COMPARISON OF THE RELATIVE SYNTHESIS OF THE PROTEINS OF THE 50S RIBOSOMAL SUBUNIT IN GROWING AND VALINE-DEPRIVED HeLa CELLS

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ABSTRACT

Quantitative studies of the synthesis of the ribosomal proteins of the 50S ribosomal subunit have been made with growing *versus* valine-deprived HeLa cells. The synthesis of total cell protein and 50S subunits was also compared between the growing and nongrowing cells. It was found that between 12 and 20 hr of valine deprivation the net synthesis of 50S subunits drops to approximately 8% of that in control cells while the over-all synthesis of 50S subunit ribosomal proteins declines to approximately 12% of that in the controls. However, the synthesis rates for each of two particular 50S subunit proteins decline to approximately 8% of the rates in the growing cells, indicating that the synthesis of one of these proteins may be rate limiting for 50S subunit biosynthesis in valine-deprived HeLa cells. Other evidence indicates that the regulation of synthesis of the ribosomal proteins in valine deprivation depends on control at the level of transcription or translation rather than being a function of the relative valine content of these proteins.

INTRODUCTION

It has been shown that deprivation for essential amino acids provides a convenient model system for studying the regulation of synthesis of macromolecules in cultured human cells (Eagle, 1955; Vaughan et al., 1967 *b*). During such deprivation, cells retain their viability for several days (Cohen et al., 1961), while the rate of protein synthesis declines to 25–30% of the rate in active growth. This low level of synthesis is maintained by endogenous protein turnover (Eagle et al., 1959).

HeLa cells deprived of methionine continue to synthesize significant quantities of 45S ribosomal precursor RNA (r-pre-RNA) which is submethylated and cannot be processed into ribosomal subunits (Vaughan et al., 1967 *b*). During this stress the synthesis of ribosomal proteins has been shown (Maden and Vaughan, 1968) to be uncoordinated with the cellular capacity to synthesize ribosomes. The cells were found to continue the synthesis of the ribosomal proteins required for the manufacture of the 50S[1] ribosomal subunit, despite the lack of capacity to utilize them.

Deprivation of cells for valine constitutes a growth stress which is readily reversible and, unlike methionine deprivation, involves only the cellular supply of a protein constituent. Valine-deprived HeLa cells continue synthesis of 45S r-pre-RNA at 30–40% the rate in normal growth but manufacture complete ribosomal subunits at only about 10% the normal rate (Maden et al., 1969). In the present work we have studied the

[1] The large and small riibosome subunits are referred to as the 50S and 30S subunits, respectively.

synthesis of the 50S subunit quantitatively in order to determine whether the rate of synthesis of ribosomal proteins might be the factor limiting the completion of subunits in valine deprivation.

METHODS

Cell Growth Conditions

HeLa S3 cells were routinely grown in suspension culture in Eagle's medium (Eagle, 1955) with 5% calf serum. Valine deprivation and recovery was initiated as previously described (Vaughan et al., 1971), except that incubation was carried out at a cell density of 6×10^5 cells/ml unless otherwise stated. During valine deprivation experiments extending for more than 6 hr, the cultures were kept tightly closed to keep the pH within the normal range, pH 7.2–7.4.

Labeling Conditions

Cells were labeled with uridine-^{14}C, leucine-^{14}C, or leucine-^{3}H (New England Nuclear Corp., Boston, Mass.) as described in Results. To ensure adequate incorporation of labeled leucine, the amino acid–deprived cultures and the control, growing cultures were incubated in medium containing 1/20th the normal concentration of leucine. For chase purposes leucine-^{12}C was added to 4 mM, giving a 200-fold chase.

Cell Fractionation and Ribosome Preparation

Cells were collected by centrifugation and fractionated into cytoplasm and nuclei according to methods previously described (Penman, 1966; Penman et al., 1966). Ribosomes were purified from the cytoplasmic fraction, converted to subunits by EDTA treatment, and analyzed on sucrose gradients as described by Warner (1966). Before precipitation of the ribosomes, with $MgCl_2$, 10% of the cytoplasm was made 1% in sodium dodecyl sulfate (SDS) and sedimented separately on a 15–30% sucrose gradient in NETS (0.5% SDS, 0.01 M Tris, 0.01 M ethylenediaminetetraacetate (EDTA), 0.1 M NaCl, pH 7.4). Since the absorption at 260 mμ of 50S subunits is negligibly changed by dissociation of rRNA from protein with SDS, the 28S rRNA UV absorption peak from gradient sedimentation analysis of this sample could be used as a reference in calculating the final yield of purified 50S subunits.

Purification and separation of the 50S particles was accomplished by dissolving the $MgCl_2$-precipitated pellet in NEB (0.01 M Tris, 0.01 M EDTA, 0.01 M NaCl, pH 7.4) before sedimentation on 15–30% sucrose gradients containing NEB 0.2 (0.01 M Tris, 0.01 M EDTA, 0.2 M NaCl, pH 7.4). The absorption profiles of these gradients at 260 mμ were recorded with a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), and the absorption specifically due to the 50S subunits was estimated as described (Warner, 1966). This method of preparation has been shown (Warner and Pene, 1966) to remove proteins loosely bound to the subunit.

