The rate of protein synthesis in a number of mammalian tissues is known to vary with the nutritional state of the animal. Although the mechanisms underlying the variation remain to be elucidated, nutrition is known to alter several factors that would be expected to influence the rate per cell. Fasting, for example, leads in the liver to decreases in the ribosome content (1, 2), in the fraction of ribosomes that are polyribosome-associated (3, 4, 5), hence potentially active in synthesis, and in the amino acid-incorporating activity of the polyribosomes as measured in vitro (6) and in vivo (7).

To study the nutritional modulation of protein synthesis, we have turned to a cell culture system in which one can define and vary the composition of the extracellular medium and measure protein synthesis much more readily than in an animal. This report presents the results of work with intact cells in which we have measured the fraction of ribosomes in polyribosomes, together with the protein synthesis rate per cell and per unit of polyribosomal RNA, during the interval between additions of nutrients to the culture. In this interval, during which glucose in the medium is depleted and glutamine is appreciably diminished, changes occur in the ribosome distribution and in the activity of the polyribosomes which resemble those observed in the liver during fasting. From these changes, it is evident that several sorts of rate limitation at the translational level are involved, and it appears that with the cell culture system one has a useful tool for investigating these translational controls.

**Materials and Methods**

**Reagents**—We purchased the tissue culture media and the minimum essential and nonessential amino acid mixtures from Grand Island Biologicals, cycloheximide from Sigma, and uniformly labeled L-lysine-\(^{14}\)C (specific activity about 250 Ci per mole) from New England Nuclear. The lysine, supplied in 0.01 N HCl, was neutralized before use by dilution with an equal volume of 0.01 N NaOH. The Glytel reagent was purchased from Pfizer. Buffer T consists of 10 mM triethanolamine, adjusted with HCl to pH 7.4 at 25\(^\circ\)C, 5 mM MgCl\(_2\), and 10 mM KCl.

**Cells**—The cells used are a strain of Ehrlich ascites tumor cells adapted to culture by Dr. Bunsiti Simizu in this laboratory from a mouse-carried linekindly supplied by Professor Donald Wallach. They grow readily both in monolayer and suspension culture, and retain the ability to produce tumors in mice.

**Cultures**—Cells were maintained in spinner flasks (Belco Glass, Inc.) at 37\(^\circ\)C in Eagle’s minimum essential spinner medium with Earl’s salts, 10\% heat-inactivated calf serum, and 100 \(\mu\)g per ml and 100 \(\mu\)g per ml, respectively, of penicillin and streptomycin. Under these conditions, the cell count doubles within 24 hours. The cell concentration was maintained in the range 4 to 8 x 10\(^5\) per ml by diluting the cultures each morning with an approximately equal volume of new medium, an operation subsequently referred to as replenishment.

**Cell Sap**—Livers of approximately 300-g male CD rats (Charles River Laboratories) were homogenized in 1.5 volumes of Buffer...
acids, the amount of added isotope was adjusted to give the
pellet, the cell sap, was removed and kept frozen in liquid nitro-
gen until just before use.

**Rate of Protein Synthesis**—The method was based on that of
Mans and Novelli (8). A 10-ml sample of the culture to be tested,
containing about 5 × 10^6 cells, was spun down (1500 × g for 2
min) at room temperature and 9.0 ml of the supernatant solu-
tion were removed. The cells were resuspended in the remaining
1.0 ml of medium and transferred to a chilled 25-ml glass-stop-
pered flask on ice, and neutralized L-lysine-14C solution was
added. Generally, 10 μl of the isotope solution (containing 0.5
μCi) were used but, in cases in which the lysine concentration in
the culture had been increased by the addition of essential amino
acids, the amount of added isotope was adjusted to give the
same lysine specific activity in all of the incubations. Otherwise,
it was not necessary to compensate for differences in the lysine
concentration in the media since it was found that even after a
24-hour growth period the decrease was negligible. After a
zero time sample was taken, the flasks were gassed with 95% O_2
| 5% CO_2 (the CO_2 being required to maintain the pH in the
7.0 to 7.2 range) and stoppered, and the incubation was begun
by placing the flasks in a 37° rotary water bath shaker. Duplicate
50-μl samples, usually taken at 10 and 20 min, were placed on
2-cm discs of Whatman No. 3MM paper, which were processed
as described (8) except that a less elaborate washing procedure
was used. After initial immersion in 10% trichloracetic acid +
0.1% L-lysine, the discs were washed, heated to 95° for 15 min,
and again washed, all in 5% trichloracetic acid + 0.1% L-
lysine, and finally washed with ethanol and dried.

