Kinetic Studies of New Ribosome Utilization in KB Cells*

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ABSTRACT: We have analyzed the kinetics of new RNA entry into three categories of cytoplasmic KB ribosomes, polysomes, 74S ribosomes, and free subunits. Our data show that: (1) the newly synthesized small 45S ribosome subunit appears in the cytoplasm before the large 60S subunit. (2) These newly synthesized particles sediment primarily as free subunits rather than as polysomes or monomers. (3) The new RNA appears in polysomes before in monomers. Six hours elapsed before polyribosomes and free subunits contained the same ratio of new to old ribosomes. The 6-hr equilibration time is too short to be compatible with a ribosome cycle in which new subunits enter polyribosomes only by net growth, but is longer than would be expected if complete exchange occurred after each passage of ribosomes across mRNA. It seems likely that the ribosomes dissociate, but perhaps not as often as every round of protein synthesis. The kinetic data suggest that some feature of cell physiology in mammalian systems segregates a part of the ribosomes in the form of inactive 74S ribosomes.

Polyribosomes of bacterial and yeast cells frequently dissociate into free subunits, presumably in conjunction with polypeptide-chain termination. The natural occurrence of ribosome dissociation has been deduced from two types of *in vivo* experiments. (1) Kinetic analysis of the distribution of new ribosomes in bacterial cells showed that polyribosomes and free subunits contain equal fractions of newly made subunits even after very brief labeling times (Mangiarotti and Schlessinger, 1967). These data strongly suggested that all subunits, those free and those in polyribosomes, participate in a common intracellular pool and that polyribosomes contribute subunits to this pool by ribosome dissociation. (2) Utilizing density-transfer experiments it has been possible to demonstrate directly that ribosome dissociation occurs *in vivo* both in bacteria (Kaempfer *et al.*, 1968) and yeast (Kaempfer, 1969). In such experiments cells are grown first in a medium containing heavy isotopes and then transferred to a light isotopes medium. The ribosomes synthesized in the two media have different densities and can be separated in density gradients. Following transfer to light medium all the heavy ribosomes are replaced by ribosomes of intermediate density containing one heavy and one light subunit. Such ribosome couples can be formed only if ribosome dissociation occurs *in vivo*.

Ribosome dissociation has not been demonstrated in mammalian systems, primarily because density-transfer experiments, which require a completely synthetic growth medium, are not technically feasible. Therefore, as an alternative, we have studied the kinetics of new rRNA entry into the different ribosome fractions of mammalian cells. For these experiments we have utilized an established tissue culture cell line of human origin, KB, which grows in suspension cultures with a generation time of approximately 28 hr. We have analyzed three categories of cytoplasmic KB ribosomes, polysomes, 74S ribosomes, and free subunits; each category in turn includes two species, one having 28S RNA (60S subunits), the other containing 18S RNA (45S subunits). The quantitative kinetic data obtained indicate that the mammalian ribosome cycle includes a ribosome dissociation step, but the dissociation may not occur as frequently as in unicellular organisms. In addition the results suggest that some feature of cell physiology in mammalian systems segregates a portion of the ribosomes in the form of inactive 74S particles.

Materials and Methods

Exponentially growing KB (Eagle, 1955) cell suspension cultures (2.5×10^5 cells/ml) were labeled with [^{14}C]uridine (57 mCi/mmole; 0.015 μCi/ml) for 24 hr and then resuspended in fresh nonradioactive medium. About 18 hr later the culture was concentrated in the same medium to a cell density of 10^6/ml; [^{3}H]uridine (20 Ci/mmole; 3 μCi/ml) was then added, and portions of the culture were harvested at subsequent times. The RNA containing [^{14}C]uridine represents uniformly labeled cellular RNA, whereas the [^{3}H]uridine label is found in newly synthesized RNA molecules.

