

Control of Production of Ribosomal Protein

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The ratio, α (rate of synthesis of ribosomal protein/rate of synthesis of total protein) was measured in *Escherichia coli* B/r with the following results.

(1) In balanced exponential growth in succinate minimal medium, $\alpha = 0.08 \pm 0.01$ at 37°C.

(2) In balanced exponential growth in glucose minimal medium, $\alpha = 0.15 \pm 0.015$ at 37°C.

(3) During the transition period after glucose is added to a culture growing exponentially in succinate, α shifts from 0.08 to 0.15 in two to five minutes.

(4) α does not vary with time in a synchronized population growing in glucose minimal medium.

Measurement of α required a modification of the sucrose gradient ultracentrifuge technique to allow separation of ribosomes from the soluble proteins without loss of the latter.

At 37°C the doubling times in succinate and in glucose are 100 minutes and 50 minutes, respectively. Combining these results with the steady-state values of α and using the known protein content of a ribosome, the average growth rate of a polypeptide is computed to be 13 ± 2 amino acids per second per ribosome in succinate, glucose, or during the transition between the two media.

1. Introduction

Control systems regulating synthesis and activity of bacterial enzymes have been studied very successfully, but comparatively little is known about controls governing the production of structural cell components, often because they are difficult to assay quantitatively. Ribosomal protein is a relatively abundant material which carries out an important cellular function and can be assayed by rather straightforward methods. Quantitative measurements of the rates of synthesis of ribosomal protein under various conditions would complement the rather extensive data concerning synthesis of ribosomal RNA and provide a useful background for constructing models of the ribosomal control system.

The present experiments include separate measurements of the rate of synthesis of ribosomal protein relative to total protein and of the rate of synthesis of total protein. Thus the rate of synthesis of ribosomal protein can be calculated:

$$\alpha(t) = \frac{\text{rate of synthesis of ribosomal protein at time } t}{\text{rate of synthesis of total protein at time } t}$$

To measure $\alpha(t)$, the cells may be pulsed at time t with a radioactive amino acid. If the label is chased by non-radioactive amino acid long enough for it to have reached its ultimate cellular position, the fraction of the total incorporated radioactivity which is found in ribosomes represents $\alpha(t)$.

Of the numerous parameters on which α may depend, the two selected for this study were cell age and growth rate. The first was chosen because of the great interest in a possible relationship between synthesis of structural components and cell age. Growth rate was observed to affect ribosome level in the work of Schaechter, Maaløe & Kjeldgaard (1958), Ecker & Schaechter (1963), Kennel & Magasanik (1962), Kjeldgaard (1961) and Neidhardt (1964), who found that the ratio of the number of ribosomes to the mass of protein in a cell population was nearly proportional to the growth rate. A ribosome synthesizing protein at a speed independent of growth rate would give this result which can be summarized as $\frac{R}{P} = \frac{C}{T}$ where R is the number of ribosomes in the culture, P is the mass of protein, T is the doubling time, and C is some constant. For a population of cells in balanced exponential growth, the same relation must hold for the rates of synthesis of ribosomes and proteins, $\frac{dR}{dT} = \frac{C}{T}$. When cells are shifted between media in which the definitive doubling times are T_1 and T_2 , respectively, the relative rate of production of ribosomes and hence ribosomal protein must shift from $\frac{C}{T_1}$ to $\frac{C}{T_2}$ when equilibrium has been reached.

In cells recovering from abnormal growth conditions the relative rate of production of ribosomal protein may also change; thus Nakada (1965) has observed that an RC^{rel} strain recovering from amino acid starvation preferentially synthesizes ribosomal protein. For a population of perfectly synchronized cells, $\alpha(t)$ is the relative rate of production of ribosomal protein as a function of cell age. In the present experiments α has been measured for cells in balanced exponential growth, during the transition between growth rates, and as a function of cell age. It was found that during the transition between growth in succinate and in glucose medium, α shifts within two to five minutes from its succinate value to its glucose value, and that α is independent of cell age.

2. Materials and Methods

(a) Media and cell measurement

Escherichia coli B/r/1 was grown on a mineral minimal medium A+B; A is 2.0 g (NH₄)₂SO₄, 6.0 g Na₂HPO₄, 3.0 g KH₂PO₄, 3.0 g NaCl, 0.011 g Na₂SO₄/200 ml. water, and B is 0.20 g MgCl₂, 0.010 g CaCl₂, 0.0005 g FeCl₃·7H₂O/800 ml. water, which are mixed shortly before use. Succinate or glucose is at 0.2%. All cultures used were in exponential growth for at least three generations when they were used in a shift experiment at a density of 1 × 10⁸ cells/ml. or selected for synchronization at an input density of 2 × 10⁷/ml. and output density of 2 × 10⁸/ml. of synchronized cells by the method of Helmstetter & Cummings (1963).

Cell numbers and volumes were measured on a modified Coulter counter with special electronics which measure cell concentrations and the distribution of individual cell volumes in the culture. Figure 1(a) shows the distribution of cell volumes present in an exponentially growing population, and Fig. 1(b) shows the distribution of measured volumes of 1.305 ± 0.016 μ diameter polystyrene latex spheres. The peak at smaller volume is from single spheres, and the peak at larger volume is from the dimeric species existing in the suspension. The precision of the volume measurements can be estimated from the width of the monomer peak in excess of that caused by the distribution in volumes of the spheres marked by the error flag. For most measurements of cell populations only the volume at the peak of the volume distribution is recorded.

For measurements of synchronized populations, D. J. Clark provided the cells synchronized by the Helmstetter-Cummings technique (Helmstetter & Cummings, 1963). The cell volume distribution of synchronized cells 46 min old and undergoing division is shown in

No. of polystyrene
No. of cells



Fig. 1. (a) Volume distribution of *E. coli* in glucose medium, measured with the Coulter counter. (b) Measured volume distribution of 1.305 μ diameter polystyrene latex spheres. The error flag represents the width of the distribution if the spheres were perfectly spherical. The second peak is due to the presence of dimeric species. (c) Volume distribution of synchronized cells. The peak at smaller volume is from cells about to divide. Error flag represents the width of the distribution if the spheres were perfectly spherical. The medium for 32PO₄ labeling contained 0.2% succinate or glucose, respectively.

Fig. 1(c). The peak at smaller volume is from cells about to divide. Error flag represents the width of the distribution if the spheres were perfectly spherical. The medium for 32PO₄ labeling contained 0.2% succinate or glucose, respectively.

