# ACTIVATION IN VITRO OF RAT LIVER POLYRIBOSOMES

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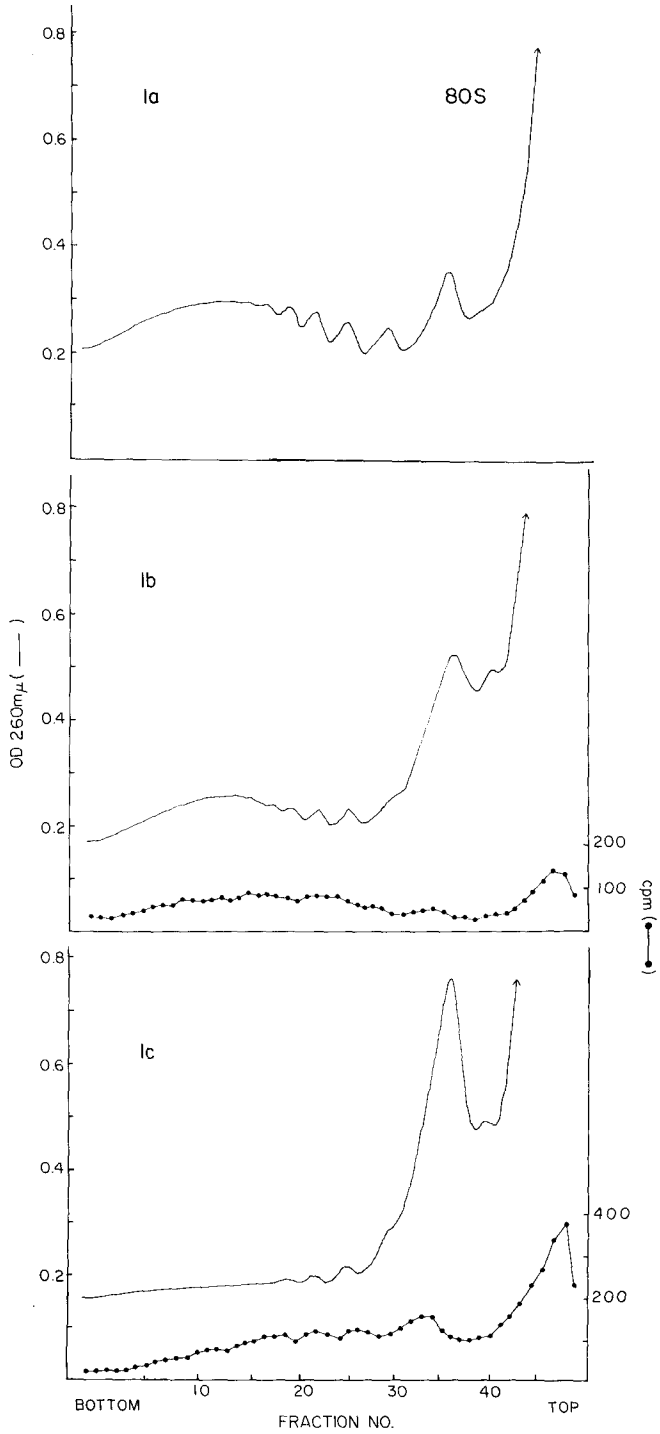
## ABSTRACT

The increase in the incorporation of amino acids into protein in vitro by preparations obtained from protein-fed rats as compared with preparations obtained from carbohydrate-fed rats has been described previously. After molecular sieving through Sephadex G-25 of cell-free preparations, the difference in incorporating activity between the two types of rats was diminished in systems containing ATP, phosphoenolpyruvate, pyruvate kinase, GTP, and a mixture of amino acids. When, after molecular sieving, a mitochondrial (15,000 *g*) supernatant was incubated for 4 min at 35°C the polysomal pattern of the preparations was unchanged. In the presence of ATP, phosphoenolpyruvate, and pyruvate kinase the polysomal incorporating activity was low and the polysomal pattern was only slightly changed. Addition of GTP increased the activity markedly, and a more pronounced activity was observed when a mixture of amino acids was added as well. As the amino acid incorporation ability increased, monosomes were formed from the polyribosomes. The activity of the polyribosomes was severalfold higher than that of non-Sephadex-treated preparations, indicating an activation of polysomal aggregates which under the usually applied conditions of incubation and prior to molecular sieving show little or insignificant activity. It was possible to activate polyribosomes from carbohydrate-fed and protein-fed rats to almost the same extent.

