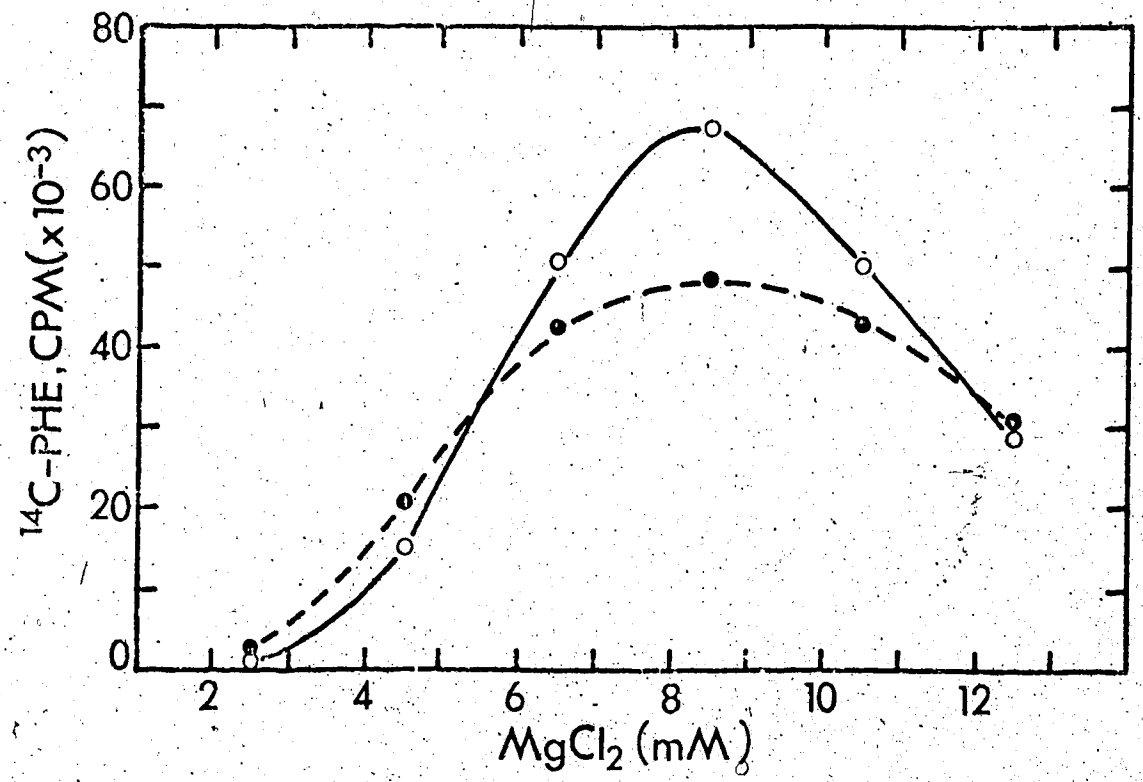


Figure 7. The effect of Mg^{2+} concentration on poly(U)-directed phenylalanine incorporation with and without ribosomal wash. The components of the reaction were the same as in Fig. 6A except that 100 mM KCl, 5 ug mouse liver tRNA and 240 ug S-30 protein were used in a final volume of 0.1 ml. The $MgCl_2$ concentration was as shown with 100 ug of ribosomal wash protein where indicated. The reaction was incubated at 30° for 15 minutes and the hot TCA precipitable counts are shown.

○—○, without ribosomal wash
●—●, with ribosomal wash

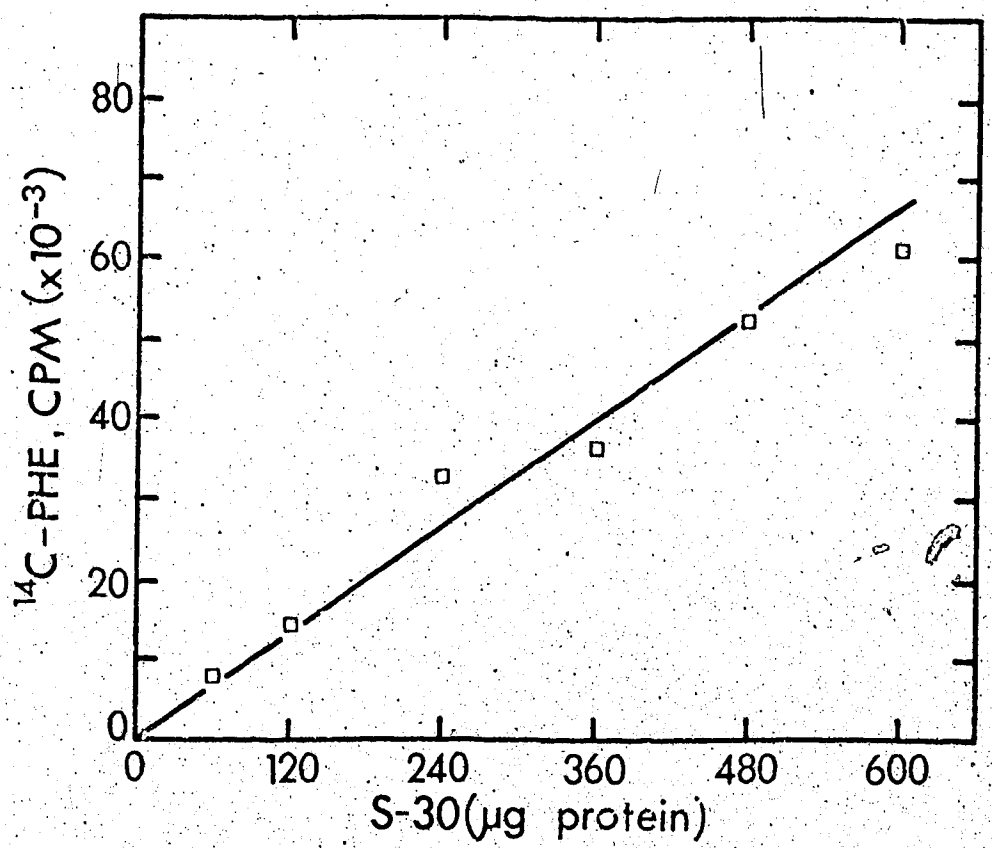


Figure 8. The effect of S-30 concentration on poly(U)-directed phenylalanine incorporation. The components in a final volume of 0.1 ml were the same as those in Fig. 6A except that 10 mM $MgCl_2$, 110 mM KCl and 8 ug mouse liver tRNA were used. The amount of S-30 protein was as indicated with a 10 minute incubation at 30°. The hot TCA precipitable counts are shown.

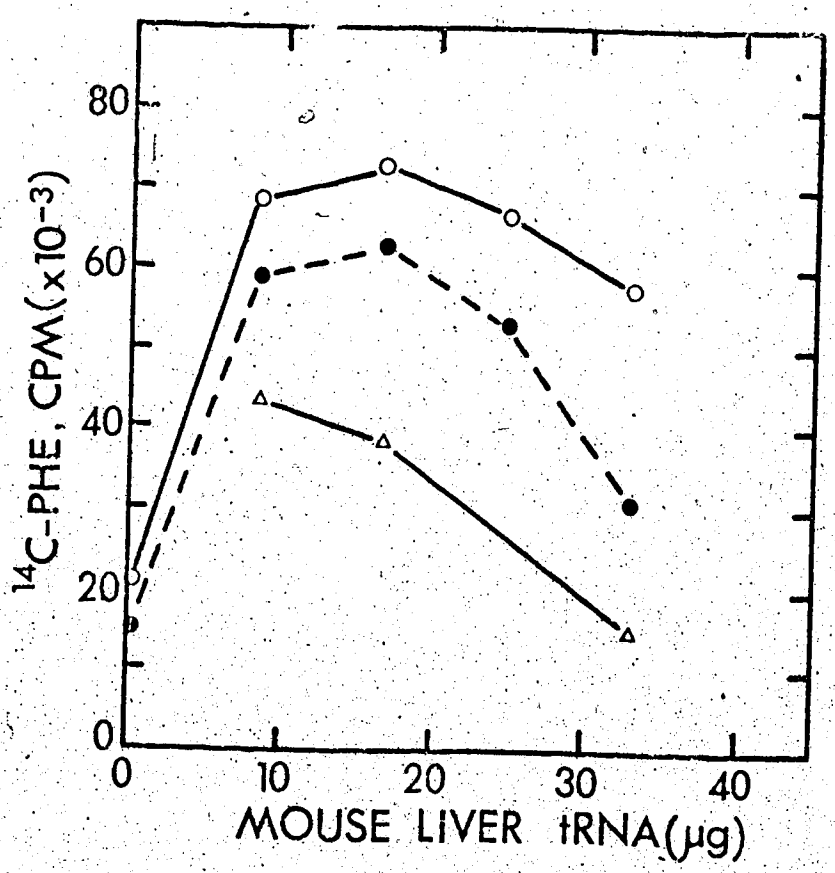


Figure 9. The effect of mouse liver tRNA on poly(U)-directed phenylalanine incorporation. The components in a final volume of 0.1 ml were the same as in Fig. 6A except that 10 mM $MgCl_2$ and 104 mM KCl were used. The reaction was incubated for 15 minutes at 30°, and the hot TCA precipitable counts are shown. The mouse liver tRNA added was as indicated.

△—△, 240 ug S-30 protein
●---●, 360 ug S-30 protein
○—○, 480 ug S-30 protein

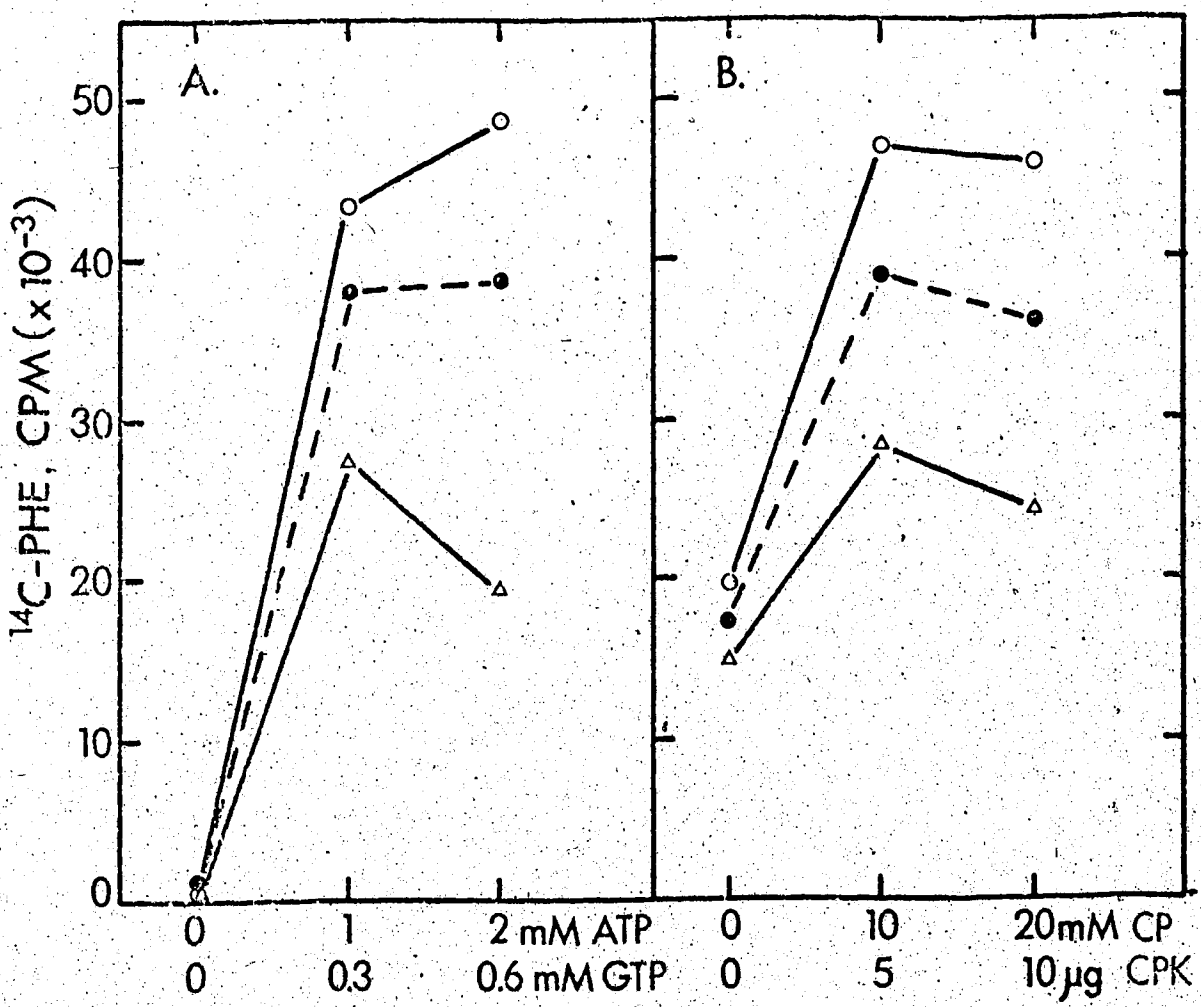


Figure 10. The effect of ATP and GTP concentrations and the effect of creatine phosphate and creatine phosphokinase concentrations on poly(U)-directed phenylalanine incorporation. The components in a final volume of 0.1 ml were the same as in Fig. 6A except that 10 mM $MgCl_2$, 104 mM KCl and 8 ug mouse liver tRNA were used. The reaction was incubated for 15 minutes at 30° with ATP and GTP concentrations as indicated in (A) and with creatine phosphate and creatine phosphokinase concentrations as indicated in (B). The hot TCA precipitable counts are shown.