(b) Labeling of proline. For measurements of proline synthesis, 5% TCA† at 0°C and after centrifugation through filters. Drops from centrifuge tubes were extracted with 0.1 M (pH 7.2)–0.0001 M-magnesium acetate, and the whole volume added to 10 ml. of scintillation fluid. Samples to be counted directly were dripped into the scintillation fluid added and counted. [14C]proline (approximately 1.0 × 10⁻⁹ g/ml. of non-radioactive proline for kinetic experiments, where the uracil were performed similar

(c) Labeling of uracil

The medium for 32PO₄ labeling contained 0.2% succinate or glucose, respectively.

† Abbreviation used: TCA, trichloroacetic acid.

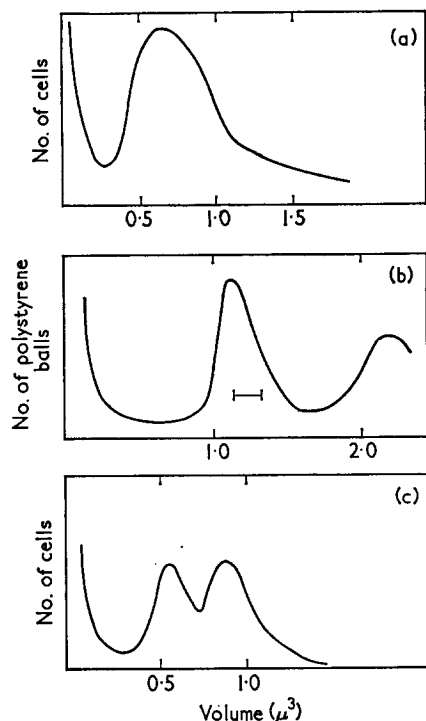


Fig. 1. (a) Volume distribution of *E. coli* B/r growing exponentially with a doubling time of 48 min in glucose medium, measured with the modified Coulter counter.

(b) Measured volume distribution of 1.305 μ diameter polystyrene latex spheres. The error flag represents the width if the only uncertainty were due to the distribution in volume of balls. The second peak is due to the presence of dimers in the suspension.

(c) Volume distribution of synchronized *E. coli* B/r at age 46 min. Newly divided cells of small volume contrast with large cells about to divide.

Fig. 1(c). The peak at smaller volume is from newly divided cells, and the peak at large volume is from cells about to divide. Except where noted, all experiments were done at 37°C where doubling times of 100 ± 10 min and 50 ± 5 min were obtained in A + B medium containing 0.2% succinate or glucose, respectively.

(b) Labeling and counting

For measurements of proline or uracil uptake, portions of cell culture were brought to 5% TCA† at 0°C and after 30 min the insoluble material was collected onto Millipore filters. Drops from centrifuged extracts were added to 1.5 ml. TM buffer (0.005 M-Tris (pH 7.2)–0.0001 M-magnesium acetate), the optical density at 260 $m\mu$ (O.D.₂₆₀) measured, and the whole volume added to 10 ml. of a dioxane-based scintillation fluid (Bray, 1960). Samples to be counted directly were dripped into counting vials, 0.75 ml. of TM and 5 ml. of scintillation fluid added and the samples counted. In all α experiments a 1-min pulse of [¹⁴C]proline (approximately 1.1×10^{-9} g/ml.) was followed by a chase with 2 to 4×10^{-5} g/ml. of non-radioactive proline for 40 min. A standard chase of 40 min was used in all but the kinetic experiments, where the chase period was the variable. Experiments with labeled uracil were performed similarly.

(c) RNA and protein measurements

The medium for ³²PO₄ labeling of RNA was 0.6 g sodium citrate, 5H₂O 0.21 g Mg₂SO₄·7H₂O, 1.0 g (NH₄)₂SO₄, 0.18 g KH₂PO₄/l. of Tris buffer, at pH 7.0 with 0.2%

† Abbreviation used: TCA, trichloroacetic acid.

succinate or glucose. The doubling times in these media were 100 ± 10 min and 55 ± 10 min respectively. Total RNA was taken to be proportional to TCA-insoluble ^{32}P incorporated into cells. This was measured by adding 1 ml. of culture to 1 ml. of TCA at 0°C for 10 min, filtering, washing, drying and counting in toluene-based scintillation fluid. Total protein was measured using the Lowry reaction. Samples were withdrawn from the culture, brought to 5% TCA and left overnight at 4°C . After centrifugation the color test was done on the pellet as described by Lowry, Rosebrough, Farr & Randall (1951).

(d) *Preparation of cell extracts*

Labeled cells in 25 ml. of culture were added to 50 ml. of carrier cells at 5×10^8 /ml. cooled to 0°C . Further preparation was done at 4°C . The cells were spun down and washed 3 times in TM, and after the last centrifugation they were resuspended in 0.3 ml. of TM and sonicated for two 5-min periods separated by 30 min to avoid excessive heating. After the first sonication period, microscopic observation showed about 1% and after the second period only 0.1 to 0.01% intact cells. Cell extracts were either centrifuged immediately or stored at -10°C until used. No change was observed in a sample stored for one month.

(e) *Centrifugation*

Preliminary studies showed that the usual centrifugation through 5 to 20% sucrose gradients did not adequately resolve ribosomes from other cell constituents due to contamination of the ribosome region by fragments of cell wall or membrane. To avoid contamination, centrifugation was done through a linear sucrose gradient in D_2O with density from 1.18 to 1.31, so that cell wall and membranes came to isopycnic equilibrium while ribosomes continued to sediment during the entire run. It has been calculated that larger protein molecules, and aggregates, with sedimentation coefficients between 20 and 40 s in the usual gradients, would sediment well behind the ribosomal subunits in the D_2O gradients. The D_2O was 0.005 M in Tris, 0.0001 M in magnesium acetate and had the D^+ concentration adjusted so that a glass electrode pH meter indicated pH 7.2 when calibrated against an H_2O buffer. The pD was then 7.6 (Glasoe & Long, 1960), but the altered pK of amino and imino groups (Maslova, Grechko & Varshavsky, 1964) give the effect of an H_2O solution at pH approximately 7.2. The centrifugation time of 3 hr with the usual 5 to 20% sucrose gradients run at 39 K (124,000 g), 4°C in the SW39 rotor must be increased to 20 hr, 36 K (107,000 g), 20°C in the high sucrose D_2O gradients to compensate for increases in density and viscosity. To permit more samples to be centrifuged, Lucite inserts accommodating 3 tubes each were made for the SW25-2 rotor, and in some experiments two separate samples, one labeled with ^3H and the other with ^{14}C were layered on each tube, allowing a total of 18 samples to be centrifuged at once. Centrifugation with the SW25-2 required 29 hr at 24 K (76,000 g), 20°C .