In order to prepare 50S subunit proteins the sucrose gradient fractions containing 50S particles were pooled and precipitated by adding trichloroacetic acid (TCA) to a final concentration of 12%. The precipitate was collected by centrifugation and washed once with cold acetone.

Ribosomal RNA was released from specified cytoplasmic and nucleoplasmic samples by adding SDS to 1% in the presence of a twofold excess of EDTA, relative to $MgCl_2$, as described previously (Soeiro et al., 1968).

Assay for Incorporation of Radioactivity

Analysis of gradient fractions for TCA-precipitable radioactivity was done as previously described (Vaughan et al., 1971).

Gel Electrophoresis of 50S Subunit Protein

Gels 20–30 cm long, 0.6 cm diameter, containing 10% acrylamide, 0.25% bis-acrylamide in 0.1 M sodium phosphate buffer (pH 7.2), 0.5 M urea, and 0.1% SDS, were made as described (Maizel, 1969). The gels were prerun with 1.5 ml of 0.1 M *N*-acetyl-L-cysteine containing 1.0% β-mercaptoethanol and 10% glycerol, at 2.5 v/cm for 1 hr.

Labeled protein samples were prepared for electrophoresis (Maizel, 1969) by dissolving them in SMP buffer (0.5% SDS, 0.1% β-mercaptoethanol, 0.1 M sodium phosphate, pH 7.5). The sample was heated for 70–80 sec at 100°C. 30% sucrose with 0.1% bromophenol blue was then added to a final concentration of 8.0% sucrose. The sample was immediately layered onto a prerun gel and electrophoresed at 2.5 v/cm until the dye marker had run to the anodic end of the gel. The running buffer employed was 0.1 M sodium phosphate (pH 7.5) containing 0.1% SDS.

Gels were fractionated according to the procedure of Maizel (1966). 30 cm gels were cut in two, at 10 cm from the cathodic end, and each length was then fractionated. To each fraction 10 ml of 4:1 (v:v) mixture of toluene-based scintillator (Omniflor, New England Nuclear Corp.) and Triton X-100 (Rohm and Haas, Philadelphia, Pa.) were added for radioactivity determination in a Packard scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

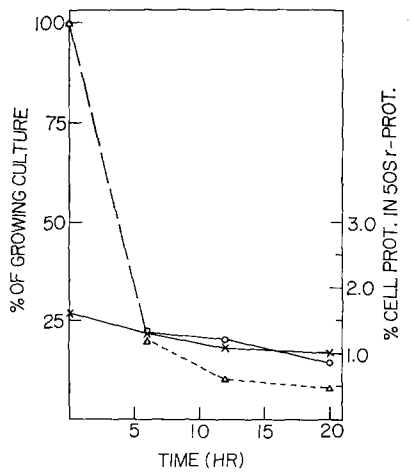

FIGURE 1 Synthesis of total cell protein, 50S ribosomal subunit protein, and 50S ribosomal subunits during valine deprivation. Total cell protein synthesis rates (○——○) were compared between cells growing in complete medium and cells incubated in medium lacking valine. Leucine-^{14}C (0.5 μCi/ml, 25 mCi/mmole, final specific activity) was added to growing cultures or to cultures deprived of valine for 6, 12, and 20 hr, and samples were taken at 30 min for determination of leucine-^{14}C incorporation into cellular protein (Warner and Pene, 1966). The observed rates of protein synthesis in the valine-deprived cells are shown as percentages of the rate in growing cells. The percentage of total cell protein synthesis represented by 50S ribosomal subunit proteins (×——×) was determined by measuring the radioactivity chased into 50S subunit proteins when growing cultures or cultures deprived of valine for 6, 12, or 20 hr were labeled with leucine-^{14}C (0.5 μCi/ml, 25 mCi/mmole) for 30 min and then given a 200-fold chase with leucine-^{12}C. Further experimental details are given in Methods and Fig. 3. The results shown are the average values from several experiments (Table II) and represent the percentage of total incorporated leucine-^{14}C in 50S subunit proteins after an 8 hr chase, corrected for sample size and subunit recoveries. The relative rates of 50S subunit synthesis (△—△) in growing and valine-deprived cultures were compared by following incorporation of uridine-^{14}C into 28S rRNA. Uridine-^{14}C (0.05 μCi/ml, 50 μCi/μmole) was added to growing cultures or valine-deprived cultures for a 3 hr labeling period. The cells were then harvested and fractionated into cytoplasmic, nucleolar, and nucleoplasmic fractions. Ribosomal RNA was released from protein in the cytoplasmic and nucleoplasmic fractions with SDS and EDTA, as indicated in Methods, before sedimentation analysis on 15–30% sucrose gradients in NETS buffer, in the SW 40 rotor of the Beckman ultracentrifuge, at 23,000 rpm for 16 hr, 18°C. The total radioactivity in cytoplasmic and nucleoplasmic

Gel Electrophoresis of Total Cell Protein

Cells were pelleted, after two rinses with cold Earle's salts solution (Earle, 1943), and were dissolved in SMP containing 1.0% SDS. The samples were then treated for electrophoresis as described above for dissolved ribosomal protein.

Measurement of Rate of Total Cell Protein Synthesis

The rate of leucine incorporation into protein in whole cells was measured as previously described (Warner and Pene, 1966; Vaughan et al., 1971).