Cytoplasmic extracts were prepared by homogenizing cells after hypotonic swelling in RSB (0.0015 M $MgCl_2$–0.01 M NaCl –0.01 M Tris, pH 7.4) for 15 min (Penman, 1966). Nuclei were removed by low-speed centrifugation and the extracts were clarified by 15-min centrifugation in the Sorvall RC2-B centrifuge at 16,000*g*. Ribosome fractions were obtained by sedimenting the extracts through 7.5–45% sucrose gradients formed on top of a 2-ml 75% sucrose cushion. Sucrose solutions were buffered with 0.01 M Tris (pH 7.4) and contained 0.0015 M $MgCl_2$–0.01 M NaCl. After centrifugation for 16.5 hr at 20,000 rpm, 5°, in the SW25.1 rotor, most of the polysomes are concentrated in the sucrose cushion and bottom of the gradient; 74S ribosomes and free 60S and 45S subunits are resolved in the remainder of the gradient. The RNA from different ribosome species was extracted by treatment with 0.5% sodium dodecyl sulfate and 0.003 M EDTA, then concentrated by alcohol precipitation together with 0.1 mg of phenol-purified cytoplasmic RNA as carrier. Just prior to zonal sedimentation in 15–30% sucrose gradients in the SW41

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rotor at 38,000 rpm, 17°, for 5.5 hr the RNA was resuspended in 0.5% sodium dodecyl sulfate, 0.003 M EDTA, 0.05 M NaCl, and 0.01 M Tris (pH 7.4). The gradients contained 0.25% sodium dodecyl sulfate, 0.001 M EDTA, 0.05 M NaCl, and 0.01 M Tris (pH 7.4).

The radioactivity in 100-μl aliquots from sucrose gradients fractions was determined by scintillation counting in 10 ml of a fluid made by mixing 600 ml of toluene, 80 g of napthalene, 400 ml of ethylene glycol monomethyl ether, and 4 g of 2,5-bis-[2-(5-*t*-butylbenzoxazolyl)]thiophene (Packard Instruments). Of the input ^{14}C counts per minute, 20% was counted in the ^{3}H channel; less than 1% of the ^{3}H counts per minute was recorded in the ^{14}C channel.

Results

Ribosome snythesis in mammalian cells takes place in the nucleus whereas most if not all functioning ribosomes are localized in the cytoplasm (Penman, 1966). Experiments utilizing several types of cells have established that the small 45S ribosome subunit appears in the cytoplasm 25 min after addition of a radioactive RNA precursor; the larger 60S particle is detected by 50 min (Girard *et al.*, 1965; Joklik and Becker, 1965a,b; Henshaw *et al.*, 1965; Perry, 1965; Hogan and Korner, 1968b; Kabat and Rich, 1969). Moreover the newly synthesized particles sediment primarily as free subunits rather than as 74S ribosomes or polysomes.

Our studies with the human cell line KB (see also Raskas *et al.*, 1970) agree with the findings previously reported for other mammalian systems. In addition we have obtained data on the kinetics of entry of new 60S and 45S subunits into all three ribosome categories, free subunits, 74S ribosomes, and polysomes. The kinetics obtained strongly suggest that at least some mammalian ribosomes dissociate into free subunits as a normal feature of ribosome metabolism.

Labeling Pattern of KB Ribosomes. For all experiments described in this report, we have utilized exponentially growing suspension cultures of KB cells. The total cellular RNA of these cultures was uniformly labeled with [^{14}C]uridine and subsequently exposed to [^{3}H]uridine for appropriate intervals. After 100 min of [^{3}H]uridine incorporation most of the newly synthesized rRNA present in the cytoplasm cosediments with the free ribosomal subunits (Figure 1A). The pattern of labeling increasingly resembles the ribosome distribution at progressively longer times of [^{3}H]uridine incorporation (Figure 1B).