Counting was done in a conventional toluene-based scintilla-
tion mixture. It was found that all of the radioactivity remained
on the disc, permitting repeated reuse of the vial and scintillation
mixture after removal of the disc. The radioactivity of zero
time samples averaged about 30 cpm and was not diminished by
the additional washing steps originally described.

The short time required for a measurement permits frequent
monitoring of the protein synthesis rate over a several hour
period. We found the rate to be constant for at least 30 min,
and to be the same as in an unconcentrated culture.

**Sucrose Gradients**—The discontinuous gradients were pre-
pared in tubes for the Spinco type 65 fixed angle rotor by layering
a mixture of 3.2 ml of 1.92 M sucrose and 0.8 ml of cell sap (final
sucrose concentration 1.6 M) over a mixture of 3.4 ml of 2.32 M
sucrose and 0.0 ml of cell sap (final sucrose concentration 2.0
M). Both sucrose solutions were made in Buffer T.

The continuous gradients consisted of 5.0 ml of a 15 to 26%
sucrose solution in Buffer T. These were overlaid with approxi-
mately 0.1 ml of sample containing about 2 A_230 units of
material to be analyzed. The tubes were spun at 39,000 rpm
in a Spinco type SW-39 rotor with the timer set for 25 min and
the brake off, and analyzed at 260 μM with a continuous flow
monitoring system (10).

**Isolation of Polyribosomes**—To 100 ml of the culture containing
about 5 × 10^6 cells, cycloheximide was added to give 100 μg per
ml. The mixture was stirred for 1 min at 37°, and then rapidly
cooled by pouring into a centrifuge bottle containing about 50 g
of crushed ice and centrifuged at 1100 × g for 5 min. After
being washed with 10 ml of Buffer T at 0°, the pelleted cells were
lysed by suspending them in 3.5 ml of rat liver cell sap (see above)
containing 0.3% Triton X-100 and 0.5% sodium deoxycholate.
As judged by microscopic examination, this procedure disrupts
virtually all of the cells, yet leaves the nuclei intact and free of
attached cytoplasm. The nuclei were removed by centrifugation
(5 min at 280 × g) and the supernatant fraction was layered over
a discontinuous gradient (see above) which was centrifuged for
15 hours at 65,000 rpm in a Spinco type 65 fixed angle rotor.
The resulting ribosome pellet was gently suspended in 0.5 ml of
Buffer T. Aggregated material was removed by centrifugation
(15 sec at 1500 × g) and a portion of the supernatant ribosome
suspension was then layered on a linear sucrose gradient (see above).

**Determination of Fraction of Ribosomes and Total RNA in
Polyribosomes**—The polyribosome fraction was taken to be the
area of the polyribosome region of the recorded absorbance
pattern divided by the total area represented by the monomers
plus polyribosomes. The total area was measured with a plani-
meter. The monomer area was determined by multiplying the
peak height by its width at one-half the distance from base to
apex; subtracting this value from the total gave the polyribosome
area. The amount of ribosomal RNA was taken to be 85% of
the total RNA (2).

**Assay**—Glucose was measured with the Glytel reagent (11),
with 50-μl samples of the undiluted culture medium and 2.0 ml
of reagent.

RNA and DNA were extracted by Schneider's procedure (12)
and assayed by the orcinol reaction (13) and Burton's modifica-
tion of the diphenylamine reaction (14), respectively. Evidently
these cells do not contain appreciable amounts of the non-DNA
diphenylamine-reacting material (15) that is extracted from
liver by the Schneider procedure, for equivalent values were
obtained if the nucleic acids were isolated by the method of
Schmidt and Thannhauser (16).