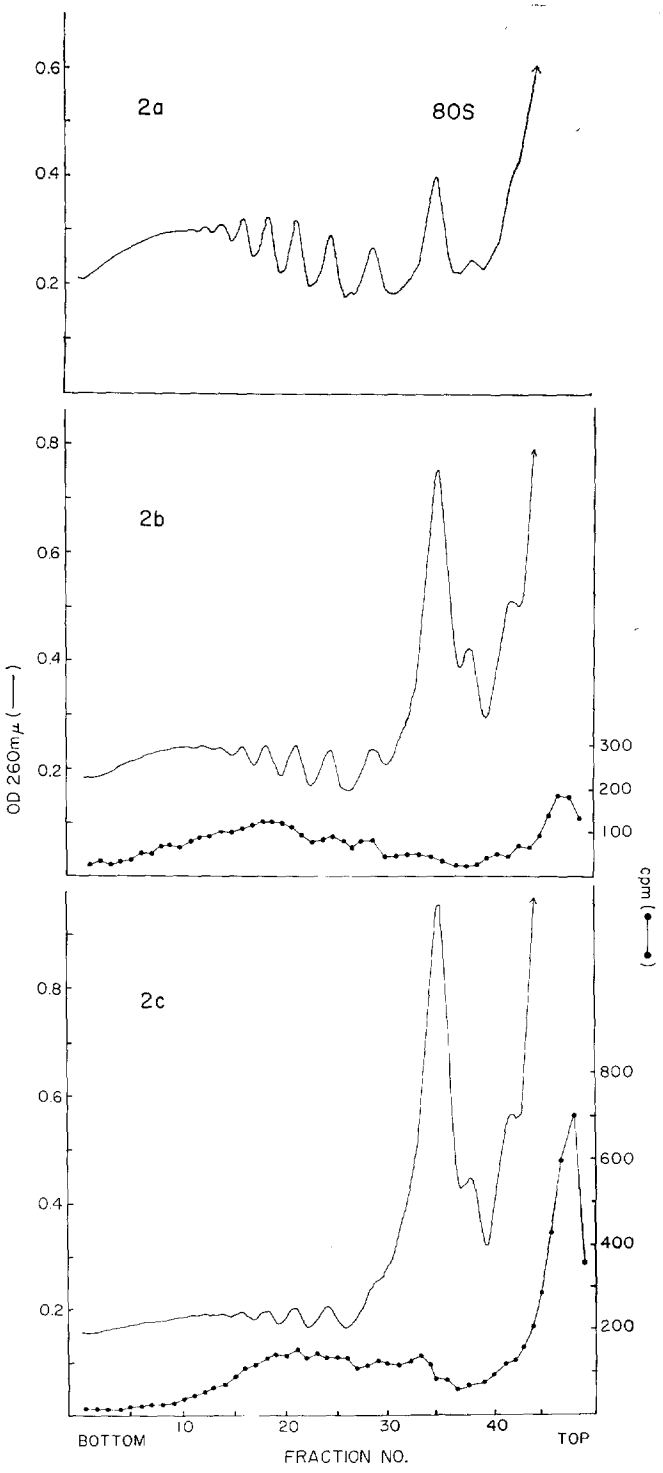
## INTRODUCTION

It is well known (11) that the rate of protein synthesis is dependent on the nutritional status of the living animal. Making use of this fact, it was possible to study some of the steps in protein biosynthesis that are concerned with the regulatory mechanism of polypeptide formation. It has been shown (3–6) that after several days of a protein-deficient diet the incorporation of amino acids into protein by liver preparations is decreased. This change in activity is apparently directed by alterations of the translation rather than of the transcription of messenger RNA. In the translation reaction a number of enzymes and cofactors are involved. When the low molecular weight components of cell-free preparations are removed and replaced by optimal concentrations of ATP, GTP, and amino acids, a marked enhancement of the incorporating activity of the system was observed (4, 5). In addition, it was found that the cell sap contains components of high molecular weight that have an inhibitory effect on the incorporating activity (6, 7). Thus it seems that a variety of components present in the cell sap influences the rate of protein synthesis. So far, it is not known which steps in protein synthesis are changed by the stimulatory and the inhibitory factors.

