LOCALIZATION OF POLYSOME-BOUND ALBUMIN AND SERINE DEHYDRATASE IN RAT LIVER CELL FRACTIONS

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ABSTRACT

The polysomes involved in albumin and serine dehydratase synthesis were identified and localized by the binding to rat liver polysomes of anti-rat serum albumin and anti-serine dehydratase [^{125}I]Fab dimer and monomer. Techniques were developed for the isolation of undegraded free and membrane-bound polysomes and for the preparation of [^{125}I]Fab monomers and dimers from the IgG obtained from the antisera to the two proteins, rat serum albumin and serine dehydratase. The distribution of anti-rat serum albumin [^{125}I]Fab dimer in the polysome profile is in accordance with the size of polysomes that are expected to be synthesizing albumin. By direct precipitation, it has been demonstrated that nascent chains isolated from the membrane-bound polysomes by puromycin were precipitated by anti-rat serum albumin-IgG at a level of 5–6 times those released from free polysomes. Anti-rat serum albumin-[^{125}I]Fab dimer reacted with membrane-bound polysomes almost exclusively compared to the binding of nonimmune, control [^{125}I]Fab dimer; a significant degree of binding of anti-rat serum albumin-[^{125}I]Fab to free polysomes was also obtained. The [^{125}I]Fab dimer made from normal control rabbit serum does not react with polysomes from liver at all and this preparation will not interact with polysomes extracted from tissues that do not synthesize rat serum albumin.

Both anti-serine dehydratase-[^{125}I]Fab monomer and dimer react with free and bound polysomes from livers of animals fed a chow diet or those fed a high 90% protein diet and given glucagon. In the latter instance, however, it is clear that the majority of the binding occurs to the bound polysomes. Furthermore, the specificity of this reaction may be further shown by the use of kidney polysomes that do not normally synthesize serine dehydratase. When these latter polysomes are isolated, even after the addition of crude and purified serine dehydratase, no reaction with anti-serine dehydratase-Fab fragments could be demonstrated. These results indicate that the reaction of the Fab fragments are specific for polysomes that synthesize rat serum albumin or rat liver serine dehydratase. Furthermore, they demonstrate that even with this high degree of specificity, some polysomes in the fraction labeled "free" are in the process of synthesizing rat serum albumin while bound polysomes to a significant, if not major, degree are the site of the synthesis of rat liver serine dehydratase.

INTRODUCTION

Rat liver, like many other tissues examined so far, contains free as well as membrane-bound polysomes (1–3). Polysomes attached to membranes are thought to be responsible for the synthesis of proteins for export, whereas free polysomes have been considered to synthesize proteins destined for intracellular use (4–8).

The use of antibodies which bind to nascent polypeptide chains is a promising approach to the identification and isolation of polysomes involved in the synthesis of specific proteins. Several studies have shown that antibodies against completed protein chains will react with polysomes synthesizing those molecules (9–14). Recently it has been demonstrated that the immunological precipitation of polysomes prepared from rabbit reticulocytes had a low specificity when unfractionated antiserum to hemoglobin was used (15, 16). One cause for this nonspecificity was ascribed to the binding of IgG to ribosomes through its Fc portion. It has since been shown that the removal of the Fc portion of the IgG molecule enhances the specificity of its interaction with polysomes (16). In this paper we describe the isolation of undegraded washed rat liver polysomes and the specific binding of ^{125}I-labeled antibody preparations to the nascent polypeptide chains on free and membrane-bound polysome populations.

MATERIALS AND METHODS

Materials

Crystalline bovine serum albumin was purchased from the Armour Pharmaceutical Co., Chicago, Ill. Crystalline bovine pancreatic ribonuclease (RNase), pepsin and yeast RNA were obtained from the Sigma Chemical Co., St. Louis, Mo., and puromycin from Nutritional Biochemicals Corporation, Cleveland, Ohio. [^{125}I]sodium iodide, L-[^{14}C]leucine, L-[^{3}H]leucine, and [^{3}H]orotic acid were obtained from the New England Nuclear Corp., Boston, Mass. sucrose (RNase-free) from Mann Research Labs, Inc., New York, and chloramine-T from Eastman Kodak Co., Rochester, N. Y.

Fractionation of Rat Liver Cells

Male Holtzman rats (Holtzman Co., Madison, Wis.) (300–350 g) which were starved overnight (16–18 h) were decapitated by using a guillotine. In some experiments as indicated in the results, rats were injected intraperitoneally with 100 μCi of [^{3}H]orotic acid/100 g body weight and then starved for 16–18 h before they were killed to isolate the labeled polysomes. The livers were rapidly removed, chilled, weighed, and minced in 2 vol of cold 0.44 M sucrose in TKM buffer (50 mM Tris-HCl, pH 7.4, 25 mM KCl, and 10 mM MgCl$_2$). Minced livers were homogenized with a Potter-Elvehjem homogenizer by using 10 strokes of a motor-driven Teflon pestle. Postmitochondrial supernate (S$_2$) was prepared by centrifuging the tissue homogenate at 20,000 g max for 10 min in a Sorvall SS-34 rotor (Ivan Sorvall, Inc., Newtown, Conn.). The upper two-thirds of the S$_2$ was removed with a pipette.

Preparation of Free and Membrane-Bound Polysomes

Polysomes were prepared by a modification of the procedure described by Blobel and Potter (1, 17). The S$_2$ fraction was adjusted to 1.35 M sucrose in TKM buffer by the addition of 1.47 vol of 2 M sucrose in the same buffer. A 20-ml portion of the S$_2$ fraction in 1.35 M sucrose was layered over 10 ml of 2 M sucrose in TKM buffer and overlaid with 4 ml of 0.44 M sucrose in the same buffer. This discontinuous gradient was centrifuged in a Beckman type 50.1 rotor (Beckman Instruments, Inc., Spinco Div. Palo Alto, Calif.) at 40,000 rpm (123,000 g max) for 6 h. The rough endoplasmic reticulum floated at the boundary between the 2 and 1.35 M sucrose layers, the smooth endoplasmic reticulum floated on top of the 1.35 M sucrose layer, and free polysomes sedimented as the pellet. The surface of the pellet was washed several times with TKM buffer. The membrane layer of the rough endoplasmic reticulum was collected after the sectioning of the tubes. In order to isolate the polysomes from this fraction, detergents (20% Triton X-100 or 20% sodium deoxycholate) were added to a final concentration of 0.5% after the addition of RNase inhibitor (10 units per ml of the membrane suspension). A 20-ml portion of the detergent-treated rough endoplasmic reticulum fraction was layered over 10 ml of 2 M sucrose in TKM buffer. The gradients were then centrifuged in a 50.1 rotor at 40,000 rpm (123,000 g av) for 6 h. The surface of each pellet (the membrane-bound polysomes) was washed several times with TKM buffer and the walls of tubes were wiped to remove membrane fragments and lipid material.

Washing of Polysomes with a High Salt Solution

To remove the soluble protein contaminants of the polysomes, 0.5 M NH$_4$Cl containing 0.44 M

sucrose, 50 mM Tris-HCl (pH 7.4), and 10 mM MgCl$_2$ was used to wash the polysomes. Each pellet of free or membrane-bound polysomes obtained by the above procedure was suspended in 8 ml of the high salt solution described above containing the RNase inhibitor (10 units per ml), homogenized gently by hand in a small glass homogenizer, and centrifuged in a 50 Ti rotor at 47,000 rpm (165,000 g av) for 6 h. The resultant pellets were washed several times with TKM buffer and stored at $-70°C$ until use.

Preparation of Nascent Polypeptide Chains

Nascent polypeptide chains were prepared from the polysomes according to the method of Blobel and Sabatini (18). Peptide chains were released from polysomes by incubation with puromycin (2 mM in final concentration) into a medium containing 0.5 M KCl, 50 mM Tris-HCl (pH 7.4), and 5 mM MgCl$_2$. In some experiments nascent polypeptide chains were labeled with L-[^{14}C]leucine which had been injected into the portal vein of rats 5 min before sacrifice.