△—△ , 200 ug S-30 protein
●—● , 300 ug S-30 protein
○—○ , 400 ug S-30 protein

Also, incorporation was very dependent on ATP and GTP, and was definitely stimulated by creatine phosphate and creatine phosphokinase (Fig.10). However, as in the case of tRNA, the amount required for maximum stimulation varied depending on the concentration of S-30 used. The optimal amount of ATP and GTP was lower at lower concentrations of S-30.

C. Studies using polysomal RNA as template

Total polysomes from fetal and adult mouse liver were the source of natural mRNA (see Methods and Materials). This polysomal RNA was incubated with S-30, ribosomal wash and other components necessary for protein synthesis, and the ^{14}C-leucine incorporated into hot TCA precipitable material was measured. Note that the polysomal RNA from mouse liver will be referred to sometimes as exogenous mRNA or natural mRNA.

The optimum concentrations of Mg^{2+} and K^{+} were 3.5 mM and 70 mM, respectively (Fig.11). The Mg^{2+} concentration curve exhibited a narrow range for leucine incorporation, while the K^{+} concentration curve showed a much broader range. These optima varied slightly from one S-30 preparation to another.

The incorporation of leucine increased linearly with S-30 concentration up to 360 ug of protein in a reaction mixture of 100 ul (Fig. 12). Approximately 250 ug were routinely used in a 100 ul reaction mixture.

Fig. 13 shows that increasing polysomal RNA concentrations increased protein synthesis linearly up to about

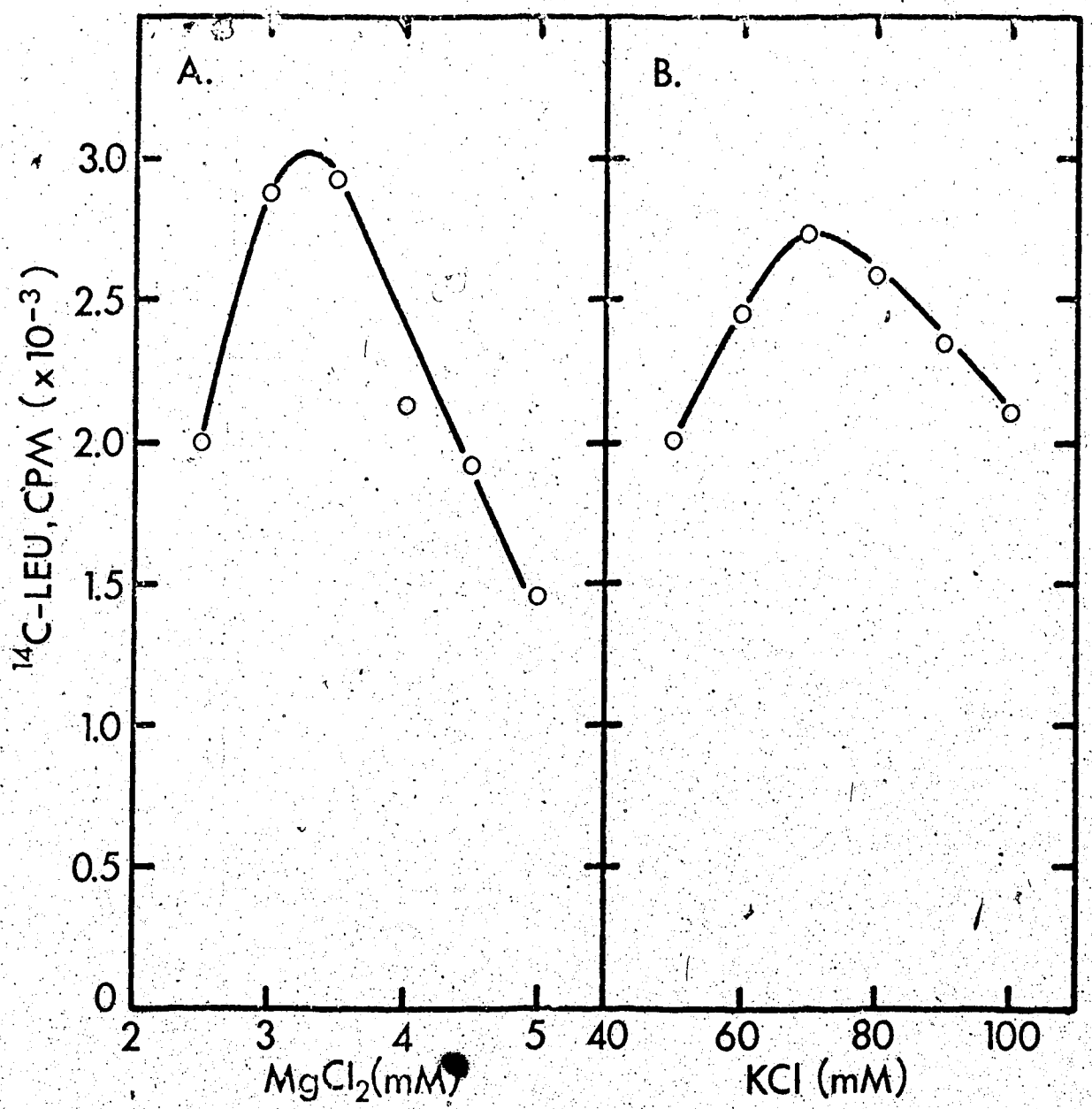


Figure 11. The effect of Mg^{2+} and K^+ concentrations on leucine incorporation using polysomal RNA.

A. The conditions of the assay were those described in Methods and Materials with the following components in 0.1 ml: 0.1 umole ATP, 0.03 umole GTP, 1 umole creatine phosphate, 5 ug creatine phosphokinase, 20 mM Tris-HCl (pH 7.8), 6 mM β-ME, 0.1 mM EDTA, 5 nmole each of 19 amino acids (excluding leucine), 93 mM KCl, 0.093 uCi of ^{14}C-leucine (311 mCi/mmole), 8 ug mouse liver tRNA, 100 ug ribosomal wash protein, 360 ug S-30 protein and 14 ug fetal polysomal RNA. The $MgCl_2$ concentration was as shown.

B. The conditions of the assay were the same as in (A) except that $MgCl_2$ was set at 3.5 mM and 300 ug S-30 protein and 9 ug fetal polysomal RNA were used. The KCl concentration and the hot TCA precipitable counts were as shown.

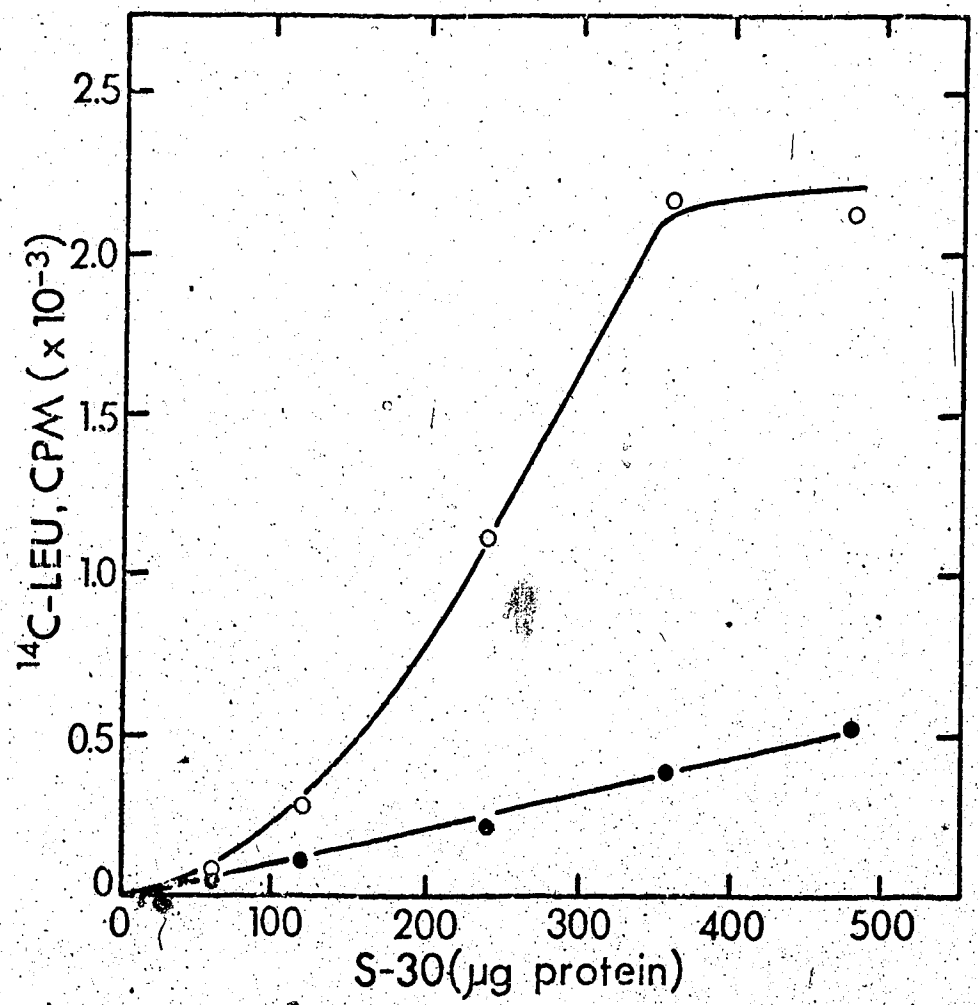


Figure 12. The effect of S-30 concentration on leucine incorporation using polysomal RNA. The conditions of the assay were the same as in Fig. 11A except that 4 mM $MgCl_2$, 109 mM KCl and 13 ug fetal polysomal RNA were used. The S-30 concentration was as indicated and the hot TCA precipitable counts were as shown.

○—○, complete system
●—●, without polysomal RNA

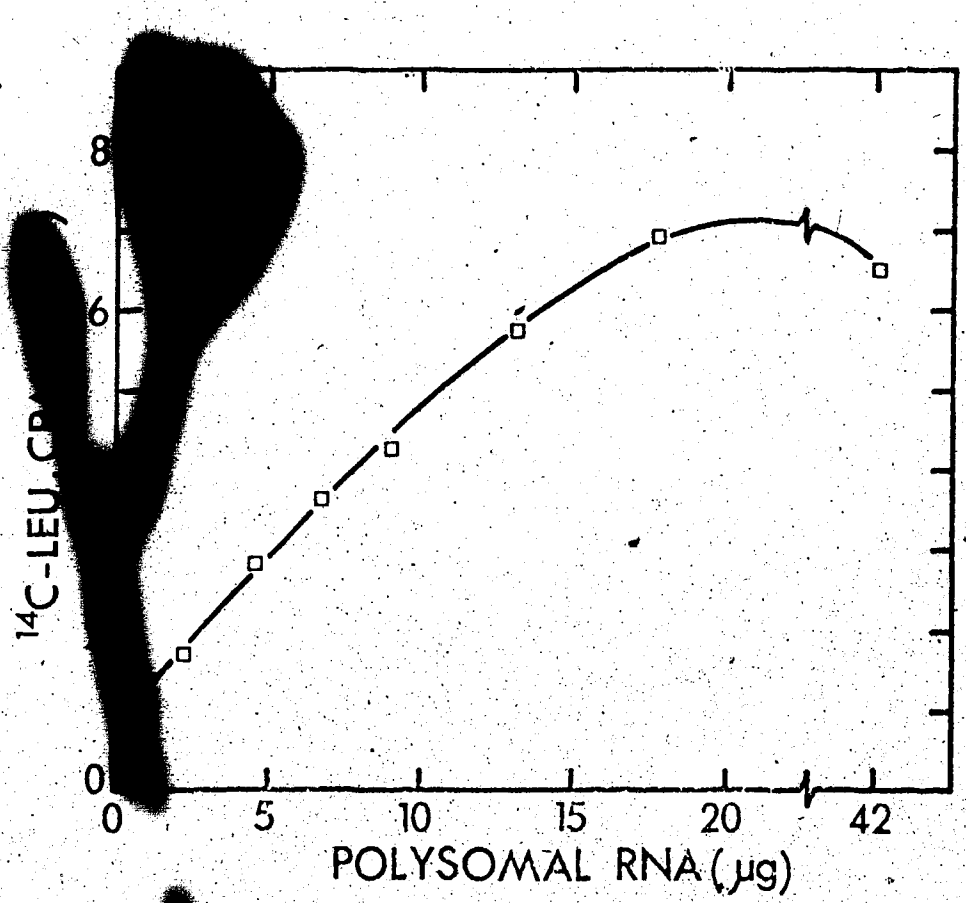


Figure [illegible] effect of polysomal RNA concentration on leuc[illegible] incorporation. The conditions of the assay were the same as in Fig.11 except no mouse liver tRNA was used and 3.5 mM $MgCl_2$, 70 mM KCl, 0.033 uCi ^{14}C-leucine (348 mCi/mmole), 120 ug ribosomal wash protein and 260 ug S-30 protein were used. The concentration of polysomal RNA used and the hot TCA precipitable counts were as shown.

