Effects of Isoleucine Deficiency on Nucleic Acid and Protein Metabolism in Cultured Chinese Hamster Cells. Continued Ribonucleic Acid and Protein Synthesis in the Absence of Deoxyribonucleic Acid Synthesis*

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ABSTRACT: Chinese hamster cells (line CHO) were cultured in F-10 medium from which isoleucine was omitted and added sera were dialyzed. This medium, which is deficient in isoleucine content in comparison to normal F-10 medium but not totally lacking isoleucine, is designated Ile$^-$ medium. The effect of reduced isoleucine concentration on DNA synthesis was measured in terms of thymidine incorporation and in terms of DNA content. Culture in isoleucine-deficient medium for 1 hr caused a decrease in thymidine incorporation to 44% of control (Ile$^+$); by 30 hr thymidine incorporation in Ile$^-$ cultures was 1% of control or less. Cell division ceased before 30 hr in Ile$^-$ medium. The DNA content at 30-hr Ile$^-$ was found to be that of a G_1 cell and remained unchanged during further culture in Ile$^-$. The effect on RNA synthesis was less pronounced. At 30-hr Ile$^-$, uridine incorporation into RNA was 55–70% of control. RNA content was equal to or slightly greater than that of a mid-G_1 cell. RNA content increased during continued culture in Ile$^-$ (15% from 30- to 48-hr Ile$^-$). RNA species whose synthesis continued included 4 S, Hn, messenger, and ribosomal at rates approximately one-third, three-fourths, one-half, and one-fifth of exponential culture values, respectively. Distribution of pulse-labeled (uridine) species among cytoplasmic materials of differing densities closely followed that found in Ile$^+$ cells. The size distribution of messenger synthesized in Ile$^-$ cultures followed that of Ile$^+$ populations. Superformation of polysomes by culture in low levels of cycloheximide showed excess messenger to be present in both Ile$^-$ and Ile$^+$ cultures. Protein synthesis rate was determined as the product of synthetic machinery (polysome content) times rate of work (translation rate). Polysome content was determined by sucrose gradient zone sedimentation analysis of cytoplasm prepared using Nonidet P-40 and sodium deoxycholate. Translation rate was estimated from analysis of the increase in specific activity of polysome species as a function of size after incorporation of radioactive amino acids for time periods shorter than needed to fully label all species analyzed. After 2 hr in Ile$^-$ medium, protein synthetic rate was equivalent to that in control populations (Ile$^+$); a polysome seven units long was translated in 42 sec. At 30 hr in Ile$^-$ medium, polysome mass per cell was over 90% of and translation rate was 60–70% of that in exponentially growing cells; respective Ile$^-$ 48-hr values were 90 and 38%. Biuret determination of protein content showed 30-hr Ile$^-$ cells to have 87–89% of the Ile$^+$ value. There was a small but definite increase in protein content during Ile$^-$ culture after 30 hr; 48-hr Ile$^-$ populations had 94% the protein content of Ile$^+$ exponential populations. These results suggest that cells accumulated in a state of G_1 arrest by culture in isoleucine-deficient medium enter a unique biochemical state totally unlike that observed in stationary-phase cultures. Our G_1-arrested cells do not enter a state of gross biochemical imbalance, even though biosynthetic capacities are maintained at much higher levels than those observed in stationary-phase cultures.

Cultures of Chinese hamster cells (line CHO) placed in isoleucine-deficient (but not totally lacking isoleucine) medium accumulate in a reversible, stable state of G_1 arrest; upon subsequent addition of isoleucine, all of the cells in the population reinitiate cell-cycle traverse, synthesize DNA, and later divide in synchrony (Ley and Tobey, 1970; Tobey and Ley, 1971). G_1 accumulation due to lowered amino acid supply is specific for isoleucine (Ley and Tobey, 1970) and has been demonstrated in other cell lines (Tobey and Ley, 1971), suggesting a general regulatory role for isoleucine in mammalian genome replication. That is, by merely manipulating the amount of the well-defined medium constituent isoleucine, genome replication, in effect, may be turned on or off.

In this report the status of macromolecular synthesis in cells grown in isoleucine-deficient medium has been examined to better define the cell's biochemical state and to investigate possible causes of G_1 arrest. Results indicate that, in contrast to stationary-phase cells or cells deprived of other essential amino acids, cells in isoleucine-deficient medium maintain high levels of macromolecular synthesis but do not enter a state of gross biochemical imbalance. The results further indicate that redistribution of cells in isoleucine-deficient medium does not result from a general reduction in polypeptide synthetic capacity.

Materials and Methods

Cells and Media. A hypodiploid (modal chromosome number of 21) line of CHO cells was maintained free of PPLO in suspension culture in F-10 medium, as described previously (Tobey and Ley, 1971). For transfer of cells to isoleucine-deficient medium, aliquots were removed from stock suspen-

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sion cultures, and the cells were pelleted by centrifugation and resuspended in isoleucine-free F-10 medium plus twice the normal glutamine concentration, dialyzed 10% calf and 5% fetal calf sera, and antibiotics. Dialyzed sera were prepared as described earlier (Tobey and Ley, 1971) except that the calf serum was heat inactivated at 56° for 30 min prior to dialysis to bring about a reduction in the amount of cell clumping in isoleucine-deficient medium.

Because the fraction of cell doublets was routinely higher (usually 0.08 *vs.* 0.03) and more variable in isoleucine-deficient cultures, the fraction of cells occurring as doublets was determined by direct microscopic observation, and this value was used to correct the cell number obtained with an electronic particle counter. Electronic particle counters score all cell doublets as a single event.

Mitotic cells (0.96–0.99 initially in mitosis) were prepared by the mitotic selection technique described elsewhere (Petersen *et al.*, 1968) and allowed to proceed into G_1 in spinner flasks (referred to as "mitotic selection G_1 cells").

Measurements of Uridine and Thymidine Incorporation and of RNA and DNA Contents. These were similar to those previously described (Enger *et al.*, 1968) except that 0.3 M KOH treatment for 18 hr at 37° was substituted for hydrolysis of RNA by ribonuclease. Also, Aquasol (New England Nuclear) was used as liquid scintillation counting solution.

Measurement of Uridine Incorporation into Unstable Nuclear and Cytoplasmic RNAs. Cultures were pretreated with 0.05 μg/ml of actinomycin D (gift of Merck & Co., Rahway, N. J.) for 30 min to preferentially reduce synthesis of ribosomal, as opposed to nuclear heterogeneous or mRNA synthesis (Perry, 1962; Perry *et al.*, 1964; Perry and Kelley, 1968; Penman *et al.*, 1968; Perry and Kelley, 1970).

Suspensions of uridine-labeled cells were treated with Nonidet P-40 and sodium deoxycholate, as previously described (Walters *et al.*, 1970), to effect fractionation into cytoplasm and nuclei.

Incorporation into nuclear heterogeneous RNA was measured as the amount of uridine incorporated into RNA (extracted from nuclei using phenol and sodium dodecyl sulfate at 58°) sedimenting faster than 10S subsequent to DNase I (Worthington, DPFF) digestion.

Polysomes and informosomes were collected from cytoplasm by centrifugation through 0.5 ml each 1 and 2 M sucrose for 3 hr at 180,000*g* av.