Purification of RNase Inhibitor

RNase inhibitor was purified from guinea pig liver by a combination of the methods of Sirakov and Kochakian (19) and Gribnau et al. (20), using ammonium sulfate fractionation (35–65% saturation), DEAE-Sephadex A-50 chromatography, and gel filtration on Sephadex G-100, Pharmacia Fine Chemicals Inc., Uppsala, Sweden. One unit of inhibitor was defined as that amount which gave 50% inhibition of the activity of 0.005 μg of crystalline pancreatic RNase. The purified RNase inhibitor thus obtained had a specific activity of 1,700 units/mg protein and could be stored at $-20°C$ for several months when it was kept in 0.5 M glycine-50 mM phosphate buffer, pH 7.8.

Preparation of Rat Serum Albumin (RSA), Serine Dehydratase, and their Antisera

Rat serum albumin was prepared by the Debro procedure (21) followed by chromatography on DEAE-cellulose with a linear phosphate (pH 7.6) gradient (0.01–0.15 M). That fraction of the eluted albumin was used as an antigen which showed only one protein band when tested by polyacrylamide gel electrophoresis (22). Serine dehydratase was purified and crystallized by the method of Inoue et al. (23). The crystalline enzyme, a mixture of the two isozymic forms, was utilized for the preparation of antisera. Rabbit antisera to albumin and serine dehydratase were prepared as described previously (22), by using foot pad injections of an emulsion of

the antigen in complete Freund's adjuvant. The IgG fraction of the anti-RSA and anti-serine dehydratase sera was prepared by ion-exchange chromatography and ammonium sulfate precipitation according to Fahey (24). IgG fractions were tested for specificity by Ouchterlony double-diffusion analysis. Normal rabbit serum was used as a control after purification of the IgG fraction.

Iodination of IgG

Iodination of the antisera and of normal IgG was carried out according to the method of Sonoda and Schlamowitz (25). To a chilled mixture of 10 mg of IgG and 100 μCi of Na^{125}I, neutralized with 0.1 N HCl to pH 7.0, in 5.0 ml of 50 mM phosphate buffer, pH 7.0, was added 5.0 ml of chilled, freshly prepared chloramine-T (133.4 μM) over a 2-min period with vigorous magnetic stirring. The reaction mixture was incubated for 30 min with stirring at 2–4°C. The excess chloramine-T was then neutralized with 1.2 ml of 667 μM NaHSO$_3$ in 50 mM phosphate buffer, pH 7.0, with continuous mixing. After 5 min, 0.2 ml of carrier KI (1.0 M) was added and the solution was passed through an AG 1-X8 anion exchange resin (1.6 $\times$ 1.0 cm column, 2.0-ml bed volume) which had been equilibrated with 50 mM phosphate buffer, pH 7.0. The column was washed with 3 ml of the same buffer. The combined eluates were dialyzed at 4°C against 4–5 batches of 3 liters of 0.154 M NaCl-10 mM phosphate buffer, pH 7.3. After dialysis, the iodinated IgG had a specific radioactivity of 10^6–10^7 cpm/mg protein.

Peptic Digestion of IgG

The globulins with or without iodine labeling were hydrolyzed by pepsin to obtain the Fab (5S fragment) described by Nisonoff et al. (26). The sample of IgG, at a concentration of 1–2%, was incubated with pepsin at a protein to enzyme ratio between 100:1 and 100:2 in 0.1 M acetate buffer, pH 4.5, at 37°C for 20 h. The turbidity which appeared on mixing pepsin with IgG persisted during the digestion period. Fractionation of the peptic digest of IgG was carried out on a Sephadex G-200 column according to Utsumi and Karush (27), who used Sephadex G-75.

Papain Digestion of IgG

To obtain the Fab monomer, anti-serine dehydratase-IgG was digested with papain according to the method of Porter (28). 50 mg of anti-serine dehydratase-IgG labeled with ^{125}I was incubated with 1 mg of papain at 37°C for 16 h and the digested IgG applied to a carboxy methyl cellulose

column (2.5 × 30 cm) which had been previously equilibrated with 0.1 M acetate buffer (pH 6.0). The proteins were eluted from the column with a linear gradient of acetate buffer (pH 6.0) from 0.1 to 0.4 N. The first two major peaks shown in Fig. 1 were combined and dialyzed against 0.9% NaCl in 10 mM sodium phosphate buffer (pH 7.4). The ^{125}I-labeled *Fab* monomer thus obtained had a specific activity of 1.2 × 10^6 cpm/mg protein.

Sucrose Density Gradients

A 10–40% isokinetic sucrose density gradient (16 ml) in TKM buffer was prepared as described before (17). Samples (10–20 A_{260}-units of polysomes in 0.5 ml) were layered on the gradients and were centrifuged in an SW 25.3 rotor at 25,000 rpm for 3.5 h. 15-drop fractions were collected by piercing the bottom of the tube and monitoring the absorbance of the gravity flow at 260 nm in a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Binding of [^{125}I]IgG and Fab Fragments from Immune Sera with Polysomes

In general, two procedures for the binding of anti-rat serum albumin and anti-serine dehydratase were employed as follows:

(a) Anti-rat serum albumin or anti-serine de-hydratase-[^{125}I]IgG and pepsin-digested [^{125}I]IgG from control and immune sera as well as the purified fragments (Fab and Fc) were used for binding to polysomes. Individual iodinated antibody preparations (2 × 10^4 cpm in 20–40 μl) were added to each fraction obtained from a gradient separation of polysomes as described above. The fraction and the labeled antisera were mixed well and incubated overnight at 2–4°C. Reaction mixtures were then
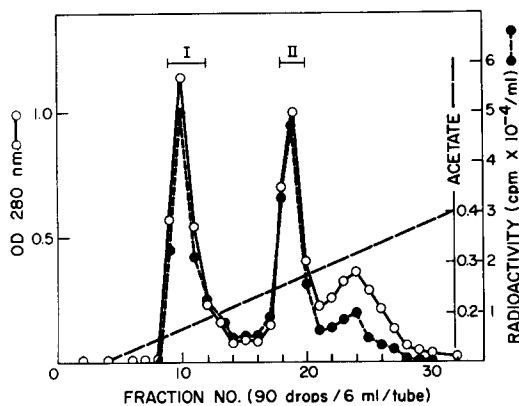

FIGURE 1 Separation of papain digest of IgG on a carboxy methyl cellulose column. See Materials and Methods for details of preparation and elution.

diluted to 11 ml with TKM buffer and centrifuged at 47,000 rpm in a 50 rotor for 1 h. Each pellet was washed six times with TKM buffer, dissolved in 1.5 ml of 97% formic acid, and used for the determination of ^{125}I radioactivity in a Packard tandem well-type spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

(b) Polysomes (10–20 A_{260}-units) were incubated with 50 μg of anti-rat serum albumin-[^{125}I]IgG or with a similar amount of anti-serine dehydratase-[^{125}I]Fab monomer which had been obtained by papain digestion. The incubation was carried out at 2–4°C overnight. The mixture of the polysomes and antibody preparations were layered on density gradients with or without precentrifugation at 47,000 rpm for 1 h to remove unreacted [^{125}I]immuno-globulin or the Fab monomers. The gradient (10–40%) was spun at 25,000 rpm for 3.5 in an SW 25.3 rotor and fractionated at 20 drops per tube.

Analytical Methods

Protein was determined by the method of Lowry et al. (29) and by the biuret reaction (30), with bovine serum albumin as standard. The RNA content of polysomes or membranes was determined by the method of Fleck and Munro (31) by utilizing a value of $E_{1\,cm}^{1\%}$ at 260 nm = 312. Polysomal RNA was also estimated by measuring A_{260}, where 25 mg of polysomal RNA/ml = 1.00 A_{260}-units (17). Inorganic phosphorus was determined according to the procedure described by Ames and Dubin (32). The amount of phospholipid was obtained by multiplying the phosphorus value by 25 (33).

