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

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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 × *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 × *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 × *g*. The supernatant fluid was centrifuged for 1 hour at 105,000 × *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 × *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 Microsomal RNA.—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 m) 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 RF [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]	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

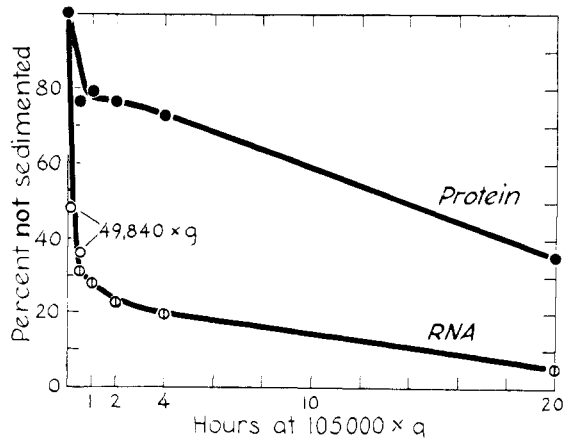

FIG. 1.—The protein and RNA content of a 17,000 × *g* supernatant of mouse liver (prepared as a 25% homogenate in 0.25 M sucrose–0.001 M $MgCl_2$) after spinning again for various times at 105,000 × *g*. Spinning at 49,800 × *g* removes almost the same percentage of RNA as the higher speed.

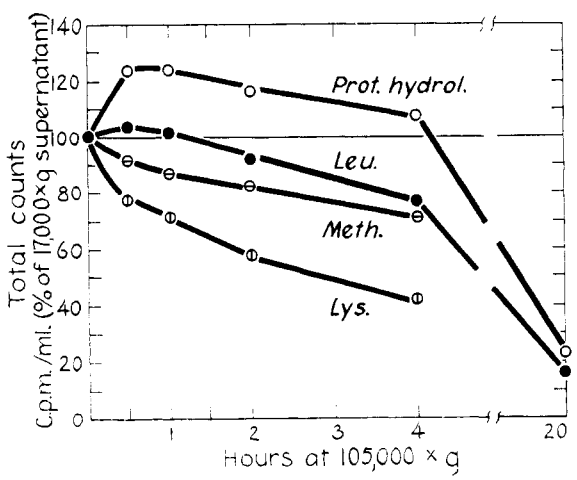

FIG. 2.—The total amount of C^{14}-amino acids incorporated into the RNA of supernatants from homogenates of mouse liver obtained after spinning at 17,000 × *g* for 10 minutes and then again for various times at 105,000 × *g*. The following abbrevations are used: Prot, hydrol. for C^{14}-algal protein 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

and 62% of the protein remained in the supernatant after 10 minutes at 17,000 × *g*. To remove this much RNA and protein from a 0.25 M sucrose (0.001 M $MgCl_2$) homogenate would require about 10 hours of spinning at 105,000 × *g* (see Fig. 1). As the concentration of magnesium was raised above 0.02 M progressively more RNA and protein remained soluble at 17,000 × *g*.

When the 17,000 × *g* supernatants prepared at these various magnesium concentrations were then spun for 60 minutes at 105,000 × *g* to obtain the soluble fraction it was found that changing the magnesium concentration from 0.001 to 0.006 M resulted in the loss of about 50% of the RNA normally present in the soluble fraction (Fig. 3) and an increase in the specific activity (cpm/OD_{260}) of the remaining RNA for accepting amino acids (C^{14} protein hydrolysate) of only 22%. It is apparent that a large proportion of the amino acid acceptor RNA had been caused to sediment with the microsomes. The degree of contamination of the microsomes by amino acid acceptor RNA is therefore also a function of the amount of magnesium in the medium used for the isolation of the microsomes.

Requirements for Amino Acid Incorporation into Washed Microsomes.—Since one wash removes some of the amino acid acceptor RNA from the microsomes but one or two subsequent washes in 0.25 M sucrose–0.001 M $MgCl_2$ remove no more of the amino acid acceptor RNA (Table I), it was of interest to determine the requirements of the washed microsomes for the incorporation of amino acids into protein. The addition of amino acid acceptor RNA to washed microsomes increases the incorporation of leucine by only about 30% (Table V). If the purified *p*H 5 enzymes are left out of the mixture only about 40% of the incorporation is achieved. Without added energy sources the reaction will not proceed at all. Without inorganic pyrophosphatase[3] present only about 30% of the activity is achieved; however, pyrophosphatase is inhibitory in the crude system

TABLE V

REQUIREMENTS FOR INCORPORATION OF C^{14}-LEUCINE INTO WASHED MOUSE LIVER MICROSOMES

Conditions are as described under Experimental. The specific activity of the C^{14}-leucine was 9280 cpm/mμmole. There were 3.9 mg of microsomal protein per assay tube. One OD_{260} unit of *S*-RNA will take up 0.121 mμmole leucine.

