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Studies on the Amino Acid Acceptor RNA in Washed Liver Microsomes*

KENDRIC C. SMITH

From the Department of Radiology, Stanford University School of Medicine, Palo Alto, California

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The amount of amino acid acceptor RNA found associated with the microsomes (or ribosomes) depends upon the nature of the medium used in their isolation and upon the time used for their sedimentation. When homogenates of mouse liver were sedimented for varying times at 105,000 $\times$ *g* and the supernatant fraction assayed for RNA and protein content and for its ability to bind amino acids to RNA, it was found that the amino acid acceptor RNA's were not "soluble" but were sedimented out of solution and at different rates. The post-microsomal particles and microsomes can be aggregated with increasing concentrations of $MgCl_2$ up to about 0.015 M and then added $MgCl_2$ causes disaggregation and loss of sedimentation properties. At 0.015 M $MgCl_2$ about half of the RNA normally found in the soluble fraction is sedimented by one hour at 105,000 $\times$ *g*. The ability of the remaining RNA to accept amino acids is increased by only about 20%, and this fact indicates that the sedimented RNA also contains a significant amount of amino acid acceptor activity. The amount of amino acid acceptor RNA associated with the microsomes due to the above conditions can be modified to some extent by the nature of subsequent washing steps. These have included multiple washings with 0.25 M sucrose–0.001 M $MgCl_2$, and treatment with 0.3% deoxycholate or with 0.5 M NaCl. However, no conditions have been found for the complete removal of all of the amino acid acceptor activity. The amino acid acceptor RNA that cannot be washed free from the microsomes does not appear to be due to simple contamination by soluble RNA since the ratios of the acceptor activities of this RNA for leucine, valine, tyrosine, and methionine are different from those found for soluble RNA. Also, the amino acid acceptor RNA from the microsomes differs chromatographically from the majority of the soluble RNA and is more similar to a large portion of that found in the post-microsomal fraction. Microsomal RNA freed of amino acid acceptor RNA (by chromatography) still contains 7% of its uridylic acid as the 5-ribosyl isomer. It thus appears extremely difficult (if not impossible) to completely wash microsomes (or ribosomes) free of amino acid acceptor RNA.

In studies on the incorporation of amino acids into microsomes, the degree of the requirement for added soluble RNA would seem to be proportional to the amount of washing that the microsomes or ribonucleoprotein particles have received. No technique of washing has been reported, however, which removes all of the residual activity of the microsomes or ribonucleoprotein particles. The fact that a portion of the amino acid acceptor activity cannot be removed has a direct bearing on the interpretation of experiments depending upon the addition of extra soluble RNA. This report outlines some of the differences between the amino acid acceptor RNA in the washed microsomes and that found in the soluble fraction and indicates some of the factors that can help shift amino acid acceptor RNA from the soluble to the microsomal fraction (and *vice versa*).

EXPERIMENTAL

Materials.—ATP (dipotassium), GTP, and UTP were purchased from Pabst Laboratories; deoxycholic acid and creatine phosphate from California Corporation for Biochemical Research; protamine sulfate and glutathione from Mann Research Laboratories; uniformly labeled C^{14}-algal protein hydrolysate (88 μc/mg), L-leucine-C^{14} (9280 cpm/mμmole), L-lysine-C^{14} (8234 cpm/mμmole), L-tyrosine-C^{14} (8109 cpm/mμmole), L-valine-C^{14} (7806 cpm/mμmole), and L-methionine-methyl-C^{14} (6721 cpm/mμmole) from Nuclear-Chicago Corporation; Cato-2, a diethyl amino ethyl derivative of starch, was from National Starch and Chemical Corporation.

Preparation of Soluble RNA.—Livers from 40–90 day old C57BL mice were homogenized in 3 volumes of 0.25 M sucrose containing 0.001 M $MgCl_2$, and centrifuged for 10 minutes at 17,000 $\times$ *g*. The supernatant fluid was centrifuged for 1 hour at 105,000 $\times$ *g*. This final supernatant was made 0.1 M in Tris buffer (*p*H 7.5) and shaken for 1 hour at 4° with an equal volume of 90% phenol (freshly distilled). After centrifugation of the mixture for 30 minutes at 3500 $\times$ *g*, the phenol layer was briefly extracted with a volume of water equal to that removed and again centrifuged. The pooled extracts were made to 2% with potassium acetate, and two volumes of cold 95%