Preparation of Cells for Electron Microscopy

Growing and valine-deprived cells were prepared for electron microscopy by rinsing in cold Earle's solution and fixation in 2% glutaraldehyde and 1% osmium tetroxide in 0.1 M sodium phosphate, pH 7.5. After centrifugation they were embedded in Epon for sectioning. Sections were stained for 15 min in uranyl acetate and for 5 min in Reynolds's (1963) lead citrate solution.

RESULTS

Effect of Valine Deprivation on General Protein Synthesis

As found in other studies on amino acid deprivation (Eagle et al , 1959), valine deprivation causes a decrease in the rate of total cell protein synthesis. At 6 hr (Fig. 1) the rate of amino acid incorporation, relative to that in growing cells, had dropped to 22%. Between 6 and 20 hr of deprivation, the relative rate of protein synthesis declined further to about 18% the rate in active growth. This residual protein synthesis has been shown to be due to the turnover of existing cell proteins (Eagle et al., 1959).

The following experiment was performed to determine whether the completed and nascent proteins being synthesized in valine-deprived cells have a normal size distribution. A culture of growing cells was labeled for 5 min with leucine-^{3}H while another, which had been deprived of valine for 2 hr, was labeled for 10 min with leucine-^{14}C.

28S rRNA was estimated (Vaughan et al., 1967 *a*) as a measure of the rate of 50S subunit synthesis and the results are given as the percentage of the rate in a growing culture. The three parameters presented in this graph are compared between growing and valine-deprived cultures on a per cell basis.

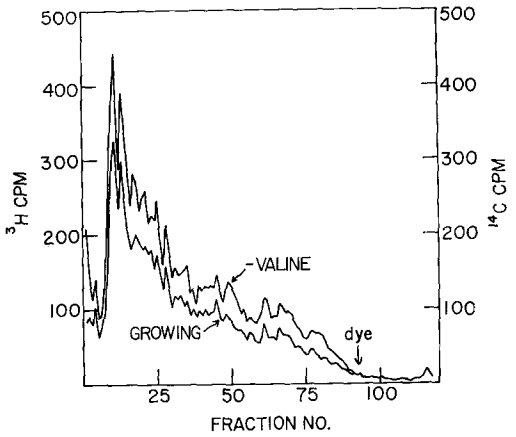

FIGURE 2 Acrylamide gel electrophoretic analysis of the total cell peptides synthesized in growing and valine-deprived cells. HeLa cells in both cultures were at a cell density of 300 × 10^4 cells/ml. Growing cells were labeled with leucine-^{3}H (5 μCi/ml, 12.5 mCi/mmole) for 5 min, and cells deprived of valine for 2 hr (Vaughan et al., 1971) were labeled with leucine-^{14}C (1 μCi/ml, 2.5 mCi/mmole) for 10 min. Processing of the samples for coelectrophoresis in SDS-urea buffer on a 10% cross-linked acrylamide gel, 20 cm by 0.6 cm, is described in Methods. The distance travelled toward the anode (to the right) by the bromophenol blue dye marker is indicated. The gel was fractionated by the method of Maizel (1966) before scintillation counting.

These culture conditions and labeling times were chosen because, in a previous study (Vaughan, et al., 1971), the average peptide synthesis time had been determined under these conditions and was found to differ by a factor of two between growing cells (50 sec) and valine-deprived cells (92 sec). At the elevated cell concentration used (Fig. 2), the effects of valine deprivation on gross protein synthesis appear within an hour after initiation of deprivation. The total cell protein was prepared from both cultures for electrophoresis in SDS-urea solution (Fig. 2) Under the conditions of electrophoresis used, the broad range of peptides in the cell, from the small nascent chains to large proteins, enter into the gel. The relative mobilities, compared to the dye marker, of peptides of molecular weights 50,000 and 22,000 are approximately 0.33 and 0.60, respectively (Warner, personal communication).

It appears that the general size distribution of nascent and recently completed peptide chains is similar in growing and valine-deprived cells. This suggests that valine deprivation does not cause significant premature release of nascent peptides.

Effects of Valine Deprivation on the Synthesis of 50 S Ribosomal Subunits

After valine deprivation periods of 6, 12, and 20 hr, uridine-^{14}C was added to cultures of HeLa cells, and 3 hr later the cells were harvested. Ribosomal RNA was released from the cytoplasmic and nucleoplasmic fractions and analyzed on sucrose gradients as described in Methods. Radioactivity in 28S rRNA was determined and compared to that entering 28S rRNA in a control culture containing valine. Previously, it was shown (Vaughan, et al., 1967 *a*) that in growing cells the 28S rRNA in nucleoplasm as well as cytoplasm is present in particles sedimenting at about 50S. This has also been shown in valine-deprived cells (Vaughan et al., 1967 *b*; Vaughan, unpublished observations). The rate of labeling of 28S rRNA in these cellular fractions is thus a direct measure of the rate of synthesis of 50S subunits. It has been shown (Maden et al., 1969) that the entry of labeled uridine into the total acid-soluble nucleotide pool is similar kinetically and quantitatively in growing and valine-deprived HeLa cells.