Reaction Mixture	Activity (cpm/mg protein)
Complete system	354
Complete, minus energy sources	4
Complete, minus inorganic pyrophosphatase[3]	105
Complete, minus purified *p*H 5 enzymes	140
Complete, minus *S*-RNA	236
Complete, minus *S*-RNA and purified *p*H 5 enzymes	106

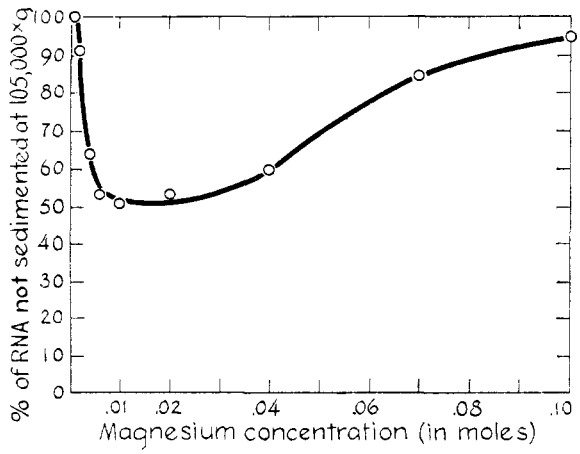

FIG. 3.—The amount of RNA remaining in solution when mouse liver homogenates (25%) prepared in 0.25 M sucrose containing various concentrations of $MgCl_2$ were spun for 10 minutes at 17,000 × *g* and then again for 60 minutes at 105,000 × *g*. The homogenate prepared in 0.001 M $MgCl_2$ is taken as the 100% control.

(substitution of supernatant fraction for purified *p*H 5 enzymes and *S*-RNA). In the presence of pyrophosphatase the washed microsomes are able to incorporate leucine to the extent of about 30% of the complete system without the addition of either the purified *p*H 5 enzymes or *S*-RNA.

Chromatographic Comparison of Soluble RNA and Microsomal RNA.—The fractions obtained when RNA is submitted to columns of Cato-2 have been shown to differ in base composition (Smith *et al.*, 1960), and when soluble RNA was used the fractions differed in their ability to take up amino acids (Smith *et al.*, 1959). Soluble RNA and microsomal RNA isolated from mouse liver have been fractionated by these techniques. The average distributions found for the various eluting conditions are given in Table VI. It has previously been pointed out that there is an apparent separation of soluble and microsomal RNA on columns of Cato-2 (Smith *et al.*, 1959, 1960; Smith and Doell,[4] 1960). This type of separation has also been reported by other investigators using different systems (Goldthwait, 1959; Mandell and Hershey, 1960; Goldthwait and Starr, 1960; Bosch *et al.*, 1960; Bloemendal *et al.*, 1960). The separation is not absolute, but it should be noted that the RNA from washed microsomes contains little or no species of RNA that are eluted by 0.15 M NaCl while better than half of the soluble RNA is eluted under these conditions. When the various chromatographic fractions were assayed for their ability to accept amino acids (C^{14}-algal protein hydrolysate) in the presence of purified

[4] The chromatographic fraction of *M*-RNA (10–20%) that is active in accepting acids is about 12% as active as soluble RNA (on basis of equal optical density units) rather than the value of 54% appearing in this abstract (see Table VI).

TABLE VI

CHROMATOGRAPHIC FRACTIONATION AND AMINO ACID ACCEPTOR ACTIVITY OF SOLUBLE RNA AND MICROSOMAL RNA

Chromatographic Fraction[a]	Recovered from Column (% of Input)	Amino Acid Acceptor Ability (cpm/OD_{260})	Acceptor Activity Recovered
S-RNA			
Unfractionated		4559	90%
0.15 M NaCl	64	4947	
1.0 M NaCl	20	4135	
1.0 M NaCl + 0.1 M NH_4OH	11	1058	
M-RNA			
Unfractionated		125	109%
0.15 M NaCl	<2[b]	---	
1.0 M NaCl	22	541	
1.0 M NaCl + 0.1 M NH_4OH	74	24	

[a] Columns 0.8 cm in diameter, containing about 10 cc of packed Cato-2 (passing $\frac{1}{32}$ in. perforated screen), have been used for the fractionation of approximately 30 optical density units (OD_{260}) of either microsomal RNA or soluble RNA by the technique of discontinuous elution. Two-ml fractions were collected at a flow rate of 0.4 ml/min, and the elution was continued until the optical density at 260 mμ fell below a value of 0.020 before the next eluting solution was substituted. The elution procedure used here was selected to show the greatest difference between the distributions for soluble RNA and microsomal RNA and do not represent the optimal subfractionation that can be obtained by this method. Both the 0.15 M peak and the 1.0 M peak could have been subfractionated by stepwise elution with intermediate concentrations of NaCl (Smith *et al.*, 1959, 1960; Smith and Doell, 1960).