Cesium chloride isopycnic analysis was similar to that described by Brunk and Leick (1969).

Methylation of 4S and rRNA. Subsequent to exposure of cultures to methionine labeled with ^3H or ^{14}C in the methyl position, the extent of methylation of 4S and rRNAs was determined as described by Enger *et al.* (1968).

Zone Sedimentation Analysis of Polysome Mass and Polysomal Amino Acid Incorporation. Labeled amino acids were added to spinner-culture aliquots quickly and at a fast stir rate to effect rapid mixing. Incorporation was terminated by pouring aliquots (usually 340 ml containing approximately 10^8 cells) over one-half volume of frozen 0.25 M sucrose cubes (cultures were never concentrated before exposure to isotopes). Cells were collected by sedimentation, washed with cold 0.25 M sucrose, suspended in 1.6 ml of $K_{100}T_{10}M_{1.5}$ buffer (100 mM KCl–10 mM Tris, pH 7.4 at 25°, 1.5 mM Mg^{2+}), and quickly frozen in Dry Ice–ethanol. Storage was at −80°. Prior to analysis, samples were thawed by immersing in room-temperature water. As soon as thaw was complete, samples were mixed well and placed in an ice bath. Nonidet P-40 (10%, 0.175 ml) was added (Borun *et al.*, 1967), and the suspension was mixed 15 sec by Vortex action. After 15 min, 0.175 ml of 5% sodium deoxycholate was added, and the suspension was mixed well again and allowed to stand an additional 20–30 min. After a final 30-sec mix, nuclei were removed by sedimentation for 30 min at 1300*g*. The supernatant cytoplasm was layered over 56-ml 10–30% sucrose gradients in Spinco SW 25.2 tubes immediately or after mixing with 0.23 ml of 8× HSB. (HSB is 0.5 M NaCl–0.05 M $MgCl_2$–0.01 M Tris, pH 7.4 at 25°.) Gradients contained buffers corresponding to those in the samples applied to the gradients. Centrifugation was for 3 hr at 25,000 rpm (4°) in the Spinco L2-65 centrifuge. Gradients were analyzed with the aid of an ISCO Model D fractionator—used to drive the gradient through the flow cell (0.5 or 1.0 cm) of a Beckman DB spectrophotometer by pumping heavy sucrose solution into the bottom of the tube. Aliquots (0.75 ml) were mixed with 0.5 ml of water and 15 ml of Aquasol (New England Nuclear) prior to liquid scintillation counting. Absorbancy base lines were determined by analysis of gradients run without sample. In the case of high-salt buffer gradients, the correction is quite significant.

Results

Effect of Culture in Isoleucine-Deficient Medium on Cell Division and Nucleic Acid Synthesis. For the purpose of this report, the medium from which isoleucine is omitted will be referred to hereafter as "Ile^- medium." Note that, in this context, Ile^- medium signifies an undetermined degree of isoleucine deficiency rather than an absolute starvation for that amino acid. Serum breakdown during cell culture could generate free amino acids (Eagle and Piez, 1960).

All isoleucine-deficient cultures were started at cell concentrations of approximately 200,000 cells/ml. This value is an approximate upper limit, since long-term cultivation of populations initially in excess of 200,000 cells/ml can result in formation of populations deficient in other nutrients as well (Ley and Tobey, 1970). The results in Figure 1A and from a large number of additional experiments indicate that, following transfer to isoleucine-deficient medium, cell division ceased within 22–25 hr, at levels varying from 42 to 46% above the initial cell concentration. Corresponding to the cell division pattern shown in Figure 1A is the pattern of incorporation of thymidine into DNA and uridine into RNA shown in Figure 1B. After only 1 hr in Ile^- medium, thymidine incorporation has been reduced to 44% of the initial value, and by 30 hr the value has decreased to 1% or less than the exponential control (Figure 1B). In contrast, uridine incorporation into RNA has dropped to 73% of initial value after 30 hr in Ile^- medium (Figure 1B). In general, thymidine incorporation was always reduced quickly and to the extent shown; uridine incorporation was always reduced less slowly and was reduced after 30 hr in Ile^- medium to 54–73% of the initial value.

Precursor incorporation as a measure of macromolecular synthesis is always subject to uncertainties because of possible changes in transport rates, pool compartmentalization, altered rates of *de novo* synthesis, turnover effects, etc. (Bölcsföldi *et al.*, 1971). Therefore, an attempt was made to confirm analyses based on precursor incorporation with other measurements. In this instance, mass data (DNA and RNA contents per culture aliquot or per cell) were obtained to enable partial confirmation of the validity of incorporation patterns; such data on content of RNA and DNA provide an alternate measurement of synthesis of *stable* species. As seen from data shown in Figure 1C, the differential effect of Ile^- culture on DNA and RNA synthesis is also evident in terms of content:

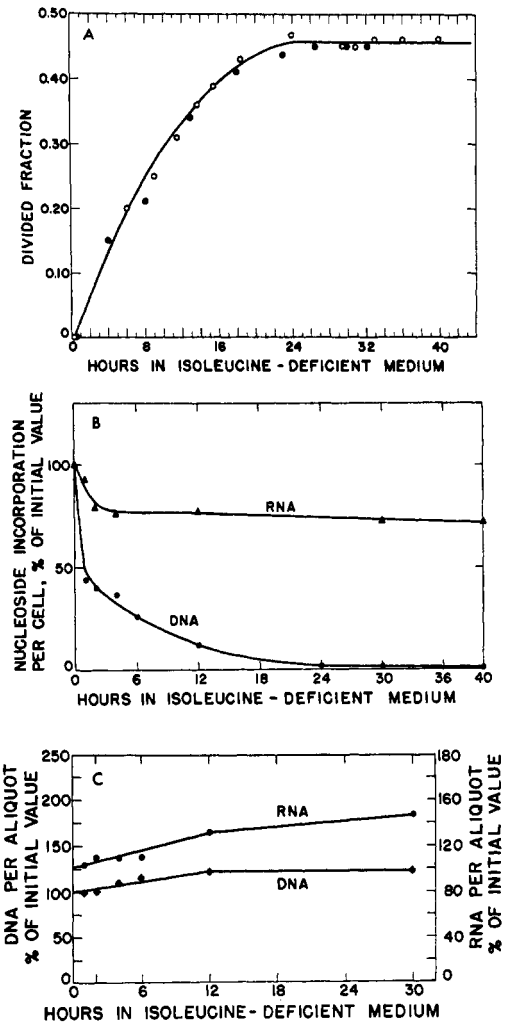

FIGURE 1: Patterns of cell division and nucleic acid synthesis during culture in isoleucine-deficient medium. (A) Increase in cell number. The divided fraction is equal to $N/N_0 - 1$. The open and closed circles represent data from two experiments. Initial cell concentrations were 199,000/ml in each experiment. (B and C) Cultures (55 ml) were exposed to 6.25 μCi of uridine-*5-t* (5 mCi/0.0433 mg, New England Nuclear) for 15 min. Aliquots (50 ml) were harvested, and incorporation into RNA (▲) and RNA and DNA content [(●) and (◆)] were determined as in Materials and Methods. Similarly, 60-ml cultures were exposed to 2 μCi/ml of thymidine-*methyl-t* (6 Ci/mmole, Schwarz BioResearch) for 15 min prior to harvesting 50 ml and determination of incorporation into DNA (●) and of RNA and DNA content [(○) and (◆)]. Content values are averages of two determinations.