Valine deprivation causes a marked decrease in the synthesis of 50S subunits (Table I; Fig. 1). After 6 hr of deprivation cells were synthesizing

TABLE I

*Effect of Valine Deprivation on 50S Ribosomal Subunit Synthesis**

Time of deprivation	In cytoplasmic 28S rRNA	In nucleoplasmic 28S rRNA	Control
hr	*cpm*	*cpm*	%
0	538,400	64,660	—
6	98,152	12,440	18.5
0	300,900	111,900	—
12	25,157	16,400	10.0
0	346,400	30,750	—
20	24,600	6070	8.1

* In these experiments cultures containing equal numbers of HeLa cells at 60 × 10^4 cells/ml, in complete or valine-deficient medium, were labeled for 3 hr with uridine-^{14}C (3–5 μCi/ml, 50 mCi/mmole). The cells were fractionated for estimation of radioactivity in 28S rRNA as previously described (Vaughan et al., 1967 *a*).

approximately 19% the amount of 50S subunits being made in normal growth. At periods of 12 and 20 hr of deprivation such synthesis had decreased to 10 and 8%, respectively. Since the relative rate of synthesis of 45S r-pre-RNA in cells deprived of valine for 6 hr is about 40% that in growing cells (Maden et al., 1969), our results indicate that only about half of the 45S r-pre-RNA made at this time is actually utilized for ribosome synthesis.

The Effect of Valine Deprivation on Synthesis of 50S Ribosomal Subunit Protein

At 6 hr of valine deprivation the relative decrease in 50S subunit synthesis is nearly the same as the relative decrease in total cell protein synthesis (Fig. 1). Thereafter in valine deprivation there is a further reduction in 50S subunit synthesis, which goes from 22% to 8% relative to growing cells by 20 hr (Fig. 1), with little accompanying decrease in the rate of total cell protein synthesis. We therefore performed experiments to determine the proportion of total cell protein synthesis devoted to proteins of the 50S subunit in growing cells and cells deprived of valine for varying periods of time.

The experiments utilized a 30 min pulse label of growing or valine-deprived cells with leucine-^{14}C, followed by a chase with a 200-fold excess of leucine-^{12}C. Valine was readded to valine-deprived cultures 15 min after the initiation of the leucine chase, so that ribosomal protein synthesized during the pulse label period could be efficiently recovered during restored ribosome synthesis. Samples were taken at various times during the chase period for determination of radioactivity in total cell protein and 50S subunit proteins. The results are shown in Fig. 3.

The data indicate that the chase with unlabeled leucine was effective. We consistently observed an initial oscillation of the radioactivity in total cell TCA-precipitate immediately after the beginning of the chase, after which the total radioactivity declined about 15% and remained constant. The percentage of radioactivity chasing into cytoplasmic 50S subunit protein is expressed relative to the radioactivity in total cell protein after 8 hr chase. As previously observed (Warner, 1966) with growing cells the chasing of pulse-labeled 50S subunit proteins into completed cytoplasmic ribosomes requires approximately 4 hr for completion, after which the radioactivity appears to be stable in ribosomes of both valine-

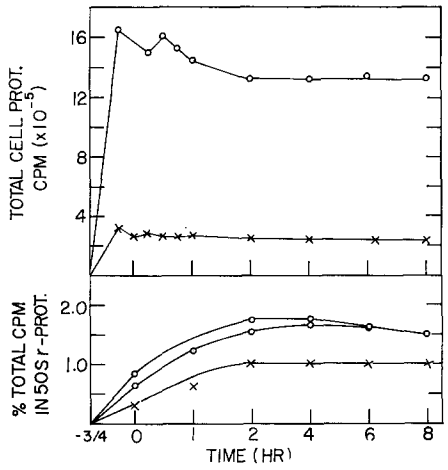

FIGURE 3 Incorporation of leucine into total cell protein and 50S ribosomal subunit proteins of growing and valine-deprived cells during pulse-chase labeling. A 200 ml culture of growing cells (○——○) or cells deprived of valine for 12 hr (×——×) was labeled for 30 min with leucine-^{14}C (0.5 μCi/ml, 25 mCi/mmole) as in Methods. Nonradioactive leucine was added for a 200-fold chase, and 15 min later valine was added to the culture (0 hr in figure). The chase was then continued for 8 hr. At the times indicated duplicate 0.5 ml samples were taken for determination of radioactivity in total cell protein (Warner and Pene, 1966), shown in the upper section corrected to total radioactivity in protein in the starting culture. Samples of 25 ml were taken for determination of the radioactivity in cytoplasmic 50S subunit proteins. The yields of 50S subunits, determined as described in Methods, were 50–80%. The corrected percentages of total incorporated leucine-^{14}C in 50S subunit protein are shown in the lower section. Chase curves for 50S subunit proteins in two separate growing cultures are shown to illustrate the reproducibility of the experiments. In calculating percentages the total cell leucine-^{14}C incorporated at 8 hr was used as the reference.

deprived and growing cells for at least another 4 hr. Data shown in Table II, and summarized in Fig. 1, indicate that 50S subunit protein synthesis declines from 1.6% of total protein synthesis in growing cells to 1.0% in cells deprived of valine for 20 hr.