Gel Diffusion and Autoradiography

Gel diffusion immunological procedures were carried out at room temperature (34). When the labeled antigens or antibodies were used, after a 2-day incubation at room temperature, gels were washed several times at 2–4°C in 2 liters of 0.154 M NaCl-10 mM phosphate buffer, pH 7.4, and finally in 2 liters of distilled water for 4–5 days to remove completely the unreacted antigens and antibodies in the gels. The washed gels were then placed on a glass plate and left to dry at room temperature for several days. Kodak Verichrome Pan film was used for the autoradiography. The dried gel film was overlapped tightly with the Kodak negative film in a dark room for 10–24 h, and the film was then developed.

RESULTS

Immunochemical Identification of Nascent Polypeptide Chains

To determine whether the antisera and other preparations used could specifically precipitate or

bind to nascent polypeptide chains on the polysomes, we labeled such chains isolated from the membrane-bound polysomes by puromycin with ^{125}I as described for the iodination of IgG and used for gel diffusion analysis. After complete formation of the immunoprecipitate between antigen and antibody in the gel, an autoradiogram was obtained and is shown in Fig. 2 a and b. Although no visible precipitin band was seen to be formed between nascent polypeptide chains and anti-RSA when the latter alone was used as antigen, a definite single visible and radioactive band was observed when the purified RSA was used as a carrier with the labeled nascent polypeptide chains. This band fused completely with the band formed between the labeled RSA and anti-RSA seen on autoradiography, as shown in Fig. 2 b. This observation shows that the nascent polypeptide chains which were reactive to anti-RSA were immunologically identical with the purified RSA.

While experiments were carried out with antiserine dehydratase-IgG and Fab monomer and dimer preparations with essentially similar results, earlier investigations (35) reported in this laboratory demonstrated that both free and bound

polysomes, isolated from rats which had been injected into the portal vein with a labeled amino acid, contain radioactive serine dehydratase antigen which could be released by incubation with puromycin in vitro. As expected, and in contrast to the puromycin release of nascent albumin chains, nascent serine dehydratase chains released by puromycin in vitro enter the extravesicular compartment (36).

Relative Amounts of Albumin Nascent Chains in Rat Liver

The relative amounts of albumin nascent chains were measured on the free and membrane-bound polysomes. Nascent polypeptide chains which were labeled with [^{14}C]leucine as described in Materials and Methods were released separately from the polysome preparations with puromycin, and then the radioactivity precipitated by anti-rat serum albumin was compared to the total acid-precipitable radioactivity. Table I shows the percentage of the labeled nascent polypeptide chains precipitated by anti-rat serum albumin. Method II using antigen + anti-rat serum albumin + anti-rabbit-IgG shows some variation
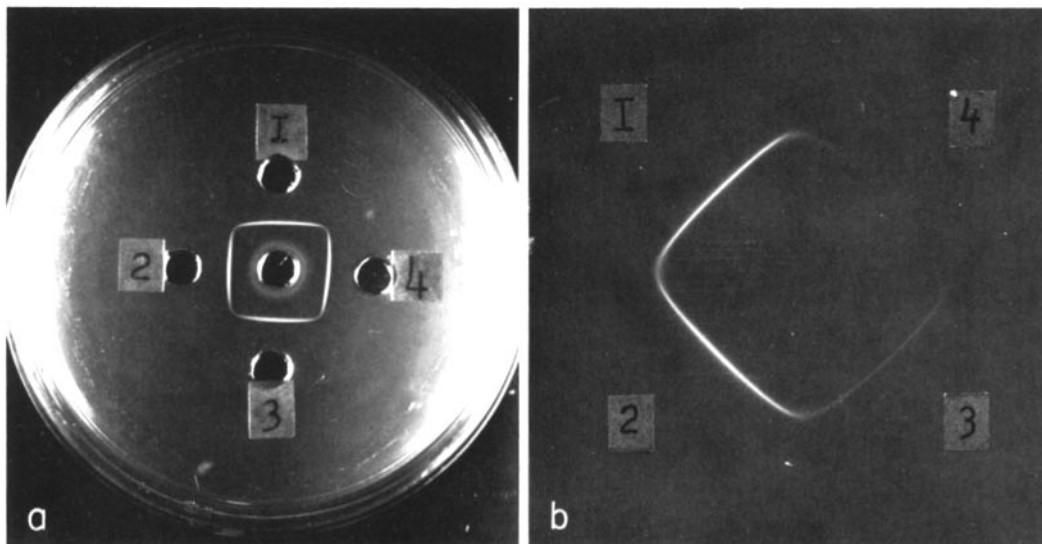

Figure 2   Immunodiffusion and its autoradiography. Immunodiffusion on agar gel and its autoradiography were carried out as described in the Materials and Methods. The center well contains 40 μl of purified anti-RSA (25 mg/ml), and in the peripheral wells the following antigens were added: (1) purified RSA (0.5 mg/ml) + [^{125}I]RSA (28,000 cpm); (2) purified rat liver albumin (0.5 mg/ml) + [^{125}I]RSA (30,000 cpm); (3) purified RSA (0.5 mg/ml) + nascent ^{125}I-labeled polypeptide chains (25,000 cpm); (4) nonlabeled purified RSA (0.5 mg/ml). (a) precipitin band in agar; (b) positive photograph of autoradiogram of gel diffusion.

TABLE I

	Nascent chains used (μg)	Method*	Total cpm	cpm precipitated with antibody	Percentage of total cpm precipitated
Nascent polypeptide Chains from bound-polysomes	10	I	1,350	159	11.8 (11.0)‡
		II	1,325	272	20.5 (19.7)
	20	I	2,630	320	12.1 (11.5)
		II	2,660	407	15.3 (14.7)
	30	I	3,650	420	11.5 (11.0)
		II	3,760	470	12.5 (12.0)
	50	I	6,360	750	11.9 (11.6)
		II	6,500	786	12.1 (11.8)
Nascent polypeptide chains from the free-polysomes plus anti-RSA + carrier rat serum albumin	10	I	1,205	28	2.4 (1.6)
	20	I	2,150	45	2.1 (1.5)
	30	I	3,645	91	2.5 (2.0)
	50	I	6,130	123	2.0 (1.7)
Control (nascent chains from bound polysomes with normal IgG plus anti-rabbit-IgG [goat])	10	II	1,340	11	0.8
	20	II	2,650	18	0.6
	30	II	3,690	20	0.5
	50	II	6,430	19	0.3

* Two procedures to precipitate the labeled nascent polypeptide chains were employed: (I) nascent chains were first incubated with 1 mg of anti-RSA at 37°C for 20 min and at 2–4°C for 4 h, and then incubated at 2–4°C overnight. 40 μg of purified RSA was added as a carrier; (II) the first step was the same as above, followed by addition of anti-rabbit IgG serum (0.1 ml of goat serum) to the reaction mixture instead of carrier RSA. After the complete precipitation of the antigen antibody complex, each precipitate was sedimented by centrifugation at 3,000 rpm for 10 min. Precipitates were resuspended in 3 ml of 0.154 M NaCl 10 mM phosphate buffer, pH 7.4, and centrifuged as above. This washing procedure was repeated five times to remove adsorbed proteins.

‡ Values in the parentheses represent the percentages corrected for the normal IgG control.

in the counts precipitated. The percent of counts precipitated was higher (20.5%) at the lower amounts of antigen used and was in the same percentage range (11–12%) as with that of the other method (Method I) at higher amounts of antigen. Method I using antigen + anti-RSA + carrier showed a constant percentage of precipitated counts (11.0–11.6%) with all amounts of antigen (nascent polypeptide chains) used.

Preparation of Fab and Fc by Peptic Digestion

Rabbit IgG (anti-rat serum albumin, anti-serine dehydratase, and normal serum) were subjected to peptic digestion at pH 4.5 after or before iodination of the IgG preparation. The resulting fragments were separated into three major populations by gel filtration through Sephadex G-200, as shown in Fig. 3. The largest fragment was identified as *Fab* with a molecular weight of 92,000. This peptide retained the ability to precipitate with a specific antigen (Fig. 4). The Fab fragment had an identical equivalence point on the quantitative precipitin curve (1.0 mg of anti-rat serum albumin-*Fab* fragment precipitated 40 μg of purified rat serum albumin) to that of whole anti-rat serum albumin-IgG. A second eluted peak was identified as the *Fc* fragment with a molecular weight of 27,000, which was easily crystallized in ammonium sulfate solution. The third peak contained smaller fragments of IgG and possibly other proteins not exceeding 5,000 in molecular weight.