3. Results

(a) *Measurement of α*

To measure α at a chosen time the radioactive label must be incorporated into protein for only a short period. In these experiments radioactive proline was added to a culture growing in the absence of proline, and the labeling period was terminated by adding a large excess of non-radioactive proline. The incorporation of [^{14}C]proline into TCA-insoluble material under such conditions is shown in Fig. 2, where it can be seen that (a) appreciable amounts of proline enter TCA-insoluble material within 15 seconds, (b) the internal pool of proline is adequate for five to ten seconds of growth, (c) addition of excess non-radioactive proline effectively stops further incorporation of radioactive proline.

Selective loss of cell components that might affect estimates of α was avoided by analyzing whole cell extracts. The necessary complete dissociation of ribosomes from cell membranes appears to be achieved by the sonication treatment, since (a) less than

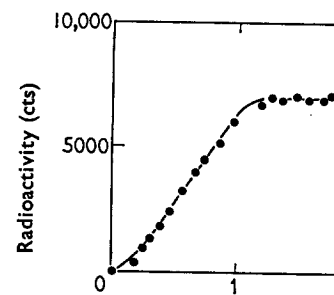


FIG. 2. Uptake of [^{14}C]proline into TCA-insoluble material. Cells with a doubling time of 48 min. At zero time, the cells were added to 50 ml. of culture; 1 min later, [^{14}C]proline was added to 50 ml. of culture; 1 min later, at the times indicated, 1-ml. vol. were mixed with 5% TCA, filtered, washed and the radioactivity measured.

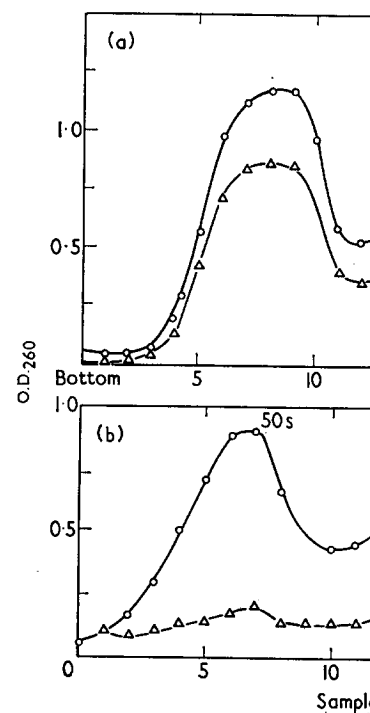


FIG. 3. (a) Optical density at $\lambda = 260 \text{ m}\mu$ of cell extracts of cells labeled with [^{14}C]uracil for 1 min, centrifuged for 3 hr at 36K (107,000 g). (b) Optical density at $\lambda = 260 \text{ m}\mu$ (—○—) of cell extracts of cells labeled with [^{14}C]proline for 1 min, centrifuged for 3 hr at 36K (107,000 g).

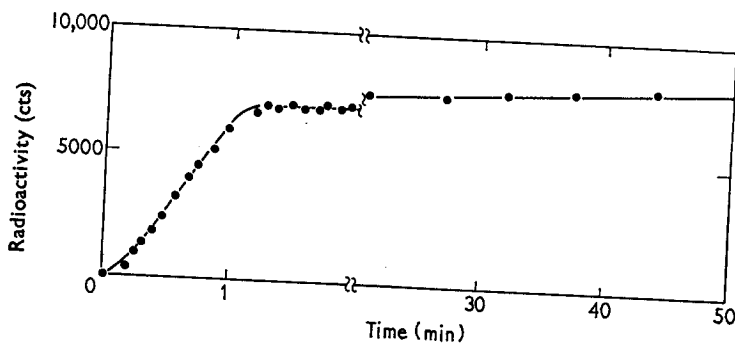


Fig. 2. Uptake of [¹⁴C]proline into TCA-insoluble material. *E. coli* B/r was growing with a doubling time of 48 min. At zero time, the cells were at 5×10^7 /ml. and 6.43×10^{-8} g of [¹⁴C]proline was added to 50 ml. of culture; 1 min later 1 mg of non-radioactive proline was added. At the times indicated, 1-ml. vol. were mixed with 10% TCA at 0°C. The cells were collected on Millipore filters, washed and the radioactivity measured in a liquid-scintillation counter.

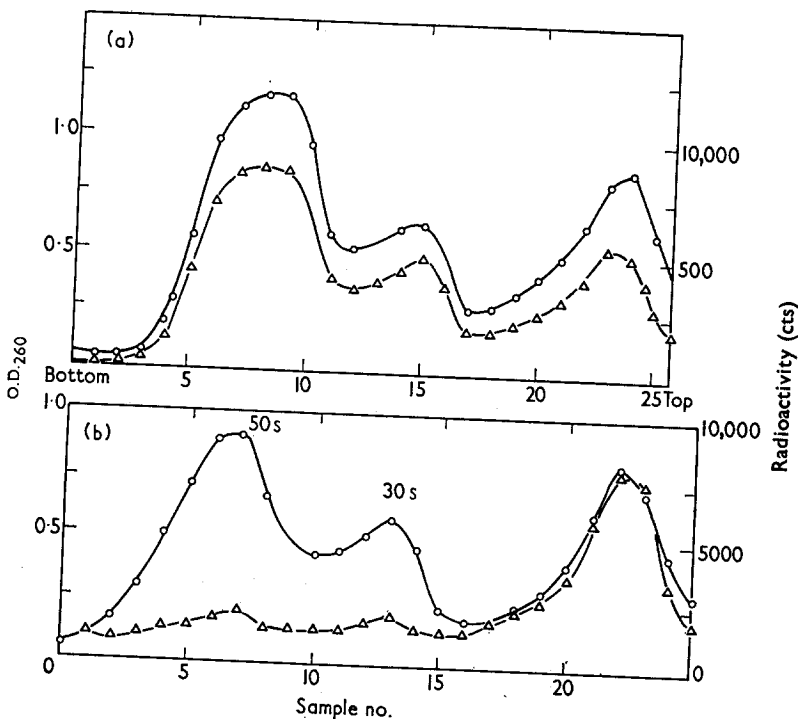


Fig. 3. (a) Optical density at $\lambda=260 \text{ m}\mu$ (—○—○—) and [¹⁴C]uracil (—△—△—) profiles of extracts of cells labeled with [¹⁴C]uracil for 1 min followed by 40 min with excess non-radioactive uracil, and centrifuged for 3 hr at 36K (107,000 g) at 4°C through a 5 to 20% sucrose gradient. (b) Optical density at $\lambda=260 \text{ m}\mu$ (—○—○—) and [¹⁴C]proline (—△—△—) profiles of extracts of cells labeled with [¹⁴C]proline for 1 min followed by 40 min with excess non-radioactive proline, and centrifuged for 3 hr at 36K (107,000 g) at 4°C through a 5 to 20% sucrose gradient.