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ethanol were added. The mixture was allowed to stand overnight at 4° (see footnote 6). Precipitate was recovered by spinning for 2 hours at 2000 rpm in an International Centrifuge. The precipitate was suspended in cold 70% ethanol–0.1 M NaCl and again centrifuged. The well-drained precipitate was dissolved in 0.1 M NaCl, and two volumes of cold 95% ethanol were added and the mixture allowed to stand overnight. After recovery of the precipitate by centrifugation the residual alcohol was removed under vacuum and the RNA was dissolved in 0.01 M NaCl and stored at −15°. This material had an $E(P)$ at 260 mμ of 6191 ± 296 (average for four preparations).

Preparation of Microsoma lRNA.—The 105,000 × *g* pellet as described above was resuspended by homogenization in its original volume of the sucrose-$MgCl_2$ solution and again spun for 1 hour at 105,000 × *g*. After decanting and draining, the tops of the pellets were rinsed with the sucrose solution. The pellets were again suspended in their original volume of sucrose solution. The solution was made to 0.1 M in Tris buffer, *p*H 7.5, and the isolation of the RNA was carried out as described above for soluble RNA. The final solution, however, was spun at 105,000 × *g* for 1 hour to remove glycogen before the sample was frozen. This material had an $E(P)$ at 260 mμ of 7496 ± 205 (average of four preparations).

From 1 g of liver approximately 13 OD_{260} units[1] of microsomal RNA and 7 units of soluble RNA were recovered.

Isolation of Purified pH 5 Enzyme.—The 105,000 × *g* supernatant, as described above, was made to *p*H 5.2 with 1 M acetic acid. This solution was then spun for 10 minutes at 3500 × *g*. The pellets were suspended in one fourth of their original volume of 0.1 M Tris buffer at *p*H 7.5, and again precipitated at *p*H 5.2, centrifuged, and redissolved in 0.1 M Tris. This solution was spun for 10 minutes at 3500 × *g*. The milky supernatant was diluted 1:50 with 0.1 M Tris and the amount of nucleic acid[2] present was estimated by optical density readings at 260 and 280 mμ (Warburg and Christian, 1942). To this solution was added an amount of protamine sulfate (5 mg/ml in 0.1 M Tris at *p*H 7.5) equal in weight to 1.5 times the weight of RNA present. After stirring for 15 minutes at 4° the solution was spun for 10 minutes at 3500 × *g*. The clear supernatant was dialyzed overnight against 2 liters of 0.02 M Tris at *p*H 7.5; 0.001 M glutathione; 0.0001 M versene. The dialyzed solution was clarified by centrifugation and lyophilized. This enzyme preparation is essentially free of RNA (Warburg and Christian, 1942) and is stable for months when kept cold and dry. A solution of the enzyme frozen overnight at −15° loses most of its activity. Solutions therefore are made up fresh as needed from the dry powder. Approximately 1.8 mg of protein (3.9 mg of lyophilized powder) was recovered from 1 g of liver.

Measurement of Incorporation of Labeled Amino Acids into RNA.—The method was essentially that of Schweet *et al.* (1958). The reaction mixture contained 10 μmoles of potassium ATP (*p*H 7.5); 100 μmoles Tris buffer (*p*H 7.5); 10 μmoles $MgCl_2$; 40 mμmoles of uniformly labeled C^{14}-L-amino acid; 1.0 OD unit (260 mμ) of soluble RNA or 5.0 units of microsomal RNA; an amount of purified enzyme to give maximum incorporation for the particular amino acid; and water to a final volume of 1.5 ml. The mixture was incubated for 20 minutes at 37°; 7.5 mg of casein (15 mg/ml in 0.1 N NaOH) was added and the mixture precipitated with 10 ml of cold 3.5% trichloroacetic acid. The precipitate was washed two times with cold 0.2 N perchloric acid, then partially dissolved in 2 ml of ethanol–0.2 N perchloric acid (5:1) and reprecipitated with 5–7 volumes of ether, and finally washed once in hot ethanol–ether (3:1) and twice with ether.