It is possible, a priori, that ribosomal protein newly synthesized in valine-deprived cells might be more unstable than in growing cells. Since turnover of such protein would affect the results obtained in our experiments, a check of the stability of the protein was made by determining the effect of delaying the addition of valine for 2 hr

TABLE II

*Synthesis of 50S Ribosomal Subunit Proteins**

Culture	In total cell protein	In 50S subunit proteins	50S subunit protein
	cpm	*cpm*	%
Growing, 30 min label	530,760	8780	1.6 (1.6)
Growing, 24 hr label	2,851,000	46,850	1.64
Valine-deprived 6 hr, 30 min label	315,260	4140	1.3 (1.3)
Valine-deprived 12 hr, 30 min label	223,620	2230	1.0 (1.1)
Valine-deprived 20 hr, 30 min label	142,950	1450	1.0 (1.0)

* These experiments were performed as described in the legend to Fig. 3, except for the 24 hr label of growing cells which was not chased with leucine-^{12}C. The number of cells used was varied in different experiments. In all cases except the 24 hr label of growing cells, the numbers shown are the average of the radioactivity determined in the samples from 4, 6, and 8 hr of chase with leucine-^{12}C, corrected to the initial culture size. The percentages in brackets are the average values found from two or more separate experiments.

after the beginning of a leucine chase. As shown in Table III the same amount of radioactivity was recovered in cytoplasmic 50S subunit protein after a 2 hr delay as when valine was added soon after the initiation of the chase. This suggests that the newly synthesized 50S subunit protein in valine-deprived cells is sufficiently stable to support the validity of our method for estimating it. Since the duration of the pulse label is short, 30 min, at a time when protein synthesis is greatly reduced, some fivefold, we assume that the small amount of labeled 50S ribosomal subunit protein will be efficiently utilized in the recovery period, where 50S subunit synthesis recovers relatively rapidly (Fig. 6) to about 50% or more of the rate in normal growth.

The results summarized in Fig. 1 indicate that by 12 and 20 hr of valine deprivation the synthesis of 50S subunits is decreased more, relative to growing cells, than can be accounted for by the total decrease in synthesis of 50S subunit proteins. This suggests that the decline in synthesis of certain 50S subunit proteins might be particularly great, possibly making one of these proteins limiting for subunit synthesis. To examine this question, the leucine-^{14}C labeled 50S subunit proteins synthesized in valine deprivation and recovered in 4 and 8 hr chases after valine addition were coelectrophoresed on 10% acrylamide gels, in SDS-urea buffer (Warner, 1966), with leucine-^{3}H labeled 50S subunit proteins prepared in growing cells. The results of analyses of 50S subunit proteins recovered after a 4 hr chase in the presence of valine after 12 and 20 hr of valine deprivation are shown in Fig. 4. The gel electrophoresis analysis showed no significant differences in the pattern of 50S subunit proteins recovered after 4 hr *versus* 8 hr of leucine chase.

The bulk of the 50S subunit proteins, located in fractions 70–140 of the gel analyses, appear to be synthesized in more or less similar proportions in growing and valine-deprived cells. However, the relative synthesis of two large proteins, in fractions 40–50, is markedly reduced in the valine-deprived cells. These two proteins have molecular weights of about 50,000 daltons (Kumar and Warner, personal communication) and, in our gel analyses, together constitute about 8.1% of the 50S subunit protein. On the assumption that

TABLE III

*Effect of Delayed Addition of Valine on the Chase Recovery of Newly Synthesized 50S Ribosomal Subunit Proteins**

Time after chase initiation of valine addition	Chasing into 50S subunit protein
min	*cpm*
15	1022
120	989

* The experiment was performed as in the legend to Fig. 3 except for the culture size, 30 ml, the leucine-^{14}C specific activity (0.15 μCi/ml, 8 μCi/μmole), and the time of addition of valine after the initiation of the leucine-^{12}C chase. The cells had been deprived of valine for 12 hr before labeling, and in both cases the samples were taken 6 hr after addition of valine.