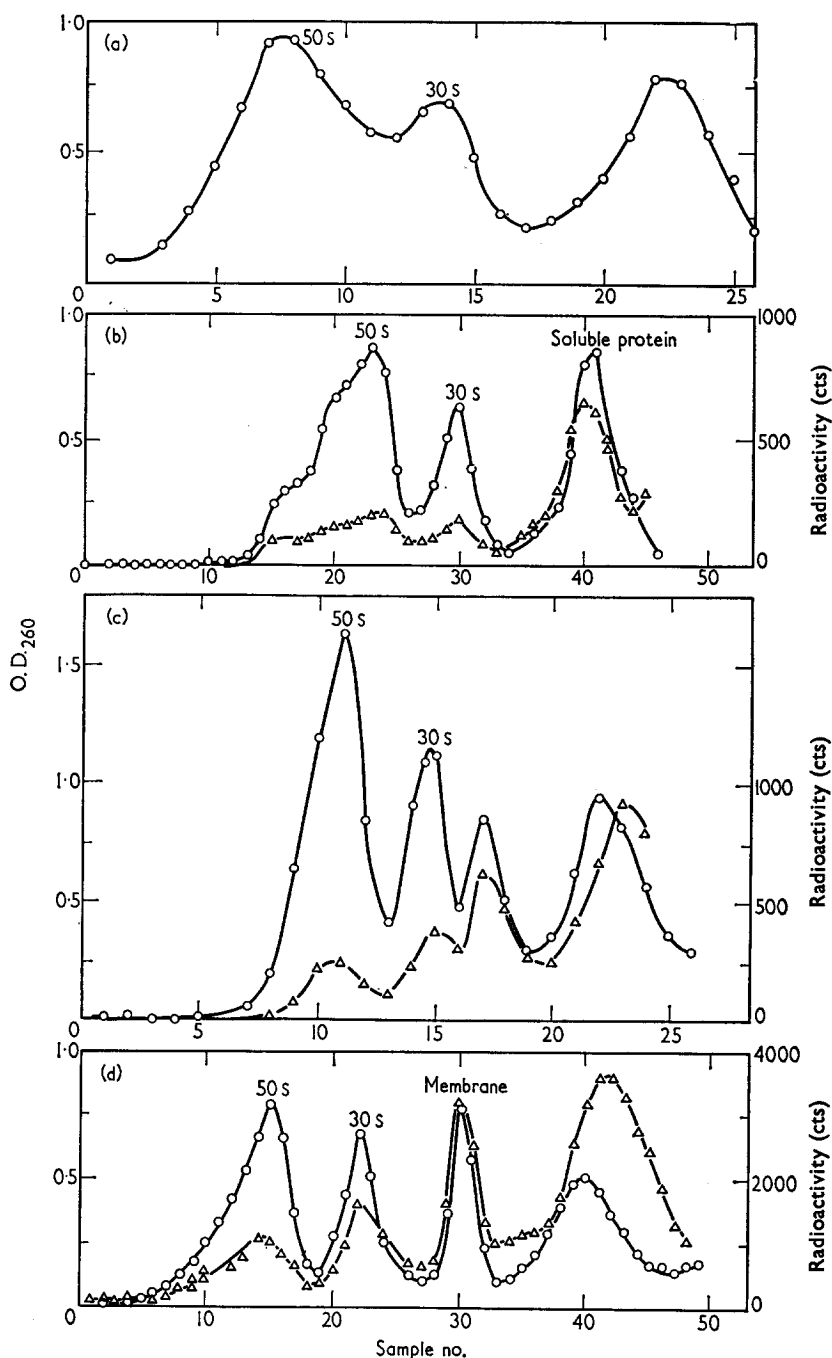


FIG. 4. (a) Optical density at $\lambda=260 \text{ m}\mu$ of the same cell extract as Fig. 3(b) centrifuged for 2 hr at 36K (107,000 g) at 20°C through a 5 to 20% sucrose gradient.

(b), (c), (d) Optical density and [^{14}C]proline profiles of the same extract as Fig. 4(a) centrifuged at 36K (107,000 g), 20°C through linear D_2O sucrose gradients. (b) 6 hr through density $\rho=1.181$ to 1.25; (c) 11 hr through $\rho=1.18$ to 1.30; and (d) 18 hr through $\rho=1.18$ to 1.30. Optical density at $\lambda=260 \text{ m}\mu$ (—○—○—), [^{14}C]proline (—△—△—).

4% of incorporated [^{14}C]uracil counts extract for 20 minutes at 10,000 g and peak seen, e.g. in Fig. 4(d). Measurement of protein into the TM rinses, and bet

The o.d.₂₆₀ and [^{14}C]uracil profile to 20% sucrose gradient at 4°C is p resolution of the 30 and 50 s rib labeled with [^{14}C]proline is shown in are poorly resolved. To prevent no ribosome region, the density of the density and [^{14}C]proline profiles of th for two hours through a linear 5 to 1.081; (b) for six hours through a g through a gradient with $\rho = 1.18$ to gradient as (c). The increase in densi and exclusion of a large fraction of n The additional protein peak, which w been identified as membrane and it di if any degradation of ribosomes seem calculate α , the counts in the ribosome

It has been observed repeatedly i counts (β) in the 30 and 50 s regions have also been seen in 5 to 20% suc (1962), and have been ascribed partly (Osawa, 1965), and partly to a 16% (Gavrilova, Ivanov & Spirin, 1966). T observed in the present experiments, 10% of the total protein in the ribosome calculating α . A number of possible an

(1) Quenching of counts was found

(2) The high β -values observed mi and 50 s subunits with regard to the s rates of turnover. These possibilities ca dent of the length of the chase (from 1 t A chase period of one minute or m ribosomes do not carry nascent polype

(3) Different amino acid composition since it would imply that Spahr's (1962) of the ribosomal subunits be incorrect viz. proline, leucine and lysine.

(4) Sonication is probably not rem extraction by grinding in alumina and same result.

(5) Binding of non-ribosomal protei unlikely since extract prepared in the p cells gave unchanged β -values.

We conclude that under the extract study, more protein sediments with the

4% of incorporated [^{14}C]uracil counts are pelleted by centrifugation of the sonication extract for 20 minutes at 10,000 g and (b) no uracil is associated with the "membrane" peak seen, e.g. in Fig. 4(d). Measurement of [^{14}C]proline indicated less than 5% losses of protein into the TM rinses, and better than 95% recovery of counts from the gradient.