The RNA was extracted from the washed and ether-dried reaction mixture by heating in 1.0 ml of 0.5 M perchloric acid at 80° for 30 minutes. An aliquot was used for determining the amount of RNA present (optical density), and 0.5 ml was counted in a Packard Tri-Carb Liquid Scintillation Counter. The counting vial contained 0.5 ml of sample in 0.5 M perchloric acid; 0.5 ml of 95% ethanol; and 15 ml of counting solution composed of 2.15 g PPO (2,5-diphenyloxazole), 17.5 mg POPOP [1,4-bis-2(5-phenyloxazolyl)-benzene], 22 g naphthalene, 250 ml 1,4-dioxane, and 50 ml xylene.

Measurement of Incorporation of Labeled Amino Acids into Protein.—The complete reaction mixture contained 3 μmoles potassium ATP (*p*H 7.5); 3 μmoles potassium GTP (*p*H 7.5); 30 μmoles creatine phosphate (*p*H 7.5); 30 μmoles Tris buffer (*p*H 7.5); 0.23 mmole KCl; 0.02 mmole $MgCl_2$; 80 mμmoles uniformly labeled C^{14}-L-amino acid; 0.2 mg crystalline inorganic pyrophosphatase[3]; 2.0 ml of washed microsomes from a 25% homogenate in 0.25 M sucrose (0.001 M $MgCl_2$); 0.5 mg purified *p*H 5 enzymes; three optical density units (260 mμ) of amino acid acceptor RNA; and water to a final volume of 3.0 ml. The mixture was incubated for 20 minutes at 37° and cooled in ice, and 3 volumes of 0.25 M sucrose–0.001 M $MgCl_2$ were added and the mixture spun at 105,000 × *g* for 60 minutes. The tops of the pellets were rinsed with the sucrose solution. The pellets were transferred to glass tubes and precipitated with cold 5% trichloroacetic acid, washed twice with cold 0.2 M perchloric acid and once with warm alcohol-ether (3:1), and then extracted with 0.5 M per-

[1] OD_{260} units are defined as the absorption of a solution of nucleic acid at neutrality at 260 mμ (1-cm cell).

[2] The nucleic acids present are considered to be primarily RNA since the nuclei had previously been removed by centrifugation.

[3] A generous gift of Dr. Moses Kunitz (see Kunitz, 1961).

chloric acid at 80° for 30 minutes. The residue was dissolved in 2.0 ml of 0.1 N NaOH. One aliquot was used for the determination of protein (Lowry *et al.*, 1951) and another for the determination of radioactivity. The counting vial contained 0.5 ml of protein solution in 0.1 N NaOH, 1 ml Hyamine (Packard Instrument Co.); 4 drops glacial acetic acid; and 15 ml of counting solution (see above).

Results

An Attempt to Wash Out the Amino Acid Acceptor RNA from the Microsomes.—The RNA isolated by phenol extraction from multiply-washed microsome preparations has been tested for its amino acid acceptor RNA content (Table I).

Table I

The Amino Acid Acceptor Activity of RNA from Washed Microsomes

A C^{14}-algal protein hydrolysate was used in the assay for amino acid acceptor activity. Further experimental conditions are described in the text.

RNA from Washed Microsomes (Times Washed)	Amino Acid Acceptor Ability (cpm/OD_{260})	% Amino Acid Acceptor RNA as *S*-RNA
0	439	9.3
1	233	4.9
2	198	4.2
3	216	4.6
	Soluble RNA: 4722	

Once-washed microsomes would correspond to our standard isolation procedure. Subsequent washings were merely repetitions of the first washing procedure. The first wash in 0.25 M sucrose–0.001 M $MgCl_2$ removes about half of the amino acid acceptor RNA from the microsomal preparation, but subsequent washings do not reduce this value. The amino acid acceptor RNA present in the washed microsomes does not seem to be due to a simple contamination by loosely associated soluble RNA.

In another series of experiments somewhat more drastic conditions were used in the hope of removing the amino acid acceptor RNA from the microsomes (Table II). The once-washed microsomes were further treated with 0.3% sodium deoxycholate (Siekevitz and Palade, 1959) and the RNA was isolated from the deoxycholate soluble and insoluble fractions by extraction with phenol. Only about 50% of the RNA was recovered by phenol extraction from the microsomes treated with deoxycholate as compared to the control microsomes. The RNA lost was apparently inert in accepting amino acids since the remaining RNA still contained an appreciable amount of amino acid acceptor activity, and at a specific activity about twice that of the control. It has been reported that ribonucleoprotein particles which were isolated with deoxycholate con-

Table II

The Amino Acid Acceptor Activity of the RNA from Chemically Treated Microsomes

Control microsomes were treated either with 0.3% deoxycholate (Siekevitz and Palade, 1959) or with 0.5 M NaCl (Littlefield and Keller, 1957) and reisolated. RNA was isolated by phenol extraction from both the pellet and supernatant fractions and assayed for amino acid acceptor activity using a C^{14}-algal protein hydrolysate (see Experimental).