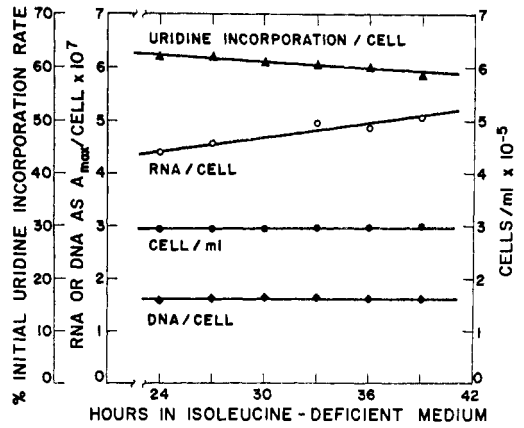

FIGURE 2: Cell concentration, uridine incorporation into RNA, and RNA and DNA contents in Chinese hamster cells during prolonged culture in isoleucine-deficient medium. Procedures were as described in Figure 1 and in Materials and Methods.

RNA mass (RNA per culture aliquot) continues to increase after 12 hr in Ile$^-$ culture and attains a level comparable to the increase in cell number, while DNA mass (DNA per culture aliquot) attains a constant value at less than 25% increase over initial value. These synthetic patterns are seen in more detail in Figure 2, where values are shown for the period 24–39 hr after transfer to Ile$^-$ medium. Notice that cell number and DNA content (DNA per cell or per culture aliquot) are constant throughout. Second, the DNA content (DNA per cell) is equal to that of cells in a G_1 population. In cultures of cells maintained 30 hr in isoleucine-deficient medium, the DNA from 10^7 cells produces in an acid hydrolysate of 1-ml volume A_{max} of 1.61, $\sigma = \pm 0.05$. The DNA value for G_1 cells produced by the mitotic selection technique is 1.62, $\sigma = \pm 0.04$ and for exponential populations is 2.01, $\sigma = \pm 0.04$. These DNA values show variation from experiment to experiment that is $< \sigma$. That the DNA content of 30-hr Ile$^-$ cells should mimic that of cells in a G_1 population was predicted by data from microfluorometric analyses reported earlier (Tobey and Ley, 1970). This G_1 DNA content was obtained in all experiments run in this series by 30-hr Ile$^-$ and remained constant during continued culture in Ile$^-$ medium.

Exponential populations of cultured Chinese hamster cells have an RNA content such that an acidified alkaline hydrolysate (*cf.* Materials and Methods) of RNA from 10^7 cells, when contained in 1 ml, has an absorbancy (at λ_{max}) of 5.08, $\sigma = \pm 0.24$. The corresponding value for mitotic-selected cells allowed to proceed into G_1 for 1 hr (*i.e.*, early G_1) is 4.39, $\sigma = \pm 0.1$; for 30-hr Ile$^-$ cells it ranges from 4.4 to 5.0. The lower values are seen in experiments where lower Ile$^-$ uridine incorporation values are noted. Continued net RNA synthesis, expressed as increased RNA content, is seen up to 48-hr Ile$^-$ in all experiments run to date.

Incorporation of uridine into RNA continues at significant levels after many hours in Ile$^-$ culture. At 30-hr Ile$^-$ the average rate of incorporation is about two-thirds of the initial exponential Ile$^+$ value, the range being 54–73% of initial value. This may be compared with a level of 20–40% for high cell-density, stationary-phase cultures of CHO cells (Tobey and Ley, 1970) and 62–64% for cells prepared by mitotic selection and allowed to enter G_1 for 1 hr (unpublished results).

We next ask whether the synthesis of any species of RNA is reduced to the point of inadequacy in isoleucine-deficient cells. We have seen that 30-hr Ile$^-$ cells have approximately the same *content* of stable RNA as exponentially growing populations. Analysis of methylation rates for 4S RNA and rRNAs in cells exposed to methionine-*methyl-t* was performed as previously described (Enger and Tobey, 1969) to determine if continued stable RNA synthesis included both 4S and rRNAs. Such analysis showed a specific methylation rate (as judged by analysis subsequent to 40-min incorporation) of approximately one-third and one-fifth of exponential culture values for 4S and ribosomal methyl incorporations, respectively. These values are in accord with the rate of mass increase (RNA per aliquot per time) in Ile$^-$ culture of about one-fourth of that observed in an exponential population (in the exponential culture, mass increase per aliquot corresponds to the increase in cell concentration—doubling each 16.5 hr;

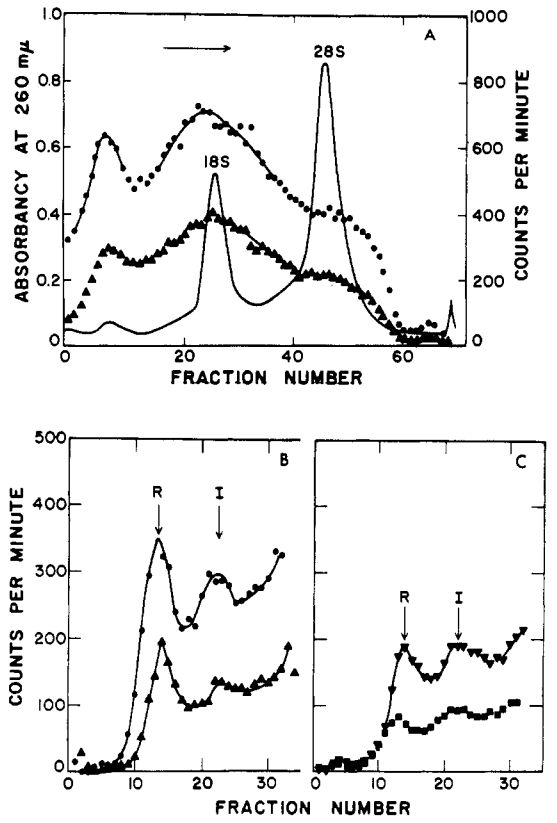

FIGURE 3: (A) Zone sedimentation analysis of uridine-labeled material extracted with phenol–sodium dodecyl sulfate from rapidly sedimenting cytoplasmic nucleoproteins. Cultures (250 ml, 30-hr Ile$^-$ or Ile$^+$) were pretreated with 12.5 μg of actinomycin D for 30 min prior to exposure to 1 μCi/ml of uridine-*5-t* for 40 min. Cell concentrations were 302,000/ml for Ile$^+$ and 288,000/ml for Ile$^-$ cultures. Four-fifths of rapidly sedimenting cytoplasmic material was phenol extracted. Incorporation data were normalized to the same number of cells: (—) A_{260} Ile$^+$, (●) Ile$^+$ counts per minute, (▲) Ile$^-$ counts per minute. (B and C) Cesium chloride isopycnic analysis of rapidly sedimenting (pelleted through 0.5 ml each 2 and 1 M sucrose by sedimenting 3 hr at 180,000*g* av) uridine-labeled, formaldehyde-fixed, cytoplasmic material. Data are normalized to the same number of cells: (●) one-tenth and (—▲—) one-twentieth of Ile$^+$ material; (▼) one-tenth and (■) one-twentieth of Ile$^-$ material. (R) The position of ribosomal material ($\rho \simeq 1.52$) and (I) that of informosome-like material ($\rho \simeq 1.4$).