The O.D.₂₆₀ and [^{14}C]uracil profile of a sedimentation through the commonly used 5 to 20% sucrose gradient at 4°C is presented in Fig. 3(a), which shows only moderate resolution of the 30 and 50 s ribosomal subunits. An identically treated extract labeled with [^{14}C]proline is shown in Fig. 3(b) where ribosomes and soluble proteins are poorly resolved. To prevent non-ribosomal protein from sedimenting into the ribosome region, the density of the gradient was increased. Figure 4 shows optical density and [^{14}C]proline profiles of the same extract centrifuged at 107,000 g, 20°C, (a) for two hours through a linear 5 to 20% sucrose gradient with density $\rho = 1.018$ to 1.081; (b) for six hours through a gradient with $\rho = 1.18$ to 1.25; (c) for 11 hours through a gradient with $\rho = 1.18$ to 1.30; and (d) for 18 hours through the same gradient as (c). The increase in density leads to increased resolution of the ribosomes and exclusion of a large fraction of non-ribosomal protein from the ribosome region. The additional protein peak, which with this strain bands at $\rho = 1.22$, has tentatively been identified as membrane and it disappears with more extensive sonication. Little if any degradation of ribosomes seems to occur during centrifugation at 20°C, and to calculate α , the counts in the ribosome region are simply divided by the total counts.

It has been observed repeatedly in these experiments that the ratio of protein counts (β) in the 30 and 50 s regions is approximately 0.8. β -values in excess of 0.5 have also been seen in 5 to 20% sucrose gradients (Britten, McCarthy & Roberts, 1962), and have been ascribed partly to a 10% excess of 30 over 50 s subunits (Osawa, 1965), and partly to a 16% higher protein to RNA ratio of 30 s subunits (Gavrilova, Ivanov & Spirin, 1966). These two facts do not fully account for β -values observed in the present experiments, but since the unaccountable residue is less than 10% of the total protein in the ribosome region, no correction was attempted when calculating α . A number of possible artifacts, however, were examined.

- (1) Quenching of counts was found to be uniform for all samples.
- (2) The high β -values observed might have reflected differences between the 30 and 50 s subunits with regard to the size of their respective precursor pools, or their rates of turnover. These possibilities can be excluded since β was found to be independent of the length of the chase (from 1 to 50 minutes) that followed the pulse labeling. A chase period of one minute or more is believed to ensure that the extracted ribosomes do not carry nascent polypeptide chains labeled during this pulse.
- (3) Different amino acid compositions of the two ribosomal subunits is improbable since it would imply that Spahr's (1962) measurements of the amino acid composition of the ribosomal subunits be incorrect for all three amino acids tested in this study; viz. proline, leucine and lysine.
- (4) Sonication is probably not removing protein from the 50 s subunit since extraction by grinding in alumina and treating with DNase (Nakada, 1965) gives the same result.
- (5) Binding of non-ribosomal proteins to the 30 s subunit after cell disruption is unlikely since extract prepared in the presence of a fivefold excess of nonradioactive cells gave unchanged β -values.

We conclude that under the extraction and sedimentation conditions used in this study, more protein sediments with the 30 s subunits than is usually seen, but it has

Radioactivity (cts)

Radioactivity (cts)

Radioactivity (cts)

ed for 2 hr
centrifuged
y $\rho = 1.181$
al density

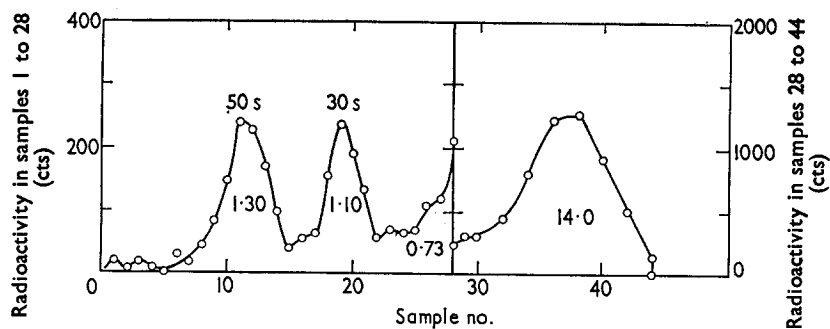


FIG. 5. Radioactive [^{14}C]proline profile of cells chilled 9.5 min after excess non-radioactive proline was added. This shows the usual resolution obtained by centrifugation through the D_2O sucrose gradients described in Materials and Methods. Note the scale change at drop 28. The numbers under the peaks represent the areas under the peaks.

not been possible to decide whether this "excess protein" is ribosomal in the the strict sense, or whether other cell proteins cosediment with the ribosome.

(b) Kinetics of amino acid appearance in ribosomes

It has been reported previously that amino acid label appears in ribosomal subunits soon after its addition to a culture (Osawa, 1965), and the kinetics of its appearance was studied by Britten *et al.* (1962). This rapid entry of label indicates that the pool of free ribosomal protein in the cells is small; on the other hand, the accumulation, in the presence of chloramphenicol, of precursor particles containing appreciable amounts of protein synthesized before the drug was added seems to require a large pool (Hosokawa & Nomura, 1965). This problem has been re-examined using the high-resolution D_2O gradients to measure kinetically the size of the pool of free ribosomal protein.

Cells in balanced exponential growth were exposed to radioactive proline, and one minute later excess non-radioactive proline was added. At intervals thereafter 25 ml. were pipetted into centrifuge bottles containing 100 ml. of medium at 0°C . Subsequent treatment was done at 4°C and all samples were washed, sonicated and frozen within three hours. The next day they were centrifuged. Figure 5 presents the profile of the 9.5-minute sample, which shows typical resolution, and Fig. 6 presents the kinetics of appearance of label in the 30 and 50 s subunits. The ratio of counts in the two sub-

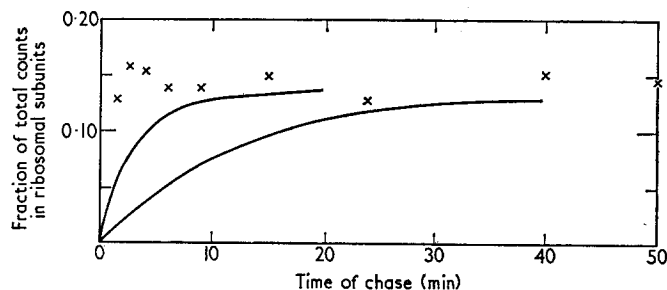


FIG. 6. Measured kinetics of appearance of label in mature ribosomal subunits as a function of time after a chase beginning 1 min after addition of the pulse label (\times). The two curves show the expected kinetics of label entry if the pool contained 4% (upper curve) and 20% (lower curve) of the amount of protein already present in mature ribosomes.

units remained appropriate ribosomes suggests ribosomal protein. These experiments are not a pool effect.