[b] No ultraviolet-absorbing peak was obtained in this fraction. The value listed is probably not RNA but represents background material eluted from the exchanger; a small value of optical density multiplied by a large volume factor. This value is the highest we have obtained for similar experiments. Usually values of 1% or less were obtained. The absence of RNA in the 0.15 M peak is also supported by the recovery of amino acid acceptor ability in the other peaks.

*p*H 5 enzymes, it was found that the 0.15 M NaCl peak of *S*-RNA was the most active in incorporating amino acids (Table VI). The fractions eluted by the higher salt concentrations and more alkaline *p*H showed a progressive decrease in specific activity (based on UV absorption). Most (if not all) of the amino acid acceptor RNA found in the washed microsomes is eluted between 0.15 and 1.0 M NaCl. The bulk of the microsomal RNA can thus be freed of amino acid acceptor RNA by this procedure.

The RNA isolated from a post-microsomal pellet obtained by respinning the soluble fraction for an additional hour at 105,000 × *g* (corresponding to 13% of the RNA originally in the soluble fraction) had a chromatographic distribution somewhat intermediate between that for soluble RNA and microsomal RNA. It is of significance that less than 5% of this RNA was eluted by 0.15 M NaCl (*vs.* more than 50% for *S*-RNA). About 35% was eluted by 0.2–1.0 M NaCl. Only about 60% of this post-microsomal RNA required the use of alkali to remove it from the column (as compared to 74% for microsomal RNA and 11% for soluble RNA [Table V]). This RNA (unfractionated) had a specific activity for accepting amino acids of 1200 cpm/OD_{260} unit (compare with Table VI) or about nine times that found for microsomal RNA. Had the microsomes been prepared by a 2-hour spinning instead of our usual 1-hour spinning at 105,000 × *g*, they would have been contaminated by this post-microsomal fraction containing an appreciable amount of amino acid acceptor activity.

DISCUSSION

The presence of amino acid acceptor RNA in washed mouse liver microsomes is not due to a simple contamination with soluble RNA, since it cannot be completely removed by repeated and varied washing procedures (0.25 sucrose–0.001 M $MgCl_2$; 0.3% deoxycholate; 0.5 M NaCl). Furthermore, the ratios of the amino acid acceptor activities toward leucine, valine, tryosine, and methionine for this RNA are different from those found for soluble RNA (Table IV). If it is ordinary soluble RNA that is bound to microsomal RNA then it is bound by a very strong bond, since the isolation of the RNA by phenol extraction does not make it revert to the chromatographic behavior of the bulk of the soluble RNA (Table VI). On the basis of its chromatographic properties one would judge its molecular weight to be intermediate between that of the bulk of the soluble RNA and of the microsomal RNA,[5] and more similar to a large portion of the RNA found in the post-microsomal fraction. If it were strongly bound to the microsomal RNA then one would expect to find it where the major fraction of the microsomal RNA is found on the chromatograms rather than in its intermediate position (Bloemendal *et al.*, 1960); however, in this form it might be inactive in incorporating amino acids with purified *p*H 5 enzymes. If some were present in this form it might explain the residual content of 5-ribosyl uracil (7% of total uridylic acid) found in this fraction even though it exhibits no amino acid acceptor activity (Table III). The presence of amino acid acceptor RNA in sucrose-

[5] RNA's fractionated on Cato-2 columns and eluted between 0.1 and 1.0 N NaCl have molecular weights of the order of 1×10^5, while those eluted in NaCl-NH_4OH range as high as 1×10^6 (N. P. Salzman, personal communication). Bradley and Rich (1956) have also reported that for another ion exchange resin, Ecteola, "In general the fractions (RNA) eluted at higher salt concentrations have higher sedimentation coefficients."

washed microsomes is further evidenced by the fact that only a little over a 3-fold stimulation of amino acid incorporation into protein is observed when S-RNA and pH 5 enzymes are added (Table V). Similar data have been reported for reticulocyte ribosomes (Schweet *et al.*, 1961; Allen and Schweet, 1962).

