REVERSIBLE DEGRADATION OF POLYRIBOSOMES IN CHANG CELLS CULTURED IN A GLUTAMINE-DEFICIENT MEDIUM

EVA ELIASSON, G. ERIC BAUER, and TORE HULTIN

From The Wenner-Gren Institute, Stockholm, Sweden. Dr. Bauer's present address is Department of Anatomy, Medical School, University of Minnesota, Minneapolis

ABSTRACT

The effects of temporary glutamine deficiency on the protein and nucleic acid metabolism of Chang's liver cells in suspension cultures have been studied. It was observed that cells maintained in a glutamine-free medium showed a reduced incorporation of labeled precursors into protein and RNA. At the same time, the activity of the ribosomes and the proportion of polyribosomal aggregates in cell extracts diminished. These effects were reversed when the glutamine content of the medium was restored. The restoration of a normal rate of amino acid incorporation by intact cells as well as by cell-free systems was time dependent, and took place within a few hours after glutamine addition without preceding increase in the prevailing low rate of RNA synthesis. The addition of actinomycin D at concentrations that strongly inhibited the RNA metabolism of the cells did not prevent the increase in protein synthesis or the reappearance of polyribosomal aggregates. These facts suggest that the restoration of protein synthesis in the cells after glutamine starvation was not dependent on a production of new messenger RNA. The experimental data are consistent with the hypothesis that previously synthesized messenger RNA, preserved in the cells in a stable form, was brought into action in response to the reestablishment of an adequate cellular environment.

INTRODUCTION

In the last few years it has been repeatedly demonstrated that specific changes in the environment of animal cells may produce rapid and reversible alterations at the level of the ribosomes. These alterations involve the sedimentation pattern of the isolated particles, as well as their ability to incorporate labeled amino acids into protein (3, 8–10, 14, 15, 19, 21). Factors interfering with the energy production of the cells have given particularly clear-cut results in this direction (8, 9, 14, 15, 21). Recent experiments with whole animals, refed after amino acid starvation, suggest that amino acid deficiency may evoke similar, reversible effects on the ribosomes (5).

The aim of the present investigation was to find out whether the omission of single essential amino acids from the medium of a cell culture would reversibly affect the protein and nucleic acid metabolism of the cells and the activity and state of aggregation of the isolated ribosomes. Although glutamine is utilized by tissue culture cells as a precursor not only of proteins (11) but also of purine and pyrimidine nucleotides (18), this amino acid was chosen in the first series of experiments, since other effects of glutamine starvation had previously been studied in this laboratory (20, also data in preparation by E. E. Eliasson). A few hours of glutamine starvation

markedly reduced the incorporation of labeled precursors into the protein and RNA of the cells. At the same time, the proportion of polyribosomes in cell extracts diminished. These effects were reversed when the glutamine content of the medium was restored.

A question of particular interest in experiments of this kind concerns the fate of the messenger RNA from the temporarily degraded polyribosomes. It was found that the restoration of active polyribosomes took place within a few hours after glutamine addition, and without preceding increase in the prevailing low rate of RNA synthesis. The addition of actinomycin D (6, 17) at concentrations that strongly inhibited the remaining RNA metabolism of the cells did not prevent the increase in the activity of the ribosomes or the reappearance of polyribosomal aggregates.

MATERIALS AND METHODS

Cell Cultures

Chang's liver cells, originally obtained from the Microbiological Associates, Inc., Bethesda, Md., were grown as spinner cultures in Eagle's medium for suspension cultures (2), with the modifications that the $NaHCO_3$ concentration was increased to 0.033 M and the NaCl concentration decreased to 0.10 M. The medium contained 10% whole horse serum, previously heated at 56°C for 3 hr. The suspensions were incubated at 37°C in an atmosphere of 5% CO_2 in air. Twice a week the stock cultures were diluted with fresh medium to a concentration of 0.25×10^6 cells per ml. During exponential growth the generation time was about 35 hr. The absence of microbiological contaminations was ascertained by test procedures described previously (4).

For glutamine starvation, exponentially growing cultures (48–64 hr after dilution) were centrifuged at 250 *g* for 20 min, and the cells were transferred to a glutamine-free Eagle's medium. These glutamine-deficient cultures either were used as such, or were supplemented with the lacking glutamine 20–24 hr later.

Incorporation of Labeled Precursors into Protein and RNA of Intact Cells

From exponentially growing cultures of Chang cells, or from cultures at different stages of glutamine starvation or restoration, $2.5–3.5 \times 10^6$ cells were collected by centrifugation at 500 *g* for 7 min and were resuspended in 250 μl of ice-cold, serum-free[1] medium. The labeled components were added in a volume of 25 μl. The final concentration of L-leucine-^{14}C (2 mc/mmole) was 0.4 mM and that of uridine-^{14}C (4 mc/mmole) was 0.2 mM. The concentration of L-phenylalanine-^{14}C was 0.2 mM (2 mc/mmole) or in one experiment (Fig. 1 *a*) 0.1 mM (8 mc/mmole). The cell suspensions were incubated in a shaking bath at 37°C and were continually aerated with 5% CO_2 in air. At zero time, and after 10 and 20 min of incubation, 25-μl samples in duplicate were withdrawn from the suspensions and placed on filter paper discs. These were dropped into ice-cold TCA[2] and processed as will be described below. In isotope experiments involving density gradient centrifugation of ribosomes, approximately 50×10^6 cells were incubated for 4 min in 5 ml medium containing 0.45 mM L-leucine-^{14}C (4 mc/mmole).