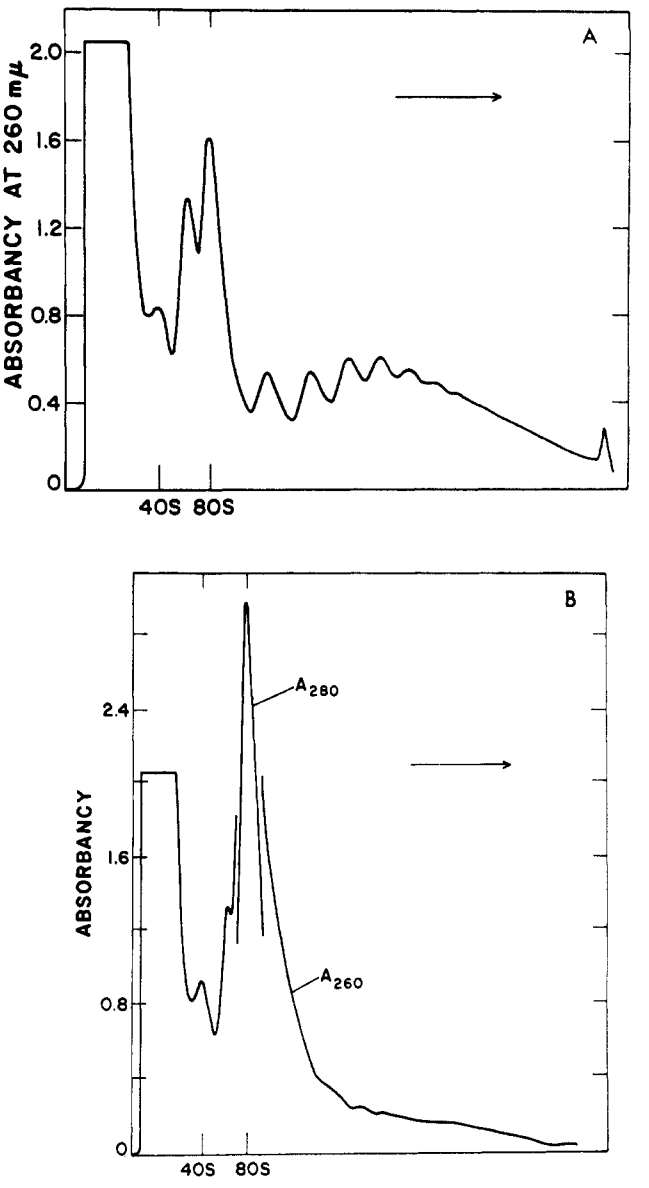

FIGURE 4: Polysomes of (A) exponentially growing cells and (B) stationary-phase culture cells. The exponential culture aliquot was 490 ml at 290,000 cells/ml, and the stationary-phase culture aliquot was 240 ml at 630,000 cells/ml. Preparation and analysis were in $K_{100}T_{10}M_{1.5}$ as described in Materials and Methods.

in Ile$^-$ cultures, the doubling of RNA per aliquot culture approximates 67 hr but cell concentration is constant).

Analysis of the rate of incorporation of uridine into high molecular weight (>4 S), heterogeneous nuclear RNA after pretreatment with low levels of actinomycin to suppress rRNA synthesis gave values of 72.5 and 73% those of exponential cultures subsequent to 15- and 40-min incorporation periods, respectively. It appears then that 4S RNA (presumably transfer) is synthesized at ~33%, ribosomal at ~25%, and heterogeneous nuclear at 73% the rates obtaining in Ile$^+$ cultures. The preferential reduction of ribosomal over heterogeneous nuclear RNA synthesis mimics the observations of Tiollais *et al.* (1971), who studied the effect of valine deprivation on RNA synthetic patterns in L5178Y cells. Bölcsföldi *et al.* (1971) have noted that the rate of uridine incorporation into RNA may be proportional to the rate of accumulation of stable species. That is, when stable RNA synthesis is reduced, less exogenous uridine is incorporated and unstable species preferentially reutilize products of the catabolic stage of turnover. The consequence of this is that uridine incorporation provides an *underestimate* of the rate of Hn or mRNA synthesis in systems such as the Ile$^-$ system where stable RNA synthesis is significantly reduced.

mRNA synthesis was measured as follows. First, cultures were pretreated with low levels of actinomycin D to preferentially reduce rRNA synthesis. Second, cells were fractionated into nuclei and cytoplasm to separate heterogeneous nuclear from cytoplasmic species. Next ribosomal, polysomal, and other rapidly sedimenting materials were separated from 4S RNAs by differential centrifugation. A portion of sedimentable material was extracted with phenol–sodium dodecyl sulfate and its RNA analyzed by sucrose gradient zone sedimentation. As seen in Figure 3A, this analysis shows that (1) there was no significant incorporation into rRNA, (2) the distribution of incorporated uridine among species of differing sedimentation rates was similar for samples from Ile$^-$ and Ile$^+$ (exponential cultures), and (3) approximately one-half