20-25 ug of polysomal RNA per 100 ul reaction mixture. At higher levels of polysomal RNA some inhibition occurred.

With the S-30 preparation used, it was found that ribosomal wash from mouse liver was required for significant leucine incorporation. Fig. 14A shows the effects of various concentrations of ribosomal wash on leucine incorporation, with and without exogenous mRNA present. The translation of exogenous mRNA was highest in the presence of 150-200 ug of ribosomal wash protein. With exogenous mRNA present, leucine incorporation in the absence of ribosomal wash was approximately 5% of the incorporation when ribosomal wash was present. So, for the translation of natural mRNA from mouse liver, the system was dependent on ribosomal wash. Ribosomal wash prepared from Sarcoma 180 cells was also active in stimulating translation of mouse liver polysomal RNA (Fig. 14B).

The concentration of ^{14}C-leucine was shown to affect the incorporation of radioactivity into hot TCA precipitable material (Fig. 15). The uptake of ^{14}C-leucine increased with increasing concentrations of the isotope up to 6 uM, although, this was accompanied by an increase in endogenous incorporation. However, the total incorporation due to the added polysomal RNA was greater at high leucine concentrations.

Fig. 16 shows that leucine incorporation directed by natural mRNA did not depend upon added tRNA. This was in contrast to poly(U)-directed phenylalanine incorporation

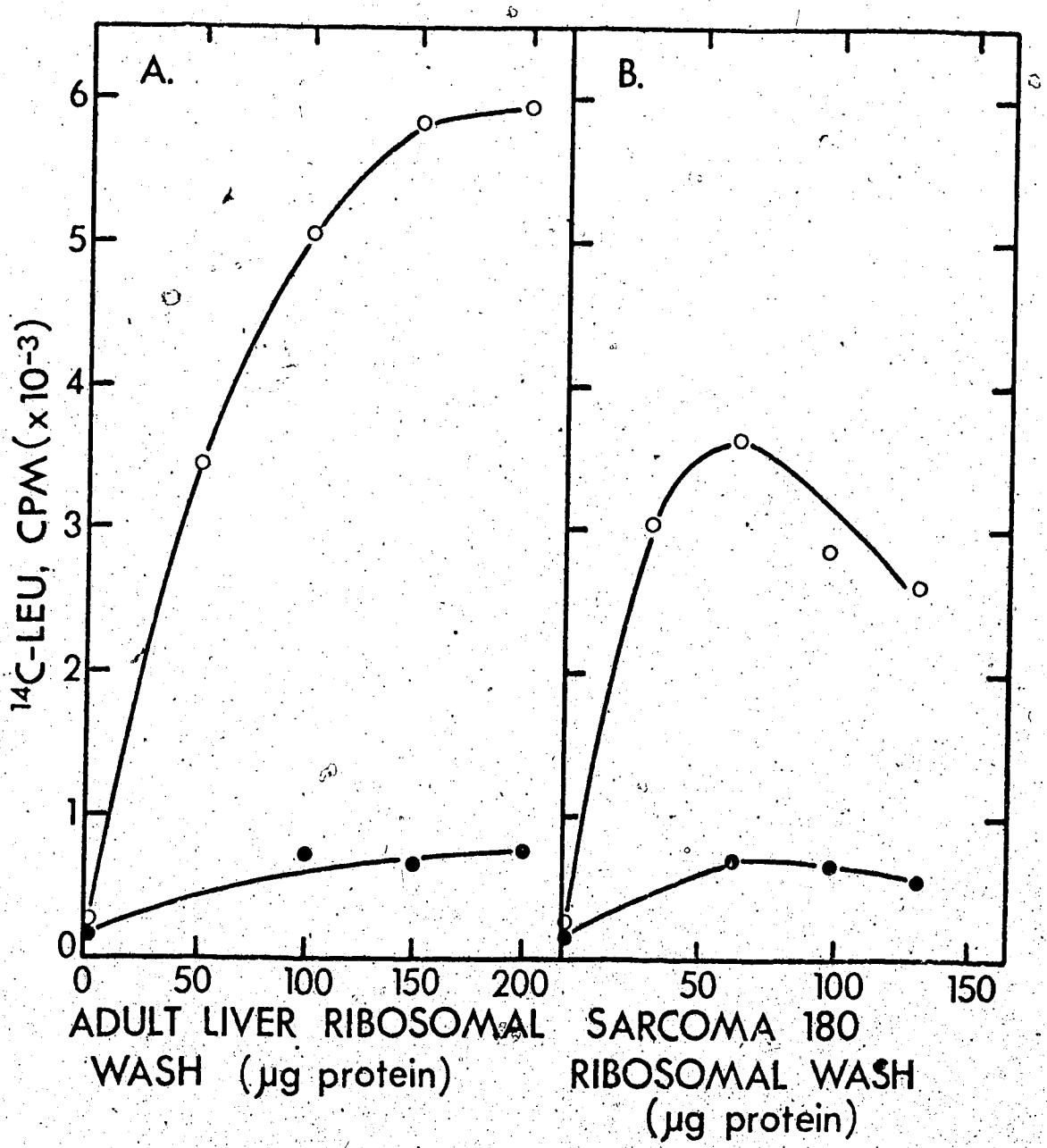


Figure 14. The effect of ribosomal wash concentration on leucine incorporation.

A. The conditions of the assay were the same as Fig. 11 except no mouse liver tRNA was used and 3.5 mM $MgCl_2$, 70 mM KCl, 0.033 uCi ^{14}C-leucine (343 mCi/mmole), 240 ug S-30 protein and 13 ug polysomal RNA were used. The concentration of adult liver ribosomal wash and the hot TCA precipitable counts were as shown.

B. The conditions of the assay were the same as in (A) except 260 ug S-30 protein and 9 ug polysomal RNA were used. The concentration of Sarcoma 180 ribosomal wash and the hot TCA precipitable counts were as shown.

O—O, complete system
●—●, without polysomal RNA

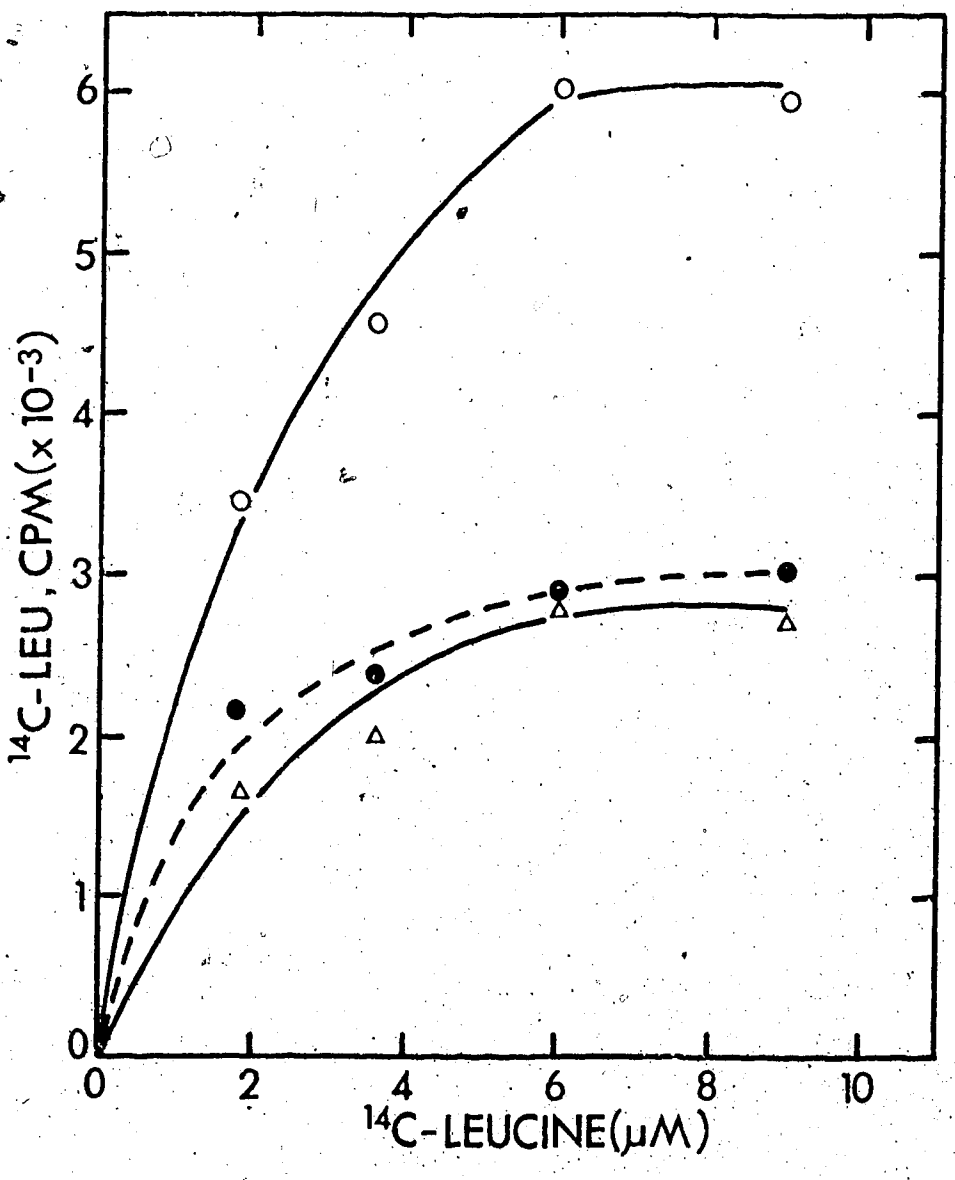


Figure 15. The effect of ^{14}C-leucine concentration on incorporation. The reaction mixture was similar to that of Fig.11 except that 3.5 mM $MgCl_2$, 70 mM KCl, 5 ug mouse liver tRNA, 100 ug ribosomal wash protein, 600 ug S-30 protein and 10 ug adult polysomal RNA were used in a final volume of 0.12 ml. The ^{14}C-leucine (343 mCi/mmole) concentration and the hot TCA precipitable counts were as shown.

○—○, complete system
●—●, without polysomal RNA
△—△, without polysomal RNA and ribsomal wash

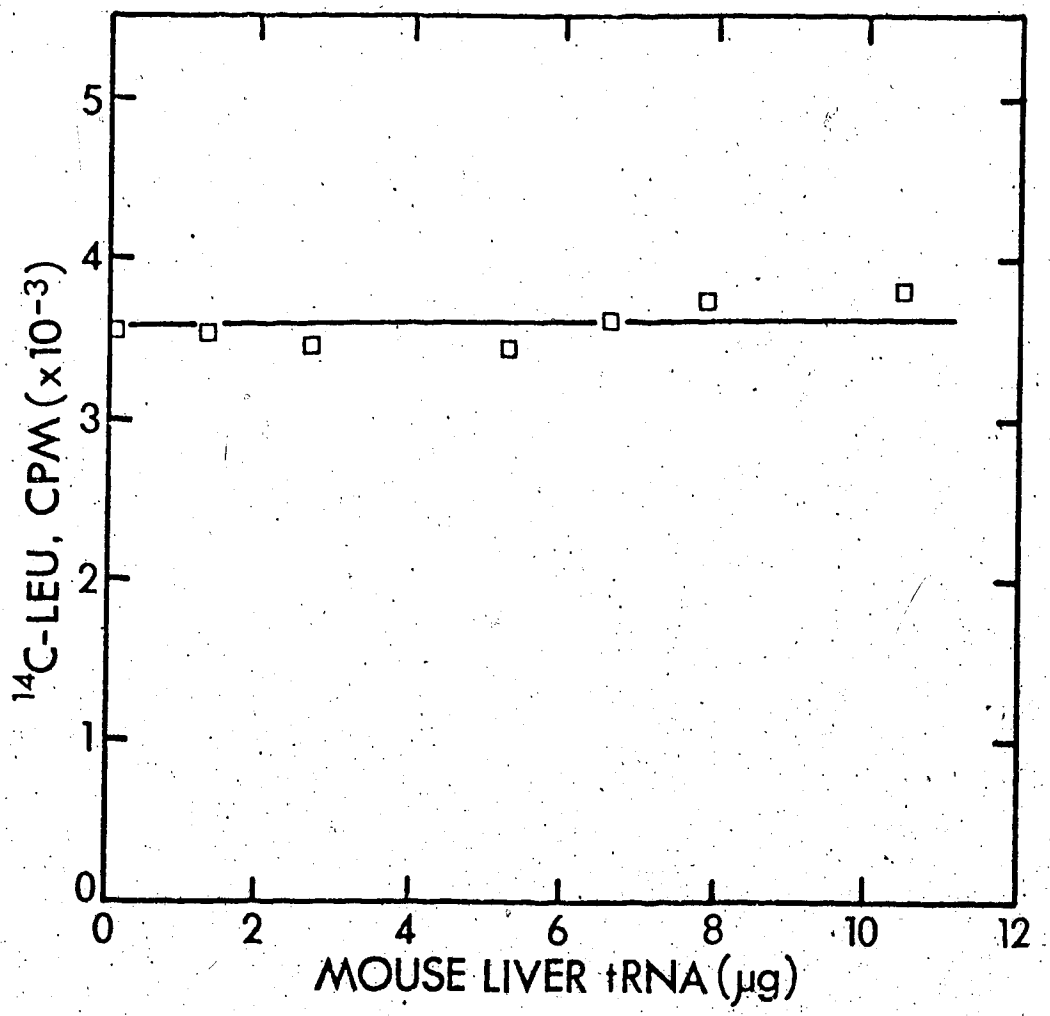


Figure 16. The effect of mouse liver tRNA on leucine incorporation. The conditions were identical to those described in Fig.11 except that 3.5 mM $MgCl_2$, 70 mM KCl, 0.083 uCi ^{14}C-leucine (343 mCi/mmole), 125 ug ribosomal wash protein, 240 ug S-30 protein and 13 ug adult polysomal RNA were used in a final volume of 0.12 ml. The amount of added mouse liver tRNA and the hot TCA precipitable counts were as shown.

which was highly dependent upon added tRNA (Fig. 9).