Preliminary experiments showed that the rate of incorporation of labeled amino acids into protein was linear from the start of the incubation, indicating a rapid equilibration between the amino acids in the medium and the intracellular amino acid pool. The incorporation of uridine started after a lag of 3 to 5 min and was linear thereafter. The uridylic acid pool of the cells was not significantly different under the various conditions used in these experiments[3]

Preparation of Subcellular Fractions

Cells from 200–500 ml culture were collected by centrifugation at 250 *g* for 20 min. After swelling in ice-cold 0.005 M $MgCl_2$ they were centrifuged at 2000 *g* for 3 min at 4°C. The firmly packed cells were resuspended in 2 volumes of cold 0.005 M $MgCl_2$, and were disrupted by 10 strokes of a tight-fitting all-glass Dounce homogenizer. To the homogenate was immediately added 0.1 volume of a concentrated medium containing 1.5 M sucrose, 0.25 M KCl, 0.005 M $MgCl_2$, and 0.35 M Tris buffer pH 7.8 (as measured after dilution at 20°C). The homogenate was centrifuged at 15,000 *g* for 6 min, and the sediment was discarded. For amino acid incorporation the supernatants were diluted with the light medium, specified below, to a protein concentration of 10.0 mg/ml.

Ribosomes were prepared by treating the 15,000 *g* supernatant fractions with 1% sodium deoxycholate

[1] The incorporation of amino acids and uridine was the same whether or not 10% serum was included in the incubation mixture.

[2] Abbreviations: PEP, phosphoenol pyruvate; RNase, ribonuclease; TCA, trichloroacetic acid; Tris, tris-(hydroxymethyl)amino methane.

[3] In two independent experiments, the uridylic acid content in exponentially growing and glutamine-starved cells was 0.010 and 0.011 μmoles/mg protein, respectively, as determined by column chromatography.

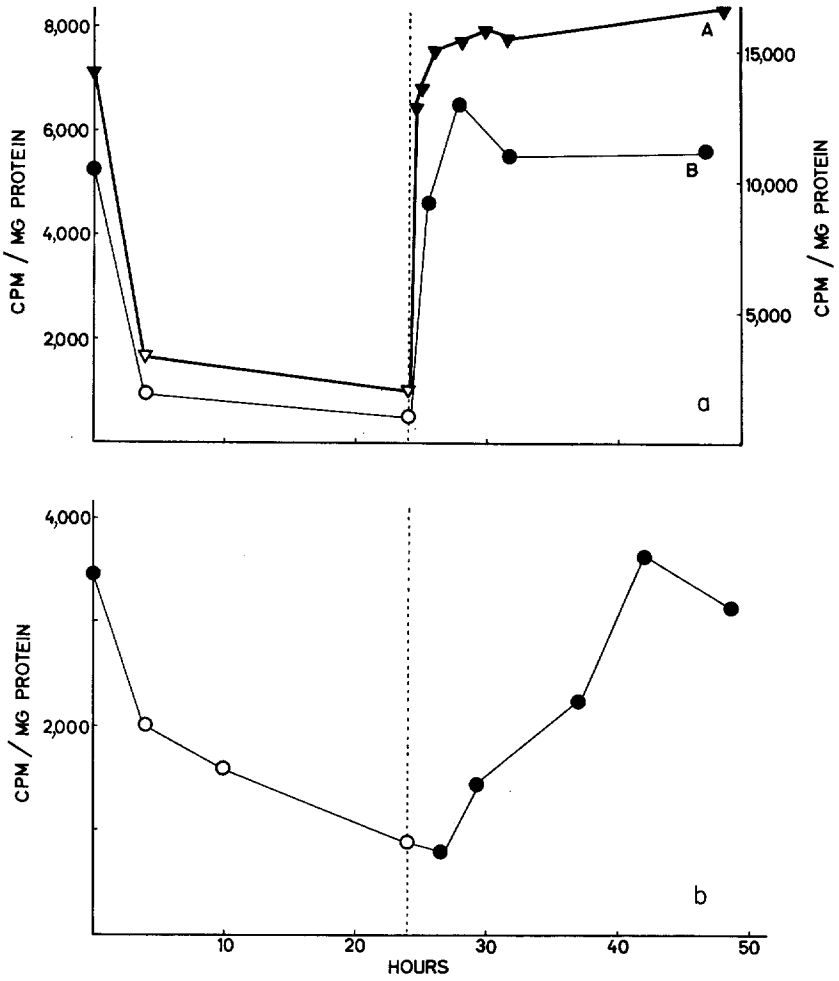

FIGURE 1 Incorporation of labeled amino acids and uridine into the protein and nucleic acid of glutamine-starved and refed Chang cells. The cells were incubated in a glutamine-deficient medium for 24 hr, after which glutamine was again added. For the incorporation tests 2.5–3.5 × 10^6 cells were incubated with the labeled components in a serum-free medium. The final volumes were 275 μl. *Fig. 1 a.* The cells were incubated for 10 min (37°C) with *A*, 0.4 mM L-leucine-^{14}C (2 mc/mmole); or *B*, 0.1 mM L-phenylalanine-^{14}C (8 mc/mmole). Curve ▽, ▼ should be read from left scale; curve ○, ● should be read from right scale. *Fig. 1 b.* The cells were incubated for 20 min (37°C) with 0.2 mM uridine-^{14}C (4 mc/mmole). Open symbols indicate cells labeled in a glutamine-free medium. Filled symbols indicate cells labeled in the presence of glutamine.