Other authors have been unable to remove amino acid acceptor RNA from microsomal and ribosomal preparations. Thus, Kirsch *et al.* (1960) report "a reduced but still significant incorporation [of leucine into guinea pig liver ribonucleoprotein particles] in the absence of supernatant." Rendi and Warner (1960) report that "this RNA is part of the ribosomes and is not contaminating cytoplasmic RNA." Allen *et al.* (1960) found that microsomal RNA had a maximum amino acid acceptor activity of about 3% of that found for their pH 5 RNA. This is comparable to that reported here (Table I). The absolute requirement of transfer RNA for the *in vitro* synthesis of hemoglobin has not been shown, probably because it has not been possible to remove small molecular weight RNA from the ribosomes (Schweet *et al.*, 1961; Allen and Schweet, 1962). Elson (1961) separated a ribonucleic acid particle from ribosomes with 0.1 M NaCl whose base composition was similar to that of transfer RNA. We have found that 0.5 M NaCl was more effective in removing amino acid acceptor RNA from sucrose-washed microsomes than was 0.3% deoxycholate (Table III). Bosch *et al.* (1961) failed to find amino acid acceptor RNA in their microsomal RNA preparations; however, a probable explanation can be offered.[6]

Besides the amino acid acceptor RNA which by the above criteria appears to be intimately associated with the microsomes (even after treatment with deoxycholate) it can be shown that, depending upon the time of centrifugation and the nature of the medium used for suspension of the tissue, one can cause as much as 50% of the RNA in the soluble fraction to shift to the microsomal fraction. Spinning the liver homogenates (25% in 0.25 M sucrose–0.001 M $MgCl_2$) at 105,000 × *g* causes the non-uniform sedimentation of the various amino acid acceptor RNA's (probably bound to protein). Thus, 29% of the activity toward lysine and 13% of the activity toward methionine is sedimented out by spinning under conditions used for the isolation of the microsomes, yet the activity toward leucine is unchanged and does not begin to be sedimented until after 1 hour at 105,000 × *g* (Fig. 2). Longer spinning times of course accentuate these differences. This may have some bearing upon the data in Table IV, which shows that compared to the amount of amino acid acceptor RNA in the soluble fraction the activity toward methionine in the microsomes is proportionally higher than that found for leucine.

The magnesium content of the suspending medium plays an even more important role in the partition of RNA between the soluble and microsomal fractions. As much as 50% of the RNA normally found in the supernatant fraction can be aggregated in the presence of 0.01 M $MgCl_2$ (Fig. 3). Thus it would take 14.5 hours of spinning at 105,000 × *g* in 0.25 M sucrose in the presence of 0.001 M $MgCl_2$ to sediment the amount of RNA removed by a 1-hour spin in the presence of 0.01 M $MgCl_2$. The sedimented RNA appears to be of a larger average molecular weight than the bulk of the soluble RNA, yet it has amino acid acceptor activity. Hess *et al.* (1961) have also found that the amount of RNA appearing in the 105,000 × *g* pellet and supernatant varies inversely with the ion concentration of the medium. They found more RNA in the pellet in the presence of 0.003 M $CaCl_2$ than in 0.25 M sucrose. Also in this connection it is interesting to note that messenger RNA is found in the supernatant fraction along with soluble RNA when the cells are extracted in 0.0001 M magnesium but is almost completely associated with the microsomes (ribosomes) in 0.01 M magnesium (Gros *et al.*, 1961; Nomura *et al.*, 1960; Brenner *et al.*, 1961).

Since it has been shown both by the present author and by the other authors cited that it is extremely difficult, if not impossible, to remove all of the amino acid acceptor RNA from the microsomes (ribosomes), and since it has also been shown that the partition of RNA between the microsomal (ribosomal) pellet and supernatant is inversely proportional to the ion content (particularly metal ion content) of the extraction medium and to the centrifugation time, great care must be exercised in the interpretation of experiments which depend upon the addition of soluble components until we know more the true chemical nature of the microsomes (ribosomes).

ADDENDUM: The Cato-2 presently available differs from the Cato-2 used in our experiments and is no longer satisfactory for chromatographic use. Large mesh DEAE-cellulose of about the same nitrogen content (0.23%) should give rather comparable results to Cato-2 (a DEAE-starch).