The time course of leucine incorporation (Fig. 17) shows that the reaction with exogenous mRNA was linear up to about 60 minutes. The incorporation was slower from 60 to 120 minutes beyond which the assay was not continued.

The effect of added ribosomal RNA on the system is shown in Fig. 18A. Ribosomal RNA from wheat germ and mouse liver gave similar results. There was a slight stimulation of the endogenous protein synthesis in the absence of exogenous mRNA. However, rRNA inhibited protein synthesis when exogenous mRNA was present. A similar experiment was reported by Jacobs-Lorena and Baglioni (1972) for a Krebs II system and mRNA for globin. Their data is shown in Fig. 18B. Their results show rRNA stimulated endogenous protein synthesis and also stimulated protein synthesis when exogenous mRNA was present.

In summary, several parameters of mouse liver mRNA translation by S-30 were studied. The optimum concentrations of Mg^{2+} and K^{+} were 3.5 mM and 70 mM, respectively. For a 100 ul reaction mixture, about 360 ug of S-30 protein, 25 ug of polysomal RNA, 150 ug of ribosomal wash protein and 6 uM ^{14}C-leucine resulted in the maximum leucine incorporation. The translation of polysomal RNA was not dependent on the addition of mouse liver tRNA. The kinetics of the reaction showed protein synthesis continuing for at least 120 minutes. Finally, the addition of ribosomal RNA inhibited

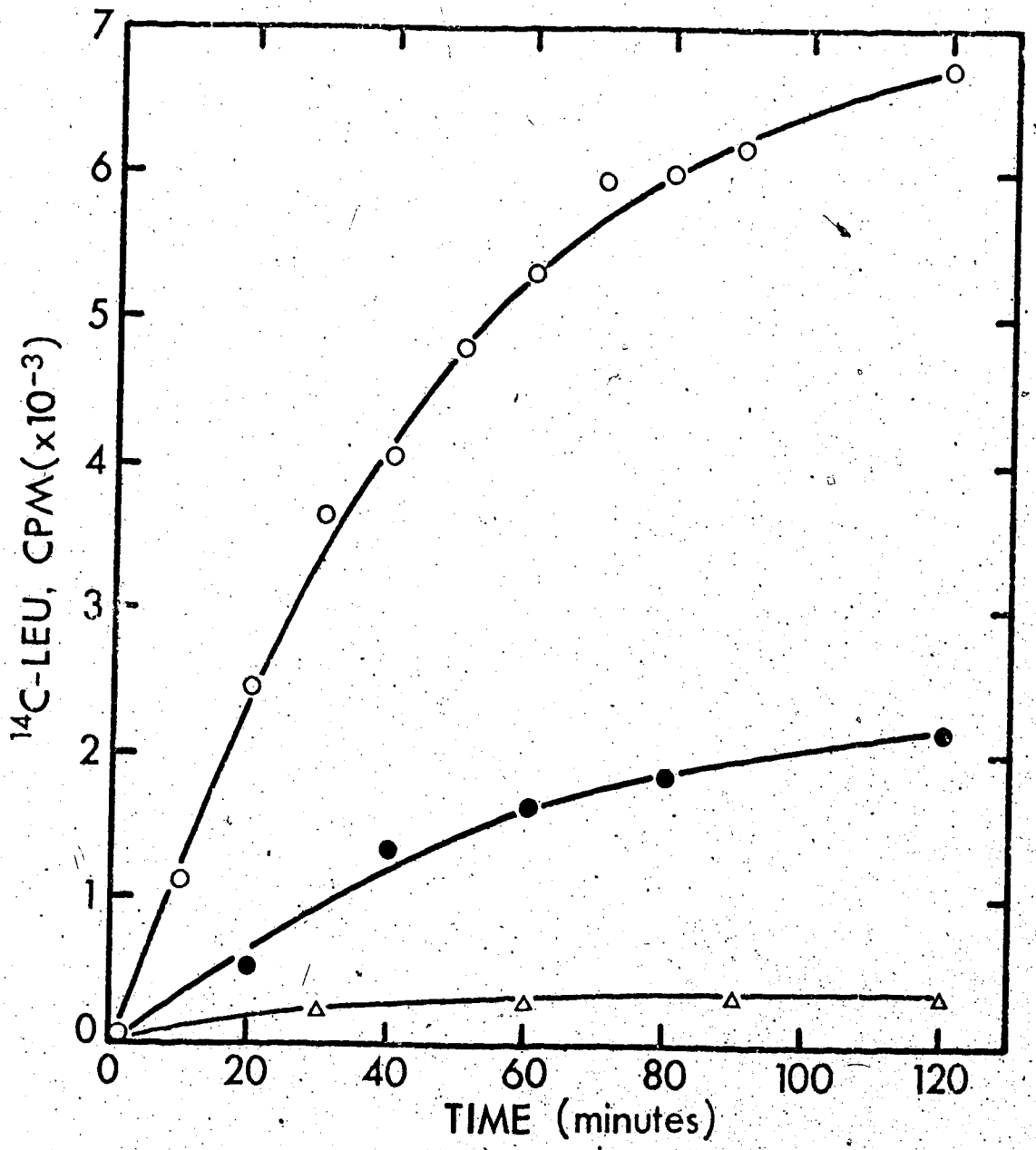


Figure 17. The time course of leucine incorporation using polysomal RNA. The components in a 0.1 ml final volume were the same as Fig. 11 except that 3.5 mM $MgCl_2$, 70 mM KCl, 5 ug mouse liver tRNA, 0.083 uCi ^{14}C-leucine (343 mCi/mmole), 125 ug ribosomal wash protein, 240 ug S-30 protein and 10 ug adult polysomal RNA were used. The time of incubation at 30° and the hot TCA precipitable counts were as shown.

O—O, complete system
●—●, without polysomal RNA
Δ—Δ, without polysomal RNA and ribosomal wash

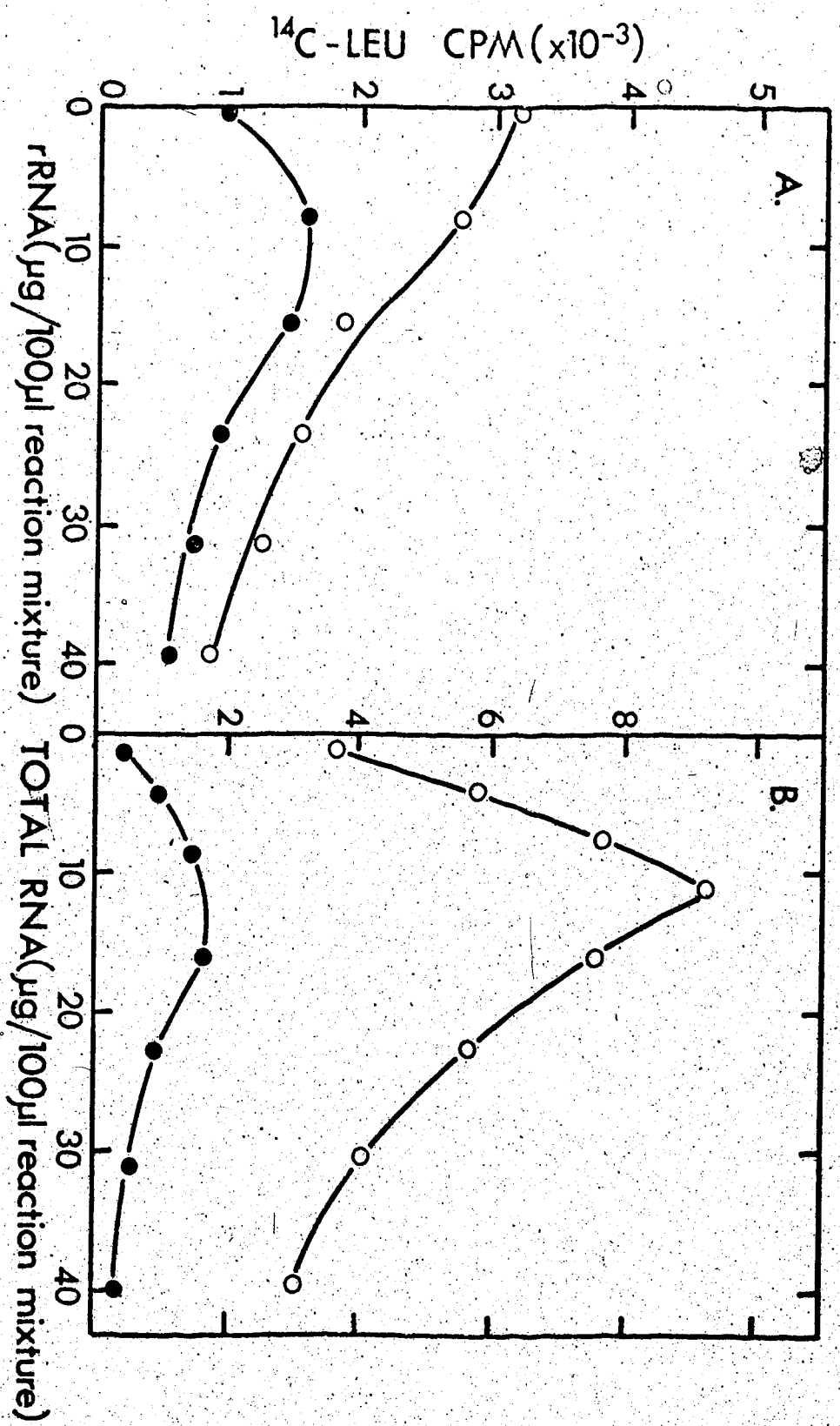
Figure 18. The effect of rRNA on leucine incorporation using polysomal RNA.

A. The conditions were similar to those described in Fig. 11 except that 3.5 mM $MgCl_2$, 70 mM KCl, 5 ug mouse liver tRNA, 0.033 uCi ^{14}C-leucine (348 mCi/mmole), 85 ug ribosomal wash protein, 260 ug S-30 protein and 10 ug fetal polysomal RNA were used. The amount of mouse liver rRNA added and the hot TCA precipitable counts were as shown.

O—O, 10 ug fetal polysomal RNA
●—●, without polysomal RNA

B. Effect of the addition of E. coli rRNA on protein synthesis by the ascites cell-free system. For each point 0.22 ug of globin mRNA were incubated 60 minutes at 30° with different amounts of E. coli rRNA and 2.7 uCi of ^{3}H-tyrosine in a final volume of 25 ul. The first point shows incorporation obtained with mRNA alone or with no addition of RNA. (From Jacobs-Lorena and Baglioni, 1972)

O—O, with mRNA
●—●, without mRNA


^{14}C-LEU CPM (x10^{-3})
A.
B.
rRNA(µg/100µl reaction mixture)
TOTAL RNA(µg/100µl reaction mixture)

translation of exogenous polysomal RNA.

D. Product analysis

With the optimal conditions for cell-free protein synthesis determined, the question arises as to whether or not α-FP and albumin are synthesized with the liver polysomal RNA preparation employed. α-FP and albumin are two major proteins synthesized in the fetal liver. In adult liver, up to one-third of the protein synthesized is albumin, while only trace amounts, if any, α-FP are synthesized (Peters, 1970).

Initially, it was attempted to identify albumin and α-FP by direct analysis of the reaction mixture by polyacrylamide gel electrophoresis at pH 8.9 . Fig. 19 shows gel patterns of proteins synthesized in Sarcoma S-30 with and without exogenous polysomal RNA. A comparison of the gel patterns shows that with polysomal RNA there is a general increase in radioactivity in the regions of α-FP and albumin, but no apparent peaks were observed. It is possible that newly formed α-FP and albumin may have been masked by other proteins that migrate near α-FP and albumin. Thus the direct analysis by gel electrophoresis was not suitable for determining the synthesis of α-FP or albumin. For this reason, precipitation with antibodies was used to determine the synthesis of α-FP and albumin.