and layering the lysate onto 10 ml of a heavy medium (0.3 M sucrose, 0.025 M KCl, 0.005 M $MgCl_2$, and 0.035 M Tris buffer pH 7.8) in Spinco 50 centrifuge tubes (cf. 22). After centrifuging at 165,000 *g* for 50 min the supernatants were aspirated, and the pellets were suspended in a light medium containing 0.15 M sucrose, 0.025 M KCl, 0.005 M $MgCl_2$, and 0.035 M Tris buffer pH 7.8. The suspensions were adjusted to a final RNA concentration of 2.0 mg/ml.

For the preparation of heavy and light ribosomes the 15,000 *g* supernatant fractions were treated with 1% sodium deoxycholate, and 0.4 ml aliquots were placed on 4.6-ml 9–36% sucrose gradients in 0.025 M KCl, 0.005 M $MgCl_2$, and 0.035 M Tris buffer pH 7.8. After centrifuging for 2.5 hr at 120,000 *g* (Spinco head SW 39), 2.0 ml of the surface layer was discarded, while the rest of the supernate was diluted with light medium and centrifuged for 50 min at 165,000 *g*. This pellet was called light ribosomes, while the pellet obtained during the initial centrifuga-

tion in the swinging bucket rotor was called heavy ribosomes. The separation between these two fractions roughly corresponded to that observed in Fig. 3, *A*.

For the preparation of rat liver cell sap the livers of 150–200 *g* male albino rats were perfused in vitro with ice-cold 0.15 M NaCl, washed and minced in light medium, and homogenized in 3 volumes of the same medium by use of a Teflon homogenizer of the Potter-Elvehjem type. After centrifugation at 165,000 *g* for 50 min a sample of the upper part of the clear supernatant was collected and diluted with light medium to a protein concentration of 10 mg/ml

Amino Acid Incorporation by Cell-Free Preparations

The amino acid incorporating systems contained, in final volumes of 125 μl, either 50–100 μl 15,000 *g* supernatant fraction, or 25–50 μl ribosome suspension in combination with 50 μl rat liver cell sap. The incubation medium had the following composition: 10 mM PEP, 1 mM ATP, 0.2 mM GTP, 20 μg/ml pyruvate kinase, 0.1 mM glutamine, 9 mM $MgCl_2$, 100 mM KCl, and 30 mM Tris buffer pH 7.8. The labeled amino acid, L-leucine-^{14}C or L-phenylalanine-^{14}C, was added at a concentration of 0.045 mM and had a specific activity of 36 mc/mmole. After incubation for 10 min at 35°C, 100-μl samples were transferred to filter paper discs, and the proteins were precipitated with 10% TCA.

Determination of Radioactivity

Protein samples were prepared for radioactivity measurement by the method of Mans and Novelli (13).

In the experiments with uridine-^{14}C the filter paper discs were extracted twice for 15 min with ice-cold 5% TCA and treated for 5 min at 0°C with a mixture (1:2) of 2 M potassium acetate buffer (pH 5) and ethanol. The samples were subsequently extracted at room temperature with ethanol, with a mixture of ethanol, ether, and chloroform (1:2:1), and finally with ether. Each of these extractions was for 15 min or more.

The dry filter papers were placed in vials containing 5 ml of a solution of 0.5% 2,5-diphenyloxazole and 0.015% 1,4-di-2-(5-phenyloxazolyl)benzene in toluene, and read in a Packard Tri-Carb liquid scintillation counter. The efficiency of the counter was 55% for ^{14}C.

Density Gradient Centrifugation

For determining the ribosomal sedimentation pattern by density gradient centrifugation the 15,000 *g* supernatant fractions were treated with 1% sodium deoxycholate, and 1.5-ml samples of these suspensions were layered onto 9–36% scurose gradients in Spinco SW 25 tubes. The gradient media had the following ionic composition: 0.025 M KCl, 0.005 M $MgCl_2$, and 0.035 M Tris buffer pH 7.8 (20°C). The gradients were centrifuged at 53,000 *g* for 135 min. After centrifugation the bottoms of the tubes were punctured with a hypodermic needle through which 8-drop fractions were collected. The optical density of these fractions was measured at 260 mμ in a Beckman DU spectrophotometer.

The distribution of radioactive protein among the ribosomal fractions of pulse-labeled cells was determined by essentially the same technique. The gradients (4.6 ml) were prepared in Spinco SW 39 tubes, on top of which 0.4 ml samples of deoxycholate-treated 15,000 *g* supernatant fractions were layered. In order to indicate the position of the 80 S peaks, duplicate samples were incubated for 2 min (35°C) with 10 μg RNase before layering. The tubes were centrifuged for 60 min at 120,000 *g*, and 3-drop fractions were collected from the bottom of the tubes onto filter paper discs. These were processed for determination of protein-bound radioactivity as previously described.