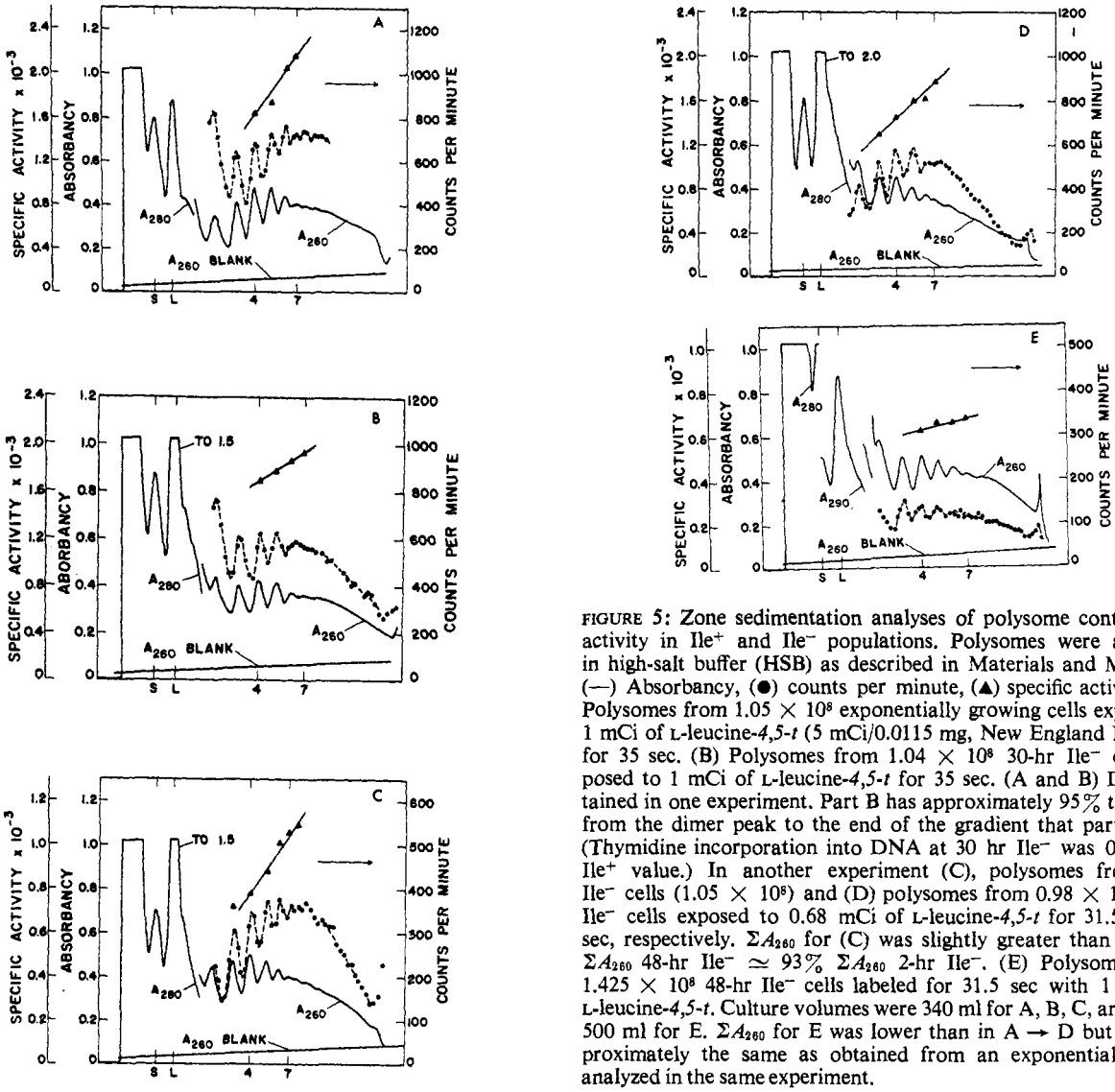

FIGURE 5: Zone sedimentation analyses of polysome content and activity in Ile$^+$ and Ile$^-$ populations. Polysomes were analyzed in high-salt buffer (HSB) as described in Materials and Methods. (—) Absorbancy, (●) counts per minute, (▲) specific activity. (A) Polysomes from 1.05×10^8 exponentially growing cells exposed to 1 mCi of L-leucine-*4,5-t* (5 mCi/0.0115 mg, New England Nuclear) for 35 sec. (B) Polysomes from 1.04×10^8 30-hr Ile$^-$ cells exposed to 1 mCi of L-leucine-*4,5-t* for 35 sec. (A and B) Data obtained in one experiment. Part B has approximately 95% the ΣA_{260} from the dimer peak to the end of the gradient that part A has. (Thymidine incorporation into DNA at 30 hr Ile$^-$ was 0.6% the Ile$^+$ value.) In another experiment (C), polysomes from 2-hr Ile$^-$ cells (1.05×10^8) and (D) polysomes from 0.98×10^8 48-hr Ile$^-$ cells exposed to 0.68 mCi of L-leucine-*4,5-t* for 31.5 and 60 sec, respectively. ΣA_{260} for (C) was slightly greater than for (A). ΣA_{260} 48-hr Ile$^-$ $\simeq$ 93% ΣA_{260} 2-hr Ile$^-$. (E) Polysomes from 1.425×10^8 48-hr Ile$^-$ cells labeled for 31.5 sec with 1 mCi of L-leucine-*4,5-t*. Culture volumes were 340 ml for A, B, C, and D and 500 ml for E. ΣA_{260} for E was lower than in A → D but was approximately the same as obtained from an exponential culture analyzed in the same experiment.

as much uridine was incorporated into the sample from Ile$^-$ as in Ile$^+$ cultures. The rapidly sedimenting cytoplasmic material contains rapidly labeled RNA, only part of which is associated with ribosomes or polysomes (Penman *et al.*, 1968). Some is associated with protein to form material of lower density—referred to as informosomes (Spirin, 1969). The apportionment of uridine incorporation between polysome-associated (R) and lower-density (I) material, in the case of Ile$^-$ and Ile$^+$ cultures, is shown by isopycnic analysis of formaldehyde-fixed samples (Figure 3B,C). Again, Ile$^-$ samples show about one-half the incorporation shown by exponential Ile$^+$ samples. The apportionment of label into polysomal and lower density material is but slightly different in the two samples. We infer that messenger synthesis in Ile$^-$ cultures is approximately 50% that seen in Ile$^+$ cultures and that its association with polysomes occurs to about the same extent relative to its synthesis rate as in Ile$^+$ cultures. In the section on polysome analysis, we will further demonstrate that sufficient messenger is present to maintain most of the ribosome population in polysomes.

Polysome Metabolism–Protein Synthesis. In cells arrested in G_1 by growth to high density (Tobey and Ley, 1970), one of the most striking effects is upon polysome metabolism. As shown in Figure 4B, polysomes in high-density, stationary-phase cultures are almost completely converted to 80S ribosomes. This observation is consistent with the low rate of amino acid incorporation into protein in stationary-phase cultures of CHO cells (Tobey and Ley, 1970).

As seen from the zone sedimentation analyses (Figure 5) of polysomes prepared from cells growing in exponential culture and for periods of 2–48 hr in Ile$^-$ culture, the isoleucine-deficient condition, which has such a pronounced effect upon cell division and DNA synthesis, does not cause significant diminution of polysome content. In 30-hr Ile$^-$ (Figure 5B) or even 48-hr Ile$^-$ (Figure 5D,E) cultures, the summation of A_{260} contained in the region of the gradients from the peak of the first polysome to the end of the gradient shows Ile$^-$ cells to have at least 90% the content of Ile$^+$ cells. There is perhaps a shift toward smaller average polysome size, but the effect is not pronounced.

The zone sedimentation analyses illustrated in Figure 5 were performed on cytoplasm treated with high-salt buffer. This treatment serves several purposes. First, 80S ribosomes which do not have messenger and peptidyl-tRNA are converted to 40S (S) and 60S (L) subunits; 80S ribosomes produced by breakdown of polysomes *via* nuclease activity, etc., do not dissociate (Fan and Penman, 1970). Thus, analyses performed in high salt buffer allow detection of polysome

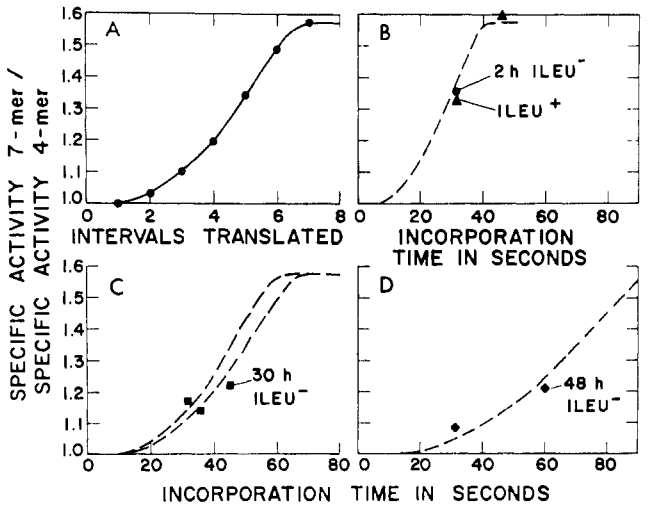

FIGURE 6: Increase in specific activity as a function of time for pulse leucine-labeled polysomes carrying seven ribosomes compared to those carrying four ribosomes: (A) model; (B) best fit to data from Ile$^+$ or 2-hr Ile$^-$ cultures; (C) best-fit for 30-hr Ile$^-$ data; and (D) 48-hr Ile$^-$ data.

breakdown, should any occur during polysome preparation or analysis. Second, adsorption of adventitious protein is reduced (Warner and Pene, 1966). This is especially important when one wishes to quantitate incorporation of labeled amino acids into incomplete, nascent polypeptides.