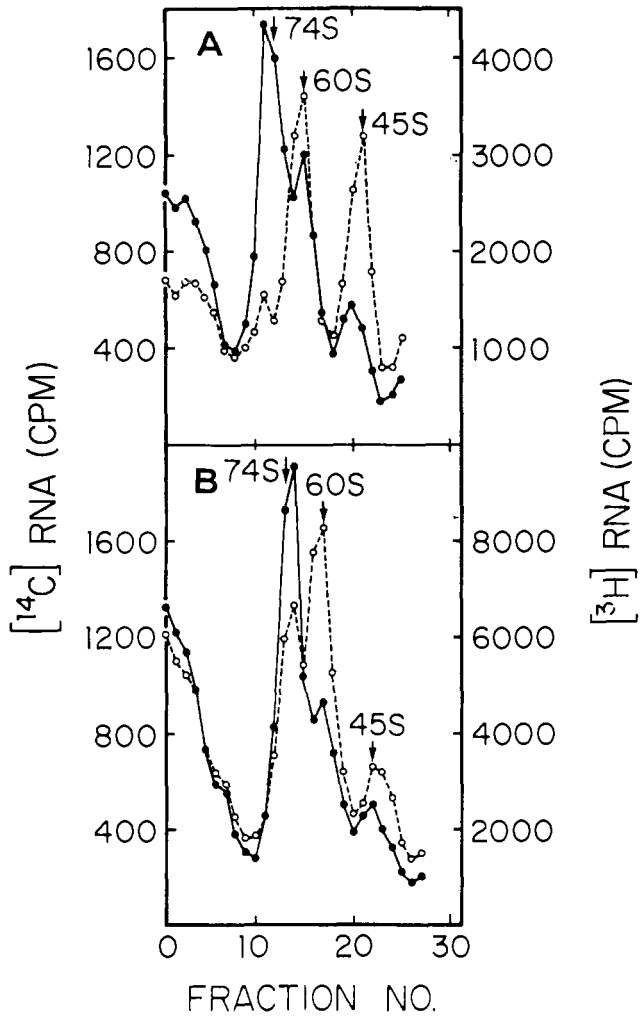

FIGURE 1: Sucrose gradient analysis of KB ribosomes. Cytoplasmic extracts of KB cells uniformly labeled with [^{14}C]uridine and subsequently labeled with [^{3}H]uridine for either 100 min (A) or 240 min (B) were prepared by homogenization in RSB and analyzed in sucrose gradients as described in Materials and Methods. Aliquots (100 μl) of each 0.75-ml fraction were counted in a scintillation counter. Approximately 10–20% of the polysomes was in the pellet in all experiments. ^{14}C counts per minute (●—●) and ^{3}H counts per minute (○—○).

The types of newly synthesized RNA cosedimenting with the different classes of ribosomes in Figure 1 was determined by sucrose gradient analysis. We analyzed the RNA content of particles in the peak fractions of the 74S ribosome and free subunit regions and in combined aliquots from the fractions in the polysome region. The RNA analysis confirmed the identity of the free subunits; the 60S subunits contain 28S RNA (Figure 2A,B) whereas the 45S subunits contain 18S RNA (Figure 2C,D). In both instances contamination of one species by the other was less than 10%. Since the total cellular RNA was prelabeled with [^{14}C]uridine, an increasing ^{3}H:^{14}C ratio for rRNA is a measure of increasing amounts of new free subunits in the cytoplasm. At 240 min (Figure 2B,D) the ^{3}H:^{14}C ratio for 28S RNA in 60S subunits and for 18S RNA in 45S subunits was greater than at 100 min (Figure 2A,C).

A similar analysis of the RNA in 74S ribosomes and in polysomes is presented in Figure 3. These ribosomes contain both 28S and 18S RNA, and the accumulation of new subunits in these entities is reflected in a ^{3}H:^{14}C ratio that increases from 100 min (Figure 3A,C) to 240 min (Figure 3B,D). The dotted lines in Figure 3 show the likely division of the [^{3}H]RNA into ribosomal and nonribosomal components (as in Girard *et al.*, 1965). The validity of this division was confirmed by treating polyribosomes with EDTA to obtain 50S and 30S subunits (Girard *et al.*, 1965). Non-rRNA is released from polysomes with EDTA treatment, and subunits so prepared had the same ^{3}H:^{14}C ratio as calculated after subtracting the heterogeneous background in the RNA sedimentation analysis. It should also be noted that at 100 min and even at 240 min the 28S [^{3}H]RNA obtained in the 74S ribosome fraction was to a large extent derived from free 60S subunits sedimenting in the adjacent region of the gradient. In the kinetic analysis described below