RNA Sample	Fraction of Total *M*-RNA (%)	Amino Acid Acceptor Ability (cpm/OD_{260})	Acceptor Activity Recovered (%)
M-RNA (Control)	100.0	190	
(A) Deoxycholate-soluble	4.2	1017	138[a]
Deoxycholate-insoluble	48.0	460	
(B) NaCl-Soluble	1.4	2587	
NaCl-Insoluble	98.4	142	93
S-RNA		8668	

[a] Calculated as the sum of (fraction of total *M*-RNA/100) × (cpm/OD_{260}) divided by (cpm/OD_{260}) for the control.

taining a stabilizing concentration of magnesium were still able to incorporate amino acids at a reduced but significant rate without added soluble fraction (Kirsch *et al.*, 1960). Deoxycholate treatment therefore does not seem to remove all of the amino acid acceptor RNA from the microsomes or ribonucleoprotein particles.

Treating the microsomes with 0.5 M NaCl according to the conditions of Littlefield and Keller (1957) also failed to remove all of the amino acid acceptor RNA but appeared to be more efficient than the deoxycholate treatment (Table II).

5-Ribosyl Uridylic Acid Content of Soluble and Microsomal RNA.—Soluble RNA has been shown to contain a high proportion of its uridylic acid as the 5-ribosyl isomer, whereas microsomal RNA contains only a few per cent (Dunn, 1959). It has been suggested (Dunn, 1959), that the few per cent of 5-ribosyl uridylic acid found in the microsomes might be due to contamination and that pure microsomal RNA might contain none. Since we were able to separate the amino acid acceptor RNA found in the microsomes from the bulk of the microsomal RNA on columns of Cato-2 (see below), it seemed that this would offer the chance to test this hypothesis, for if the hypothesis were true, the non-amino acid acceptor RNA would contain no 5-riboysl uridylic acid. Table III shows that contrary to what was expected the microsomal RNA was not devoid of 5-ribosyl uridylic acid. The alkaline chromatographic peak of microsomal RNA, which is essentially devoid of amino acid acceptor activity (see below),

Table III

5-Ribosyl Uracil Monophosphate Content of Soluble RNA and Microsomal RNA from Mouse Liver

Approximately 25 mg each of soluble RNA and microsomal RNA were separated on large columns of Cato-2 (2.2 × 27.0 cm) into two fractions by elution first with 1 M NaCl and then with 1 M NaCl containing 1 M NH_4OH (Smith *et al.*, 1960). The neutral salt and alkaline salt peaks were recovered by alcohol precipitation. The 1 M NaCl peak contains essentially all of the amino acid acceptor activity of the RNA isolated from washed microsome preparations (see Table VI). The RNA fractions were hydrolyzed in alkali and the nucleotides separated by paper electrophoresis in 0.4 M ammonium formate at *p*H 2.5. The uridylic acid band for each RNA fraction was extracted from the paper with water and the extracts taken to dryness under vacuum. This material was chromatographed in isopropyl alcohol–acetic acid–water (60:30:10, v/v/v) containing 0.004 M EDTA. The oligonucleotide material remaining at the origin (Smith and Allen, 1953), the 5-ribosyl uridylic acid (identified by its spectral shift and R_F [Davis and Allen, 1957]), and uridylic acid were eluted from the paper and assayed, and the data were expressed as the per cent of the total ultraviolet-absorbing material present in the latter two fractions. 100 μg uridylic acid was assayed for its ψ-UMP content for *S*-RNA and 176 and 292 μg, respectively, were assayed for *M*-RNA.

Chromatographic Fraction	Recovered from Column (% of Input)	% of Uridylic Acid ψ-UMP[a]	% of Uridylic Acid Oligo
M-RNA			
Unfractionated		9[b]	4
Neutral peak	11	—[c]	—
Alkaline peak	90	7	2
S-RNA			
Unfractionated		19	7
Neutral peak	85	22	5
Alkaline peak	10	—[c]	—

[a] ψ-UMP: 5-ribosyl uracil monophosphate. [b] Values are the average of duplicate determinations. [c] Sample too small to analyze.

contains 7% of its uridylic acid as the 5-riboysl isomer. It seems improbable, therefore, that the amount of this compound found in the microsomes could be due entirely to contamination with loosely associated amino acid acceptor RNA. Dunn *et al.* (1960) have recently reported that treatment of microsomes with 0.5% deoxycholate fails to remove all of the RNA containing 5-ribosyl uridylic acid.