(c) Ribosomes

Cells in balanced growth were exposed to radioactive proline, and one minute later excess non-radioactive proline was added. At intervals thereafter 25 ml. were pipetted into centrifuge bottles containing 100 ml. of medium at 0°C . Subsequent treatment was done at 4°C and all samples were washed, sonicated and frozen within three hours. The next day they were centrifuged. Figure 5 presents the profile of the 9.5-minute sample, which shows typical resolution, and Fig. 6 presents the kinetics of appearance of label in the 30 and 50 s subunits. The ratio of counts in the two sub-

Figure 7 shows the growth of cells in balanced exponential growth. Cells in balanced growth were exposed to radioactive proline, and one minute later excess non-radioactive proline was added. At intervals thereafter 25 ml. were pipetted into centrifuge bottles containing 100 ml. of medium at 0°C . Subsequent treatment was done at 4°C and all samples were washed, sonicated and frozen within three hours. The next day they were centrifuged. Figure 5 presents the profile of the 9.5-minute sample, which shows typical resolution, and Fig. 6 presents the kinetics of appearance of label in the 30 and 50 s subunits. The ratio of counts in the two sub-

1×10^7

Cells per ml.
 1×10^7

1×10^7

FIG. 7. Growth of cells in balanced exponential growth. Cell number ($-\circ-$) Coulter counter.

shift; previously, a shift in glucose medium to bro the cell volume at the and an increase in this

The total volume of al

where v is volume of a the modified Coulter ce O.D. $_{450}$ or O.D. $_{650}$, which response in terms of ce change until the large medium has been attain

Figure 8 shows $\alpha(t)$ du this measurement is tha

units remained approximately the same for all samples. The rapid entry of label into ribosomes suggests that the pool contains less than a five-minute supply of free ribosomal protein. These results also show that the 40-minute chase routinely used in these experiments is more than adequate to make the measurement independent of any pool effect.

(c) *Ribosomal protein production in balanced exponential growth and during the shift from succinate to glucose*

Cells in balanced growth give $\alpha = 0.08$ and 0.15 in succinate and glucose media, respectively.

Figure 7 shows the growth curve of cells diluted from storage at 4°C , grown in succinate and shifted to glucose. Repeated measurements with the modified Coulter counter show a distinct change in the rate of cell division about 75 minutes after the

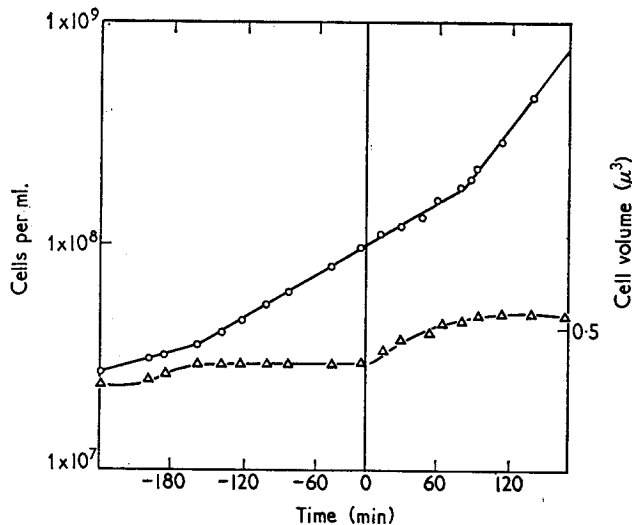


Fig. 7. Growth of cells of *E. coli* B/r in succinate medium to which glucose was added at zero time. Cell number (—○—○—) and cell volume (—△—△—) were measured with the modified Coulter counter.

shift; previously, a similar change was observed 60 to 70 minutes after a shift from glucose medium to broth (Maaløe & Kjeldgaard, 1966). In the present measurements the cell volume at the maximum of the volume frequency distribution was recorded, and an increase in this volume becomes apparent about ten minutes after the shift.

The total volume of all cells in a fixed volume of culture is $V(t) = \int_0^{v_{\max}} vn(v,t)dv$,

where v is volume of a cell and $n(v,t)$ is the number of cells of volume v at time t . With the modified Coulter counter, $V(t)$ was found to vary with time in the same way as O.D.₄₅₀ or O.D.₆₅₀, which shows a rapid response to the addition of glucose; the delayed response in terms of cell division shows that the pre-shift rate of division does not change until the larger average cell volume characteristic of growth in the new medium has been attained.

Figure 8 shows $\alpha(t)$ during a shift from succinate to glucose. The important aspect of this measurement is that, after addition of glucose, α rapidly reaches a value close to

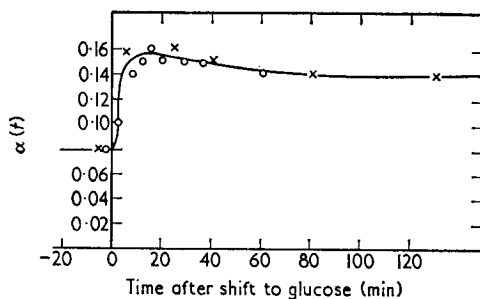


FIG. 8. $\alpha(t)$ for two separate series of measurements after a shift from balanced growth in succinate medium to growth with succinate plus glucose.