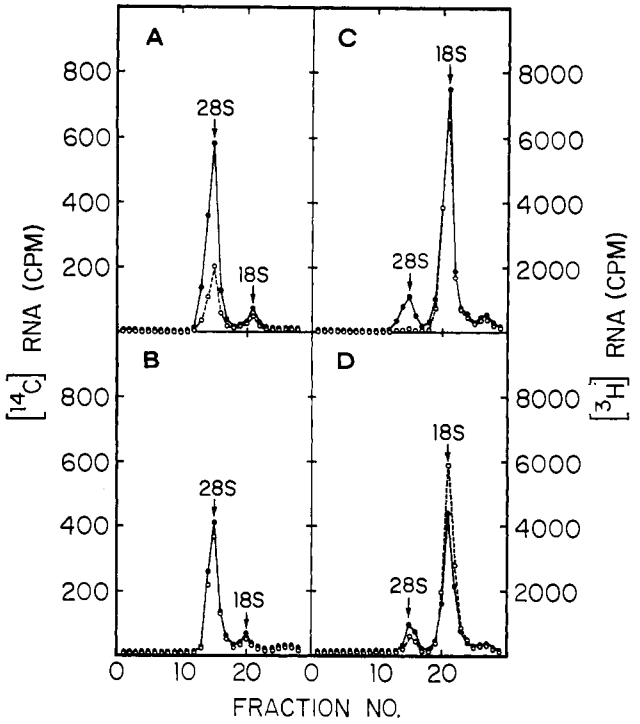

FIGURE 2: Sucrose gradient analysis of the RNA in free ribosomal subunits. The RNA of the peak fractions from the free 60S and 45S ribosome subunits shown in Figure 1 was precipitated and then sedimented through sucrose gradients to resolve 28S and 18S rRNA as described in Materials and Methods. The RNA analyzed included the free 60S subunits labeled with [^{3}H]uridine for 100 min (A) and 240 min (B) as well as the free 45S subunits labeled for 100 min (C) and 240 min (D). ^{14}C counts per minute (●—●) and ^{3}H counts per minute (○—○).

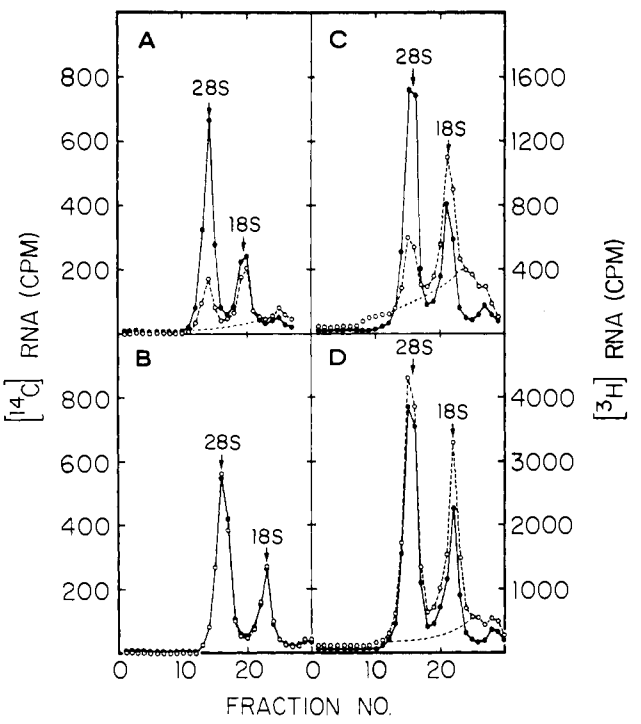

FIGURE 3: Sucrose gradient analysis of the RNA in polyribosomes and 74S ribosome monomers. The RNA in the 74S peak fraction and in combined aliquots from the fractions in the polysome region shown in Figure 1 was precipitated and then sedimented through sucrose gradients to resolve 18S and 28S rRNA as described in Materials and Methods. The RNA analyzed included 74S monomers labeled with [^{3}H]uridine for 100 min (A) and 240 min (B) as well as polysomes labeled for 100 min (C) and 240 min (D). The dotted lines indicate the likely division of newly made [^{3}H]RNA into ribosomal and nonribosomal fractions as in Girard *et al.* (1965). ^{14}C counts per minutes (●—●) ^{3}H counts per minute (○—○).

the ^{3}H:^{14}C ratio for 28S RNA in 74S ribosomes has been corrected for this factor.

Kinetics of Ribosome Synthesis. The complete kinetic data obtained from the experiment shown in Figures 1–3 are presented in Figure 4. The RNA in the different ribosome categories at different times after addition of [^{3}H]uridine to cells prelabeled with [^{14}C]uridine was analyzed by sucrose gradient centrifugation (as in Figures 2 and 3); from these gradients we obtained the ratio of new to old 18S or 28S rRNA (^{3}H:^{14}C) for each species at each sampling time. The increasing ^{3}H:^{14}C values represent increases in the numbers of new ribosomes, for the [^{14}C]RNA remains constant during these labeling times. We note that: (1) the newly synthesized 45S ribosome subunit appears in the cytoplasm before the 60S subunit. (2) These newly synthesized particles sediment primarily as free subunits rather than as polysomes or 74S ribosomes. (3) The rRNA in polysomes has a consistently higher ^{3}H:^{14}C ratio than that in 74S ribosomes.