The antibodies used were monospecific as tested by double immunodiffusion and immunoelectrophoresis (see Methods and Materials). The specificity of these

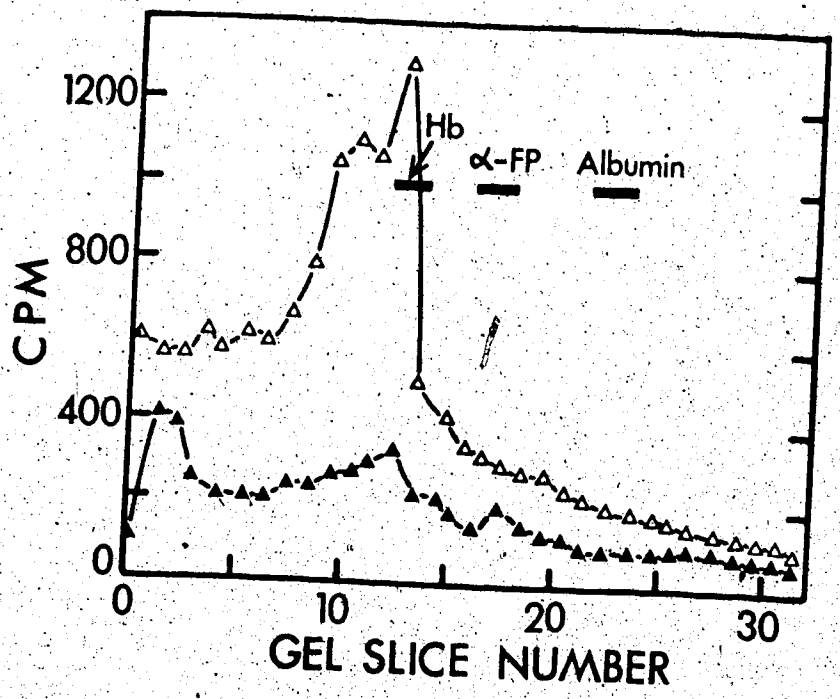


Figure 19. Analysis of cell-free products by polyacrylamide gel electrophoresis. The reactions had the components described in Methods and Materials with 37 uCi of ^{3}H-leucine (1 Ci/mmole), 17 uCi of ^{3}H-valine (2 Ci/mmole), 17 uCi of ^{3}H-phenylalanine (1 Ci/mmole) and 0.05 umole of glutamic acid per ml of reaction mixture. Following incubation at 30° for 60 minutes and centrifugation at 125,000 xg (av) for 120 minutes, an aliquot of the supernatant was run on a 7% polyacrylamide gel, pH 8.9. Carrier α-FP and albumin were present. The gel was sliced, digested and counted as described in Methods and Materials. The position of hemoglobin (Hb), α-FP and albumin are indicated.

△—△, Reaction with fetal polysomal RNA added

▲—▲, Reaction without fetal polysomal RNA

antibodies was also tested using samples containing radioactive α-FP and albumin. Gel analysis of the samples before and after incubation with antibodies revealed that anti-α-FP and anti-albumin reacted specifically and quantitatively with α-FP and albumin, respectively, (Koga and Tamaoki, 1974).

1) Concentration of polysomal RNA

Using immunoprecipitation the synthesis of α-FP and albumin in the presence of different concentrations of polysomal RNA was determined (Fig. 20). The synthesis of α-FP and albumin reached a maximum at about 250 ug of fetal polysomal RNA per ml of reaction.

2) Time course of α-FP synthesis

The time course of α-FP synthesis as determined by immunoprecipitation is presented in Fig. 21 along with the total hot TCA precipitable counts (total protein synthesis). α-FP synthesis continued linearly for 60 minutes while total protein synthesis stopped after about 90 minutes.

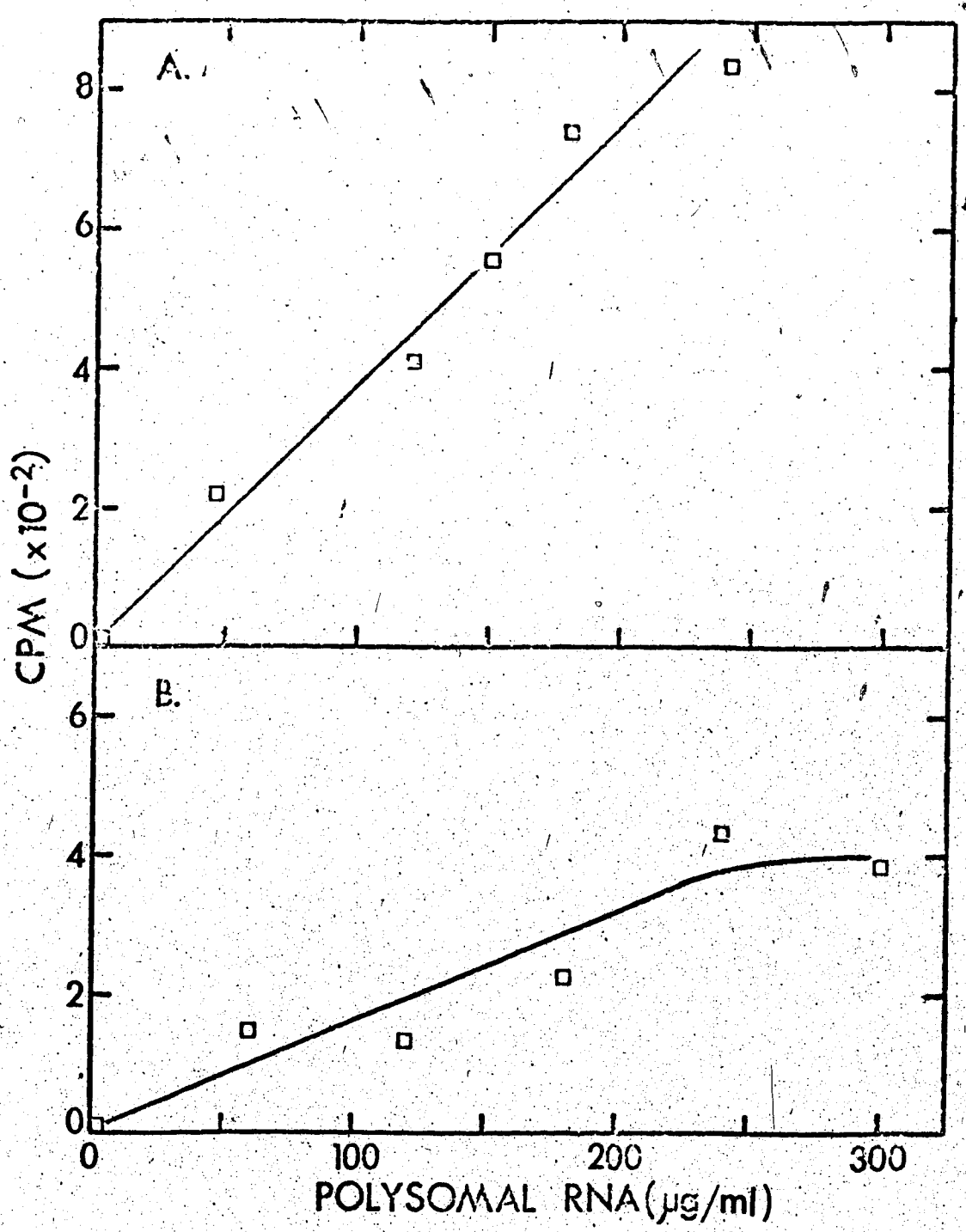
3) Source of polysomal RNA and ribosomal wash

Leucine incorporation into hot TCA precipitable radioactivity in the presence of mouse liver polysomal RNA in the Sarcoma S-30 depended upon ribosomal wash. However, as described previously the source of ribosomal wash was not confined to the mouse liver, as Sarcoma 180 cells also gave active ribosomal wash preparations. This suggests non-specific functions for the ribosomal wash factors. In orier to test further whether mRNA specific

Figure 20. The effect of fetal polysomal RNA concentration on α-FP and albumin synthesis. The reaction mixtures were as described in Methods and Materials except that the concentration of fetal polysomal RNA was varied and also that 75 uCi of ^{3}H-leucine (190 mCi/mmole), 20 uCi of ^{3}H-valine (17.7 Ci/mmole), 20 uCi of ^{3}H-glutamic acid (1.4 Ci/mmole) and 10 uCi of ^{3}H-phenylalanine (20 Ci/mmole) were used per ml. Centrifugation was at 125,000 xg (av) for 2 hours in a Type 50 Rotor and the immunoprecipitation procedure for both α-FP and albumin was performed as described in Methods and Materials. The background counts for α-FP (264 cpm) and for albumin (255 cpm) obtained from a reaction mixture with no polysomal RNA added have been subtracted for each value.

A. α-FP synthesis

B. albumin synthesis


A.
B.
8
6
4
2
0
6
4
2
0
CPM (x10-2)
0
100
200
300
POLYSOMAL RNA(µg/ml)

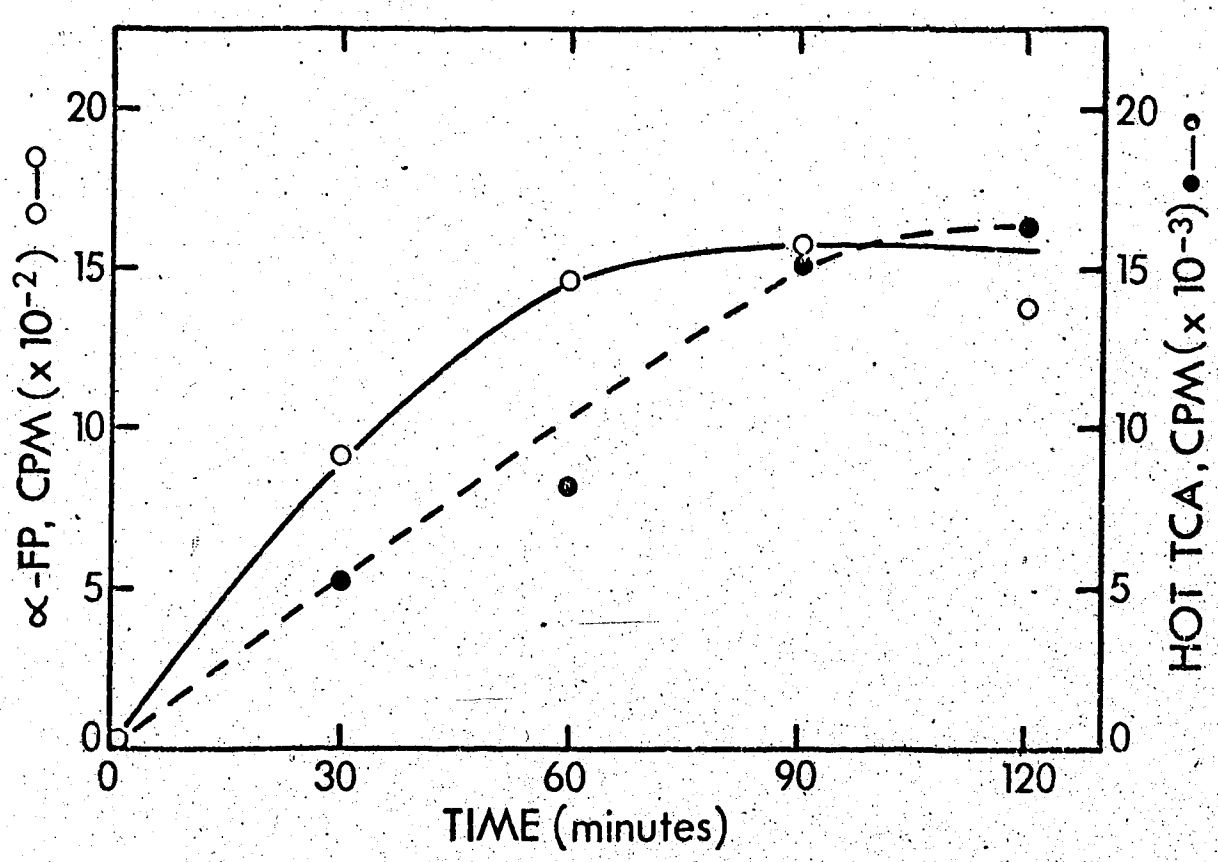


Figure 21. The time course of α-FP synthesis. The reaction mixture contained components as described in Methods and Materials, using fetal polysomal RNA and 30 uCi of ^{3}H-leucine (1 Ci/mmole), 20 uCi of ^{3}H-valine (2 Ci/mmole), 20 uCi of ^{3}H-glutamic acid (34 Ci/mmole) and 10 uCi of ^{3}H-phenylalanine (20 Ci/mmole) per ml. Aliquots were removed after 30, 60, 90 and 120 minutes of incubation at 30°. α-FP immunoprecipitable counts were determined for the high speed supernatant of each aliquot. A background of 481 cpm (obtained from a reaction with no polysomal RNA added) was subtracted from each. Also, hot TCA precipitable counts were determined for 25 ul of reaction mixtures.

o—o, α-FP immunoprecipitable counts
•---•, hot TCA precipitable counts

factors are involved in the synthesis of α-FP, the effect of ribosomal wash from fetal and adult liver and Sarcoma 180 cells on the synthesis of α-FP was studied. Albumin synthesis was also assayed as this served as an internal control.