Note that in the preparations analyzed (Figure 5) 80S peaks are absent. The amount of subunits shown thus indicates the relative amount of nonpolysomal material. The proportion of these free ribosome forms relative to polysome forms does increase during Ile$^-$ culture. Evidently the increased RNA content observed during Ile$^-$ culture is expressed as an increase in nonpolysomal forms.

Polysome content is not greatly diminished in Ile$^-$ culture and, therefore, the protein synthetic machinery is maintained. Is it functioning as rapidly as in Ile$^+$ culture? One could most easily measure such rate by comparing rates of amino acid incorporation into polysomes or into total peptides. However, such measurements are susceptible to altered transport rates, "pool effects," etc. It would appear quite likely that such effects upon the relationship of precursor incorporation to true synthesis rate would be altered in cells whose supply of an essential amino acid is greatly diminished. Translation rate has been measured by determining incorporation into free (completed) and polysome-associated peptide material as a function of time (Stanners, 1968; Fan and Penman, 1970). Here again, the fact that the culture is deficient in an essential amino acid makes one consider the probability of rapid protein turnover (*cf.* Hershko and Tomkins, 1971). Rapid catabolism of released peptides could affect such an analysis. What is required is a measure of translation rate relatively unaffected by pool effects, protein catabolism, etc.

Subsequent to exposure to labeled amino acids, the specific activity of polysomes of a given size increases with time. This value reflects synthetic rate only if dilution of labeled precursor is unaltered, etc. However, the specific activity of polysomes labeled for a given amount of time also increases as a function of *size*. Since all size species involved probably see the same precursor source, measurement of the rate of increase in specific activity of polysomes as a function of size subsequent to incorporation for periods of time less than needed to fully label all species in question should provide a measure of relative translation rate unaffected by effects on transport or dilution of precursor. Application of such an analysis to determine translation rate is illustrated in Figure 6. First, we consider the effect of increasing the length of incorporation times upon the specific activity of a larger polysome relative to that of a smaller species. We have chosen the 7-ribosome unit in the polysome (7-mer) as the larger species as it is the largest species sufficiently resolved to allow determination of its position in the gradient. The 4-ribosome unit (4-mer) is chosen as the smaller species because it is sufficiently far removed from free (low molecular weight) material such that spill over of top-gradient material should not affect its specific activity. Extrapolation of values determined for 5- and 6-mers aids in the analysis. We may calculate that the specific activity of the 7-mer will increase relative to that of the 4-mer as shown in Figure 6A, where "interval translated" represents the time needed to translate the amount of messenger contained per attached ribosome. The calculation is an average of the numbers obtained using those polysomes which have just released and those which are just about to release a peptide. For sufficiently short incorporation periods, the greater average peptide length of the larger polysome has no effect (<one interval). For longer periods, the longer average peptide length endows the larger polysome with a greater specific activity. The slope of the curve increases upon attainment of a fully labeled condition by the smaller species. The ratio attains a maximum and then remains constant for incorporation periods greater than needed to fully label the larger species. Figure 6B–D involves application of the analysis. The 7:4 specific activity ratio is determined for a given period of incorporation. The theoretical curve is then best fitted to the experimental point by assigning differing values for seconds needed to translate one interval, and the resultant plot provides an estimation of interval time in seconds—or an inverse measure of translation rate.

Translation rate in exponentially growing cultures or in 2-hr Ile$^-$ cultures is such that best fit is obtained by assigning a value to interval translation time of 6 sec (42 sec to fully label a 7-mer). Pulse times of 50 and 75 sec gave specific activity ratios which were within experimental error of the theoretical plateau value, indicating that peptide length is proportional to polysome length in the case of polysomes 4–7 units long.

Analysis of 30-hr Ile$^-$ populations (Figure 6C) results in significantly lower values for (specific activity of 7-mer)/(specific activity of 4-mer) after incorporation periods of 30–45 sec. Variations seen are noted from experiment to experiment—not within a given experiment. These data indicate that the time to translate one interval is 9–10 sec in 30-hr Ile$^-$ cultures. According to the analysis shown in Figure 6D, the corresponding value for 48-hr Ile$^-$ cultures is 14 sec. Translation rates for 30- and 48-hr Ile$^-$ cultures are thus estimated as 63 and 38% of the rates possessed by Ile$^+$ or 2-hr Ile$^-$ cultures. Correspondingly, 30-hr Ile$^-$ protein synthesis is approximately 0.90 (relative polysome mass) $\times$ 0.63 (translation rate) = 57% of Ile$^+$, and 48-hr Ile$^-$ is 0.9 $\times$ 0.38 = 34% of Ile$^+$.

The above analyses provide estimations of protein synthesis rate in an absolute rather than *net* sense. In cultures deficient in an essential amino acid, one might expect a corresponding rate for catabolism (*i.e.*, turnover of protein with no net synthesis). Biuret protein values were obtained to check this prediction. Surprisingly, 30-hr Ile$^-$ cultures have fully 86–90% the protein content of Ile$^+$ exponential cultures. Had there been no net protein synthesis in Ile$^-$ culture, the ~44% increase in cell number would have diminished protein content

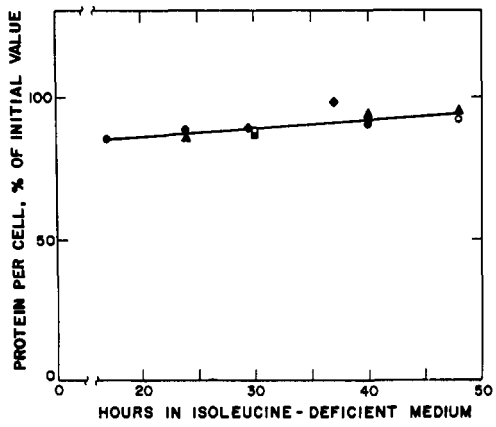

FIGURE 7: Protein content of cells cultured in Ile⁻ medium as per cent of initial exponential culture (Ile⁺) value. Cells from a measured aliquot of culture were harvested by centifugation, washed twice by suspension in and sedimentation through 0.25 M sucrose at 4° and stored as frozen cell pellets. Upon thawing, cell pellets were suspended in chloroform–methanol (3:1, v/v). After 15 min at room temperature, five-tenths volume of methanol was added (to facilitate sedimentation by lowering solution density), the suspensions were chilled in an ice bath and insoluble material was removed by centrifugation. After thorough drying, sediments were dissolved in 0.5 ml of 0.3 M KOH prior to addition of 2 ml of biuret reagent (Gornall *et al.*, 1949). Absorbancy was then determined at 530 mμ and converted to milligram of protein using a bovine serum albumin calibrated standard curve of A_{540} *vs.* milligram of protein. Each symbol represents data from a single experiment. "Per cent of initial value" relates to the relative Ile⁻ and Ile⁺ contents determined within each experiment, not Ile⁻ contents compared to an average Ile⁺ value.

to 100%/(1 + 0.44) or 70% Ile⁺. Further, determination of protein content as a function of Ile⁻ culture time shows that content increases in Ile⁻ culture to ~94% of Ile⁺ by 48 hr (Figure 7).