Ribosome Equilibration. When different ribosome categories contain equal fractions of new ribosomes, they are equilibrated. Thus polysomes and free subunits are equilibrated when their RNA components have the same ^{3}H:^{14}C ratio. As shown in Figure 5A,C mammalian ribosomes do not equilibrate quickly, for the new 60S and 45S ribosome subunits are not distributed equally among the different species of ribosomes until 6 hr after addition of the radioactive RNA precursor. Equilibration of both 28S and 18S RNA between monomers and polysomes requires several hours (Figure 5B,-D) but less time than subunit–polysome equilibration.

Discussion

Ribosome Equilibration and the Ribosome Cycle in Mammalian Cells. Our data show that ribosome synthesis in the tissue culture cell line KB follows the pattern observed in all mammalian systems reported to date. In addition we have obtained a quantitative estimate of the time required for equilibration of new ribosomes with those preexisting in the cell. Following addition of a radioactive RNA precursor, 6 hr elapsed before polysomes and free subunits contained equal fractions of new ribosomes (Figure 5).

The observed equilibration time suggests that at least some of the ribosomes in polysomes dissociate into subunits. An alternative ribosome cycle is one in which subunit dissociation does not occur and new and old ribosomes mix as 74S ribosomes, not as free subunits. Since the per cent of the total ribosomes present as single ribosomes plus polyribosomes is constant in mammalian cells (Hogan and Korner, 1968a), this model would require that new subunits enter polyribosomes and 74S ribosomes only by cellular growth. We have calculated the amount of ^{3}H-containing RNA that would be introduced into polysomes by exponential growth of the