The aim of this work is to study the activation process brought about by the stimulatory components. As will be shown below, the reactions of protein synthesis taking place at the polyribosomes are markedly enhanced. In fact, we will demonstrate an activation of those polyribosomal aggregates which under normal conditions show a depressed activity.




FIGURES 1 *a–c* Polysomal distribution and amino acid incorporating activity of polyribosomes from carbohydrate-fed animals. The system contained a mitochondrial supernatant from carbohydrate-fed rats in the absence or the presence of energy. After incubation, DOC was added, and the polysomal distribution was studied by means of sucrose gradient centrifugation. Input to gradient contained 77 μg of RNA. 1 *a*, incubation in the absence of energy for 4 min at 35°C; 1 *b*, incubation in the presence of ATP, PEP, pyruvate kinase, GTP, and leucine-$^{14}$C for 2 min at 35°C. 1 *c*, as in *b*, but incubation was for 4 min at 35°C.




FIGURES 2 *a–c* Polysomal distribution and amino acid incorporating activity of polyribosomes from protein-fed rats. For details see Fig. 1. The mitochondrial supernatant from carbohydrate-fed rats was replaced by that from protein-fed rats. Input to gradient contained 76 μg of RNA.

TABLE I

*Incorporation of Leucine-$^{14}C$ into Protein under Various Conditions of Incubation*

| Preparation | System | Specific radioactivity | | | | | |
|---|---|---|---|---|---|---|---|
| | | Carbohydrate-fed (C) | Protein-fed (P) | Ratio P:C | Carbohydrate-fed (C) | Protein-fed (P) | Ratio P:C |
| | | *CPM/mg RNA* | *CPM/mg RNA* | | *CPM/mg protein* | *CPM/mg protein* | |
| Untreated mitochondrial supernatant | Complete with or without GTP | 68,000 | 90,000 | 1.32 | 4,100 | 6,000 | 1.46 |
| Sephadex-treated mitochondrial supernatant | Complete | 46,500 | 59,000 | 1.26 | 2,150 | 3,200 | 1.50 |
| Sephadex-treated mitochondrial supernatant | Complete plus GTP | 152,000 | 152,000 | 1.00 | 7,000 | 8,200 | 1.17 |
| Sephadex-treated mitochondrial supernatant | Complete plus GTP plus amino acids | 206,000 | 225,000 | 1.10 | 9,500 | 12,100 | 1.27 |

Untreated mitochondrial supernatant from carbohydrate-fed and protein-fed rats (first line) was incubated for 4 min at 35°C in a complete system containing 1 mM ATP, 10 mM PEP, 40 μg/ml pyruvate kinase, and 0.01 mM leucine-$^{14}C$. After treatment with DOC, the polyribosomes were separated by sucrose density gradient centrifugation. Fractions were collected, and the radioactivity was measured. The specific activities per unit RNA or protein of the mitochondrial supernatant are computed from the total radioactivity obtained. The results are the mean values of three independent experiments.

Sephadex-treated mitochondrial supernatant from carbohydrate-fed and protein-fed rats (lines 2–4). The mitochondrial supernatant was passed through a column of Sephadex G-25 before incubation for 4 min at 35°C in a complete system as described above, and after addition of GTP (0.1 mM), and GTP and an amino acid mixture (12.5 μg/ml). Radioactivity was measured in the fractions obtained after sucrose density gradient centrifugation. The specific activity values are computed from the total radioactivity obtained. The results are the mean values of three independent experiments.

A preliminary report of this work has appeared (8).