The distribution of ^{125}I in the fragments showed that the smallest fragment peak was most heavily labeled and the Fc peak, the least. The specific radioactivity of these fragments was as follows: $Fab = 1.6 \times 10^6$ cpm/mg protein and $Fc = 0.9 \times 10^6$ cpm/mg protein. To obtain an Fc fraction
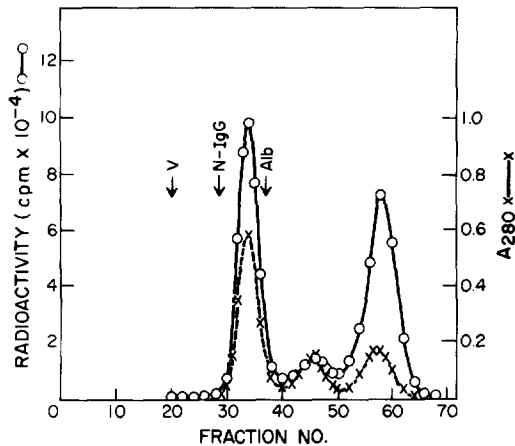
FIGURE 3 Gel filtration profile of peptic digest of ^{125}I-labeled anti-RSA eluted from Sephadex G-200. 10 mg of a peptic digest of ^{125}I-labeled anti-RSA which was prepared as described in the Materials and Methods was applied onto a Sephadex G-200 column (1.0 × 100 cm), previously equilibrated with 0.154 M NaCl–10 mM phosphate buffer, pH 7.8, containing 2 mM EDTA. 2-ml fractions were collected. A_{280}, (×—×) and ^{125}I radioactivity (o—o) are shown. V, void volume; N-IgG, nontreated IgG; Alb, rat serum albumin.

FIGURE 4 Immunodiffusion of pepsin-digested fragments of anti-RSA. (1) fraction directly precipitated with 12–19% Na_2SO_4 after peptic digestion, 6.8 mg/ml; (2) purified anti-RSA (nontreated), 15 mg/ml; (3) peak 1 from G-200 (Fab), 9.7 mg/ml; (4) same as (2); (5) peak 2 from G-200 (= Fc), 7 mg/ml. The center well contains purified RSA (40 µl of 250 µg/ml).

having a similar specific activity to that of the Fab fragment, the Fc fragments that had not been prelabeled were used for iodination separately in some experiments.

Separation of the Free and Membrane-Bound Polysomes

The relative RNA, protein, and phospholipid composition of the free (F-PR) and membrane-bound polysomes (B-PR) of rat liver is shown in Table II. It may be seen that the RNA to protein ratio of B-PR increased from 0.202 to 0.683 and the RNA to phospholipid ratio increased from 0.75 to 10.15 after detergent treatment and washing with high salt solution (0.5 M NH_4Cl). The F-PR had a similar composition before and after such treatment, this ratio being almost identical to that of the B-PR after detergent treatment. Since the membranes of the endoplasmic reticulum and the ribosomes contain high concentrations of phospholipid and RNA, respectively (37), the above data show that the F-PR fraction was not significantly contaminated by membrane components and thus was essentially free of any B-PR.
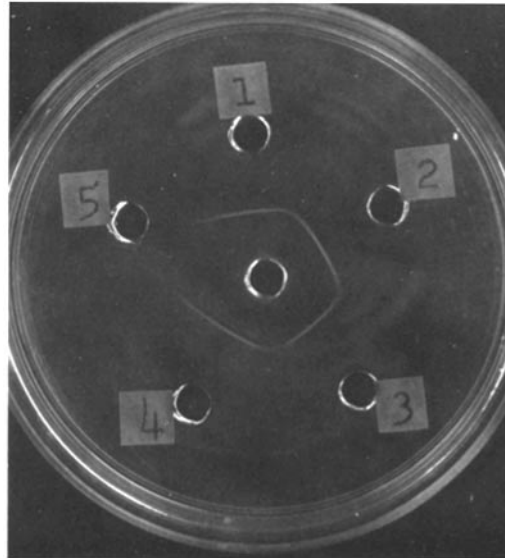
The B-PR fraction may become contaminated with the F-PR if the latter become entrapped in the matrix of the membranes at the interphase during discontinuous sucrose density centrifugation. This possibility was tested as follows. The F-PR and B-PR were labeled with [^{3}H] orotic acid in vivo by intraperitoneal injection. The labeled polysomes were incubated with unlabeled fractions according to the protocol shown in Table III. After keeping the mixture at 0–2°C for 30 min, the F-PR and B-PR were reisolated after various periods of centrifugation and the radioactivity in each fraction was determined in order to estimate the degree of cross-contamination between these two polysomal species.

When unlabeled F-PR were mixed with labeled B-PR, only 1–2% of the labeled polysomes sedimented with the free polysomes after a 4-h centrifugation, while 8–15% of the radioactivity sedimented with the F-PR after a 14-h spin. After a 6-h centrifugation of the mixture, 2–7% of the B-PR sedimented with the free polysomes. When unlabeled B-PR were mixed with labeled F-PR about 89% of the radioactivity sedimented to the bottom of the centrifuge tube after 4 h of centrifugation, while more than 95% of the radioactivity sedimented after 6–14 h of

TABLE II

Chemical Composition of the free and membrane bound polysomes of Rat Liver

	Ratio of RNA to protein	Ratio of RNA to phospholipid
Membrane bound polysomes		
Before detergent treatment and washing with 0.5 M NH_4Cl	0.202 ± 0.02	0.75 ± 0.07
After detergent treatment and washing with 0.5 M NH_4Cl	0.683 ± 0.07	10.15 ± 0.65
Free polysomes		
Before detergent treatment and washing with 0.5 M NH_4Cl	0.651 ± 0.06	8.65 ± 0.70
After detergent treatment and washing with 0.5 M NH_4Cl	0.704 ± 0.09	11.51 ± 0.75

The procedures for this experiment were as described under Materials and Methods. The values represent the average ± standard deviation for from four to six experiments.

TABLE III

Cross contamination of free (F-PR) and membrane bound (RER) polysomes

Combinations studied	Conditions of isolation	Centrifugation period					
		4 h		6 h		14 h	
		Free	Bound	Free	Bound	Free	Bound
		cpm		*cpm*		*cpm*	
F-PR (UL)* + RER (L)*	40,000 rpm 4 h	1,550 (1.9%)	78,100 (98.1%)	5,250 (6.6%)	74,600 (95.4%)	12,050 (14.8%)	69,500 (85.2%)
F-PR (UL) + per (L)	40,000 rpm 4 h	820 (1.0)	79,200 (99.0)	1,890 (2.3)	79,500 (97.7)	6,450 (8.1)	73,500 (91.2)
RER (UL)* + F-PR (L)*	40,000 rpm 4 h	90,500 (89.4)	10,800 (10.6)	95,600 (93.6)	6,500 (6.4)	98,050 (95.6)	4,500 (4.4)
RER (UL) + F-PR (L)	40,000 rpm 6 h	89,300 (88.5)	11,650 (11.5)	97,050 (95.0)	5,050 (5.0)	98,900 (95.8)	4,400 (4.2)

* The labeled (L) polysomes and rough endoplasmic reticulum (RER) were prepared by injection in vivo of [³H]orotic acid and were isolated by discontinuous density centrifugation at 40,000 rpm for 4 h or 6 h. The unlabeled (UL) polysomes and RER were also prepared in the same way. After mixing both preparations in the combinations noted above, the F-PR and RER were reisolated by 4-, 6-, and 14-h centrifugations and the radioactivity in each fraction was determined. See text for further details.

centrifugation. These observations show that the contamination of F-PR and B-PR with each other could be controlled to less than 5% by the 4-h centrifugation for the F-PR and by the 6–14-h centrifugation for the B-PR under these experimental conditions. These experiments do not exclude the possibility that some polysomes which are bound to membranes of the endoplasmic reticulum in vivo could have been removed from the membrane surfaces during homogenization. Furthermore, Murty and Hallinan (37) were able to demonstrate the presence of small amounts of agranular reticulum in preparations of free polysomes from rat liver.