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[6] Since these authors (Bosch *et al.*, 1960) state that "higher amounts of RNA were consistently obtained (when precipitated with ethanol) at 2–4° than at 20°," it might be pertinent to point out that besides the above observation it is also necessary to allow the RNA to stand at 4° overnight in the presence of salt and ethanol to obtain quantitative precipitation (unless the RNA is very concentrated). Furthermore, a centrifugation time of up to 2 hours at 2000 rpm (International Centrifuge) was necessary for quantitative sedimentation of the product even after standing overnight. The RNA that is lost if these precautions are not observed is the soluble RNA. It is inferred from the authors' remarks that these precautions were not observed, and this may be why they found no amino acid acceptor RNA in their preparations of microsomal RNA.

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REFERENCES

Allen, E. H., Glassman, E., Cordes, E., and Schweet, R. S. (1960), *J. Biol. Chem. 235*, 1068.
Allen, E. H., and Schweet, R. S. (1962), *J. Biol. Chem. 237*, 760.
Bloemendal, H., Bosch, L., and Sluyser, M. (1960), *Biochem. et Biophys. Acta 41*, 454.
Bosch, L., Bloemendal, H., and Sluyser, M. (1960), *Biochim. et Biophys. Acta 41*, 444.
Bosch, L., Van Der Wende, G., Sluyser, M., and Bloemendal, H. (1961), *Biochim. Biophys. Acta 53*, 44.
Bradley, D. F., and Rich, A. (1956), *J. Am. Chem. Soc. 78*, 5898.
Brenner, S., Jacob, F., and Meselson, M. (1961), *Nature 190*, 576.
Crestfield, A. M., Smith, K. C., and Allen, F. W. (1955), *J. Biol. Chem. 216*, 185.
Davis, F. F., and Allen, F. W. (1957), *J. Biol. Chem. 227*, 907.
Dunn, D. B. (1959), *Biochim. et Biophys. Acta 34*, 286.
Dunn, D. B., Smith, J. D., and Simpson, M. V. (1960), *Biochem. J. 76*, 24P.
Elson, D. (1961), *Biochim. et Biophys. Acta 53*, 232.
Goldthwait, D. A. (1959), *J. Biol. Chem. 234*, 3245.
Goldthwait, D. A., and Starr, J. L. (1960), *J. Biol. Chem. 235*, 2025.
Gros, F., Hiatt, H., Gilbert, W., Kurland, C. G., Risebrough, R. W., and Watson, J. D. (1961), *Nature 190*, 581.
Hess, E. L., Herranen, A. M., and Lagg, S. E. (1961), *J. Biol. Chem. 236*, 3020.
Holley, R. W., Apgar, J., and Doctor, B. P. (1960), *Ann. N. Y. Acad. Sci. 88*, 745.
Kirsch, J. F., Siekevitz, P., and Palade, G. E. (1960), *J. Biol. Chem. 235*, 1419.
Kunitz, M. (1961), *Arch. Biochem. Biophys. 92*, 270.
Littlefield, J. W., and Keller, E. B. (1957), *J. Biol. Chem. 224*, 13.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Mandell, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1*, 66.
Nomura, M., Hall, B. D., and Spiegelman, S. (1960), *J. Mol. Biol. 2*, 306.
Petermann, M. L. (1960), *J. Biol. Chem. 235*, 1998.
Rendi, R., and Campbell, P. N. (1959), *Biochem. J. 72*, 34.
Rendi, R., and Warner, R. C. (1960), *Ann. N. Y. Acad. Sci. 88*, 741.
Roberts, R. B., editor (1958), Microsomal Particles and Protein Synthesis, New York, Pergamon Press.
Schweet, R., Bishop, J., and Morris, A. (1961), *Lab. Invest. 10*, 992.
Schweet, R., Bovard, F. C., Allen, E., and Glassman, E. (1958), *Proc. Nat. Acad. Sci. U. S. 44*, 173.
Siekevitz, P., and Palade, G. E. (1959), *J. Biophys. Biochem. Cytol. 5*, 1.
Smith, K. C. (1960), *Biochim. et Biophys. Acta 40*, 360.
Smith, K. C., and Allen, F. W. (1953), *J. Am. Chem. Soc. 75*, 2131.
Smith, K. C., Cordes, E., and Schweet, R. S. (1959), *Biochim. et Biophys. Acta 33*, 286.
Smith, K. C., and Doell, R. G. (1960), *Fed. Proc. 19*, 318.
Smith, K. C., Rebhun, S., and Kaplan, H. S. (1960), *Anal. Biochem. 1*, 202.
Takanami, M. (1960), *Biochim. et Biophys. Acta 39*, 318.
Warburg, O., and Christian, W. (1942), *Biochem. Z. 310*, 384.