Amino acid analyses were performed with Beckman model
120 C analyzer.

**Cell-free Protein Synthesis**—The partially fractionated system
of Lamfrom and Knopf (17) was used except for the magnesium
concentrations, which are given in Table I. The reaction
mixture contained ribosomes equivalent to 45 μg of ribosomal
RNA, 2 μCi of L-phenylalanine-14C (375 Ci per mole), and,
when added, 100 μg of polyuridylicate, in a total volume of 1.0
ml. Incubation was for 30 min at 37°.

**RESULTS**

**Extraction of Representative Polyribosome Preparation**—The
validity of physiological interpretations based on the relative
proportion of single ribosomes, which are inactive in protein
synthesis, and polyribosomes, which are active, depends on
obtaining subcellular preparations that exhibit a ribosome dis-
bution matching that of the intact cell. The most serious
problem in obtaining such a representative distribution is the
artefactual production of single ribosomes, which can occur in

---

1 We feel justified in assuming that the specific activity of the
intracellular lysine pool closely approaches that of the medium
(and hence is the same throughout our experiments) for, at the
concentration of lysine in the medium (0.3 mM), one can expect
the contribution of exogenous labeled lysine to be large relative
to the endogenous unlabeled contribution from protein turn-
over (9).
two ways. Polyribosomes can be broken down through ribonuclease action or by mechanical shearing of the connecting mRNA strand. Also, single ribosomes may accumulate if they run off the strand faster than they reattach should conditions permit protein synthesis at an appreciable rate during the preparative procedure (initiation apparently is a more labile step in synthesis than translation at the low temperatures used (18)).

Ribosome runoff was minimized by adding sufficient cycloheximide to the culture just before harvesting to stop movement of ribosomes along mRNA (19), and by chilling the culture to zero degrees before centrifuging the cells. The effectiveness of this treatment was evaluated with the experiment presented in Fig. 1, in which portions of a culture were processed in different ways.

Relatively slow cooling, achieved by swirling the culture flask in an ice bath, yielded Pattern A, in which the single ribosome peak comprises 12% of the total ribosome absorbance. With more rapid chilling, achieved by pouring the culture over crushed ice, the single ribosome fraction was only 5% of the total, as seen in B. When cycloheximide was added before harvesting, even though subsequent chilling was slow, the single ribosomes fraction still was 5% (C). The virtual identity of the polyribosome patterns under these latter two conditions shows the effectiveness of cycloheximide in preventing ribosome runoff. The combination of rapid chilling and cycloheximide yielded a pattern (D) not appreciably different from B and C. Thus, it is unlikely that cycloheximide, at the concentrations used here, produces any artifactual alteration of the polyribosome distribution such as might occur with low concentrations of the drug (20). Because its use makes the rate of chilling less critical, we chose to use cycloheximide routinely together with rapid chilling by pouring the culture over ice.

The problem of minimizing ribonuclease action was approached by using rat liver cell sap, which contains a potent ribonuclease inhibitor (21, 22), in both the homogenizing medium and the discontinuous gradients. Nuclease activity also was slowed by performing all preparative procedures at temperatures close to 0°C. The value of using cell sap was found to be similar to that of using cycloheximide; i.e., although good patterns were at times obtained without cell sap, the patterns never were better in its absence and generally were better in its presence. Also, preparations made with cell sap were found to be more stable on storage at 0°C. Therefore, it was used routinely.