Analysis of Polysomes

Typical polysome sedimentation profiles are presented in Fig. 5–7. There is no significant difference between the profiles of the free and membrane-bound polysomes after washing with high salt solution (0.5 M NH_4Cl) although the resolution of the small polysomes and monosomes derived from the membrane-bound polysomes
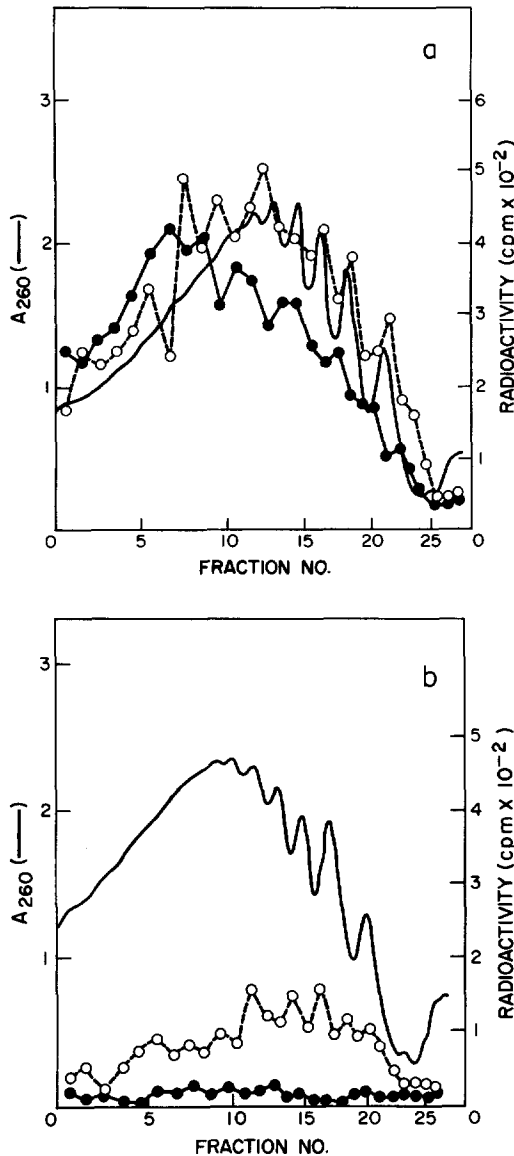
was poorer than those of the free polysomes before they were washed with a high salt solution. Most of the ribosomes (approximately 70%) sediment in the polysome region with the highest concentration in the region that corresponds to aggregates of 5–10 ribosomes. When nascent polypeptide chains were labeled in vivo with [14C]leucine, the radioactivity was predominantly in the polysome region. The absence of a radioactive peak in the monosome region indicates that the polysomes were not degraded extensively during isolation and analysis. Polysomes frozen at −70°C have the same sedimentation properties as the fresh preparation of polysomes; hence, the polysomes were frequently frozen before use. The $A_{260}:A_{280}$ ratio of the free polysomes varied between 1.78 and 1.84 (mean = 1.80), while that of the membrane-bound polysomes between 1.76 and 1.81 (mean = 1.79).

Binding of ^{125}I-labeled Anti-RSA to Rat Liver Polysomes

Fig. 5 a shows the distribution of radioactivity of anti-RSA-[125I]IgG and anti-RSA-[125I]Fab bound to the membrane-bound polysomes fractionated by density gradient centrifugation. An important characteristic of the binding of anti-RSA-[125I]Fab is that it is not uniformly distributed along the polysome profile but is predominantly associated with the region that corresponds to large polysomes, while anti-RSA-[125I]IgG is distributed almost uniformly along the A_{260} profile. Fig. 5 b shows the distribution of radioactivity after normal control rabbit [125I]IgG and [125I]Fab were bound to the same polysomes (B-PR) as that seen in Fig. 5 a. Although a significant amount of labeled normal control [125I]IgG is present in the oligosome region as well as in the polysome region, no binding of normal [125I]Fab is observed. These observations shown in Fig. 5 a and b indicate that nonimmune control [125I]IgG and some part of anti-RSA-[125I]IgG bind nonspecifically to liver polysomes. This evidence is compatible with the suggestions by Holme et al. (15, 16) and further argues that the Fab fragment reactive with RSA binds with a high degree of specificity to membrane-bound liver polysomes.

Fig. 6 shows similar experiments using free polysomes. A difference in distribution of IgG and

FIGURE 5 Sucrose density gradient profile of the membrane-bound polysomes and binding of rabbit anti-RSA-[125I]IgG (Fig. 5 a) and normal rabbit [125I]-IgG (Fig. 5 b) to fractionated polysomes. 20 A_{260}-units of the bound polyribosomes were applied onto a 10–40% isokinetic sucrose density gradient (16 ml/tube) and centrifuged in an SW 25.3 rotor at 25,000 rpm for 3.5 h. Fractions of 0.6 ml were collected with an accompanying A_{260} monitor (Gilford model 2400). Binding of anti-RSA-[125I]IgG and anti-RSA-[125I]Fab to each fraction of polysomes was carried out as described in the Materials and Methods. In Fig. 5 a, A_{260} (solid line), anti-RSA-[125I]IgG (○—○), and rabbit anti-RSA-[125I]Fab (●—●) are shown. In Fig. 5 b, A_{260} (solid line), normal rabbit-[125I]IgG (○—○), normal rabbit-[125I]Fab (●—●) are seen.
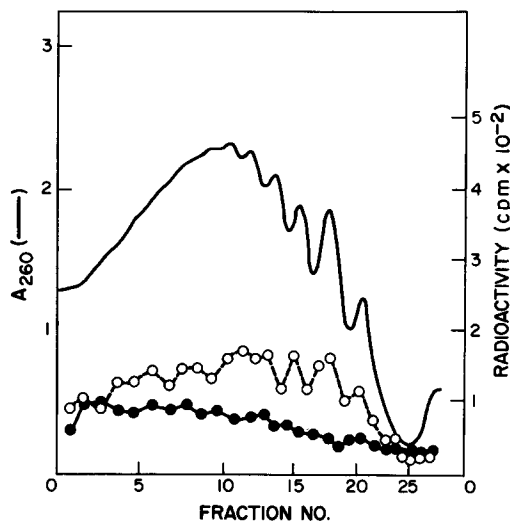
FIGURE 6 Sucrose density gradient profile of the free polysomes and binding of anti-RSA [^{125}I]IgG and -Fab. This is the same experiment using the free polysomes as described in Fig. 4 *a*. 20 A_{260}-units of the free polysomes were applied to the gradient. A_{260} (solid line), anti-RSA-[^{125}I]IgG (○—○), and anti-RSA-[^{125}I]-Fab (●—●) are shown.

FIGURE 7 Sedimentation profile of guinea pig liver bound polysomes. The polysome profile and radioactivity were determined as described in Fig. 5 *a*.

Fab of anti-RSA serum is also observed but a significant amount of anti-RSA-[^{125}I]*Fab* was bound to the free polysomes, indicating that the free polysomes are also involved in the synthesis of nascent polypeptide chains of the albumin molecule, a conclusion supported by the experiment of the direct precipitation technique using carrier RSA (Table I). To investigate further the specificity of the binding reaction of anti-RSA to polysomes, we studied guinea pig liver polysomes prepared by methods similar to those used for rat liver polysomes. Fig. 7 shows that no anti-RSA-[^{125}I]*Fab* is bound to guinea pig liver polysomes although anti-RSA-[^{125}I]IgG is bound to some extent to these polysomes. This further indicates that anti-RSA-IgG itself could not be used as a wholly specific antibody in binding experiments with liver polysomes (15).