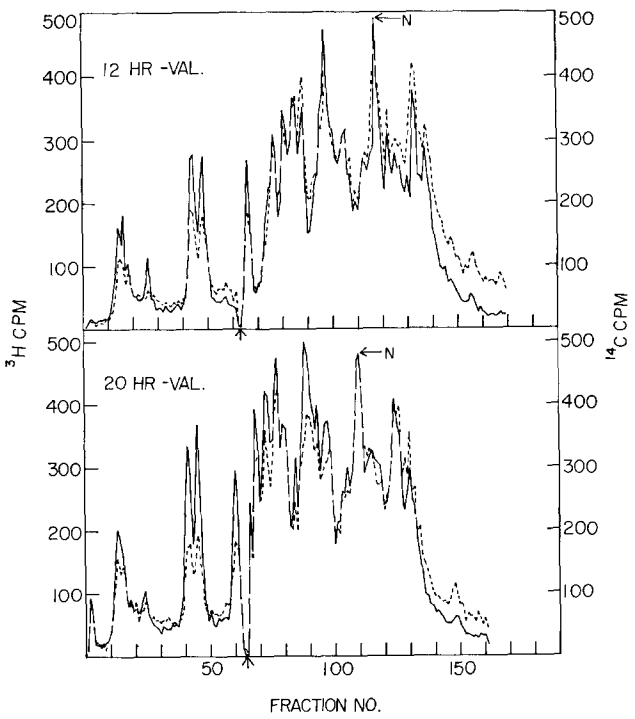

FIGURE 4 Acrylamide gel electrophoresis of 50S ribosomal subunit protein from growing and valine-deprived cells. Growing cells were labeled 24 hr with leucine-^{3}H (0.01 μCi/ml, 0.05 mCi/mmole), and valine-deprived cells were labeled 30 min with leucine-^{14}C (0.5 μCi/ml, 25 mCi/mmole) as in Methods and Fig. 3. 50S subunit protein was prepared from both cultures for coelectrophoresis on 10% acrylamide gels as described in Methods. The labeled 50S subunits from valine-deprived cells were prepared after a 4 hr chase with leucine-^{12}C in the presence of valine, as described in Fig. 3. The gel patterns compare 50S subunit proteins labeled in growing cells (——) vs. cells deprived of valine (----) for 12 or 20 hr. Arrows in the figure indicate the protein-^{3}H peak on which the protein-^{14}C patterns were normalized, and the position at which the gels were cut to permit radioactivity assay by the method of Maizel (1966).

each of these proteins is present as one molecule per 50S subunit, this value is in good agreement with the total protein composition of the subunit deduced from its estimated molecular weight, 3.2×10^6 daltons (Tashiro and Yphantis, 1965), and the estimated molecular weights of 28S rRNA, 1.9×10^6 daltons (McConkey and Hopkins, 1969), and 5S rRNA, 0.3×10^4 daltons (Rosset et al., 1964). From the data in Fig. 4, we find the synthesis of these two proteins together to be reduced to 5.7% and 5.5% of 50S subunit protein synthesis at 12 and 20 hr, respectively, of valine deprivation. At 20 hr of deprivation the relative synthesis of the *total* 50S subunit proteins compared to total protein synthesis is reduced to 63% of the proportion in normal growth (Table II). The relative synthesis of these two particular proteins is thus over-all reduced at 20 hr of deprivation to about 43% of the proportion in normal growth, i.e., $(5.5/8.1) \times 63$.

We cannot yet specify which of these two proteins is synthesized in the smaller amount, nor can we completely exclude the possibility that the relative reduction in synthesis of some other 50S subunit protein, in the region of fractions 70–140 in the gels, is even greater.

Recovery of 50S Ribosomal Subunit Synthesis after Valine Deprivation

Previously it has been shown (Maden et al., 1969) that the rate of synthesis of 45S r-pre-RNA returns to the rate in normal growth within 3 hr after the addition of valine to a culture previously deprived of valine for 6 hr. We have examined the rate of synthesis of 50S ribosomal

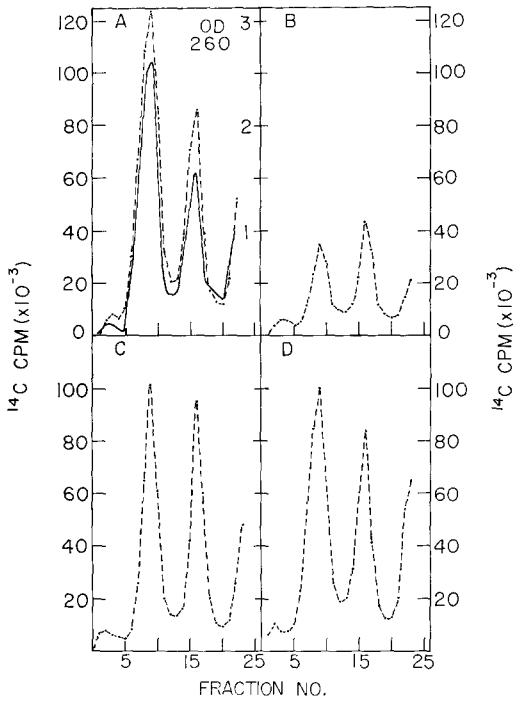

FIGURE 5 Sucrose gradient analysis of cytoplasmic ribosomal 28S and 18S rRNA from growing cells, valine-deprived cells, and cells recovering from valine deprivation. 35 ml cultures were labeled with uridine-^{14}C (3.5 μCi/ml, 50 μCi/μmole) for 3 hr, and ribosomal RNA was prepared from cytoplasm and analyzed on sucrose gradients as described in Fig. 1. The sedimentation analyses shown are of RNA from: A, labeled growing cells; B, cells labeled after 6 hr valine deprivation; C, cells valine-deprived for 6 hr and then given uridine-^{14}C and valine together; D, cells valine-deprived for 6 hr and then given valine for 5 hr before addition of uridine-^{14}C. (——), OD 260 mμ; (----), ^{14}C radioactivity. The ultraviolet absorption profiles were similar for all the cultures.

subunits in cells recovering from 6, 12, and 20 hr of valine deprivation (Figs. 5 and 6). At best, in cells recovering from 6 hr deprivation, the rate of 50S subunit synthesis reaches only 75% the rate in normal growth and then declines again. Recovery of the rate of 50S subunit synthesis reaches only about 40% within 2 hr of recovery from 20 hr of deprivation. In all cases 50S subunit synthesis reaches a maximum after about 2 hr of recovery, and then is significantly reduced by 5 hr.