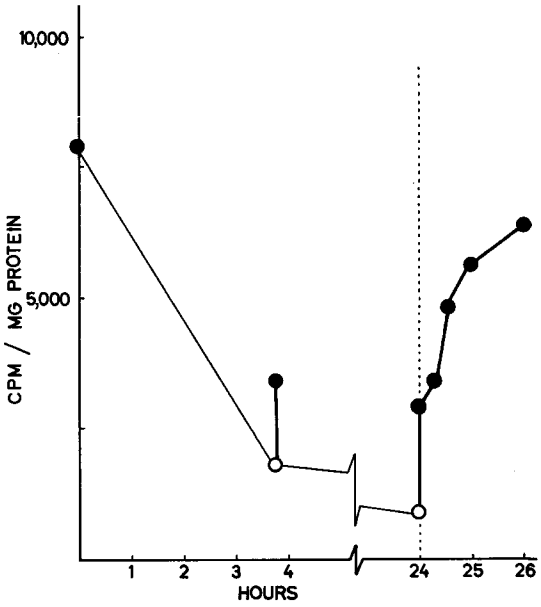

FIGURE 2 Immediate and time-dependent effect of glutamine on the incorporation of leucine-^{14}C into protein by glutamine-starved Chang cells. The incubation was as described in Fig. 1 *a*. Open symbols: the cells were incubated with leucine-^{14}C for 10 min (37°C) in a glutamine-free medium. Filled symbols: the incubation was carried out in the presence of glutamine.

Chemical Analysis

Protein was determined by the method of Lowry et al. (12). The RNA-content of ribosome suspensions was estimated from the differential ultraviolet absorption curves obtained by extraction with 0.4 M perchloric acid at 0°C and after hydrolysis for 17.5 min at 70°C (16). Free nucleotides were determined by chromatography on microcolumns of Dowex A 1 (formate form) as described previously (7).

Chemicals

ATP, GTP, and RNase were obtained from the Sigma Chemical Co., St. Louis, Mo. PEP was synthesized by the method of Clark and Kirby (1). Pyruvate kinase was purchased from the Boehringer GmbH, Mannheim, Germany, polyuridylic acid from the Miles Chemical Co., Clifton, N.J., ^{14}C-labeled leucine and phenylalanine from the Radiochemical Centre, Amersham, England, and uridine-2-^{14}C from the New England Nuclear Corp., Boston, Mass.

RESULTS

The Reversible Effect of Glutamine Deficiency on the Synthesis of Protein and RNA in Intact Chang Cells

When exponentially growing cells were transferred to a glutamine-deficient medium, their ability to incorporate labeled amino acids into protein rapidly decreased. No net gain in the protein content of the cells was observed over a period of 24 hr. Following glutamine addition the protein synthesis was restored, and after about 4 hr the rate of amino acid incorporation was comparable to, and in some experiments even exceeded, that of exponentially growing cells (Fig. 1 *a*). Preliminary experiments with similar results were carried out with arginine-deficient cells.

The lack of one essential amino acid in the cell culture might by itself be expected to restrict the rate of amino acid incorporation, in view of the well-known specificity of the incorporation process. As a preliminary test of whether or not the inherent capacity of the cells for protein synthesis was also affected by glutamine deficiency, similar incorporation experiments were carried out with glutamine added to the cell suspension just before the labeled amino acid (Fig. 2). In this case the isotope incorporation became more rapid than in the absence of glutamine, although it was still considerably smaller than in exponentially growing cells. After 24 hr of glutamine deficiency the immediate rise in the rate of amino acid incorporation following glutamine addition accounted for less than one-third of the increase attained 4 hr later, i.e. at a time when the incorporation activity had been completely restored (Fig. 1 *a*, 2).

TABLE I

Incorporation of L-Phenylalanine-^{14}C into Protein by Subcellular Fractions of Glutamine-Starved and Refed Chang Cells

The glutamine-starved cells had been incubated in a glutamine-deficient medium for 24 hr. Refed cells of the same batches had been cultured for 4 hr in a complete medium following a 24 hr glutamine starvation. Heavy and light ribosomes were separated by gradient centrifugation (2.5 hr at 120,000 *g*, Spinco head SW 39). The medium for cell-free amino acid incorporation contained 1 mM ATP, 0.2 mM GTP, 10 mM PEP, 20μg/ml pyruvate kinase, 0.1 mM glutamine, 9 mM $MgCl_2$, 100 mM KCl, 30 mM Tris–HCl buffer pH 7.8, and 0.045 mM L-phenylalanine-^{14}C (36 mc/mmole). The incubation tubes contained, in final volumes of 125 μl, either 0.5–1.0 mg 15,000 *g* supernatant protein, or 0.05–0.1 mg ribosomal RNA in combination with a standard amount of rat liver cell sap equivalent to 1 mg protein. All tests were run in duplicates. The incubation (35°C) was for 10 min (A, B) or 15 min (C). The radioactivity values are expressed as cpm/mg protein (A), or cpm/0.1 mg ribosomal RNA (B, C).