It was of interest to know which part of the IgG molecule was responsible for the nonspecific binding to ribosomes and also to which part of the ribosome the IgG molecule binds. For this purpose, ribosomes were treated with puromycin in 0.5 M KCl at 37°C, followed by 5–20% sucrose density centrifugation in an SW25.3 rotor at 25,000 rpm for 3.5 h. Large and small subunits were obtained as shown in Fig. 8. Binding experi-
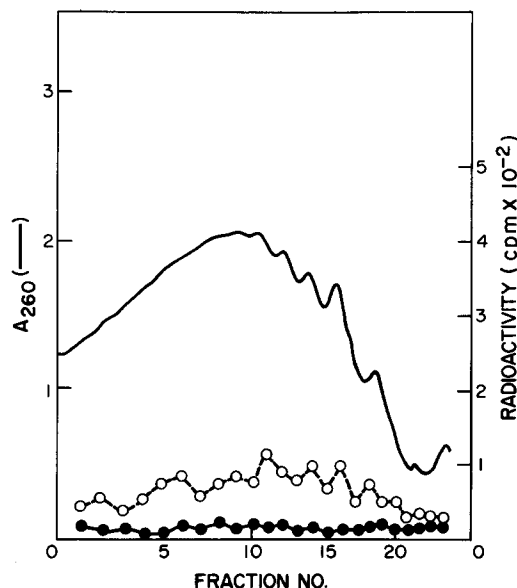
ments with anti-RSA-[^{125}I]IgG, [^{125}I]*Fab*, and [^{125}I]*Fc* were performed in the same way as described above. As shown in Fig. 8, only IgG bound to both large and small ribosomal subunits with an almost equal specific activity. Neither the *Fab* nor *Fc* fragment showed significant binding to the subunits. From these observations it is clear that both subunits have a similar affinity for the binding of IgG, but which part of the IgG molecule is responsible for this binding has not been determined.

Fig. 9 shows that after incubation of the membrane-bound polysomes of rat liver with anti-RSA-[^{125}I]*Fab*, radioactivity is distributed throughout the supernate, the polysome region, and the pellet. Most of the radioactivity is in the supernatant region and represents unreacted [^{125}I]*Fab* molecules. A significant amount of label is present in the pellet and much less radioactivity was distributed in the polysome region compared with those of Fig. 5 *a*. When the polysome-antiRSA complex was washed to remove the unreacted anti-RSA-[^{125}I]*Fab* and subjected to a precentrifugation on a density gradient (Fig. 9), it can be noted that the amount of radioactivity in the pellet was approximately 75% of the total polysome-bound radioactivity. It is possible that the radioactivity found in the pellet may be due to the immunological
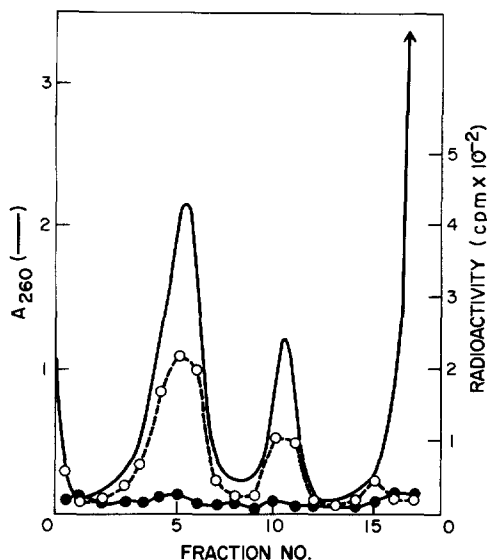
FIGURE 8 Sedimentation profile of ribosomal subunits and their reaction with anti-RSA-[^{125}I]IgG and -Fab. Ribosomal subunits were prepared by the method of Blobel and Sabatini (18), using puromycin. Approximately 10 A_{260}-units of ribosomal subunits were applied onto a 5–20% linear sucrose density gradient, followed by centrifugation at 25,000 rpm for 6.4 h at 18°C. A_{260} (solid line), anti-RSA-[^{125}I]IgG (o—o), anti-RSA-[^{125}I]Fab and -[^{125}I]Fc, (●—●) are seen.

FIGURE 9 Sedimentation profile of the bound polysomes after incubation of anti-RSA-[^{125}I]Fab and polysomes (20 units) with and without removal of unreacted [^{125}I]antibody. The conditions for binding of [^{125}I]antibody were described in Materials and Methods. A_{260} of the nonwashed sample (solid line), and of washed sample (dotted line) are shown. Radioactivity of nonwashed sample (×—×) and of washed sample (o—o) are also presented.

cross-linking of two or more polysomes that by themselves would normally sediment at a lower rate, since most of the radioactivity was distributed in the polysome region and much less radioactivity was at the bottom when method (a) was employed as the binding procedure (see Fig. 5 a and Materials and Methods). Hence, the procedure described above could be used to isolate those polysomes synthesizing a specific protein.

Binding of Anti-Serine Dehydratase
[^{125}I]Fab Monomer and Dimer to Free and Membrane-Bound Polysomes

Since rat serum albumin is a protein secreted by the liver, the binding of anti-RSA-IgG and Fab fragment (dimer) predominantly to membrane-bound polysomes is in support of earlier workers (5–8). Since the intracellular site of synthesis of enzymes for the intracellular economy is not so clear as with that of secretory proteins, we extended the studies on rat serum albumin to the hepatic enzyme, serine dehydratase. In Fig. 10 is seen the reaction of anti-serine dehydratase-
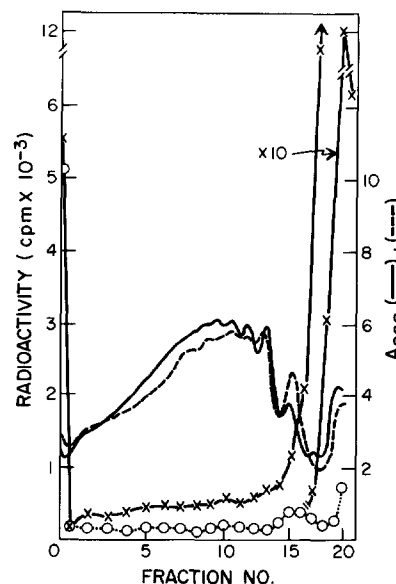
[^{125}I]Fab dimer with free (a) and membrane-bound (b) polysomes from livers of rats fed laboratory chow and fasted for 15 h. As can be seen from this data, whether it is expressed as total or specific radioactivity, a substantial amount of polysome-bound reactive serine dehydratase antigen is seen in both free and membrane-bound fractions, unlike that with rat serum albumin where the majority of the reactive albumin was associated with bound polysomes. However, in view of the fact that chow-fed animals contained variable amounts of serine dehydratase, it became of interest to determine the distribution of the polysome-bound serine dehydratase in animals where the enzyme had been induced by hormonal and dietary means. For this purpose, rats were fed a 90% protein diet for 6 days and then given a subcutaneous injection of 2 mg of glucagon. The results of this experiment and the binding of anti-serine dehydratase-[^{125}I]Fab dimer are seen in Fig. 11. In this instance, a larger proportion of the radioactivity is associated with the bound polysomes suggesting that it is this polysome
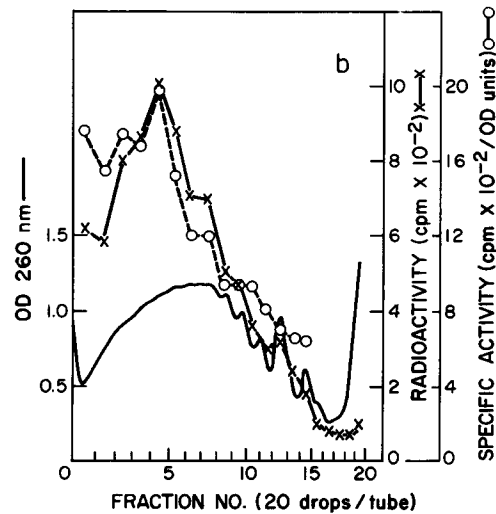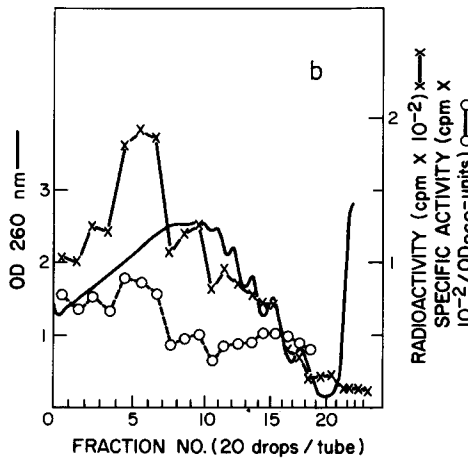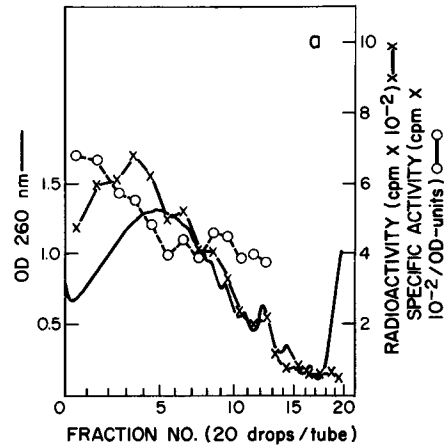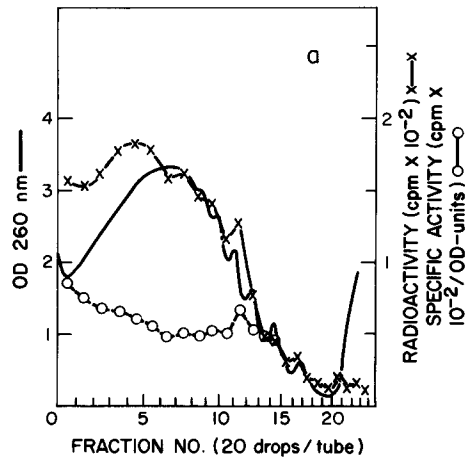
FIGURE 10   Free and membrane-bound polysome pro-
files from livers of rats fed chow and fasted 16 h.
Polysome profiles were obtained by sucrose density
gradient centrifugation and fractions obtained. The
fractions were mixed with pepsin-digested anti-serine
dehydratase-[^{125}I]Fab dimer and incubated at 2–4°C
overnight. The reaction mixtures were then centrifuged
and counted for radioactivity as described in Materials
and Methods. The specific activity was expressed as
radioactivity per OD_{260} units per fraction. The results
with free polysome (a) and membrane bound polysomes
(b) are demonstrated.