During the recovery period the relative synthesis of 50S and 30S ribosomal subunits, which becomes unbalanced during valine deprivation (Maden et al., 1969; Maden, 1969), is partially restored (Fig. 5) toward the normal ratio. However, even after 5 hr recovery from a 6 hr deprivation, significantly more 30S than 50S subunits appear to be made.

Electron Microscope Examination of Growing and Valine Deprived Cells

Fig. 7 demonstrates the typical appearance of both growing cells and cells deprived of valine for 12 hr. Valine-deprived cells showed no gross abnormalities when compared to growing cells. The only changes noted were increased convolution of the cell membrane and more densely staining mitochondria in valine-deprived cells. In other respects, including the appearance and number of nucleoli, valine-deprived cells seem to have a normal structure.

DISCUSSION

Maden and Vaughan (1969) have demonstrated that ribosomal protein is synthesized by HeLa cells even when ribosomes cannot be made.

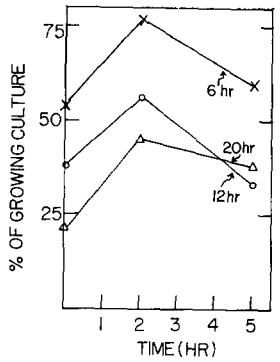

FIGURE 6 Relative synthesis of 50S subunits as a function of time after readdition of valine. Cells were deprived of valine for 6, 12, or 20 hr, and valine was then readded to normal concentration. Uridine-^{14}C (3.5 μCi/ml, 50 μCi/μmole) was added for a 3 hr labeling period beginning either at the time of valine addition or 2 hr or 5 hr after valine addition. A control culture of cells growing in normal medium was similarly labeled. The cytoplasmic and nucleoplasmic RNA was prepared and analyzed for radioactive 28S rRNA as described in Fig. 1. The total radioactivity incorporated into 28S rRNA in the cells recovering from valine deprivation is shown as the relative percentage of incorporation into 28S rRNA in the control culture vs. the time at which uridine-^{14}C was added after valine addition. Cells recovering from 6 hr valine deprivation (×——×); 12 hr (○——○); 20 hr (△——△).