## MATERIALS AND METHODS

### *Materials*

L-Leucine-$^{14}C$, UL (specific activity, 200 mc/mmole) was obtained from New England Nuclear Corp., Boston, Mass. ATP (disodium salt), GTP (sodium salt), Tris-HCl, Tris base, and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. Pyruvate kinase (EC 2.7.1.40) was obtained from C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany; DOC[1] was obtained from E. Merck A.-G., Darmstadt, Germany. PEP was synthesized as described by Clark and Kirby (2) and was recrystallized three times as its monocyclohexyl ammonium salt. Before use, the salt was dissolved in 0.2 M Tris-HCl buffer (pH 7.8) and adjusted to pH 7 with 0.7 M KOH. Unlabeled amino acids were obtained from California Corp. for Biochemical Research, Los Angeles, Calif. A mixture was prepared (13) with leucine-$^{12}C$ being replaced by leucine-$^{14}C$. Sephadex G-25 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. All solutions were made up in glass-distilled water.

### *Animals*

Sprague-Dawley female rats (Sprague-Dawley, Inc., Madison, Wis.) weighing from 120 to 150 g were kept for 4 days on a carbohydrate-containing diet consisting of 45% glucose, 45% potato starch, and 10% vegetable fat. On the next day one control group was maintained on the same diet while another group received a protein-rich diet containing 80%

[1] The following abbreviations are used: PEP, phosphoenolpyruvate; DOC, sodium deoxycholate.

casein, 10% glucose, and 10% vegetable fat. The groups of rats are referred to as carbohydrate-fed and protein-fed rats, respectively. The rats were sacrificed 17 hr after the last meal by a blow onto the head and decapitation.

### *Preparation of Subcellular Fractions*

The liver was perfused with ice-cold 0.15 M KCl and then placed into medium (0.25 M sucrose; 0.035 M Tris-HCl, pH 7.8, measured at 25°C; 0.025 M KCl; 0.05 M $NH_4Cl$; and 9 mM $MgCl_2$). The liver was homogenized in twice its weight of medium. The homogenate was centrifuged for 7 min at 15,000 *g*. The mitochondrial supernatant was used in the amino acid incorporating experiments.

### *Molecular Sieving Through Sephadex G-25*

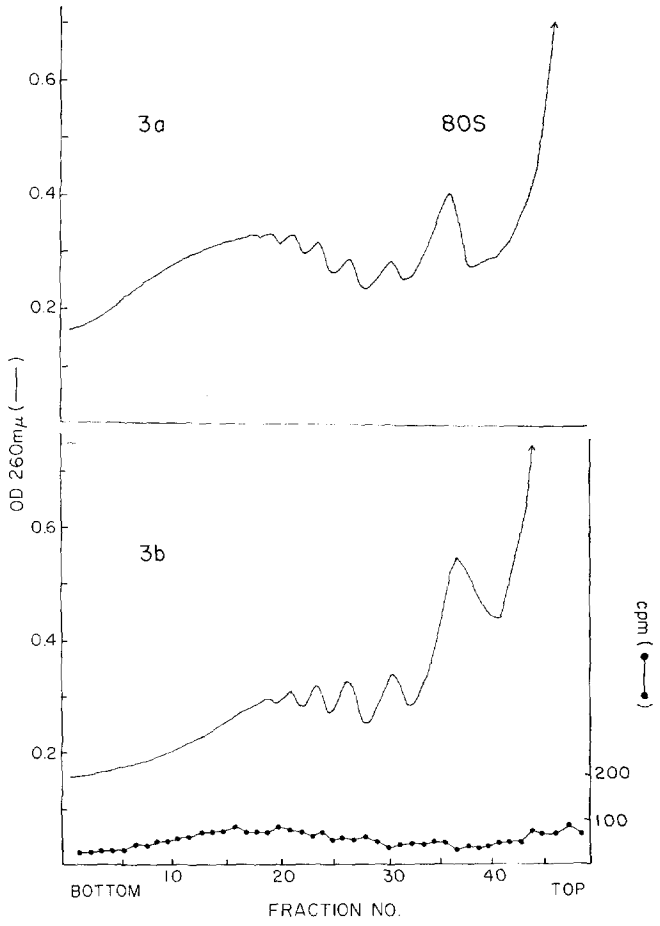
Columns of Sephadex G-25 (2.3 × 20 cm) were equilibrated with medium, and 6 ml of the mitochondrial supernatant were passed through the column as described in detail for cell sap and microsomes (4).