fraction synthesizing serine dehydratase to the
greatest degree in induced animals.

In order to substantiate further this finding,
animals that had been given glucagon were treated
in the same manner as described in Materials and
Methods and the free and bound polysome frac-
tions isolated. In this instance, however, the bind-
ing of anti-serine dehydratase-[^{125}I]Fab monomer

FIGURE 11   The free and membrane-bound polysome
profiles from livers of rats fed a 90% protein diet for
6 days followed a subcutaneous injection of 2 mg of
glucagon. The methodology is the same as in Fig. 10
showing the free polysomes (11 a) and membrane-bound
polysomes (11 b).

was determined and the data seen in Fig. 12. In
this figure it can be seen even more clearly that
the bound polyribosomes contain substantially
more of the reactive serine dehydratase antigen
than do the free polysomes. As controls for these
studies, polysomes were isolated from the kidney
of rats and purified in the presence of ribonuclease
inhibitor which had been purified from guinea
pig liver. Kidney polysomes were then mixed
with purified serine dehydratase, incubated at
2°C for 1 h, washed with 0.5 M NH Cl, and the
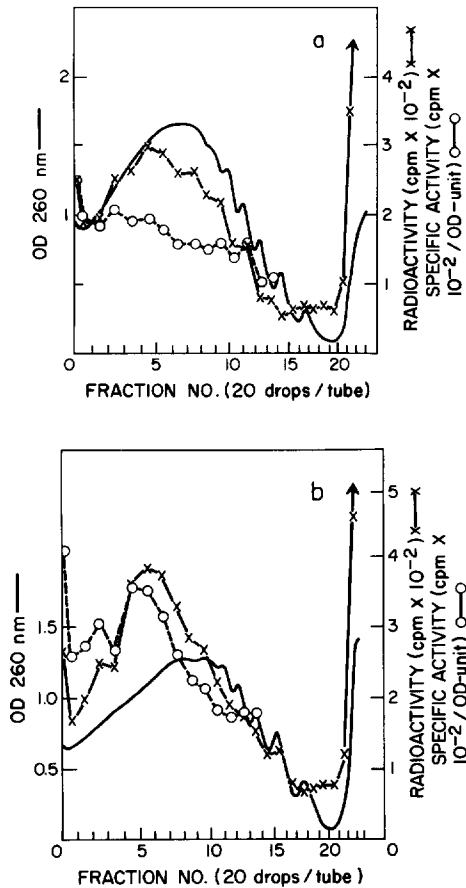polysomes separated on sucrose density gradients

FIGURE 13 Nonbinding of anti-serine dehydratase-[125I]Fab monomer and dimer to kidney polysomes. Rat kidney polysomes were isolated in the presence of ribonuclease inhibitor purified from guinea pig liver. 10 OD-units of kidney polysomes were mixed with 15 μg of purified serine dehydratase or an equivalent amount of crude supernatant fractions of liver. When the Fab dimer was used, the polysomes were incubated at 2°C for 1 h, washed with high salt solution, and separated on a sucrose density gradient as described in Materials and Methods. Polysome fractions were used for binding of anti-serine dehydratase-[125I]Fab dimer. When the Fab monomer was used, the technique employed was identical to that of Fig. 12. The single line (×—×) shows the data for both dimer and monomer.

FIGURE 12 The binding of anti-serine dehydratase-[125I]Fab monomer to free and membrane-bound polysomes from livers of rats given 2 mg of glucagon simultaneously. The Fab monomer preparation was obtained and iodinated as described in Materials and Methods. The labeled Fab monomer was mixed with the polysome suspension and incubated at 2-4°C overnight. The entire mixture was then applied to a sucrose density gradient (10-40%) spun at 25,000 rpm for 3.5 h in an SW 25.3 rotor and fractionated (20 drops per tube). The radioactivity of the fractions were then determined as described previously. (a) shows the free polyribosomes and (b) the bound polyribosomes, respectively.

as described in Materials and Methods. These experiments were carried out both with the Fab monomer and dimer using kidney polysomes premixed with serine dehydratase. The results of these experiments, seen in Fig. 13, demonstrate very clearly that neither of the Fab preparations which specifically react with serine dehydratase on liver polysomes bind significantly to kidney polysomes. Thus, from these experiments one
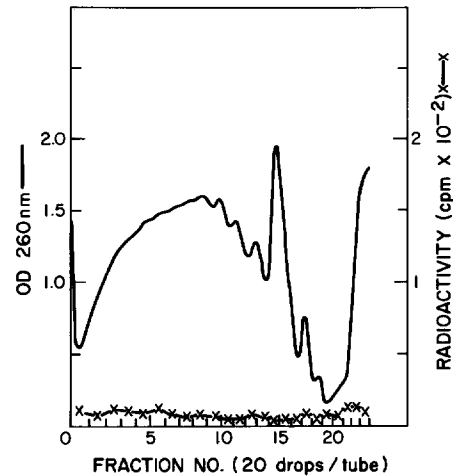
may conclude that the bound polyribosomes contribute to a very large extent in the intracellular synthesis of the hepatic enzyme, serine dehydratase.

DISCUSSION

The data reported in this paper demonstrate that polysome-associated albumin and serine dehydratase occur in liver either predominantly, or, to a large extent, in combination with polysomes bound to the membranes of the endoplasmic reticulum. These studies have attempted to control many of the problems which have beset investigators in the past in localizing the sites of synthesis of specific proteins within the cell.