Amino Acid Acceptor RNA from Washed Microsomes.—When microsomal RNA obtained from the standard washed microsomal preparation was treated with 1 M NaCl as originally described by Crestfield *et al.* (1955), it was found that nearly all of the amino acid acceptor RNA could be separated from the inert microsomal RNA on the basis of the latter's insolubility in 1 M NaCl at 0° (see also Smith, 1960). The yield was about 6% of the total RNA, but the RNA

Table IV

Comparison of Amino Acid Acceptor Activity of Soluble RNA and of Microsomal RNA Soluble in 1 M Salt at 0°

Experimental conditions are described in the text.

Radioactive L-Amino Acid	Radioactivity Incorporated into RNA (cpm/OD_{260} unit): Soluble RNA	Salt-Soluble Microsomal RNA	Ratio *S*-RNA / *SSM*-RNA
Leucine	797	123	6.5
Valine	430	89	4.8
Tyrosine	171	46	3.7
Methionine	207	107	1.9

was only about one fourth as active in accepting amino acids (C^{14}-algal protein hydrolysate) per optical density unit of RNA as was the RNA from the soluble fraction.

Such a preparation was tested for its maximum acceptance of several amino acids and compared with a preparation of soluble RNA (Table IV). These data argue further against the probability of simple contamination of the microsomes by soluble RNA since the ratios for the incorporation of the several amino acids by the two preparations of amino acid acceptor RNA are not constant.

Incorporation of Amino Acids into RNA of Liver Supernatant Fractions Centrifuged for Various Times at 105,000 × g.—The microsomal supernatant fraction or soluble fraction is normally obtained after 0.5 to 3 hours of centrifugation at 105,000 × *g* (see Roberts, 1958). If the amino acid acceptor RNA and activating enzymes were truly soluble then centrifuging for these various time periods should have very little effect on the yield of soluble fraction or on the purity of the microsomes. However, the fact that we find amino acid acceptor RNA in washed microsomes could be interpreted to mean either that this RNA is an integral part of the microsomes or that some of the amino acid acceptor RNA is normally in a particulate form and therefore follows the microsomes in their sedimentation characteristics. In order to examine this problem a 17,000 × *g* supernatant of mouse liver was prepared in the usual manner and then spun at 105,000 × *g* for 20 hours, with aliquots removed at various intermediate times. At the end of the experiment, aliquots of each supernatant were assayed for RNA (acid precipitability and extraction, see Experimental) and protein (Lowry *et al.*, 1951) content, and for the ability of the RNA to accept various radioactive amino acids.

By 30 minutes about 70% of the RNA and 25% of the protein had been sedimented (Fig. 1). After 30 minutes there was a slow steady decrease in both the RNA and protein content from the supernatant fluid until at 20 hours there was only about 5% of the RNA and 35% of the protein remaining. The concept of the soluble fraction

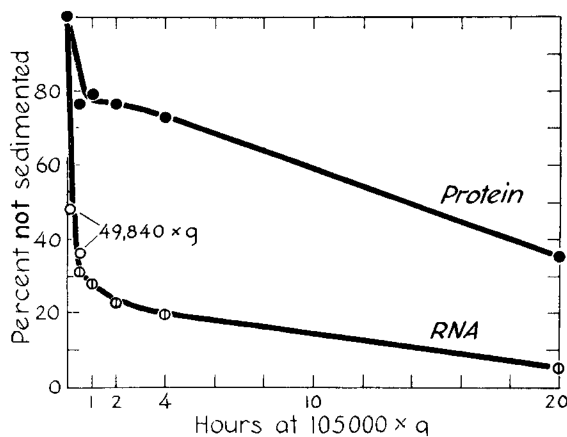

FIG. 1.—The protein and RNA content of a 17,000 × g supernatant of mouse liver (prepared as a 25% homogenate in 0.25 M sucrose–0.001 M $MgCl_2$) after spinning again for various times at 105,000 × g. Spinning at 49,800 × g removes almost the same percentage of RNA as the higher speed.