In Experiment I, in Table 4, polysomal RNA from fetal and adult liver was used with ribosomal wash from fetal and adult liver. In Experiment II ribosomal wash from Sarcoma 180 cells was used as well. The control in each case was a reaction mixture containing no polysomal RNA, and the counts obtained were subtracted from the counts of the appropriate samples. The ratio of α-FP to albumin synthesized is given for each sample. In Experiment I this ratio is 3-4 times higher for fetal RNA as compared to adult, for both ribosomal wash preparations. In Experiment II, again the fetal RNA samples have a very high ratio compared to the adult RNA. The results are similar with either fetal, adult or Sarcoma ribosomal wash. These results show that the source of RNA determined whether α-FP was synthesized; that is, only with fetal polysomal RNA was α-FP appreciably synthesized. In the adult liver the synthesis of α-FP mRNA is apparently stopped indicating transcriptional control of α-FP synthesis.

The immunoprecipitable counts in Experiment II are much higher than in Experiment I (Table 4). This is possibly due to the use of tritiated glutamic acid in Experiment II. The amino acid analysis of both α-FP and albumin shows that about

Table 4. The effect of the source of polysomal RNA and the source of ribosomal wash on α-FP and albumin synthesis

Reactions were done according to Methods and Materials using both fetal and adult polysomal RNA, and fetal, adult, and Sarcoma 130 ribosomal wash. In Experiment I, 37 uCi of ^{3}H-leucine (1 Ci/mmole), 17 uCi of ^{3}H-valine (2 Ci/mmole), 17 uCi of ^{3}H-phenylalanine (1 Ci/mmole) and 0.05 umole of glutamic acid were used per ml. In Experiment II, 30 uCi of ^{3}H-leucine (1 Ci/mmole), 20 uCi of ^{3}H-valine (2 Ci/mmole), 20 uCi of ^{3}H-glutamic acid (1.4 Ci/mmole) and 10 uCi of ^{3}H-phenylalanine (1 Ci/mmole) were used per ml. Immunoprecipitable counts were determined for α-FP and albumin as described in Methods and Materials. The control reactions with no polysomal RNA added are shown and are used as background values (-bkd indicates background subtracted). Also, the α-FP to albumin ratio is given for each reaction mixture.

Ribosomal wash	Polysomal RNA	Immunoprecipitable counts α-FP cpm	α-FP -bkd cpm	albumin cpm	albumin -bkd cpm	α-FP / albumin
		Experiment I				
Fetal	-	312	-	144	-	-
Fetal	Fetal	620	308	277	133	2.3
Fetal	Adult	575	263	466	322	0.8
Adult	-	225	-	106	-	-
Adult	Fetal	590	365	251	145	2.5
Adult	Adult	324	99	270	164	0.6
		Experiment II				
Fetal	-	220	-	262	-	-
Fetal	Fetal	1,422	1,202	378	116	10.4
Fetal	Adult	427	207	490	228	0.91
Adult	-	256	-	244	-	-
Adult	Fetal	1,809	1,553	465	221	7.0
Adult	Adult	539	283	956	712	0.40
Sarcoma 180	-	244	-	281	-	-
Sarcoma 180	Fetal	1,062	818	397	116	7.1
Sarcoma 180	Adult	440	196	646	365	0.54

13% of their amino acid residues are glutamic acid (Peters, 1970; Zimmerman, personal communication).

4) SDS gel electrophoresis

In order to determine the size of the newly made α-FP molecules, the immunoprecipitated material from a reaction mixture with fetal polysomal RNA was analysed by SDS polyacrylamide gel electrophoresis (Fig. 22). About 10% of the total radioactivity on the gel co-migrated with the carrier α-FP (MW 70,000). This value varied from 10-23% in several other experiments. There were four other peaks with molecular weights of 41,000, 23,000, 16,000 and 10,000 (as estimated from the log MW versus mobility graph). The control sample incubated without added RNA is also shown and has only background radioactivity present.

The SDS gel described above was for a sample from a reaction incubated for 120 minutes. In order to test the possibility of the breakdown of completed α-FP into the smaller molecular weight species during incubation, samples were removed from a reaction mixture after 30, 60 and 90 minutes, respectively, and the immunoprecipitate from each sample was analysed by an SDS gel (Fig. 23). The SDS gel patterns were very similar to the previous SDS gel discussed (Fig. 22) except that the radioactivity peaks were not as well resolved. With longer incubation times the total radioactivity increased but the overall gel patterns were very similar. This indicates that the breakdown of completed α-FP did not occur.

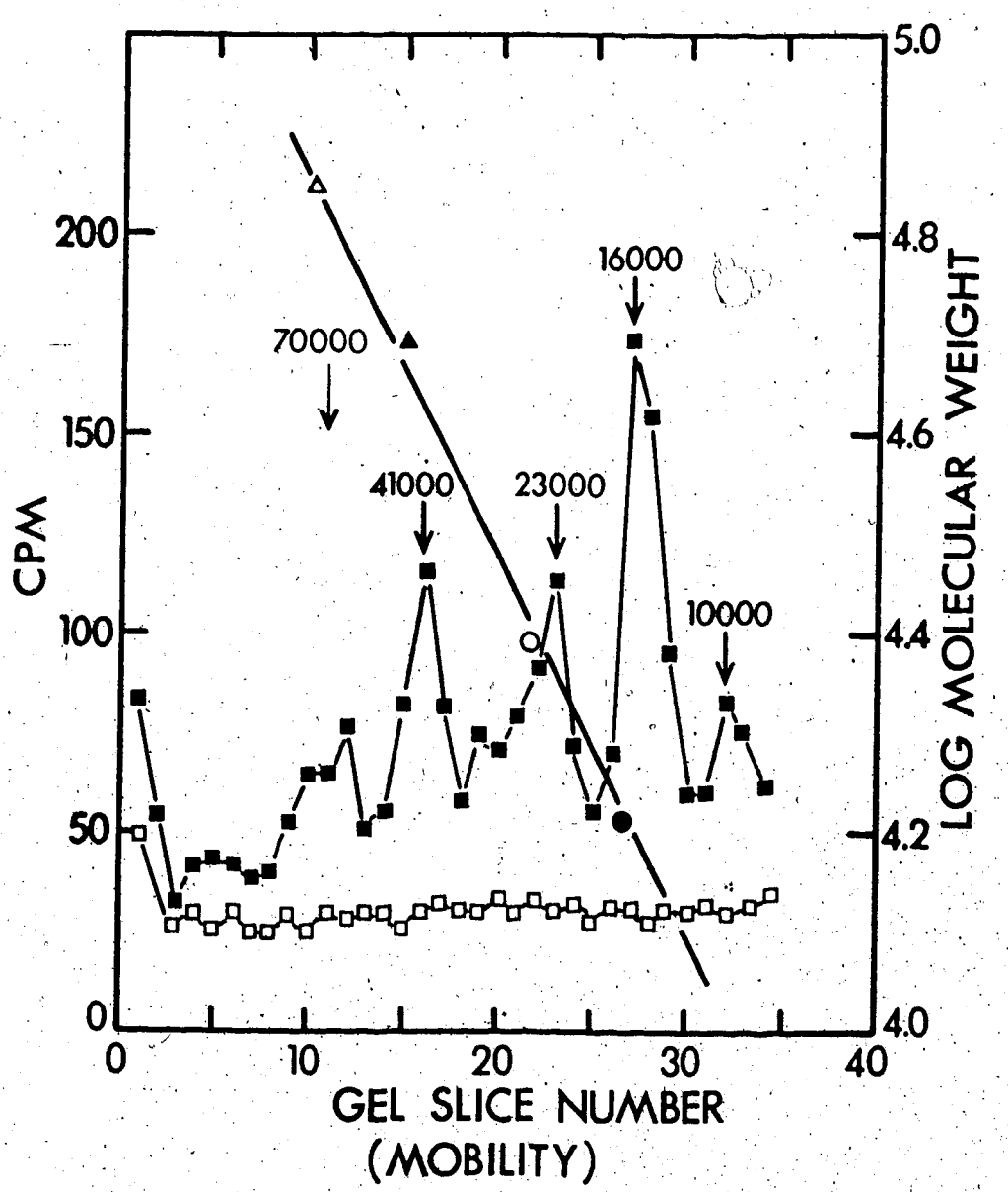


Figure 22. SDS polyacrylamide gel electrophoresis of an immunoprecipitate of α-FP. A large scale reaction was done as described in Methods and Materials with fetal polysomal RNA and 30 uCi of ^{3}H-leucine (1 Ci/mmole), 20 uCi of ^{3}H-valine (2 Ci/mmole), 20 uCi of ^{3}H-glutamic acid (1.4 Ci/mmole) and 10 uCi of ^{3}H-phenylalanine (1 Ci/mmole) per ml. The immunoprecipitation procedure (as in Methods and Materials) was carried out on the high speed supernatant of the reaction mixture. The immunoprecipitate for α-FP was dissolved in 4 M urea, 1% SDS and 1% β-ME, and run on an SDS polyacrylamide gel (see Methods and Materials). The gel was sliced and counted as described in Methods and Materials. Also, the gel for a reaction with no polysomal RNA added is shown. Also shown on this figure is a graph of log molecular weight versus mobility that was determined using molecular weight standards of: α-FP (70,000), Δ; heavy chain of immunoglobulin (50,000), ▲; light chain of immunoglobulin (25,000), O; and hemoglobin (16,000), ●.

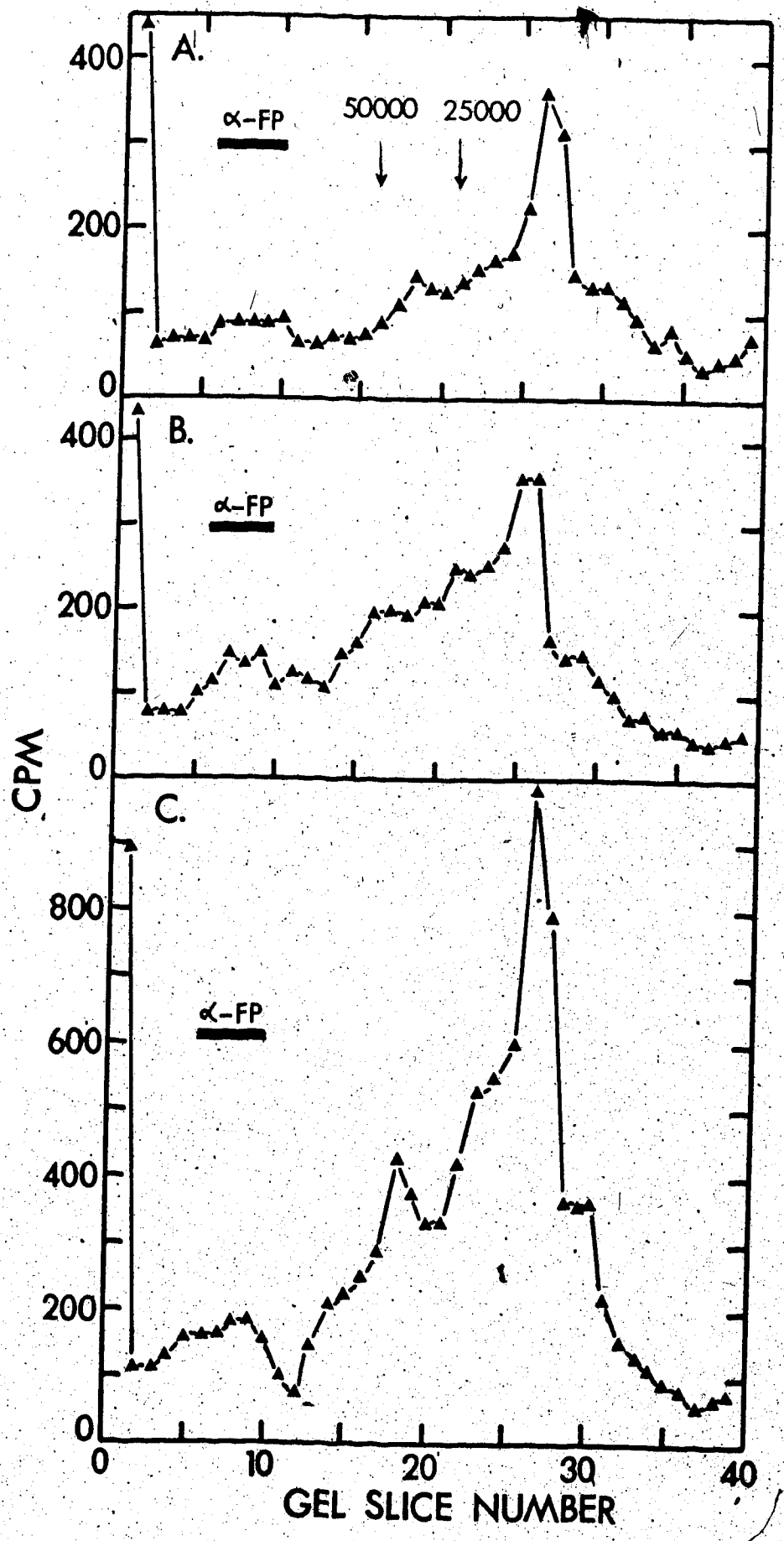
■—■, with polysomal RNA
□—□, without polysomal RNA

Figure 23. SDS gel analysis of α-FP immunoprecipitates -- time course. The reaction mixture contained fetal polysomal RNA and 30 uCi of ^{3}H-leucine (1 Ci/mmole), 20 uCi of ^{3}H-valine (2 Ci/mmole), 20 uCi of ^{3}H-glutamic acid (34 Ci/mmole) and 10 uCi of ^{3}H-phenylalanine (20 Ci/mmole) per ml. The other components were as described in Methods and Materials. Aliquots were removed after 30, 60 and 90 minutes of incubation at 30°. Immunoprecipitation of α-FP and SDS gel analysis were done as described in Methods and Materials. The position of α-FP and heavy and light chains of immunoglobulin are indicated.