The relatively small fraction of single ribosomes in Patterns B, C, and D of Fig. 1 indicates that our preparative procedure provides excellent preservation of the polyribosomes. However, as it was important to know whether apparent changes of only a few per cent in the proportion of monomeric ribosomes were significant, we sought to estimate the proportion that arose through polyribosome degradation during the isolation procedure. To do this, we labeled the nascent peptide chains of the polyribosomes by briefly incubating the cells with a radioactive amino acid and examined the distribution of radioactivity in
changes in ribosome distribution and rate of protein synthesis following medium replenishment—The above described method for obtaining representative and undegraded ribosome preparations yielded highly reproducible distribution patterns from the same culture. However, comparing different cultures, we continued to see marked variability in the patterns. Thus, it appeared that the variations were physiologically significant, and that, despite the apparent exponential multiplication of the cells, which generally doubled in number each day, the method of maintaining the cultures did not provide even approximately constant growth conditions. That the variations very likely were due to exhaustion of one or more nutrients, and possibly also to accumulation of metabolites, was indicated by the finding that by 24 hours after replenishment glucose was no longer detectable in the culture medium and the pH had decreased appreciably.

To see whether systematic variations in ribosome distribution occurred following replenishment of the culture medium, we monitored the ribosome distribution at intervals during a 24-hour period after diluting the culture with an equal volume of new medium. Fig. 3 presents the results. There was a progressive shift of single ribosomes into polyribosomes during the first 4 hours; after about 9 hours this trend reversed, and by 24 hours the pattern closely resembled that obtained initially.

We then determined not only the ribosome distribution but also the rate of protein synthesis, the RNA and DNA content of the culture, and the glucose concentration in the medium during the interval after replenishment. The results of three typical experiments, some covering only portions of the 24-hour
Changes in the rate of protein synthesis per unit of cells (A) and per unit of polyribosomes (B), in the fraction of ribosomes in polyribosomes (C), and in the glucose concentration of the medium (D) following replenishment of the medium in three experiments performed as for Fig. 3. ○—○, ●—●, and ■ ■ results of Experiments 1, 2, and 3, respectively. From Panels A and B, it can be seen that, following medium replenishment, the rates of synthesis per cell increased steadily to about 3 times the initial value after about 6 hours, remained constant for the next 8 hours, and then decreased to about the zero time level by 24 hours.

The rise and fall in the rate of synthesis per cell were due to changes of two sorts involving the protein-synthesizing apparatus. From Panel C, Experiment 3, in which early time points following replenishment were obtained, one sees that the fraction of polyribosome-associated ribosomes increases rapidly, reaching a maximum of about 90% after 2 to 4 hours. Thus, a larger fraction of the ribosomes was engaged in synthesis. In addition, the synthesis rate per unit of polyribosomal ribosomes also increased following replenishment, as seen in Panel B. After rising, this rate and the polyribosome fraction remained approximately constant for a period and then declined. Although these changes occurred roughly in parallel, the polyribosome fraction consistently increased more rapidly than did the synthesizing activity of the polyribosomes. From Panel D, one sees that the glucose concentration fell steadily and was too low to be detected after 12 to 16 hours. In both experiments, synthesis per cell began to decline at the same time that glucose was exhausted.

**Effects of Adding Various Nutrients to Depleted Cultures**

The above results indicate that changes in glucose concentration could have been responsible for the observed effects on the protein-synthesizing apparatus, but it remained possible that depletion of other nutrients and accumulation of metabolic products also were involved. With the exception of glutamine, the amounts of the essential amino acids in the medium considerably exceed those required to yield the amount of protein synthesized in the culture during a 24-hour period. Glutamine depletion remained a possibility since it not only is incorporated into protein and serves as a nitrogen source for synthesis of the nonessential amino acids, but also is subject to degradation in the medium through the glutaminase activity associated with serum. Depletion of an essential serum factor was also a possibility.

To determine the relative importance of glucose, serum, and essential amino acids in maintaining protein synthesis, we added the three components singly and in combinations to a culture that had been replenished with complete medium 24 hours before; after a 4-hour incubation period, the ribosome distribution patterns were obtained and the rates of protein synthesis and the RNA and DNA content were measured. Also, to evaluate the possibility that accumulation of metabolites was a factor, the same measurements were made after dilution of the culture with fresh medium.