### *Amino Acid Incorporation In Vitro*

The final volume was 240 μl. The incubation mixture contained 200 μl of the mitochondrial supernatant, corresponding to 100–150 μg of RNA, 1 mM ATP, 10 mM PEP, 40 μg/ml pyruvate kinase, and 0.01 mM leucine-$^{14}$C. GTP (0.1 mM) and a mixture of amino acids (12.5 μg/ml) were added when indicated. Incubation at 35°C was carried out for the time indicated and DOC at a final concentration of 0.8% was added after incubation and before the density gradient centrifugation.

### *Sucrose Gradient Centrifugation*

A linear gradient from 0.4 to 1.3 M sucrose in 0.035 M Tris-HCl, pH 7.8, 25 mM KCl, 0.05 M $NH_4Cl$, and 6 mM $MgCl_2$ was layered into tubes of the SW 39 rotor of the Spinco Model L centrifuge and equilibrated overnight. A volume of 150

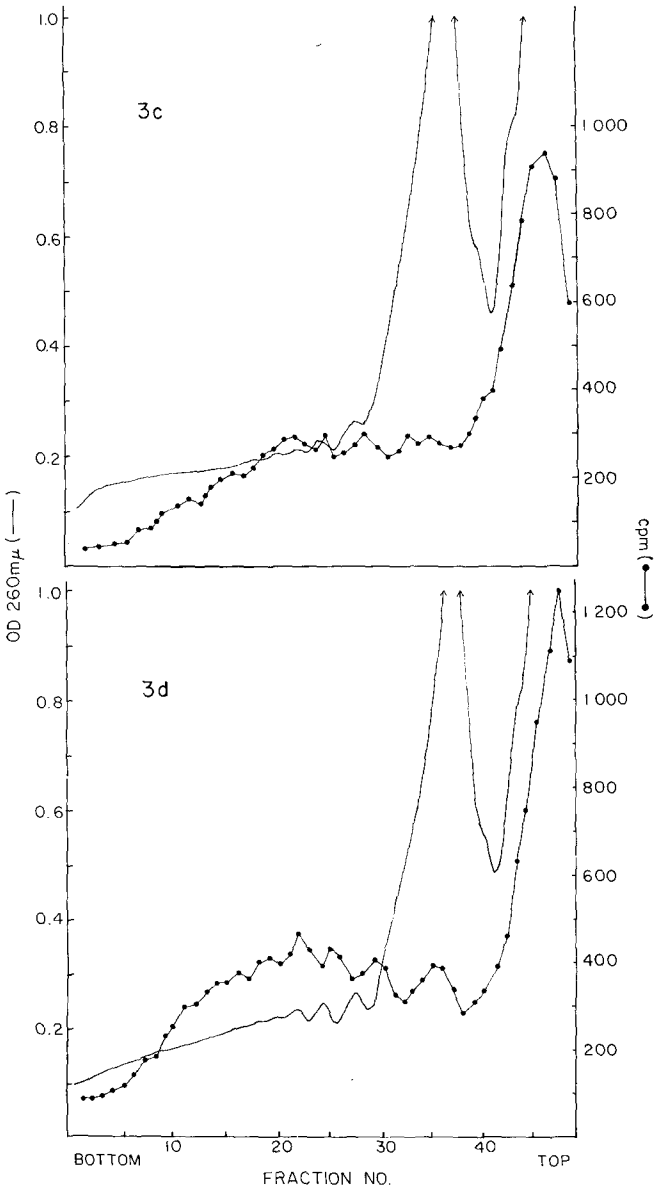



FIGURES 3 *a–d* Activation of polyribosomes from carbohydrate-fed rats after molecular sieving through Sephadex G-25. A mitochondrial supernatant from carbohydrate-fed rats was passed through a column of Sephadex G-25 and incubated for 4 min at 35°C. After addition of DOC, the polysomal distribution and radioactivity values along the gradient were determined. Input to gradient contained 94 μg of RNA. 3 *a*, incubation in the absence of energy; 3 *b*, incubation in the presence of ATP, PEP, pyruvate kinase, and leucine-$^{14}$C.