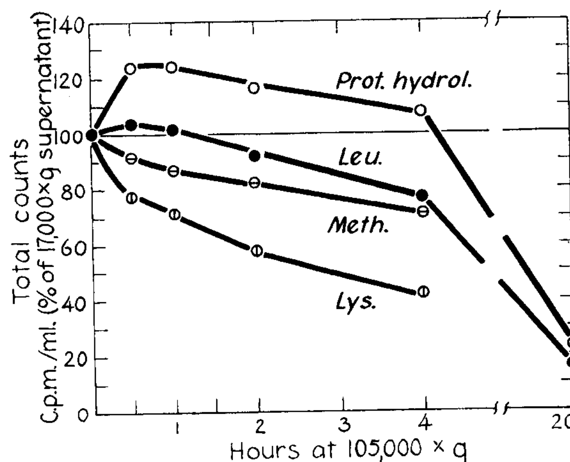

FIG. 2.—The total amount of C^{14}-amino acids incorporated into the RNA of supernatants from homogenates of mouse liver obtained after spinning at 17,000 × g for 10 minutes and then again for various times at 105,000 × g. The following abbrevations are used: Prot, hydrol. for C^{14}-algal protein hydrolysate; Leu. for C^{14}-leucine; Meth. for C^{14}-methionine; and Lys. for C^{14}-lysine. The data for lysine and leucine are from duplicate experiments. Further details are given in the text.

thus loses most of its meaning. One should specify not only the time of centrifugation but also the metal ion content of the medium, since about half of the so-called soluble fraction can be aggregated with the microsomes in the presence of 0.01 M $MgCl_2$ (see below).

The assay for the incorporation of amino acids into RNA was the same as described under Experimental, except that the supernatant fraction was used in place of purified RNA and enzymes. If the amino acid acceptor RNA and activating enzymes were truly soluble then the total counts incorporated per ml of supernatant should remain constant even though inert RNA was removed by centrifugation. This does not prove to be the case. When a C^{14}-algal protein hydrolysate was used as the amino acid source the total counts incorporated into RNA increased to a maximum at about 1 hour and then decreased with longer times (Fig. 2). Similar results were obtained for leucine. However, for methionine and to a greater extent for lysine, the 17,000 × g supernatant showed the greatest incorporation of amino acids into RNA and the activity decreased steadily with time of centrifugation. After 4 hours at 105,000 × g the incorporation of lysine was reduced by 60%.

Although the soluble RNA's are generally considered to have a uniform sedimentation coefficient of around 4 S, the sedimentation coefficients of the amino acid acceptor activities described in these experiments would appear to be both somewhat greater and heterogeneous in their distribution. This could well be due to the association of this RNA with protein or small particles. Regardless of the mechanism, the implication is clear that depending upon the time of centrifugation used in the isolation of the microsomes there will be more or less contamination with amino acid acceptor RNA (and activating enzymes). Since the amino acid acceptor RNA and/or activating enzymes are distinguishable on the basis of their sedimentation characteristics from sucrose homogenates, some use of this could complement the chromatographic (Smith *et al.*, 1959) and countercurrent distribution (Holley *et al.*, 1960) techniques that have been used for the fractionation of amino acid acceptor RNA.

The Aggregation and Disaggregation of Microsomes and Post-Microsomal Particles by Increasing Concentrations of Magnesium.—Microsomes are known to be aggregated by high concentrations of magnesium (Petermann, 1960; Rendi and Campbell, 1959; Takanami, 1960), and, since about 20% of the RNA in the soluble fraction is chromatographically similar (see below) to a fraction found in washed microsomes and to a majority of the RNA associated with the post-microsomal fractions, it seemed possible that this material might be aggregated by high concentrations of magnesium and thus result in a purer sample of soluble RNA free of post-microsomal-particle-like RNA. The effect of magnesium on the sedimentation of microsomes and post-microsomal material was therefore studied.

Homogenates of mouse liver were prepared (25% in 0.25 M sucrose) and then diluted with an equal volume of 0.25 M sucrose containing various amounts of magnesium chloride. These solutions were allowed to stand in the cold for 30 minutes with frequent mixing and then centrifuged at 17,000 × g for 10 minutes. As the concentration of magnesium was raised, more and more RNA and protein were sedimented until, at a concentration of 0.006 M, only 15% of the RNA