**Table II**

<table>
<thead>
<tr>
<th>Experiment and additions</th>
<th>Rate per unit of cells</th>
<th>Rate per unit of polyribosomes</th>
<th>Polyrribosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. None..........................</td>
<td>6.6 6.3 47</td>
<td>10 6.4 72</td>
<td>7.4 5.8 56</td>
</tr>
<tr>
<td>Glucose.......................</td>
<td>18 8.4 93</td>
<td>18 9.6 91</td>
<td>11 7.7 70</td>
</tr>
<tr>
<td>Essential amino acids........</td>
<td>24 10 93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum..........................</td>
<td>11 7.1 71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose + serum................</td>
<td>15 8.4 85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose + essential amino acids</td>
<td>18 9.6 91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum + essential amino acids</td>
<td>11 7.7 70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose + essential amino acids + serum</td>
<td>17 9.6 87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum + nonessential amino acids</td>
<td>13 7.6 80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**a** Counts per min of L-lysine-14C incorporated per min per µg of DNA.

**b** Counts per min of L-lysine-14C incorporated per min per µg of polyribosomal RNA.

**c** Fraction of total ribosomes in polyribosomes.

**d** Dialyzed calf serum, 1 ml/9 ml of medium, was used in each case.
The results of two experiments are given in Table II. Added singly, essential amino acids, dialyzed serum, and, most markedly, glucose, increased the polysome fraction, and thereby the rate of protein synthesis per cell, without significantly altering the rate of synthesis per unit of polysomes. However, addition of the three supplements together produced a nearly 3-fold increase in synthesis per cell due to a marked increase in the polysome fraction and to the activity per polysome. Dilution with fresh medium was somewhat more effective in restoring synthesis than merely supplementing the culture, presumably because accumulated metabolites were diluted (23). (From the high glycolytic activity of these cells, one would assume lactate to be a major accumulated product.) When the supplements were added in pairs, glucose plus essential amino acids gave maximum restoration of both the polysome fraction and the rate per polysome; the further addition of serum did not significantly alter the rate. Thus, depletion of glucose and one or more essential amino acids, but not serum factors, was responsible for the changes observed in the 24-hour experiments. Since the combination of nonessential amino acids plus serum produced an effect which was slight compared with that of glucose plus serum, glucose apparently was more important as an energy source than as a source of carbon for biosynthesis.

In order to determine which of the essential amino acids fell to suboptimal concentrations, we measured the amino acid content of the medium after 24 hours of cell growth. Of the 13 amino acids originally present, only glutamine had decreased appreciably, although interference by an unidentified component prevented a precise quantitative determination. This observed depletion of glutamine but not of the other amino acids is in agreement with the findings of others (23).

**DISCUSSION**

With a commonly used schedule of suspension culture maintenance and at cell densities within a range often used, we have found unexpectedly wide fluctuations in the protein-synthesizing activity of Ehrlich ascites tumor cells during the 24-hour interval between replenishment of the medium. Although the cell number generally doubled during this interval, the culture clearly was not in a steady state of continuous exponential growth throughout the period. If optimal concentrations of nutrients are supplied, the generation time of these cells is about 16 hours; thus with our original maintenance schedule, the actual growth curve during what might be termed a growth cycle must have been sigmoid rather than exponential.

To obtain cells that are closely similar from day to day in protein-synthesizing activity, exhibiting a low fraction of single ribosomes and a consistently high rate of synthesis per unit of polysomes, we now incubate the cells in fresh medium for 2 to 4 hours prior to use, following overnight growth. Our present culture maintenance schedule also includes adding 1 mg per ml of glucose and 0.3 mg per ml of L-glutamine at about 5 p.m. each day in addition to dilution with fresh medium at about 9 a.m. (The addition of glucose in this way is preferable to doubling the amount incorporated directly into the medium, for glycolysis was found to be increased by increasing the glucose concentration.)

Although the changes observed during the growth cycle present a practical problem if one wishes to obtain cells in a reproducible physiological state, the changes are of considerable interest, closely resembling those seen in certain tissues of an intact animal during fasting and refeeding. In the liver of a rat or mouse deprived of food, polysome activity measured both in vitro (6) and in vivo (7) decreases substantially after several days, followed by a progressive shift in the ribosome distribution pattern to monomeric ribosomes and smaller polyribosomes (3-5, 7), changes that are reversed within several hours after refeeding (4, 5).