μl of mitochondrial supernatant in 0.8% DOC was layered on top. Centrifugation was for 60 min at 125,000 *g* without braking during deceleration. The gradient was displaced by heavy sucrose and was monitored at 260 mμ in a double-beam photoelectric ratio recording Beckman spectrophotometer, Model DK-2, supplied with an LKB rectangular continuous flow cell, type 4712 A-4, 4 mm light pass. 2-drop fractions were collected on filter paper discs, which were processed as described by Mans and Novelli (10) and were counted in a Packard Tri-carb liquid scintillation counter at 40% efficiency (4).

### *Analyses*

Protein was determined by the method of Lowry et al. (9) with bovine serum albumin as a standard. RNA was extracted from the mitochondrial supernatant in 0.4 M $HClO_4$, and the absorption at 260 mμ was measured (12). The extinction coeffi-

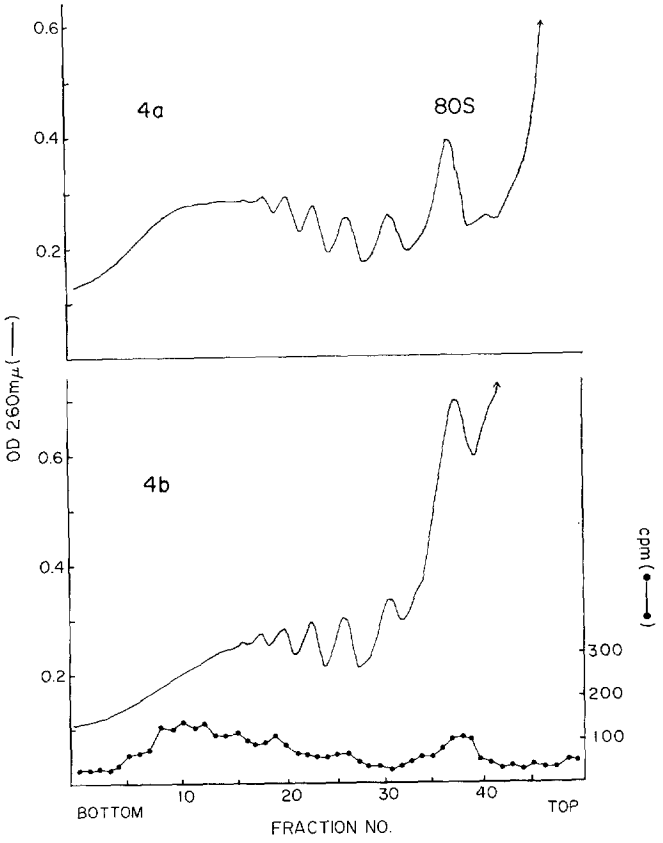



FIGURES 3 *c* and *d* See legend for Figs. 3 *a* and *b*. Fig. 3 *c*, incubation in the presence of ATP, PEP, pyruvate kinase, GTP, and leucine-$^{14}$C; 3 *d*, incubation in the presence of ATP, PEP, pyruvate kinase, GTP, amino acid mixture, and leucine-$^{14}$C.

cient of 34.2 $mg^{-1}$ $cm^2$ was used for calculating the amount of RNA (14).