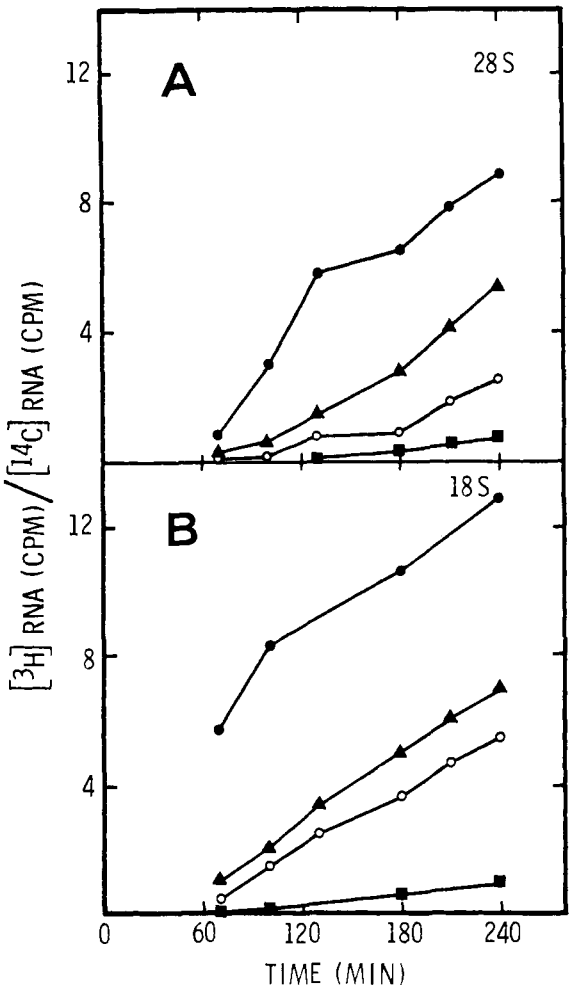

FIGURE 4: Kinetics of ribosome synthesis in KB cells. Cells were labeled and extracts prepared and fractionated on sucrose gradients as described for Figure 1. Polyribosomes, monomers, and free 60S and 45S subunits were treated with sodium dodecyl sulfate, and the RNA was analyzed in surcrose gradients as in Figures 2 and 3. For each ribosome category the ratio of new/old 28S (A) or 18S(B) rRNA ($^3H:^{14}C$) was determined from the peak fraction in the sodium dodecyl sulfate gradient. New [^3H]polysomal 18S RNA was corrected for contamination by mRNA as shown in Figure 3. Polysomal rRNA (▲-▲-▲); monomer rRNA (O-O-O); free subunit rRNA (●-●-●). Also shown is a calculated curve (■-■) for the polysomal $^3H:^{14}C$ ratio that would be obtained if new subunits could enter polyribosomes only in proportion to cell growth. These values were obtained by assuming the ribosome pool increases exponentially with the observed generation time of 28 hr and that new 18S rRNA enters the cytoplasm 25 min after addition of radioactive precursor whereas 28S RNA is detected at 50 min. We have also assumed that all subunits entering the polyribosomes during a given time interval have the $^3H:^{14}C$ ratio measured for the free subunits at the end of that time period and that all free subunits are equally competent as precursors to polysomes.

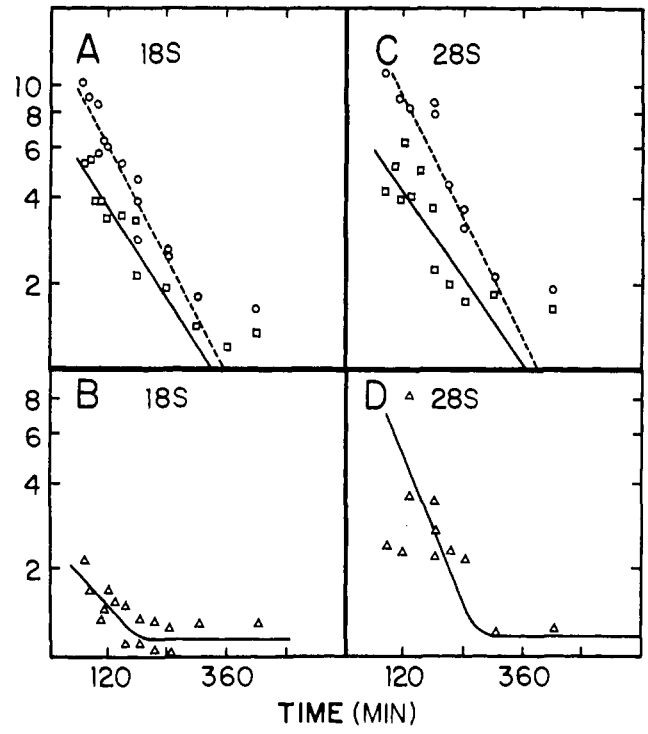

FIGURE 5: Equilibration of newly synthesized with preexisting KB ribosomes. The relative new ribosome content of the different ribosome species at times after addition of [^3H]uridine is presented in this figure. When different ribosome species have the same $^3H:^{14}C$ value at the same time, they are considered equilibrated and have the same fractional content of new particles. At each sampling time the $^3H:^{14}C$ value for one ribosome category divided by that for another class of ribosomes is a measure of the extent of equilibration. These ratios are plotted on the ordinate. These numbers are from data of four experiments including that presented in Figure 4. The lines drawn in A and C are the calculated least-squares lines utilizing all data except the values for 420 min; data from the individual experiments yield a similar least-squares line. ($^3H:^{14}C$ in free subunit RNA): ($^3H:^{14}C$ in polysomal RNA) (□—□); ($^3H:^{14}C$ in free subunit RNA): ($^3H:^{14}C$ in monomer RNA) (O- -O); ($^3H:^{14}C$ in polysomal RNA): ($^3H:^{14}C$ in monomer RNA) (△—△). (A and B) 18S rRNA; (C and D) 28S rRNA.

ribosome pool. For this calculation (see legend to Figure 4), we assumed that all subunits entering the polysomes during a given interval have the $^3H:^{14}C$ ratio of the free subunits at the end of the time period. In addition we assumed that free subunits labeled with ^3H and ^{14}C are equally competent as precursors to polyribosomes. As seen in Figure 4, this model is not compatible with the kinetic data; only a fraction of the new ribosomes in polyribosomes can be accounted for by the increase in the total number of ribosomes.

Thus, if ribosome dissociation did not occur, new subunits would not enter polyribosomes and monomers as rapidly as observed and ribosome equilibration would require much longer than 6 hr. The data could be consistent with an absence of *in vivo* dissociation only if unknown factors favor polyribosome formation from newly synthesized rather than preexisting subunits. However, there is no evidence that new free subunits are especially competent as polysome precursors; in fact the opposite has been suggested (Perry and Kelley, 1966).