These changes in polyribosome activity and ribosome distribution indicate clearly that protein synthesis in mammalian cells can be modulated by translational controls acting at several points. Rate limitation of ribosome movement along the mRNA strand was indicated in our experiments by the changes observed in the rate of peptide synthesis, i.e. peptide chain elongation, per unit of polyribosome-associated ribosomes. That the rate of chain initiation also is influenced by cell nutrition can be inferred from the accumulation of single ribosomes during nutritional deprivation in our cultures even though ribosome movement, hence runoff, had slowed. Further, it appears that the processes of initiation and elongation can be modulated independently. This is indicated by the results of Experiment A, Table II, showing that, after addition of supplements singly to the medium, the fraction of ribosomes in polysomes increased without any change in the synthesis rate per polyribosomal ribosome. Thus, the initiation rate had increased without a change in the rate of peptide chain elongation. A similar dissociation of the two processes, with initiation increasing more rapidly than elongation after replenishment of the culture medium, was seen in Experiment 3 of Fig. 4.

However, the means by which the reaction rates are varied in response to the extracellular concentration of nutrients remain obscure even though the reactions involved in peptide synthesis now are understood in some detail. Substrate concentrations below saturating amounts must necessarily slow the reactions, and one would expect that, with glucose depletion, the intracellular ATP concentration, and secondarily the GTP and aminoacyl-tRNA levels, would fall. However, it is not understood why limitations of these substrates, essential for both initiation and elongation, should influence one process more than the other. Nevertheless, when the energy supply is restricted, chain initiation is slowed to a greater extent than elongation (24-26).

It is clear also that control is exerted through changes in the amounts or activities (or both) of macromolecular components of the synthesizing apparatus. That such changes were at least in part responsible for the alterations in peptide initiation and elongation observed in our cultures, and for impaired initiation seen in those of Eliasson, Bauer, and Hultin (27) after prolonged glutamine deprival, can be inferred from the relatively slow restoration of these processes after replenishment of the medium; if substrate limitation alone were involved, a very rapid reversal would be expected (27-29). More direct evidence of impaired function of, or deficiency in, the macromolecular factors involved in peptide elongation is the finding (6, 7) that polyribosomes from the liver of fasted animals, compared with those from fed animals, incorporate amino acids into peptide less actively in vitro even though supplied with optimal concentrations of substrates. A similar diminution of polyribosome activity in vitro has been observed (30) comparing preparations from yeast in the stationary growth phase with those from exponentially growing cells. Evidence has recently been presented (31) indicating that the presence of an inhibitor rather than lack of
some essential component may account for the reduction in peptide elongation rate that develops during nutritional deprivation. In the case of initiation, although the availability of mRNA conceivably could be a limiting factor, reversal of the apparent impairment has been observed (5, 27, 28) even when RNA synthesis is effectively inhibited.

In any event, our observations indicate that mammalian cells in culture afford a system for studying, with greater ease and precision than is possible in an intact animal, the ways in which nutritional factors modulate protein synthesis. Experiments aimed at determining the individual effects of limiting the supply of energy sources, amino acids, and serum constituents are now in progress.

Acknowledgments—We thank Dr. Howard H. Hiatt for his advice and encouragement and Miss Carol Kleiner for her skillful assistance.

REFERENCES


Downloaded from http://www.jbc.org/ by guest on August 15, 2017
Nutritional Effects on the Polyribosome Distribution and Rate of Protein Synthesis in Ehrlich Ascites Tumor Cells in Culture
Walther J. W. van Venrooij, Edgar C. Henshaw and Carl A. Hirsch


Access the most updated version of this article at [http://www.jbc.org/content/245/22/5947](http://www.jbc.org/content/245/22/5947)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/245/22/5947.full.html#ref-list-1](http://www.jbc.org/content/245/22/5947.full.html#ref-list-1)