The final note on polysome metabolism concerns availability of mRNA. We ask if the small decrease in polysome content in Ile⁻ culture is due to insufficient mRNA to support full formation. Availability of unassociated mRNA has been demonstrated using low levels of cycloheximide to reduce the rate of translation relative to rate of initiation or ribosome attachment (Stanners, 1966; Fan and Penman, 1970). This type of analysis was applied to Ile⁻ cells. In this experiment, both Ile⁺ and Ile⁻ cultures were showing relatively large proportions of 80S ribosome forms. The extensive conversion of such 80S forms to polysomes, effected as shown in Figure 8, indicates that mRNA is *not* in short supply in Ile⁻ culture.

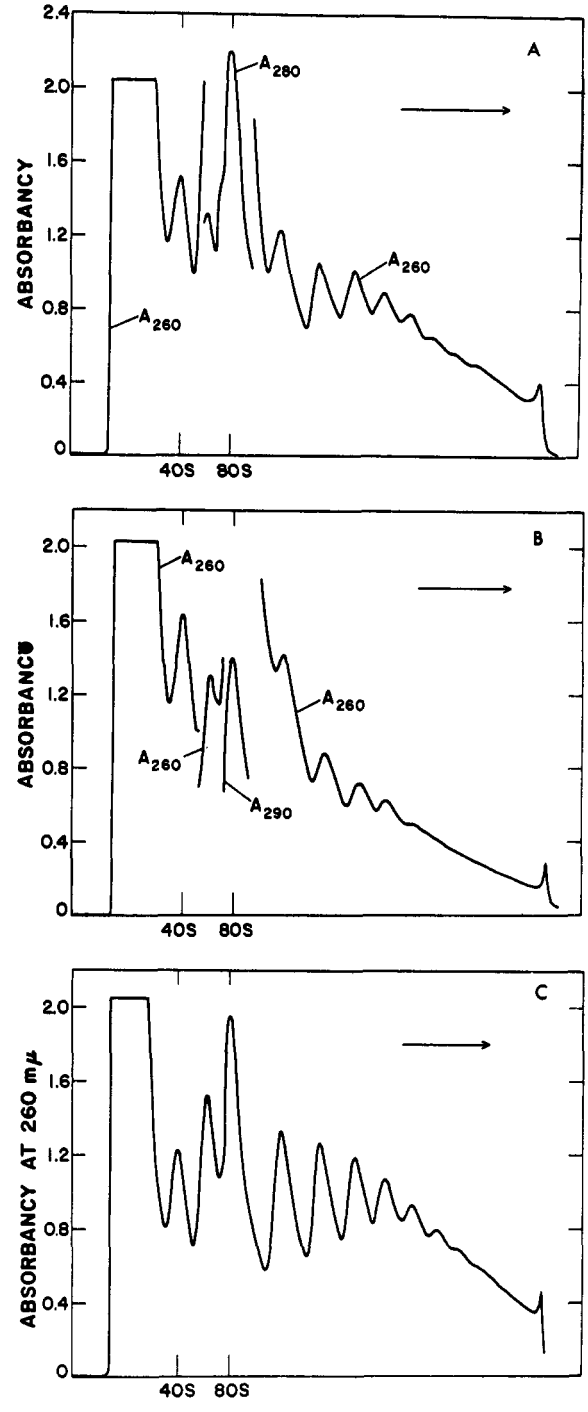

FIGURE 8: Superformation of 48-hr Ile⁻ polysomes: (A) polysomes of Ile⁺ cells; (B) polysomes of 48-hr Ile⁻ cells; and (C) polysomes of 48-hr Ile⁻ cells after culture in 1 μg/ml of cycloheximide for 45 min. Preparation and analysis were in $N_{100}T_{10}M_{1.5}$ (10 mM NaCl–10 mM Tris, pH 7.4 at 25°, 1.5 mM Mg^{2+})

Discussion

Upon transfer to isoleucine-deficient medium, there is an abrupt reduction in rate of DNA synthesis and an accumulation of cells with the G_1 content of DNA. In contrast, the effect of Ile⁻ culture upon RNA synthesis is much less pronounced. Synthesis continues at about two-thirds Ile⁺ rate long after DNA synthesis and cell division have ceased. The reduction is apportioned among species such that no major species is completely shut off. Synthesis of stable moieties is reduced to the greatest extent: to about one-fifth the Ile⁺ rate for rRNA and to about one-third the Ile⁺ rate for 4S RNA. Since cell division ceases in Ile⁻ cultures, a full complement of these species is maintained. Continued synthesis at higher rates would have resulted in a gross imbalance of RNA relative to DNA, but this did not occur in the Ile⁻ cell. Synthesis of unstable moieties is less reduced: to a little over 70% of the value in the Ile⁺ control for Hn and to ~50% of the control rate for mRNA. These values agree well with expected values for early G_1 cells calculated from data obtained in exponential cultures (synthesis of unstable species may triple during interphase (Enger and Tobey, 1969)). Further, superformation of polysomes in Ile⁻ cultures using low levels of cycloheximide indicates that the amount of available mRNA is not a limiting factor, although the possibility exists that the synthesis

of mRNA species necessary for cell-cycle traverse is selectively inhibited.

Protein synthesis was also much less affected than was genome replication. In particular, note that after 2 hr in isoleucine-deficient medium, when the rate of thymidine incorporation has been decreased by 60% (Figure 1B), the rate of protein synthesis was unchanged from that of the control culture (Figure 5C). After 30 hr in isoleucine-deficient medium the rate of protein synthesis was still 60–70% of that obtained in exponentially growing cultures. Even after 48 hr in isoleucine-deficient medium, the synthesis rate was still 34% of the rate in exponential cultures. Thus, it is apparent that the synthesis of DNA is much more rapidly and completely inhibited than are the syntheses of RNA and protein.