Subcellular fractions	Glutamine-starved	Refed
A. 15,000 *g* supernatant fractions	63	223
B. Whole ribosomes + rat liver cell sap	295	635
C. Heavy ribosomes + rat liver cell sap	1520	1630
Light ribosomes + rat liver cell sap	142	98

The ability of the same cells to incorporate ^{14}C-labeled uridine into nucleic acid was studied in parallel. Irrespective of whether or not glutamine was added to the test suspension together with the isotope, glutamine starvation produced a marked decrease in the rate of labeling (Fig. 1 *b*). This

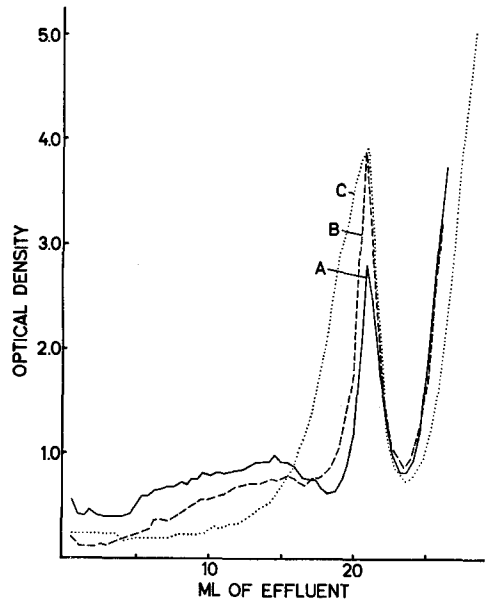

FIGURE 3 Short-term effect of glutamine deficiency on the gradient sedimentation pattern of ribosomes. The 15,000 *g* supernatant fractions of the cell homogenates were treated with 1% deoxycholate and placed on 9–36% sucrose gradients containing 0.025 M KCl, 0.005 M $MgCl_2$, and 0.035 M Tris buffer pH 7.8. The centrifugation time (53,000 *g*) was 2¼ hr. Curve *A*, exponentially growing cells. Curve *B*, cells of the same batch, but after a 4 hr glutamine starvation. Curve *C*, the 15,000 *g* supernatant fraction of cells *A* was treated for 10 min at 0°C with 10 μg/ml ribonuclease.

Amino acid incorporation data (cpm/mg protein per 10 min incubation): Incorporation of phenylalanine-^{14}C (0.2 mM, 2 mc/mmole) by whole cells (cf. Fig. 1 *a*): *A*, 2710; *B*, 750 (in the presence of glutamine 1700). Incorporation of leucine-^{14}C by 15,000 *g* supernatant fractions: *A* 342; *B*, 182. The cell-free incorporation system was as described in Table I.

could not be accounted for by an increased pool size of pyrimidine nucleotides in the cells (cf. Methods). When glutamine was restored to the culture medium, the activity of the cells of uridine incorporation was gradually restored. The time course of this restoration was, however, entirely different from that just described for the amino acid incorporation. While in the latter case there was a complete recovery within 4 hr, the restoration of the uridine incorporation was not complete until about 16 hr. During the first 4 hr in the complete medium the rate of uridine incorporation remained at the same low level as in the glutamine-starved cells, and in several experiments it even showed a slight further decrease.

The Activity of Cell-Free Amino Acid Incorporating Systems from Glutamine-Starved Chang Cells

Cell-free amino acid incorporating systems prepared from Chang cells which had been transferred to a glutamine-free medium showed a continual decline in activity. The reduced incorporation was not due to a lack of glutamine in the assay system, since the incubation was carried out in the presence of this amino acid. When the glutamine content of the cell culture was restored, the incorporation activity of the cell-free preparations gradually increased. These alterations were essentially a changing property of the ribosomes, since similar effects were observed when isolated ribosomes from Chang cells were combined with cell sap from rat liver (Table I).

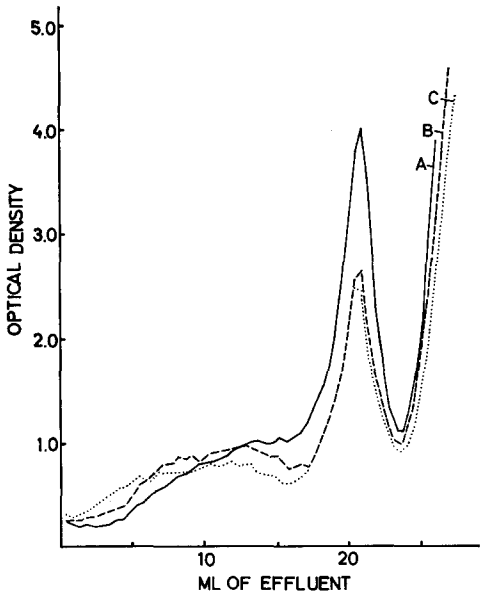

FIGURE 4 Reappearance of polyribosomes after glutamine starvation. The experimental procedure was as described in Fig. 3. Curve *A*, cells incubated for 22 hr in a glutamine-deficient medium. Curve *B*, glutamine-starved cells of the same batch incubated in a complete medium for 2 hr. Curve *C*, glutamine-starved cells of the same batch incubated in a complete medium for 24 hr.