A. 30 minutes

B. 60 minutes

C. 90 minutes


A.
α-FP
50000
25000
400
200
0
B.
α-FP
400
200
0
C.
α-FP
800
600
400
200
0
CPM
0
10
20
30
40
GEL SLICE NUMBER

5) Affinity column chromatography

Another approach used to obtain newly made α-FP from reaction mixtures was affinity column chromatography, with antibody against α-FP attached to the column material. The preparation of the affinity column (i.e., binding antibody to the cyanogen bromide-activated Sepharose 4B), the binding procedure and the elution of the column are described under the Methods and Materials section. After washing off non-bound material, the elution of bound material resulted in a single peak whether using unlabelled α-FP or a reaction mixture with radioactive α-FP. Eluted bound material was pooled, concentrated by freeze drying and analysed by gel electrophoresis. The results show that protein produced through translation of endogenous mRNA in a fetal liver lysate contains a major peak co-migrating with α-FP (Fig. 24A). The peak contained 50% of the total radioactivity on the SDS gel. The product resulting from translation of exogenous mRNA in S-30 also shows a radioactive α-FP peak on an SDS gel which amounted to 22% of the total radioactivity recovered (Fig. 24B). There are several other peaks present with the overall pattern very similar to that observed in the SDS gel of an immunoprecipitate of α-FP (Fig. 22).

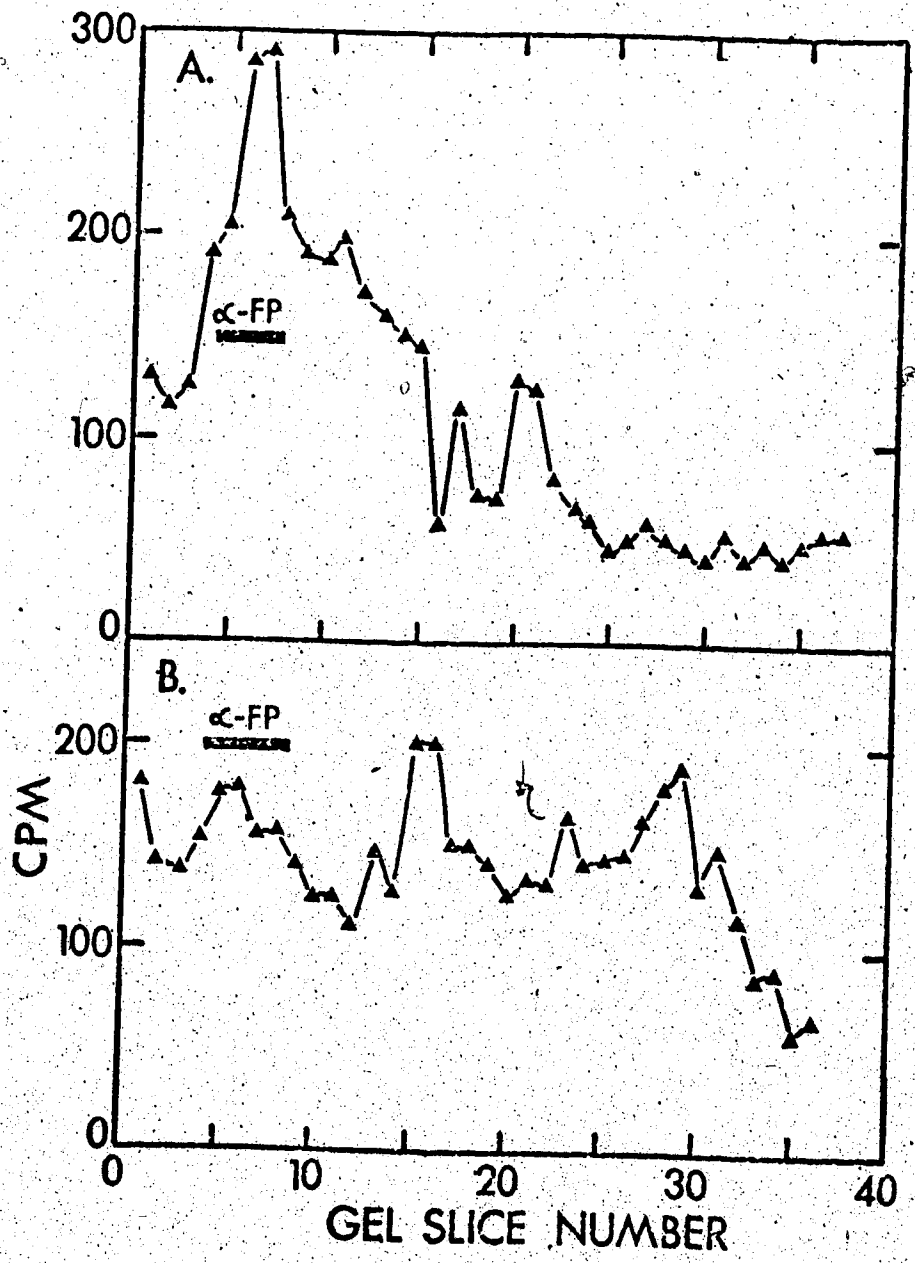
6) Cyanogen bromide cleavage of α-FP and albumin

The material immunoprecipitated by anti-α-FP and retained by the affinity column that was of lower molecular

Figure 24. Polyacrylamide gel analysis of affinity column bound material synthesized in cell-free reactions. The material bound to the affinity column was analysed by SDS gel electrophoresis.

A. The reaction mixture with endogenous synthesis of a fetal liver lysate (Tamaoki et al., 1974) was applied to the affinity column. SDS gel analysis of the bound material is shown. Carrier α-FP was also present on the gel.

B. The reaction mixture was with exogenous fetal polysomal RNA and S-30 using 75 uCi of ^{3}H-leucine (190 mCi/mmole), 20 uCi of ^{3}H-valine (17.7 Ci/mmole), 20 uCi of ^{3}H-glutamic acid (34 Ci/mmole) and 10 uCi of ^{3}H-phenylalanine (20 Ci/mmole) per ml. The SDS gel of the affinity column bound material is shown.


A.
B.
α-FP
α-FP
300
200
100
0
200
100
0
CPM
0
10
20
30
40
GEL SLICE NUMBER

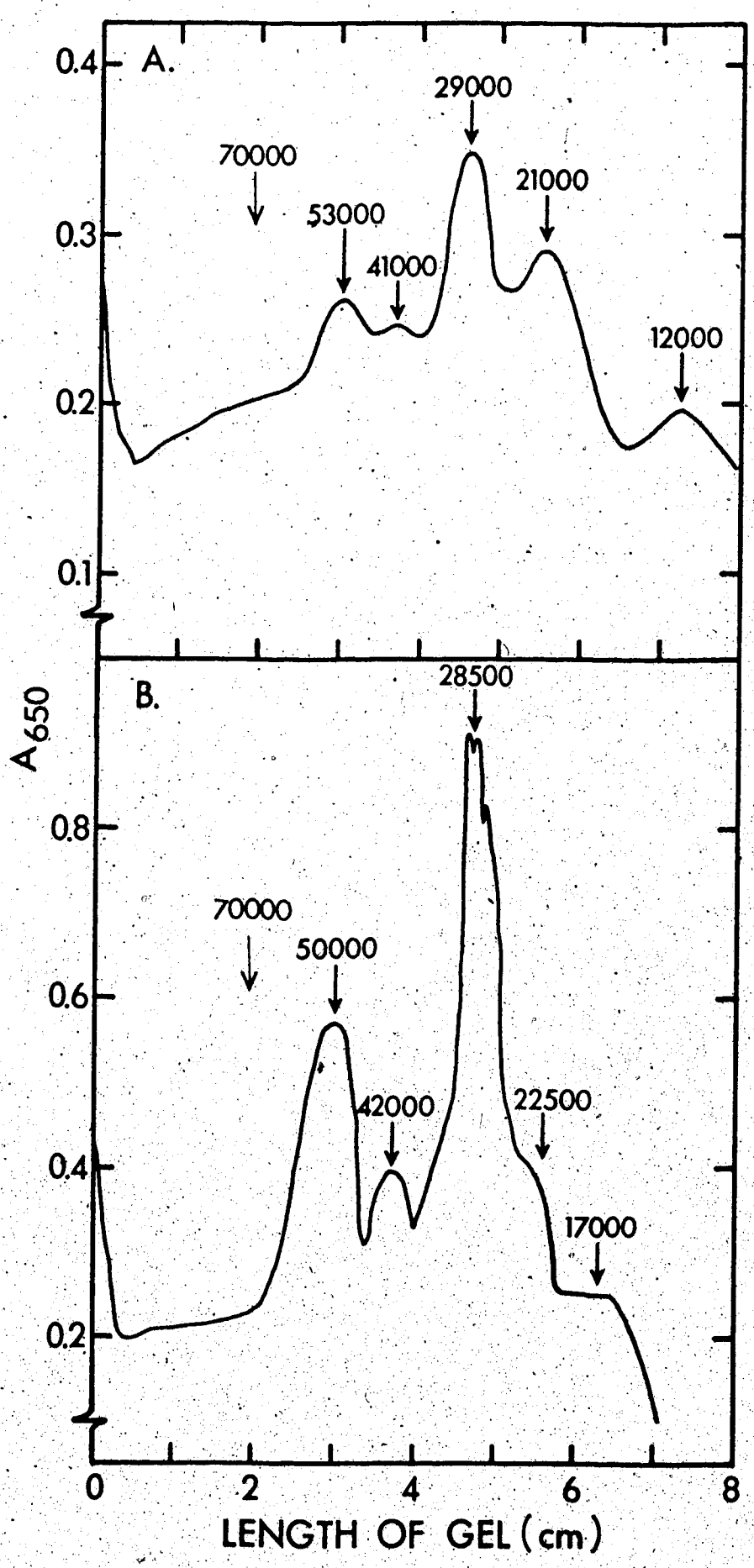
weight than α-FP probably represents fragments of α-FP. It has repeatedly been shown in several systems that antibodies against complete protein will react with fragments of proteins being synthesized on polysomes (Warren and Peters, 1965; Hartlief and Koningsberger, 1968; Schecter 1974). To demonstrate directly that anti-α-FP reacts with fragments of α-FP, the reactivity of cyanogen bromide generated fragments of α-FP with anti-α-FP was tested. Mouse α-FP was cleaved with cyanogen bromide as described in Methods and Materials, and the breakdown products were analysed on an SDS gel. The results of three separate experiments showed protein peaks with approximate molecular weights of 53,000, 41,000, 29,000, 21,000 and 12,000 (Fig. 25A). The 29,000 and 21,000 molecular weight peaks are the most prominent on the gel. No complete α-FP (MW 70,000) was remaining. Fig. 25B shows the cyanogen bromide cleavage products of mouse albumin. The approximate molecular weights of these protein peaks were 50,000, 42,000, 28,500, 22,500 and 17,000. Again no complete albumin (MW 70,000) was remaining.

The cyanogen bromide cleavage products were found to be reactive with specific antibodies as analysed by double immunodiffusion (Fig. 26). The single spur in both cases demonstrates that cyanogen bromide cleavage of the complete α-FP or albumin destroys some antigenic determinants. No reaction was observed between cleavage products of α-FP with anti-albumin and vice versa.

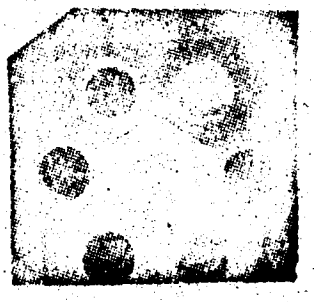
Figure 25. SDS gel analysis of cyanogen bromide cleavage products of α-FP and albumin. The cyanogen bromide cleavage procedure followed is described in Methods and Materials. SDS gel analysis of the cleavage products was done as described in Methods and Materials. The arrows indicate the protein peaks and the molecular weight estimates are based on the mobility and Fig. 22.

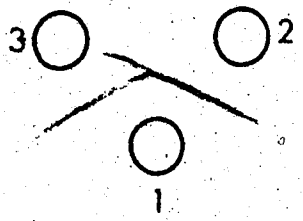
A. House α-FP

B. House albumin

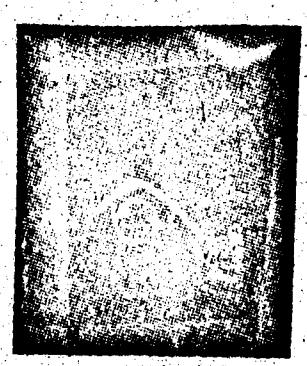

A.
B.
29000
70000
21000
53000
41000
12000
28500
70000
50000
42000
22500
17000
0.4
0.3
0.2
0.1
0.8
0.6
0.4
0.2
A_{650}
0
2
4
6
8
LENGTH OF GEL (cm)

A





B



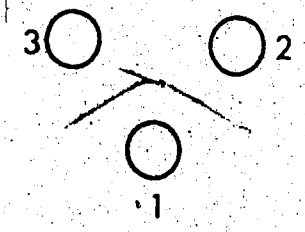


Figure 26. Double immunodiffusion plates of antibodies and cyanogen bromide cleavage fragments of α-FP and albumin.