The RNA, protein, and phospholipid composition of ribosomal fractions has been used as a criteria to measure the degree of contamination with membranous material and nonribosomal proteins (3, 37). Murty and Hallinan (37) have reported RNA to phospholipid ratios of 3.8 and 36, respectively, for the bound polyribosomes of rat

liver before and after deoxycholate treatment. Andrews and Tata (3) found that the RNA to phospholipid ratio of hepatic membrane-bound polysomes was 2.4. This value was obtained for the heavy rough microsomal membranes. The same authors demonstrated that the ratio for free polysomes was 21.0. Studies by Webb et al. (38) and Bloemendal et al. (39) demonstrated RNA to phospholipid ratios in rough microsomal membrane to be 6.2 and 6.7, respectively. A comparison of these values with those obtained in Table II indicates that the membrane-bound polysomes of our experiments had a higher phospholipid content than those reported. Even after 0.5% deoxycholate treatment the residual phospholipid was higher than that of the previous observations. On the other hand, Olsnes (40) demonstrated that treatment of polysomes with concentrations of 0.5 M salt completely removed all adsorbed protein from their preparations. In our studies, both 0.5 M KCl and NH_4Cl washes were used routinely for all preparations. The efficacy of this treatment may be seen in the removal of serine dehydratase from kidney polysomes (Fig. 13).

While the phospholipid analysis indicated that the free polyribosomes prepared by discontinuous density gradient centrifugation were as free from membrane contamination as polysomes prepared by detergent treatment, the absence of any significant cross-contamination between the two classes of polysomes was directly shown by incubations of the mixtures of unlabeled and radioactively labeled polysomes (Table III). This is further evidence that the occurrence of these two classes of polysomes in the sucrose gradient is not an artifact of preparative procedures, but corresponds to the free and bound polysomes, observed in vivo by electron microscopy.

Recently, Holme et al. (15, 16) demonstrated that when whole antisera (anti-rabbit hemoglobin) were used in reactions between polysomes and antibody, the interaction was not totally specific. One cause for this nonspecificity was ascribed to the binding of IgG to ribosomes through the Fc portion. Their results clearly demonstrated that the digestion of IgG with pepsin leads to a more specific precipitation of polysomes. In the present experiments we confirmed their finding in that the reaction of polysomes bearing RSA or serine dehydratase with Fab fragments exhibits greater selectivity than with IgG. Although it was suggested by Holme et al. (16) that nonspecific

binding of IgG to the ribosomes was through the Fc portion, our experiments showed that the Fc fragment which was digested with pepsin and isolated from Sephadex G-200 (or G-100) did not bind to ribosomes (Fig. 8). One of the other possibilities for the nonspecific binding is the involvement of the third component of peptic digests of IgG (Fig. 3). Since this component(s) is dialyzable, its molecular size must be less than 6,000 and it is difficult to obtain enough concentrated material for a binding experiment. As yet we have not obtained conclusive results in localizing the nonspecific binding site of IgG.

In the determination of polysomal-bound antigenic protein by immunochemical means, specific reactions of antibody with antigen on polysomes must be clearly differentiated from the nonspecific binding of antibody. Indeed, previous studies of antibody binding to polysomes have generally been difficult to interpret because of insufficient controls to show polysomal integrity and specificity of binding. One of the main emphases of this paper is to demonstrate the specificity of this binding reaction by IgG and Fab fragments. Several lines of evidence support the proposal that the anti-RSA- and anti-serine dehydratase-[^{125}I]Fab is reacting only with those polysomes that synthesize these respective proteins. The ^{125}I radioactivity in polysomes is not distributed randomly, but rather, it is associated mainly with large polysomes (Fig. 5 a). The Fab prepared from normal rabbit IgG does not bind to rat liver polysomes (Fig. 5 b), and conversely, anti-RSA-[^{125}I]Fab does not react with polysomes extracted from guinea pig liver (Fig. 7). When rat liver supernate is mixed with a homogenate of guinea pig liver before the isolation of polysomes, the anti-RSA-[^{125}I]Fab does not react with the polysomes of guinea pig liver, indicating that the binding is not due to a nonspecific association of albumin with the polysomes. Moreover, when polysomes were incubated with radioactive albumin, no radioactivity was found in the polysome region (30). The same conclusions may be stated with respect to anti-serine dehydratase-[^{125}I]Fab monomer or dimer as seen in Fig. 13. In this instance, kidney polysomes, even when premixed with serine dehydratase and subsequently washed, did not react at all with this specific anti-serine dehydratase-Fab preparation. In studies not described in this paper, both anti-serine dehydratase-IgG and Fab dimer were reacted with both whole polysomes and the puromycin-

released material. Reactions completely analogous to those with serum albumin (Table I, Figs. 5–7) were elicited. Furthermore, earlier studies (35) from this laboratory have shown by means of radioactive labeling in vivo the presence of immunoreactive serine dehydratase antigen in a nascent form bound to polysomes of the rough endoplasmic reticulum. In other experiments not reported here, serine dehydratase antigen added at the initiation of homogenization of either kidney or of liver from an animal fed 0% protein for 5 days (41) was completely removed from polysome preparations by washing in 0.5 M salt. These studies further support the contention that the immunoreactive serine dehydratase associated with free and bound polysomes and reacting with the antiserine dehydratase-[^{125}I]Fab monomer is in fact the nascent chain of the enzyme. Furthermore, these studies demonstrate that this technique is extremely sensitive being able to detect nascent chains which constitute as little as 1% of the total population on the polysomes.

In addition to identifying specific polysomes, the binding of antibodies to polysomes has important consequences such as: (a) the possibility of quantitating the relative number of specific nascent chains in a polysomal preparation and hence indirectly measuring the amount of translatable messenger RNA (mRNA), and (b) the precipitation of specific polysomes, either directly with antigen or indirectly with anti-immunoglobulins. Previous studies have shown that ribosomes from liver, muscle, and plasma cells can be precipitated with antibodies to albumin (6), myosin (14), and immunoglobulins (12, 13), respectively. However, the specificity of the precipitation reaction has not been demonstrated completely in most of these studies. The ability to isolate undegraded polysomes synthesizing a single polypeptide chain has already been demonstrated as a valuable tool for isolating and characterizing the components (mRNA and mRNA-specific proteins) involved in the synthesis of specific proteins (42).

Other workers have demonstrated that about 15% of albumin-synthesizing polysomes appeared to be free polysomes (8, 43) while Takagi et al. (44) presented evidence that virtually all albumin synthesis occurred on bound polysomes of rat liver. The possible role of the small number of free polysomes synthesizing serum albumin in liver reported by these authors and also demon-

strated in this paper (Fig. 6) is of potential interest. Both Jungblut (45) and Peters (46) demonstrated low levels of serum albumin present free in the cytoplasm of hepatic cells. In both instances the amount of albumin in the cytosol amounted to between 7 and 15% of the total intracellular albumin. Recently Uenoyama and Ono (47) showed that in the Morris 5123 hepatoma, in which albumin synthesized is not exported but remains within the cell in the cytosol, albumin synthesis occurs almost entirely on free polyribosomes. Ferritin (8, 43, 48) and arginase (49) are synthesized on free polysomes to a large extent. On the other hand, significant amounts of ferritin are associated with bound polysomes (8, 43, 48) as is the case also with arginase (49). Furthermore, Andrews and Tata (3) demonstrated that not all proteins synthesized on bound polysomes are secreted and Ragnotti et al. (50) demonstrated that NADPH-cytochrome c reductase, which is associated with the microsomal membrane, is synthesized from both free and bound polysomes to essentially the same extent. The demonstration by these studies that an intracellular enzyme of the cytosol, serine dehydratase, is synthesized on both free and membrane-bound polysomes (Fig. 10) and to a greater extent on membrane-bound polysomes in the livers of rats in which the synthesis of the enzyme is induced to a high rate by glucagon and a 90% protein diet (Fig. 11) further demonstrates in support of these latter papers that enzymes important in the intracellular economy are synthesized on both free and membrane-bound polysomes. Furthermore, if the experiments reported in this paper are considered in light of the data of Blobel and Potter (51), serine dehydratase synthesis probably occurs predominantly on bound polysomes. How this fact relates to the stabilization of the serine dehydratase template as discussed in an earlier paper from this laboratory (52) will be the subject of future experimentation.

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