Amino acid incorporation data (cpm/mg protein): Incorporation of phenylalanine-^{14}C by whole cells: *A*, 1110; *B*, 2150; *C*, 2420. Incorporation of leucine-^{14}C by 15,000 *g* supernatant fractions: *A*, 295; *B*, 519; *C*, 612.

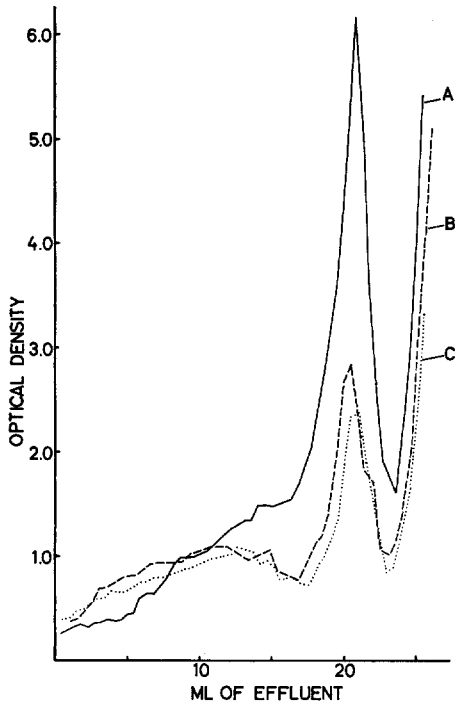

FIGURE 5 Effect of actinomycin D on the reappearance of polyribosomes after glutamine starvation. The experimental procedure was as described in Fig. 3. Curve *A*, cells incubated in a glutamine-deficient medium for 24 hr. Curve *B*, glutamine-starved cells from the same batch incubated for 4 hr in a complete medium. Curve *C*, glutamine-starved cells from the same batch incubated for 4 hr in a complete medium containing 0.2 μg/ml actinomycin D.

Incorporation data (cpm/mg protein); Incorporation of phenylalanine-^{14}C by whole cells: *A*, 336; *B*, 1840; *C*, 1970. Uridine-^{14}C incorporation by whole cells: *A*, 614; *B*, 697; *C*, 226. (The uridine-^{14}C incorporation of exponentially growing cells of the same batch was 1845.) Incorporation of leucine-^{14}C by 15,000 *g* supernatant fractions: *A*, 148; *B*, 432; *C* 373.

The Sedimentation Pattern in Ribosomes from Chang Cells Cultured in the Presence and Absence of Glutamine

Ribosomes from exponentially growing Chang cells had a characteristic sedimentation pattern with a distinct separation between a moderate 80 S peak and a broad polyribosomal region (Fig. 3). A significant amount of heavy, RNA-containing material sedimented at the bottom of the gradient tubes. When the 15,000 *g* supernatant fraction was treated with RNase before the sedimentation analysis, the amount of RNA in the sediment and in the polyribosomal region of the gradient was strongly reduced, while the 80 S peak showed a corresponding increase. In the experiment shown in Fig. 3, the RNase treatment was carried out under particularly mild conditions. The 80 S peak showed a broad shoulder at the heavy side obviously representing polyribosomal fragments, including dimers and trimers.

Within 4 hr after the cells had been transferred to a glutamine-free medium the sedimentation pattern of the ribosomes was significantly altered. The proportion of heavy polyribosomes was reduced, while at the same time the 80 S peak increased and became less distinctly separated from the polyribosomal region (Fig. 3). With continued glutamine starvation this shift became increasingly pronounced (Figs. 4, 5).

After restoration of the glutamine content of the medium the shift in the sedimentation pattern of the ribosomes towards smaller aggregates and monomers was reversed (Figs. 3–5). As soon as 2 hr after glutamine addition the reduction in the size of the 80 S peak was quite marked, and the shape of the sedimentation pattern in the polyribosomal region attained a normal appearance 2 hr later (cf. 15). The reversible changes in the sedimentation properties of the ribosomes during glutamine starvation were not associated with any significant accumulation of active ribosomal monomers in the 80 S peak (Table I). In experiments with pulse-labeled cells the particle-bound, radioactive protein was, under all experimental conditions, concentrated in the heavy polyribosomal fractions (Fig. 6). Likewise, the incorporation activity in vitro was almost exclusively found in the heavy particles. On the basis of their RNA content, preparations of polyribosomes from exponentially growing, glutamine-starved and refed cells had similar specific incorporation activities (Table I).

Effect of Actinomycin D on the Recovery of Chang Cells from Glutamine Starvation

In the experiment shown in Fig. 7, Chang cells had been preincubated in a glutamine-free medium for 24 hr. Actinomycin D and glutamine were added at the start of the experiment. After 4 hr the rate of amino acid incorporation into the control culture had increased by a factor of four,