## RESULTS

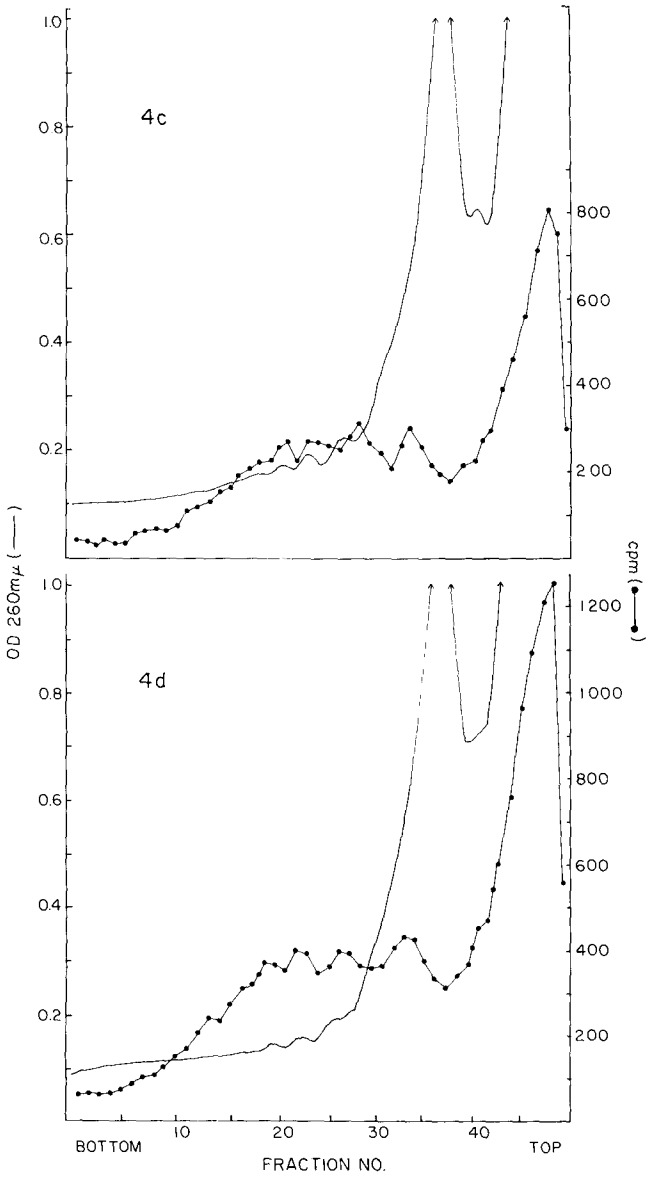
Separation of polyribosomes on a sucrose gradient after various treatments of a mitochondrial supernatant is shown in Figs. 1–4. In Figs. 1 *a* and 2 *a* are illustrated the polysomal patterns after incubation of a mitochondrial supernatant for 4 min at 35°C before treatment with DOC. No energy was added to the system and, therefore, most of the ribosomes remained as polysomal aggregates. This is true for preparations from both carbohydrate-fed and protein-fed rats. When the system contained ATP, PEP, pyruvate kinase, GTP, and leucine-$^{14}$C, there was a continuous increase with time in the number of monoribosomes from polysomes. An increase in monoribosomes was observed already after 2 min of incubation (Figs. 1 *b* and 2 *b*) and was more pronounced after 4 min at 35°C (Figs. 1 *c* and 2 *c*). Simultaneously with the formation of monoribosomes, there was an incorporation of labeled amino acid into protein by the polysomal fractions. As the time of incubation was prolonged, a shift in radioactivity from the heavy toward the light polysomal aggregates was observed. In addition, the radioactivity at the top of the gradient in the soluble fraction increased. The increase in labeling with time, and the production of labeled soluble proteins was more pronounced in preparations obtained from protein-fed rats as compared with preparations from carbohydrate-fed rats. While Figs. 1 and 2 represent one typical experiment, the mean values of the radioactivity data of three independent experiments are summarized in Table I (first line). The difference in specific activity between carbohydrate-fed and protein-fed rats is in agreement with earlier results (3, 4). Although the presence of GTP had no effect on the polysomal pattern or labeling of proteins, it was included in the amino acid incorporating systems for reason of comparison with the experiments that will be described below. The addition of a




FIGURES 4 *a–d* Activation of polyribosomes from protein-fed rats after molecular sieving through Sephadex G-25. For details see Fig. 3. The mitochondrial supernatant from carbohydrate-fed rats was replaced by that from protein-fed rats. Input to gradient contained 77 μg of RNA.

mixture of amino acids inhibited the incorporating activity and was therefore omitted.

When microsomes and cell sap are passed through a column of Sephadex G-25 the rate of amino acid incorporation into protein is enhanced by GTP and amino acids (4). As shown in Table I (lines 2–4), the enhancement was more pronounced with preparations obtained from carbohydrate-fed rats as compared with preparations from protein-fed rats. It was of considerable interest to know in what way the cell-free preparations were affected by the following procedures applied to obtain a stimulation of the rate of amino acid incorporation: (*a*) by molecular sieving and (*b*) by the cofactors needed for an optimal incorporation of amino acids into protein. One of the procedures included in the experiments for obtaining the mean values of Table I is shown in detail in Figs. 3 and 4. Treatment with Sephadex G-25 followed by incubation for 4 min at 35°C in the absence of energy (Figs. 3 *a* and 4 *a*) gave a polyribosomal pattern comparable to that shown for non-Sephadex-