Continued protein and RNA synthesis subsequent to cessation of DNA synthesis in cells grown in isoleucine-deficient medium is not unexpected in view of the requirement for continuous synthesis of functional RNA and protein throughout interphase up to within 114 and 8 min of mitosis, respectively (Tobey *et al.*, 1966). That is, in order for cells to accumulate in G_1 in Ile^- medium, G_1 cells must be prevented from initiating DNA synthesis, while cells initially in S, G_2, and M must be capable of synthesizing functional RNA and protein species required for cell-cycle traverse in order to allow them to complete interphase and accumulate in G_1. Indeed, transfer to isoleucine-deficient medium brings about an extremely rapid reduction in rate of DNA synthesis (Figure 1B)—the result of a shutting off of the G_1-to-S transition, coupled with a reduced rate of DNA synthesis by cells completing S in Ile^- medium. Note the immediate reduction in *rate* of cell division following transfer of cells to Ile^- medium (Figure 1A). With increasing exposure period to Ile^- medium, the rate of cell-cycle traverse continues to decrease, suggesting that the cells completing interphase in Ile^- medium are experiencing increasing difficulty in obtaining traverse-essential polypeptide species. However, once again note that initiation of DNA synthesis has long since ceased, implying that the effects of limiting amounts of isoleucine on DNA initiation and completion of interphase *are two separate phenomena.*

For a culture deficient in an essential amino acid, protein synthesis continues at a surprising rate. The high rate of protein synthesis, coupled with the low net increase in protein mass during culture in Ile^- medium, suggests that (1) the rate of polypeptide turnover must increase appreciably in G_1-arrested cells in view of the high synthetic rate and low availability of isoleucine and (2) the small but definite increase in protein content suggests that the cells are obtaining additional isoleucine, most likely through the catabolism of serum polypeptides (Eagle and Piez, 1960). Although proteolysis of serum polypeptides during dialysis of serum could liberate additional isoleucine, this possibility is unlikely in view of amino acid analyses of our dialyzed serum which revealed an absence of free isoleucine.

The limiting quantities of available isoleucine may well explain our observation of reduced polypeptide synthesis in 30-hr Ile^- culture—not through translation at a normal rate employing fewer polysomes as is characteristic of stationary-phase cultures (Stanners and Becker, 1971) but, rather, a *reduced rate of translation* utilizing normal numbers of polysomes. Our reduction in translation rate may indicate that the rate-limiting step is availability of isoleucine such that partially completed polypeptide chains must stop at isoleucine-requiring sites and await the catabolic liberation of isoleucine from preformed moieties.

Our results are in stark contrast to those obtained in studies of stationary-phase cultures (Ward and Plagemann, 1969; Becker *et al.*, 1971) in which rates of macromolecular synthesis and quantities of biosynthetic machinery are much more greatly reduced in high-density, stationary-phase monolayers (Stanners and Becker, 1971) or in suspension cultures (Tobey and Ley, 1970; Figure 4B above). Rather, our results more closely resemble those of recent studies of contact- or confluency-inhibited cells in monolayer culture. For example, in confluency-inhibited mouse 3T3 cells RNA synthesis continues at 75% of the rate of subconfluent cells (Weber and Edlin, 1971). However, note that our system has two major advantages over confluency-inhibited cell cultures. (1) Virtually any number of cells for biochemical studies may be simply produced in suspension culture with our technique, whereas large numbers of confluency-inhibited cells are produced only with great difficulty and manipulation of large numbers of culture vessels. (2) In contrast to confluency-inhibited cultures in which the process bringing about accumulation in G_1 arrest is modulated by ill-defined, poorly characterized "serum components" and is further complicated by effects of pH (Ceccarini and Eagle, 1971), in our system the active agent is the well-defined, readily manipulated amino acid isoleucine.

It appears that removal of isoleucine from the culture medium results in a unique biochemical state in which biochemical machinery remains essentially intact and functioning at relatively high levels of activity; however, these cells do not appear to enter a state of gross biochemical imbalance in regard to their content of protein and nucleic acid species. We conclude that G_1 arrest in Chinese hamster cells cultured in medium deficient in isoleucine is not due to lack of protein or RNA synthetic capability, since cessation of DNA synthesis precedes any appreciable effect upon RNA and protein synthesis. Rather, some mechanism whose functioning is essential to G_1 traverse or initiation of S is more sensitive to lowered isoleucine supply than is RNA or protein synthesis.

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Effect of Growth Conditions on Peptidoglycan Structure and Susceptibility to Lytic Enzymes in Cell Walls of *Micrococcus sodonensis**

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ABSTRACT: Cell walls of *Micrococcus sodonensis* were isolated and purified from cultures grown in a synthetic medium and a complex medium. Chemical analyses revealed that two preparations were free of cell membrane contaminants and were qualitatively identical. Glutamic acid, glycine, alanine, and lysine were present in a 1:1:2:1 ratio. *N*-Acetylmuramic acid and *N*-acetylglucosamine were present in equimolar amounts, although walls from cells grown in synthetic medium contained twice as many hexosamine residues as those from cells grown in the complex medium. The subunit peptide structure was determined to be N^{α}-[L-alanyl-γ-(α-glutamylglycine)]-L-lysyl-D-alanine in both cases. Sequential enzymic digestions and isolation of peptide fragments revealed that peptide cross bridging was accommodated by "head-to-tail" assembly of peptide subunits. Such assembly was facilitated by N^{ϵ}-(D-alanyl)-L-lysine and D-alanyl-L-alanine linkages. The distribution and length of cross bridges varied significantly in the two preparations. Controlled digestion studies disclosed significant differences in lysozyme susceptibility of the two preparations. Forty-nine per cent of the dry weight of walls purified from cells grown in complex medium, as opposed to 75% from cells grown in defined media were solubilized by lysozyme. Analysis revealed a greater degree of peptide substitution of the glycan and shorter cross bridges in the lysozyme-insoluble portion than the lysozyme-susceptible portion of the cell walls from both sources. Overall, the peptidoglycan structure was more complex in the walls of cells grown in complex than in synthetic medium. The chemical analyses, plus electron microscopic examination led to the conclusion that in both cases the cell wall is composed of at least two peptidoglycan matrices, one of which is insensitive to lysozyme. This resistance to lysozyme results from a combination of two factors associated with this portion of the wall, (a) an increased level of *O*-acetyl substitution and (b) an increased complexity of cross-linked structure.

In several members of Micrococcaceae, the peptide subunit N^{α}-[L-alanyl-γ-(α-glutamylglycine)]-L-lysyl-D-alanine (Ghuysen, 1968; Schleifer and Kandler, 1970) is the building block of the cell wall peptidoglycan. These subunits are interlinked by peptide bridges extending from the ϵ-amino group of lysine of the one peptide subunit to the α-carboxyl group of the C-terminal alanine of another peptide subunit. "Head-to-tail" assembly of several identical peptide subunits occurs in several micrococcal cell walls and is facilitated by two types of peptide linkages: N^{ϵ}-(D-alanyl)-L-lysine linkages which extend from the ϵ-amino group of lysine to the carboxyl groups of D-alanine, and D-alanyl-L-alanine linkages which actually assemble the peptide subunits in head-to-tail sequence (Schleifer and Kandler, 1967; Ghuysen *et al.*, 1968a,b; Campbell *et al.*, 1969).

The first observation that altered cellular nutrition produces significant changes in the cell wall was made in walls obtained from *Streptococcus faecalis* grown under conditions of threonine depletion (Toennies and Shockman, 1959). Variations in peptidoglycan structure under altered conditions of growth have been documented (Smith and Henderson, 1964; Smith *et al.*, 1965) but these changes involve substitution of one amino acid for another in the peptide subunit. This paper re-

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