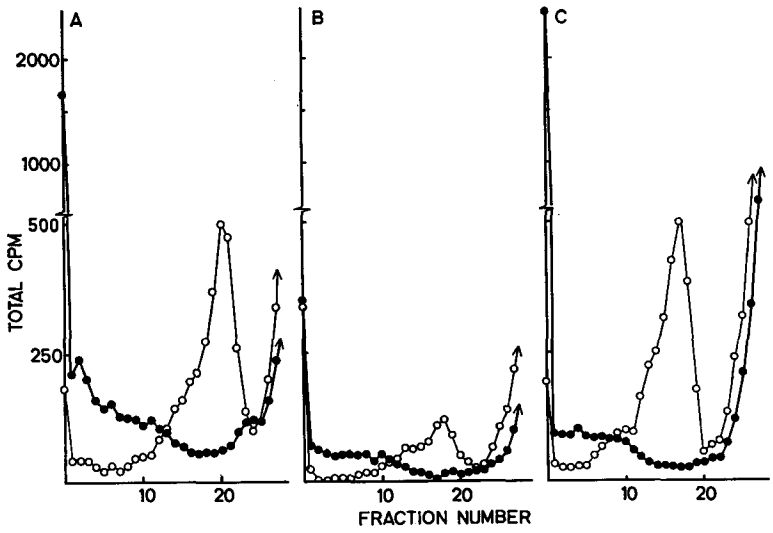

FIGURE 6 Distribution of radioactive protein among density gradient fractions after pulse-labeling of the cells with leucine-^{14}C. The cells were incubated for 4 min (35°C) with leucine-^{14}C before homogenization. The 15,000 *g* supernatant fractions were treated with 1% deoxycholate, and 0.4 ml aliquots were placed on 4.6-ml 9–36% sucrose gradients. After centrifuging for 60 min at 120,000 *g* (Spinco head SW 39) 3-drop fractions were collected from the bottom of the tubes and the radioactivity of the protein determined. A, exponentially growing cells; B, cells incubated in glutamine-deficient medium for 24 hr; C, glutamine-starved cells incubated in a complete medium for 4 hr. Curves with open symbols indicate 15,000 *g* supernatant fractions treated with RNase (10 μg/ml) for 2 min at 35°C.

while at the same time the uridine incorporation showed a slight further decrease.

Actinomycin D at a concentration of 0.2 μg/ml produced pathological alterations of the cells and prevented their further multiplication when transferred to normal medium after 4 hr. Although this concentration of actinomycin D caused a further reduction of the uridine incorporation to 10% of that of exponentially growing cells, the increase in the rate of amino acid incorporation was not inhibited, but even slightly enhanced [Figs. 5 (legend), 7]. With higher actinomycin D concentrations (0.5–1.0 μg/ml) the rate of uridine incorporation was reduced to less than 5% of the incorporation activity of exponentially growing cells. This effect was attained within 1 hr after the addition of the inhibitor. Despite the almost complete inhibition of DNA-dependent RNA synthesis suggested by the above figures (cf. 6, 17), the rate of protein synthesis in these lethal cells increased to a level not much lower than in the controls[4] [Figs. 5 (legend), 7]. Pulse-labeling experiments indicated that, as in control cells, this incorporation was essentially associated with heavy ribosomal aggregates (Fig. 8).

When the glutamine-starved cells were incubated for 4 hr in a complete medium containing 0.2 μg/ml of actinomycin D, there was essentially the same shift in the ribosomal sedimentation pattern towards a higher proportion of polyribosomal aggregates as in the absence of the inhibitor (Fig. 5). There was also a corresponding increase in the ability of cell-free preparations to incorporate labeled amino acids into protein (Fig. 5, legend). With higher concentrations of actinomycin D (0.5–1.0 μg/ml) the sedimentation pattern became increasingly abnormal, with a tendency of the ribosomes to precipitate already during the 15,000 *g* centrifugation.

DISCUSSION

The omission of the essential amino acid, glutamine, from the culture medium of Chang cells caused a rapid and extensive decrease in the

[4] When the period of actinomycin treatment was extended beyond 4 hr, the activity of protein synthesis gradually decreased, concomitantly with progressive morphological alterations and increasing cell death.

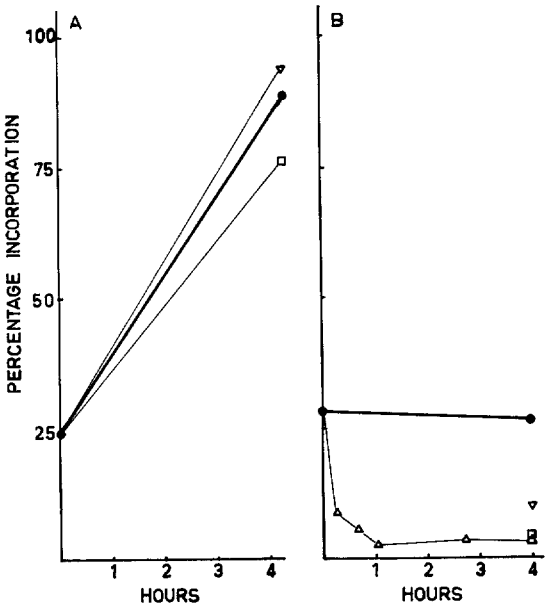

FIGURE 7 Effect of actinomycin D on the recovery of Chang cells from glutamine starvation. The cells had been preincubated in a glutamine-deficient medium for 24 hr. Glutamine was added at zero time, together with actinomycin D at the concentrations indicated. The incubations with leucine-^{14}C (A) and with uridine-^{14}C (B) were carried out as described in Fig. 1. The concentrations of actinomycin D were: ●, none; ▽, 0.2 mg/ml; △, 0.5 mg/ml; □, 1.0 mg/ml. Incorporation by exponentially growing cells before glutamine starvation (indicated as 100%): leucine-^{14}C, 3640 cpm/mg protein; uridine-^{14}C 2530 cpm/mg protein.

capacity of the cells to incorporate labeled amino acids into protein. When the glutamine content of the medium was restored, the incorporation activity rose within minutes to 30–40% of that of exponentially growing cells. After this sharp but limited initial rise, further increase became less steep, and full restoration was not attained until 2–4 hr later.