FIGURES 4 *c* and *d* See legend for Figs. 4 *a* and *b*.

treated preparations in Figs. 1 *a* and 2 *a*. After addition of ATP, an ATP regenerating system, and labeled amino acid, and after 4 min of incubation, the polysomal aggregates were only slightly reduced in concentration (Figs. 3 *b* and 4 *b*). The incorporating activity of the system was very low, indicating an efficient removal of low molecular weight components by molecular sieving. As shown in Figs. 3 *c* and 4 *c*, addition of GTP to the incubation systems caused a striking increase in the number of monoribosomes derived from the polysomal aggregates at the same time that the incorporation of amino acids into polypeptide was markedly enhanced. A more pronounced effect was observed when an amino acid mixture was added as well (Figs. 3 *d* and 4 *d*). The ultraviolet absorption in the polysomal region seemed unchanged. The polypeptide formation by the polyribosomes was strongly enhanced, as was the release of labeled proteins which were concentrated at the top of the gradient.

## DISCUSSION

Sucrose gradient centrifugation has enabled a direct study of polypeptide synthesis by the polyribosomes and of changes of the polysomal pattern during this process. Most of the polyribosomes remained intact when a liver mitochondrial supernatant was incubated for several minutes at 35°C in the absence of energy. Molecular sieving through Sephadex G-25 before the incubation did not significantly alter the polysomal pattern. After removal of the low molecular weight components and addition of energy in the form of ATP and an ATP regenerating system, only a slight breakdown of polysomal aggregates was observed, which was accompanied by a low rate of incorporation of amino acids into protein. This was expected since GTP and amino acids had been removed by molecular sieving. It is concluded from these results that energy as such had but little effect on the breakdown or stability of polyribosomes. On the other hand, it is known that rats deprived of energy by prolonged starvation or injection of 2,4-dinitrophenol contain a decreased amount of polyribosomes (15, 16). Under those conditions, however, the lack of energy is correlated with a decreased ability to incorporate amino acids into protein rather than with a direct effect on the stability of polyribosomes. In the absence of energy, initiation of peptide synthesis and thus polyribosome formation might be restricted.

In earlier studies (4, 5) we have observed a stimulation of amino acid incorporation after addition of GTP and amino acids to an otherwise complete incubation system. The effect of the cofactors was manifest only in systems that had been passed through a column of Sephadex G-25. In the present investigation, evidence of a severalfold activation of polyribosomes by the cofactors is presented. The extent of activation is remarkably high and more pronounced as compared with non-Sephadex-treated preparations (cf. Figs. 1 *c* and 2 *c* with Figs. 3 *d* and 4 *d*). At the same time, a high number of monoribosomes and of soluble proteins were formed, indicating that the rate at which the ribosomes run off the messenger RNA was greatly enhanced.

Although the radioactivity data on the polyribosomes showed a further increase in activity after addition of amino acids to a system already containing GTP, no significant increase in the amount of polyribosomes was observed. Such an increase would indicate a new formation of polyribosomes from the monosomes and initiation of polypeptide synthesis. The possibility is suggested, however, by the radioactivity data. In fact, it has been shown that in the case of C-ribosomes, (polyribosomal fraction as prepared according to Wettstein et al. [17]), initiation of peptide synthesis in the presence of an amino acid mixture can be studied by following the increase in ultraviolet absorbance of the polyribosomes (1).

The difference in the amino acid incorporating activity by microsomes between carbohydrate-fed and protein-fed rats is diminished when the preparations of both types of rats are passed through a column of Sephadex G-25 (4). Similar results were obtained here when a mitochondrial supernatant was incubated in the presence of GTP and an amino acid mixture. The effect of the cofactors confines itself to the polyribosomes, which were significantly enhanced in their capacity to incorporate amino acids into protein. The stimulation of preparations from carbohydrate-fed rats was relatively higher as compared with preparations from protein-fed rats. The present data indicate that both systems are activated to an extent which greatly exceeds the originally obtained activity of non-Sephadex-treated preparations. In addition, it is shown that the mechanism of protein synthesis is not irreversibly affected when rats are kept for a few days on a protein-free diet.

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