Other investigators have reported experiments that suggest ribosome dissociation occurs *in vivo* (Kabat and Rich, 1969). Hogan and Korner (1968b) analyzed the distribution of radioactive rRNA in ascites tumor cells several generations after a labeling period and found data consistent with the occurrence of ribosome dissociation. Also extensive studies with NaF, an inhibitor of protein synthesis, yielded results compatible with

ribosome dissociation (Colombo *et al.*, 1968; Hoerz and McCarty, 1969).

The 6-hr period required for equilibration in KB cells is much longer than would be expected if dissociation is an obligatory step in each round of protein synthesis and each free subunit is available to mix with those generated by dissociation. However, the kinetic analysis presented here yields only a maximum equilibration time and does not exclude the possibility that dissociation occurs upon completion of every ribosome transit of mRNA. By contrast *Escherichia coli* ribosomes equilibrate in less than 5% of the doubling time (Mangiarotti and Schlessinger, 1966) whereas KB ribosomes require 20% of a generation. Numerous models of ribosome function could account for the observed delay in equilibration; our kinetic data do not distinguish between these alternatives. (i) Physical compartmentalization in the intact cell may account for the delay in equilibration. The reassociation of the same 60S and 45S subunits after termination may be favored because of geographical proximity. (ii) All ribosomes dissociate but do so less frequently than at every polypeptide chain termination. (iii) Not all ribosomes in polysomes dissociate into subunits at polypeptide termination, *i.e.*, there are different classes of polyribosomes. (iv) Some free cytoplasmic subunits may be ineligible to enter polyribosomes because of immaturity, as suggested by the experiments of Perry and Kelley (1966). When *in vitro* mammalian systems are developed that utilize natural mRNA molecules, it will be possible to test the relative functional maturity of new and old free subunits.

Role of Monomers in Mammalian Cells. As described above, in exponentially growing KB cells new subunits preferentially enter polysomes rather than 74S ribosomes (see also Girard *et al.*, 1965; Joklik and Becker, 1965a, and Kabat and Rich, 1969). Several other observations indicate that a variable fraction of the 74S ribosomes present in extracts of mammalian cells are a unique ribosome species. Conditions that decrease the rate of protein synthesis in ascites tumor cells cause an increase in the per cent of ribosomes found as single ribosomes while not affecting the size of the free subunit fraction (Hogan and Korner, 1968a). Similarly, late after infection with the nuclear DNA virus adenovirus type 2, when protein synthesis is decreasing, KB ribosomes contain a greater per cent of 74S ribosomes than in uninfected cells (Raskas *et al.*, 1970). This relationship between cell physiology and single ribosome content makes less likely the possibility that some of the 74S ribosomes are an artefact arising during preparation of extracts. Two observations indicate that not all 74S ribosomes are active ribosomes. Single ribosomes isolated from rat liver contain less nascent protein than polyribosomes (Noll *et al.*, 1963). In addition recent experiments using KB cells infected with adenovirus type 2 have shown that 74S ribosomes receive at least three times less viral mRNA than polysomes (H. J. Raskas and C. K. Okubo, in preparation).

Presumably there are two kinds of 74S ribosomes in mammalian cells. A fraction of the 74S particles are polyribosomes that contain only one ribosome. These single ribosomes would contain the ribosome bound mRNA and nascent protein detected in the 74S region of sucrose gradients. The remainder of the 74S ribosomes are inactive in protein synthesis and receive new subunits at a later time than the polyribosomes. The kinetic data described here do not distinguish between two possible sources of the new subunits eventually found as these single ribosomes. Such subunits may be derived either from polyribosomes or directly from free subunits.

Many of the 74S ribosomes may be derived from dividing cells present in the nonsynchronized culture; the polyribosomes of interphase cells disaggregate to single ribosomes and both protein and RNA synthesis are greatly reduced during metaphase (Robbins and Scharff, 1966). By analogy with HeLa cells (Rao and Engleberg, 1966), a growing KB culture will contain only 6% metaphase cells at a given time. Therefore in our experiments the inactive 74S ribosomes derived from dividing cells are insufficient to account for all the delay in entry of new subunits into single ribosomes.

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