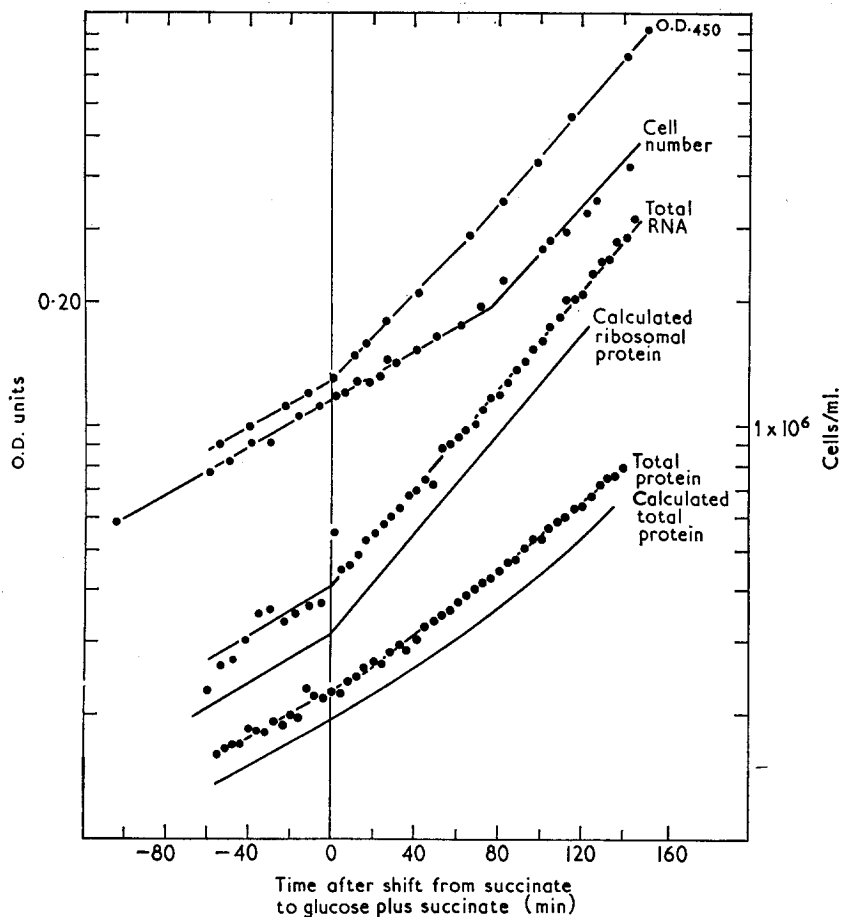


FIG. 9. Optical density at $\lambda=450 \text{ m}\mu$, cell number, TCA-insoluble ^{32}P , protein measured by the Lowry reaction, calculated ribosomal protein and calculated total protein as a function of time on a logarithmic scale. The cells were grown in succinate medium until glucose was added at zero time. Note that the ratio of RNA to protein has very nearly doubled between the first and the last measurements, as required by the relation $\frac{R}{P} = \frac{C}{T}$.

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Figure 10 shows together with the rel age. Cell number an the time of cell divi

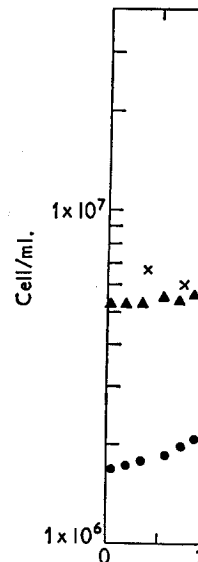


FIG. 10. Cell number (Methods). Relative cell v

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its new definitive one. Figure 9 shows cell number, $O.D._{450}$ total acid-insoluble ^{32}P in cells (taken to be RNA), and total protein during the transition period. The cell number showed the usual change to the new division rate 75 minutes after the shift, whereas the optical density almost immediately assumed the rate of increase corresponding to balanced growth in glucose. Measurements of glucose and succinate utilization made with generally ^{14}C -labeled compounds show that a smooth transition takes place during the two hours after the shift from the use of succinate as the sole carbon source to the use of both glucose and succinate in the ratio of carbons of approximately 6:1.

(d) Ribosomal protein production as a function of cell age

Figure 10 shows the step-wise increase in cell number in a synchronized culture together with the relative rate of production of ribosomal protein $\alpha(a)$ where a is cell age. Cell number and size were again measured on the modified Coulter counter. At the time of cell division the small, newly divided cells can be clearly distinguished

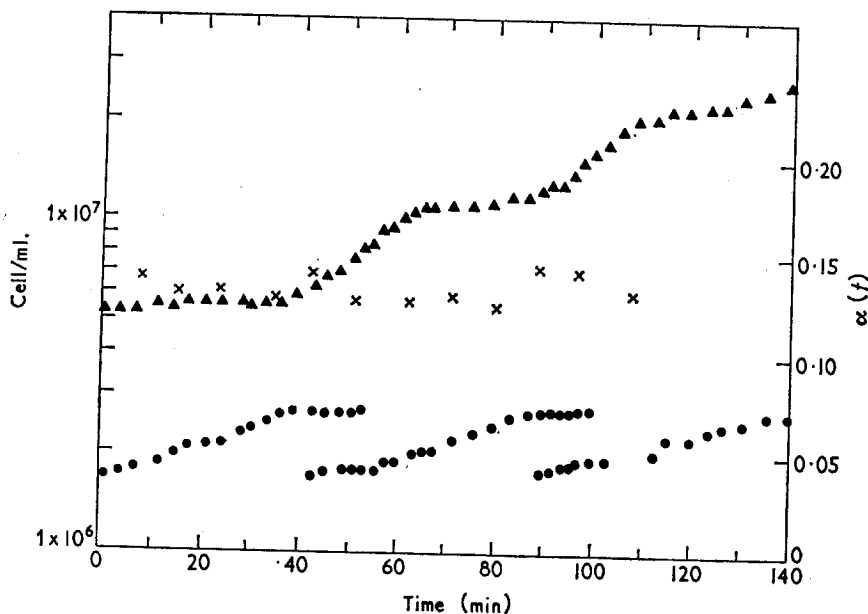


Fig. 10. Cell number (▲) and α (x) at various ages of synchronized cells (see Materials and Methods). Relative cell volume is on a linear scale (●).

from cells about to divide (see Fig. 1(c)). The relative rate of production of ribosomal protein was calculated from the radioactivity profiles of centrifuged cell extracts. Within experimental error α is independent of cell age. It is, therefore, unnecessary to calculate the value of α which would have been observed if the cells had been in perfect synchronization.

4. Discussion

The relative rate of synthesis of ribosomal protein

$$\alpha = \frac{\text{rate of synthesis of ribosomal protein}}{\text{rate of synthesis of total protein}}$$

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and the absolute rate of synthesis of ribosomal protein were determined for *E. coli* B/r growing in succinate minimal medium, glucose minimal medium, during a transition from succinate minimal to glucose plus succinate minimal, and as a function of cell age for cells growing in glucose minimal medium. The measurements of α were made by pulsing the growing cells with radioactive amino acids, then separating ribosomes from other cell protein and measuring their relative radioactivity. Adequate separation of ribosomes from other cell constituents was made possible by sedimentating cell extracts through a D_2O -sucrose solution the density of which is adjusted to prevent non-ribosomal protein from sedimenting with ribosomes as occurs in gradients of lesser density. This method for measuring α circumvents the problem of solubilizing the ribosomal proteins, and after the long chase period no small labeled molecules remain to diffuse down the centrifuge tube, thus eliminating the need for acid precipitation and washing prior to counting. Finally, the abundance of proline in ribosomal protein is about the same as in total protein, 3.67 and 4.17%, respectively (Spahr, 1962), so that no correction was applied when calculating α as the ratio of counts in the ribosome region to total counts.†

Our measurements are valid only if the radioactive label stays in the structures into which it has been incorporated during the pulse, demanding that turnover of ribosomes as well as of soluble proteins can be disregarded. The measurements of Davern & Meselson (1960), Koch & Levy (1955) and Mandelstam (1958) show that this is permissible. It is assumed that external label is not incorporated preferentially into ribosomal protein or non-ribosomal protein, but that the incorporation is proportional to the synthesis rates of these proteins. In the measurements presented here, ribosomes were dissociated into their 30 and 50 s subunits to examine the possibility that the proteins of the two subunits might be under separate control. In the cases thus far examined, the two classes of protein appear to be under coordinate control.