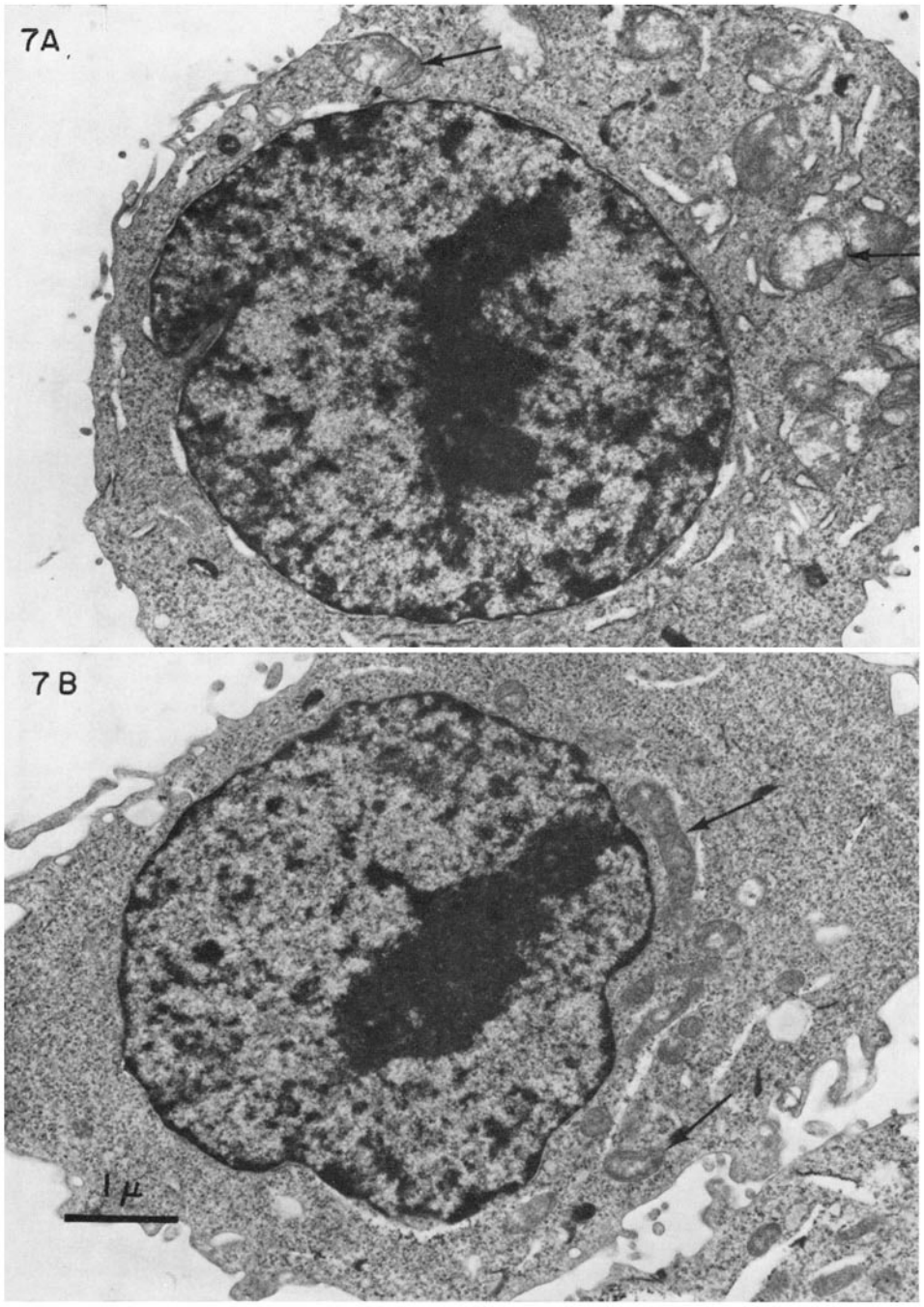

FIGURE 7 Electron micrographs of growing and valine-deprived HeLa cells. Growing HeLa cells (*A*) and cells deprived of valine for 12 hr (*B*) were pelleted and prepared as described in Methods. Arrows indicate the mitochondria which appear more densely stained in cells deprived of valine. × 14,000.

Craig and Perry (1971) have observed that ribosomal protein appears to be synthesized even when 45S r-pre-RNA is not being made. These qualitative studies raise the question whether the synthesis of ribosomal protein is normally the limiting factor in ribosomal synthesis. This is of considerable interest in view of recent demonstrations that normal, diploid human cells, like heteroploid cells, synthesize excess 45S r-pre-RNA under certain conditions, degrading the unusued material (Rubin, 1968; Cooper, 1969; Vaughan, manuscript submitted).

Our results indicate that in valine-deprived HeLa cells the rate of synthesis of ribosomal protein for the 50S subunit may in fact be limiting the rate of synthesis of these particles. By 20 hr of deprivation the synthesis of 50S subunit proteins, relative to total protein synthesis, is reduced to about 63% of that in growing cells, and the relative synthesis of at least two specific proteins is reduced more severely, to about 43%. At this time of deprivation the absolute rate of protein synthesis is 18% of that in growing cells. Thus, if ribosomal protein synthesis is limiting for 50S subunit synthesis, cells deprived of valine for 20 hr should synthesize 50S subunits at a rate not greater than 8% of the rate in growing cells. This is in agreement with the rate we obtained with uridine labeling analysis, which was 8%.

The agreement between the calculated maximal rate of 50S subunit synthesis and the observed value makes it plausible that ribosome synthesis in mammalian cells can be regulated through the rate of synthesis of ribosomal proteins. Our data show that in general the synthesis of the various 50S subunit proteins declines similarly during valine deprivation, but that there are definite variations, within a factor of two, in the reduction in synthesis of individual proteins. It is not possible at present to establish which particular 50S subunit protein is most reduced in synthesis during valine deprivation.

It should be noted that our results do *not* indicate that valine-deprived cells produce 50S subunits with abnormal proportions of the various 50S subunit proteins. To the contrary, it is known (Maden et al., 1969) that the ribosomal subunits matured and sent to the cytoplasm in valine deprivation are complete and functional in the sense that they enter polyribosomal structures as in normal cells.

These experiments have depended upon a "rescue" of ribosomal protein made during valine deprivation, by the subsequent addition of valine to allow restored cell growth. We have observed that while the synthesis rates of 45S r-pre-RNA (Maden et al., 1969) and total cell protein (Pawloswki and Vaughan, personal observations) rapidly approach normal values on the readdition of valine, the rate of 50S subunit synthesis recovers only partially in the first 5 hr of recovery and then declines significantly. The reasons for this lack of full recovery of 50S subunit synthesis are unclear at present. These observations do, however, provide a basis for considering certain features of the control of ribosome synthesis in valine deprivation. First, they make it unlikely that the particularly large decline in relative synthesis of certain 50S subunit proteins is a simple consequence of these proteins being unusually rich in valine. In this case 50S subunit synthesis should recover fully and rapidly on readdition of valine.

The second point is related to the imbalance of synthesis of 50S and 30S subunits which has been observed in HeLa cells deprived of valine and lysine (Maden et al., 1969; Maden, 1969). This imbalance is still observed in ribosome subunit synthesis after 5 hr of recovery from valine deprivation. Therefore, it also cannot be a simple consequence of 50S subunit proteins being richer than 30S subunit proteins in valine. Instead, it appears that the net rate of synthesis of the various ribosomal proteins during valine deprivation must be under controls, transcriptional or translational or both, which are not tightly coordinated. The pattern of recovery of 50S subunit synthesis on the readdition of valine suggests that the regulation of this synthesis may be complex. At present the synthesis of ribosomal protein during recovery from amino acid deprivation has not been investigated. It will be of interest to determine whether the synthesis of the individual 50S subunit proteins is rapidly restored to the proportions charteristic of normal growing cells.

Electron microscope examination of growing and valine-deprived cells indicates that, during the time periods employed in these studies, valine-deprived cells retain a normal over-all appearance. The cause for the more dense staining of mitochondria, in valine-deprived cells, is as yet not known and is being further investigated. These findings are in agreement with those of Cohen et al. (1961), in that those authors also found only slight cell alterations after valine deprivation periods of up to 24 hr. These findings strengthen the validity of valine deprivation as a useful tool

in studying macromolecular synthesis in cell culture.

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