A. 1) anti-α-FP, 2) α-FP, 3) cyanogen bromide cleaved α-FP.

B. 1) anti-albumin, 2) albumin, 3) cyanogen bromide cleaved albumin.

DISCUSSION

In recent years, proteins made by the mammalian liver have been synthesized in cell-free systems directed by exogenous messenger RNA. These include rat albumin (Taylor and Schimke, 1973; Shafritz, 1974) and ferritin (Shafritz et al., 1973) in a heterologous rabbit reticulocyte cell-free system, and mouse albumin and α-FP in an homologous mouse liver system (Faber et al., 1974; Koga et al., 1974). In the present work, attempts were made to synthesize mouse α-FP and albumin in a heterologous cell-free system using Sarcoma 130 S-30.

Some of the properties of the present cell-free system can be compared to those of Sarcoma 130 and Krebs II S-30's reported by others.

The optimum Mg^{2+} concentration for poly(U) translation was found to be in the range of 7-8 mM. Jenkins et al. (1973) found a Mg^{2+} optimum for poly(U) translation in the range of 5-7 mM for similar S-30 preparations. Ribosomal wash factors have been shown to increase incorporation at low Mg^{2+} concentration while decreasing incorporation at high Mg^{2+} concentration, thus lowering the optimum concentration of Mg^{2+} (Shafritz and Anderson, 1970; Woodley et al., 1972; Murty et al., 1974).

In the present work, the addition of ribosomal wash did not alter the Mg^{2+} optimum but resulted in a general reduction of phenylalanine incorporation. This was not due to a defect of the ribosomal wash used since the same wash preparation was found to induce a "Mg^{2+} shift" in a cell-free system derived from adult liver (unpublished observation). Presumably, the factors required for "Mg^{2+} shift" were already present in the S-30 preparation.

Ribosomal wash was essential, however, for the translation of natural polysomal RNA in this system. This is contrary to the observation of Jenkins et al. (1973) who found that ribosomal wash was stimulatory but was not essential for the translation of exogenous mRNA in their S-30 preparations. The cause of this difference is not clear at present but it may be due to the different mRNA's used. Jenkins et al. (1973) used RNA from mengovirus, reovirus, bovine entero virus-1 and sea urchin egg, while in the present work fetal and adult mouse liver polysomal RNA was used. Conflicting results with respect to the requirement for ribosomal wash have been reported for the Krebs II S-30. Several workers have shown the translation of exogenous mRNA in the absence of added ribosomal wash; e.g., pure globin mRNA (Jacobs-Lorena and Baglioni, 1972), pituitary tumour RNA (Bancroft et al., 1973) and Sindbis viral mRNA (Cancedda and Schlesinger, 1974). On the other hand, Metafora et al. (1972) and Mathews et al. (1972) reported that ribosomal wash from rabbit reticulocytes greatly

stimulated protein synthesis dependent on exogenous globin mRNA or EMC virus RNA.

Translation of natural mRNA in the present system did not depend upon added tRNA, in contrast to poly(U)-directed phenylalanine incorporation which was highly dependent upon added tRNA. The requirement for $tRNA_{phe}$ in the poly(U) system would be many fold greater than the requirement for tRNA specific for one amino acid for the translation of natural mRNA. The S-30 apparently contains enough tRNA for each amino acid for natural mRNA translation but insufficient $tRNA_{phe}$ for optimal poly(U) translation. Metafora et al. (1972) and Aviv et al. (1971) reported a Krebs II S-30 which was tRNA dependent when used to translate exogenous natural mRNA. However, Jacobs-Lorena and Baglioni (1972), also using a Krebs II S-30, failed to find any dependence on exogenous tRNA. Benveniste et al. (1973) showed that S-30 pre-incubated for 45 minutes was independent of added tRNA for protein synthesis while S-30 pre-incubated for 100 minutes was dependent upon added tRNA. Jenkins et al. (1973), using a Sarcoma 180 S-30, also reported that varying the pre-incubation time during preparation of S-30 determined whether the system was tRNA dependent or not. This is probably due to inactivation of tRNA during pre-incubation.

The time course study showed that protein synthesis in the present S-30 system continued for at least 120 minutes.

Jenkins et al. (1973) showed continued initiation of protein synthesis after 45 minutes but did not continue the assay for a longer time. Most reports on the Krebs II system show protein synthesis stopping after about 60 minutes (Jacobs-Lorena and Baglioni, 1972; Mathews et al., 1972).

An attempt was made to stimulate the system once incorporation had slowed down. The incorporation rate was slower from 60 to 120 minutes than for the first 60 minutes of incubation. After 90 minutes of incubation various components were added; e.g., mRNA, ATP, GTP and S-30. The best stimulation was achieved by the addition of mRNA and ribosomal wash, but was only a 10% increase in leucine incorporation (data not shown). It seems that the decreased rate of incorporation from 60 to 120 minutes cannot be attributed to a lack of any single component but rather results from a general tiring of the whole system.

Ribosomal RNA stimulated background endogenous protein synthesis and inhibited protein synthesis when exogenous mRNA was present. Mathews and Korner (1970) and Jacobs-Lorena and Baglioni (1972) both reported a similar stimulation of background protein synthesis by rRNA. Since rRNA would not be expected to have any messenger activity, the stimulation can be explained on the basis of protection of endogenous mRNA against nuclease action. Evidence for such a protective effect was reported by Jacobs-Lorena and Baglioni (1972).

Using purified globin mRNA they also reported, however, that rRNA stimulated globin synthesis, presumably again due to protection of mRNA against nuclease action. Their data is shown in Fig. 18 along with the results obtained with the present cell-free system. The contrasting data could be explained if one considers the ratio of total rRNA to active mRNA in the reaction mixture. Above a ratio of about 10:1, Jacobs-Lorena and Baglioni's data (Fig. 18B) begin to show inhibition. With total polysomal RNA the ratio of rRNA to active mRNA is probably at least 20:1 (if not 100:1) initially. So, with the addition of rRNA an inhibition occurs. This inhibition may be due to the presence of an inhibitory factor in the rRNA preparation or binding of rRNA to some component of the reaction mixture resulting in an inhibition.

Since the protein synthesis in the present cell-free system depended upon the presence of polysomal RNA and ribosomal wash, this system can be used to determine whether the lack of synthesis of α-FP in adult liver is due to the deficiency of mRNA or a specific initiation factor. The presence of specific initiation factors to certain eukaryotic mRNAs has been demonstrated previously (Nudel et al., 1973; Wigle and Smith, 1974; Heywood et al., 1974). The present results show that all three ribosomal wash preparations tested (fetal liver, adult liver and Sarcoma 180) were active in supporting α-FP synthesis in the presence

of fetal polysomal RNA. Adult polysomal RNA on the other hand did not direct the synthesis of α-FP in the presence of any of these wash preparations. This was not due to degradation of mRNA since albumin synthesis was observed using the same adult polysomal RNA preparations. It was concluded that active mRNA for α-FP is deficient in the adult liver. Possible limiting steps in the production of active α-FP mRNA include the synthesis of RNA on the DNA template, structural modificiation of mRNA and transport of mRNA from the nucleus of the cell.

Since the assay of α-FP and albumin was based on immunoprecipitation, it is of critical importance that the antibodies used were specific for these proteins. Two immunological tests (Ouchterlony double immunodiffusion and immunoelectrophoresis) indicated that the antibodies were monospecific (Fig. 3). Quantitative precipitation of α-FP and albumin by these antibodies has been shown using samples containing radioactive α-FP and albumin in various proportions (Tamaoki et al., 1974; Koga and Tamaoki, 1974). The rate of synthesis of α-FP and albumin in vitro as analysed by immunoprecipitation correlates well with the in vivo levels (serum concentrations) of these proteins throughout fetal development (Koga and Tamaoki, 1974). Various cell-free systems in which no α-FP synthesis was expected (adult liver lysate, free polysomes from fetal liver and Sarcoma S-30 system with adult polysomal RNA or

without any exogenous RNA) gave only background counts with anti-α-FP. Furthermore, the present results were very similar to those obtained with the homologous cell-free system (Koga et al., 1974), in which the synthesis of α-FP and albumin has been established by polyacrylamide gel electrophoresis. The immunoprecipitable counts presented above, therefore, provide a valid measure, at least proportional to the amounts of newly formed α-FP and albumin.

SDS gel analysis of immunoprecipitated protein revealed that from 10-23% of the total protein was complete α-FP molecules. However, it has repeatedly been shown in several systems that antibodies can bind to nascent polypeptide chains (Warren and Peters, 1965; Hartlief and Koningsberger, 1968; Schecter, 1974). In fact this is the basis for selective immunoprecipitation of specific polysomes. Specific mRNA can then be isolated from these polysomes (Schecter, 1974). It is therefore likely that smaller size proteins revealed by SDS gel analysis represent nascent α-FP molecules. Support for this possibility was obtained by the observation that α-FP fragments (MW 12-53,000) produced by cyanogen bromide cleavage reacted with anti-α-FP. Albumin fragments, similarly obtained, reacted with anti-albumin. There was no cross reaction between α-FP fragments and anti-albumin and vice versa, which indicates that the specificity of the antibodies

exists at the level of fragments. Specific reactions of antibodies with fragmented antigens have also been shown with other proteins treated with proteolytic enzymes. They include: human albumin (Press and Porter, 1962); myoglobin (Crumpton and Wilkinson, 1965); and TMV protein (Young et al., 1966).

The formation of polypeptide fragments appears to be a property inherent to cell-free systems employing exogenous mRNA. There are a number of examples in the literature. When EMC virus RNA was translated in a Krebs II system, proteins of different sizes were synthesized. These resulted from premature termination at many points along the RNA (Kerr et al., 1972; Boime et al., 1972). Similar findings have been reported for TMV RNA in a wheat germ cell-free system (Roberts et al., 1973) and for Sindbis virus RNA in a Krebs II system and a rabbit reticulocyte system (Cancedda and Schlesinger, 1974; Cancedda et al., 1974A, 1974B). Shafritz (1974) used a rabbit reticulocyte cell-free system to translate exogenous liver RNA. SDS gel analysis of immunoprecipitable material (for albumin) showed that greater than 90% of the counts were in polypeptide components smaller than albumin. For the Krebs II system other reports of incomplete proteins being synthesized include: lens crystallin, (Zelenka and Piatogorsky, 1974); light chain of immunoglobulin, (Schecter, 1974); tryptophan oxygenase (Schutz et al., 1973). Also, Wiche

et al. (1974) reported the immunoprecipitation of fragments of neuroblastoma tubulin synthesized in a cell-free system.

The question arises as to why incomplete proteins are synthesized in these cell-free systems. One possibility is premature termination of translation resulting in the synthesis of fragments. This could be due to a rare species of tRNA being limiting or some structural feature of certain sites on an mRNA which results in ribosomes being released. Mathews and Osborn (1974) have shown that the rate of elongation in the Krebs II system is about 25 amino acid residues per minute which is only one-tenth that in vivo. They attribute premature termination of EMC viral RNA translation (see above) to this slow elongation. If it is assumed that the rate of chain elongation in the present Sarcoma cell-free system is similar to that in the Krebs II system, the complete translation of α-FP mRNA would require about 23 minutes. Thus, large mRNAs, such as that for α-FP and albumin may be subject to a greater chance of nucleolytic attack. Partially degraded mRNA could conceivably still direct the synthesis of N-terminal fragments of proteins. Prevention of fragment formation may only be acheived through a better understanding of the protein synthesizing machinery to bring about efficient and maximum utilization of mRNA in cell-free systems.

CONCLUSION

The Sarcoma 180 S-30 system used in the present work depended upon ribosomal wash for the translation of polysomal RNA. This was used to advantage for showing that the synthesis of α-FP was dependent upon polysomal RNA from fetal liver and was unaffected by the source of ribosomal wash. The inability of adult liver to synthesize α-FP is thus due to the deficiency of active α-FP mRNA. It is concluded that α-FP synthesis is controlled primarily at at the level of transcription. Further study is necessary to determine which step involved in the synthesis of active mRNA (synthesis of RNA on the DNA template, modification or transport) is responsible for the reduction of α-FP mRNA in adult liver.

The cell-free products recovered by using antibody were found to contain a considerable amount of fragments of α-FP. Future work in this area must be directed toward increasing the percentage of complete protein molecules synthesized. This could possibly be done by improving the cell-free system to increase the stability and efficiency of translation of long mRNA's. Another approach would be to use purified α-FP mRNA rather than polysomal RNA.

With purified α-FP mRNA, complementary DNA (cDNA) could be synthesized. The cDNA could then be used to

establish whether the lack of active α-FP mRNA in adult liver results from transcription not occurring or from some defect in the modification of precursor α-FP mRNA into active mRNA. Structural studies of pure α-FP mRNA may reveal features of the molecule that affect its stability and account for the declining synthesis of α-FP in developing embryos.

These studies on α-FP may provide an opportunity for understanding mechanisms regulating protein synthesis during development and carcinogenesis.

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