The difference between the small pool size indicated by the present measurements and the larger pool size indicated by the accumulation of chloramphenicol particles could be explained in several ways: (1) the protein of chloramphenicol particles could be general cell protein which associates with rRNA in the presence of chloramphenicol, (2) the protein content of chloramphenicol particles could have been overestimated, (3) mature ribosomes could donate some of their protein to ribosomal RNA to form the chloramphenicol particles, or (4) in the present experiments the pool of ribosomal protein could have decreased due to continued maturation of ribosomes while cells were at 4°C. None of these possibilities has been excluded. The largest pool consistent with the present measurements contains less than 5%, whereas the smallest pool consistent with the measurements of Hosokawa & Nomura (1965) contains more than 15% of the total cellular ribosomal protein. Further experiments will be done on this discrepancy.

The average rate of protein synthesis per ribosome can be deduced from α measurements on steady state cultures in which the quantity per volume of culture of each cell component will increase with time as $\exp \mu t$. If we let \bar{K} be the average number of amino acids polymerized per second per ribosome, the rate of protein synthesis is

$$\frac{dP(\text{total})}{dt} = \bar{K}R, \quad (1)$$

† Fifteen independent experiments with glucose-grown *E. coli* B/r carried out over the past year gave α -values ranging from 0.14 to 0.17, with an average of 0.155.

where R is the number of ribosomes per volume of culture. If c is the number of amino acids synthesized per ribosome per second, the rate of protein synthesis is

We recall the definition of \bar{K} as the average number of amino acids polymerized per second per ribosome.

Combining equations (1) and (2) gives

Since the culture is at steady state, $dP/dt = 0$.

Differentiating with respect to time gives

Combining (4) and (6) gives

The weight of the protein in the culture is $W = P/V$ (Waller, 1964). Thus $c = \bar{K}/W$. Substituting $c = 0.15$ in this estimate is the same as substituting a value of \bar{K} of about ± 13 . The same value of \bar{K} by Kepes (1966) on the basis of measurements of $K_{1ac} = 13$. This agreement indicates that all ribosomes are indeed active in protein synthesis.

In the shift from succinate to glucose, the doubling times were approximately doubled, increased from 0.08 to 0.16 hours immediately before and after the shift. The rate of synthesis of ribosomal protein in the culture (total protein) which paralleled the rate of protein synthesis. This is not sufficient evidence to conclude that all ribosomes are active in protein synthesis.

If the rate of protein synthesis is αR , the rate of protein synthesis can be

where R is the number of ribosomes and P is the number of amino acids in proteins. If c is the number of amino acids in the protein of a ribosome, the rate of ribosome synthesis is

$$\frac{dR}{dt} = \frac{1}{c} \frac{dP(\text{ribosomal})}{dt} \quad (2)$$

We recall the definition of the measured quantity α

$$\alpha = \frac{dP(\text{ribosomal})}{dt} / \frac{dP(\text{total})}{dt} \quad (3)$$

Combining equations (1), (2) and (3) gives

$$\frac{dR}{dt} = \frac{\alpha \bar{K} R}{c} \quad (4)$$

Since the culture is assumed to be in balanced exponential growth,

$$R = R_0 e^{\mu t} \quad (5)$$

Differentiating with respect to time gives,

$$\frac{dR}{dt} = \mu R_0 e^{\mu t} = \mu R \quad (6)$$

Combining (4) and (6) yields,

$$\bar{K} = \frac{\mu c}{\alpha} \quad (7)$$

The weight of the protein in a 70 s ribosome has been estimated at 950,000 daltons (Waller, 1964). Thus $c = 8.3 \times 10^3$, and for a 48-minute doubling time, $\mu = 2.4 \times 10^{-4}$, $\alpha = 0.15$. Substituting gives $\bar{K} = 13$ amino acids/sec. The least accurately known factor in this estimate is the value of c . Its possible error introduces an uncertainty into the value of \bar{K} of about ± 2 amino acids/sec. Maaløe & Kjeldgaard (1966) obtained about the same value of \bar{K} by measuring rRNA instead of protein. It should be emphasized that the result $\bar{K} = 13 \pm 2$ amino acids per second per ribosome is an average value assuming that all ribosomes in the culture are equally active. However, measurements of Kepes (1966) on the synthesis of the enzymes of the lactose operon yield a value of $K_{lac} = 13$. This agreement could be fortuitous, but it suggests that most ribosomes are indeed active in protein synthesis.

In the shift from succinate to glucose plus succinate minimal medium, where the doubling times were approximately 100 and 50 minutes, respectively, α rapidly increased from 0.08 to 0.15. Since the rate of synthesis of *total* protein is the same immediately before and after the shift, a doubling of α implies that the rate of synthesis of *ribosomal* protein has also doubled. Figure 9 shows the amount of ribosomal protein in the culture (calculated from α and from independent determinations of total protein) which parallels the ribosomal RNA measured as ^{32}P incorporation. This is not sufficient evidence to establish that ribosomal RNA serves as messenger for ribosomal protein, but a lack of evidence against it.

If the rate of protein synthesis is assumed to be proportional to ribosomes, the protein synthesis can be calculated, and the result is plotted in Fig. 9. The close

correspondence between measured and calculated values implies that the rate of protein synthesis is at all times proportional to the number of ribosomes present, i.e. that \bar{K} is constant through the shift. The value of \bar{K} could fluctuate slightly as it is an approximation to take ribosome number proportional to RNA (Maaløe & Kjeldgaard, 1966).

These cells synchronized by the Helmstetter-Cummings method in which they have received little physiological shock show steps in the rate of DNA replication (Clark & Maaløe, 1967), but do not show steps in α . The fact that α does not vary with cell age implies that a cell devotes a constant fraction of its protein synthesis to production of ribosomal protein at all times. If α and \bar{K} are constant with cell age, the ribosome number and protein mass per cell must increase exponentially; however, only very accurate measurements could distinguish a linear increase from an exponential increase in one doubling period.

The average number of ribosomes per cell can be estimated to be 10,000 from the result that ribosomal protein is 15% of cellular protein and that the average protein per cell is 10^{-13} g. The protein per cell was calculated from the average cell volume of 7.5×10^{-13} cm³ measured with the Coulter counter, the average cell density of 1.1, and the dry weight and protein composition of *E. coli* from Roberts, Cowie, Abelson, Bolton & Britten (1955). The figure 10,000 for strain B/r, growing in glucose, is similar to the corresponding figure derived for *Salmonella typhimurium* on the basis of measurements of total ribosomal RNA (Maaløe & Kjeldgaard, 1966).

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