The characteristic biphasic recovery of protein synthesis after glutamine starvation should be considered in connection with the concomitant ribosomal alterations. During glutamine starvation the proportion of ribosomes with a sedimentation constant of approximately 80 S gradually increased to more than twice the normal level. This fraction of particles was not associated with any significant activity of amino acid incorporation. At the same time there was a marked reduction in the proportion of rapidly sedimenting, active particles, apparently representing heavy polyribosomes. After the addition of glutamine this picture was gradually reversed. We feel that during glutamine starvation two factors contributed to the reduction in the protein synthesis of the cells: (*a*) the acute shortage of the essential amino acid, glutamine, that prevented potentially active polyribosomes from working with full efficiency (as soon as glutamine was again made available, this restriction was eliminated); (*b*) the gradual loss of heavy polyribosomes.

Disaggregation of polyribosomes may in principle be mediated through either of the following two mechanisms: (*a*) breakdown of the strand of messenger RNA that connects individual ribosomes; (*b*) reduction of the number of particles simultaneously attached to the same, intact messenger RNA. Sedimentation data do not allow a distinction to be made between these two possibilities. It is obvious, however, that a decrease in the average size of the ribosomal aggregates due to messenger RNA breakage can be reversed only if new messenger is made available, e.g. through RNA synthesis. It was of a particular interest, therefore, to see whether the restoration of a normal polyribosomal pattern in glutamine-starved Chang cells was preceded by an increased rate of incorporation of labeled uridine into nucleic acid.

Our experiments clearly indicated that this was not the case. In fact, the already low uridine incorporation in the starved cells showed a further decrease upon glutamine addition, in most experiments lasting over the whole period of 2–4 hr required for the amino acid incorporation to reach its normal level. Actinomycin D proved highly toxic to Chang cells after glutamine starvation. Nevertheless, concentrations of this antimetabolite that effectively inhibited the limited RNA-metabolism still remaining after glutamine starvation had a very small effect, if any, on the increase in amino acid incorporation. This insensitivity to actinomycin D in combination with the delayed onset of the increased RNA metabolism after glutamine starvation strongly suggests that the restoration of a normal rate of protein synthesis in the cells was not dependent on the production of new messenger RNA. It is tempting to conclude that previously synthesized messenger RNA, preserved in the cells in a stabilized form (cf. 8, 10) was brought into action in response to the reestablishment of an adequate cellular environment.

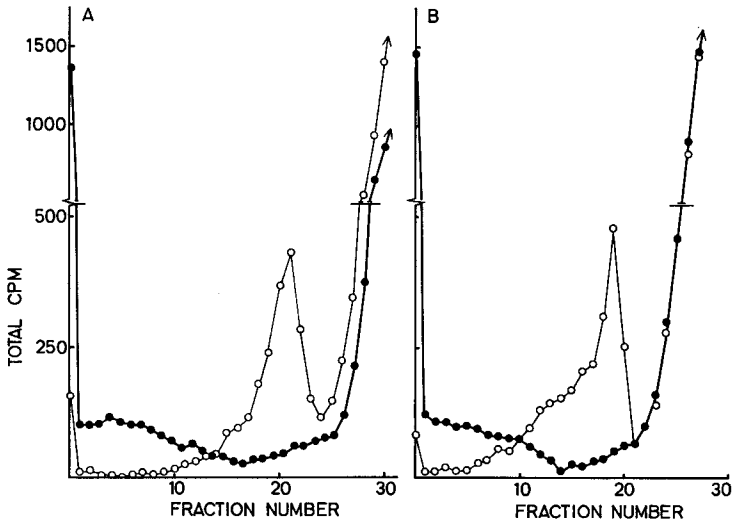

FIGURE 8 Distribution of radioactive protein among density gradient fractions of previously glutamine-starved Chang cells, recovering in the presence of actinomycin D. After 24 hr of glutamine starvation the cells were incubated for 4 hr in a complete medium containing 0.2 μg/ml (A) or 1.0 μg/ml (B) actinomycin D. Before homogenization the cells were given a 4 min pulse of leucine-^{14}C. Curves with open symbols indicate that the cleared homogenates had been treated for 2 min (35°C) with 10 μg/ml RNase before gradient analysis. For further experimental details, see Fig. 6.

The relatively long time required for the restoration of heavy polyribosomes after glutamine starvation is worthy of note. This sluggishness of the ribosomal reattachment is inconsistent with a free ribosome–messenger RNA interaction and suggests either that some ribosomes in the glutamine-starved cells had a reduced affinity for functional attachment to the messenger, or, alternatively, that the messenger RNA was not immediately available for reaction with ribosomes.

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