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Establishment of Exponential Growth After a Nutritional Shift-Up in *Escherichia coli* B/r: Accumulation of Deoxyribonucleic Acid, Ribonucleic Acid, and Protein

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The accumulation of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein was followed in cultures of *Escherichia coli* B/r during exponential growth in different media and for 2 h after a nutritional shift-up from succinate minimal medium (growth rate [μ_1] = 0.67 doublings per h) to glucose plus amino acids medium (μ_2 = 3.14 doublings per h). During postshift growth of the culture, the amounts of RNA (R), DNA (D), and protein (P) increased such that the ratios of the increments ($\Delta R/\Delta P$; $\Delta D/\Delta P$) were constants (k_1, k_2). This implies that the rates of accumulation of nucleic acids and protein form time-invariant ratios; i.e., $(dR/dt):(dD/dt):(dP/dt) = k_1:k_2:1$. These constants change from their preshift value to their final postshift value (i.e., k_1 and k_2) within a few minutes after the shift. k_1 is a function of the activity of ribosomes, whereas k_2 is related to the initiation of rounds of DNA replication. These parameters and the observed change in the doubling time of RNA ($= \mu_2/\mu_1$) were used to derive kinetic equations that describe the accumulation of DNA, RNA, protein, and cell mass during the 2- to 3-h transition period after a shift-up. The calculated kinetics agree closely with the observed kinetics.

The synthesis of nucleic acids and protein in bacteria growing exponentially at different rates or after a nutritional shift-up has been studied by numerous workers (e.g., references 11-13, 20, 21, 24-27, 30, 31). The purpose of the current investigation was to clarify two controversial questions.

(i) Does the protein synthesis rate per ribosome change after a shift-up? Earlier studies indicated that the ribosomal efficiency is constant and independent of growth rate (21, 31), whereas recent studies suggested an increase with growth rate (12). An increased activity per ribosome after a shift-up suggests a reduced idling of ribosomes and, thus, a reduced synthesis of guanosine tetraphosphate (14); this compound determines the initial response of bacteria to a nutritional shift-up (18, 28, 33).

(ii) Does the synthesis rate of stable ribonucleic acid (RNA) per genome increase immediately after a shift-up to its final postshift value? Earlier, Maaløe and Kjeldgaard concluded (page 102 of reference 20): "Thus, irrespective of the pre-shift rate of synthesis, the number of RNA units produced per second and per genome can be raised with no apparent lag to a value in the neighborhood of the definitive value in the broth culture." More recently, we inferred that the rate of RNA synthesis per genome requires several hours to reach its final postshift value (4). A fast increase in this value would require that a shift-up activate an inactive reserve of RNA polymerase (21), whereas a slower increase over several hours can be accounted for by a redistribution of RNA polymerase over ribosomal RNA (rRNA), transfer RNA (tRNA), and messenger RNA (mRNA) genes combined with an increased synthesis of RNA polymerase during postshift growth (3, 4).

Both questions can be answered by observing the accumulation of deoxyribonucleic acid (DNA), RNA, and protein after a shift-up. Previously, we followed only RNA and protein for about 30 min after the shift (11, 12). Since a theoretical analysis (4) indicated a long duration for the transition between two steady states of balanced growth, we have here extended the period of observation to 2 h and included DNA in our study. The results show that the ribosome efficiency increases and that the rate of RNA synthesis per genome requires several hours to reach its definitive value after the shift.

MATERIALS AND METHODS

Bacterial strain and growth of cells. The bacterial strain used was *Escherichia coli* B/r (ATCC 12407). Cultures were grown at 37°C in minimal C

medium (15) supplemented with either 0.45% sodium succinate or 0.2% glucose or 0.2% glucose plus 20 μg of each of the 20 standard L-amino acids per ml. Experimental cultures were inoculated by at least a 300-fold dilution of a fresh overnight culture containing the same nutritional supplements as the experimental culture. Growth was measured as the increase in the concentration of cell mass (absorbance at 460 nm [A_{460}]; see below). The doubling times of the bacteria growing in the described media are listed in Table 1 (μ = doublings per hour).

After a nutritional shift-up, the cells were diluted twice about twofold during postshift growth to prevent the cultures from entering stationary phase (Fig. 3). Control experiments (not illustrated) had indicated that a twofold dilution with prewarmed, pre-aerated medium at an A_{460} of about 0.6 does not cause a measurable lag in the accumulation of mass, nucleic acids, or protein. The exact dilution factors were determined by extrapolation of the curve log mass (A_{460}) versus time (Fig. 3, squares). Using these dilution factors, the postshift kinetics of mass increase were then constructed for a culture thought to be undiluted (Fig. 3, triangles; Table 2).

Determination of cell mass, DNA, RNA, and protein. Cell mass is determined as absorption of light at 460 nm by the culture (1-cm light path); the sum of the weights of protein, DNA, and RNA is proportional to the A_{460} (Fig. 5b; Fig. 2 of reference 13).

The amounts of DNA, RNA, and protein were determined in duplicate samples of cultures growing within a turbidity (A_{460}) range of 0.25 to 0.75. After subtraction of a blank (medium without bacteria), the values obtained were proportional to the mass of cells in that sample. The results are expressed in relative units as DNA per mass, RNA per mass, and protein per mass (Table 1). The reproducibility of each assay was generally better than 5% (see duplicate samples in Table 1).

To measure DNA, the method of Meijs and Schilperoort (23) was modified: cells in 10-ml samples of culture were precipitated with 2 ml of cold 3 M trichloroacetic acid at 0°C, filtered through glass-fiber filters (Reeve Angel, 984 H), washed three times with cold tap water, dried, and placed in scintillation vials. The filters were incubated in 1.0 ml of 1.6 M $HClO_4$ at 70°C for 30 min. After cooling to room temperature, 2.0 ml of diphenylamine reagent was added (modified from Burton [6]: 0.5 g of diphenylamine [reagent ACS, Eastman Kodak Co.] in 50 ml of glacial acetic acid, 0.5 ml of concentrated H_2SO_4, and 0.25 ml of a 16-mg/ml solution of acetaldehyde in water). After incubation for 18 to 20 h at 30°C, the assay mixture was filtered through a glass-fiber filter (same type as above), and the A_{600} of the filtrate was measured (1-cm light path). The assay was calibrated with 2'-deoxyadenosine (AdR, Sigma Chemical Co.) and with purified, high-molecular-weight *E. coli* DNA. Under our conditions, an A_{600} of 1.0 corresponds to 0.23 μmol of AdR (1.4×10^{17} molecules) per assay or to 0.4 μmol of DNA nucleotides (= 3.25 A_{260} units of undenatured DNA = 2.4×10^{17} DNA nucleotides = 1.2×10^{17} purine nucleotides: diphenylamine reacts only with purine nucleotides; reference 6), respectively. Thus, an absorbance of 1.0 (from a 10-ml sample) represents 2.6×10^{16} *E. coli* DNA nucleotides or 14.3 μg of DNA per ml of culture (average of AdR and DNA calibration).

For RNA and protein determination, 5-ml samples of culture were precipitated with 1 ml of 3 M trichloroacetic acid at 0°C. The acid-insoluble material (within whole cells) was collected on a glass-fiber filter (same as for DNA) and washed three times with cold tap water. The dried filters were placed in vials containing 2.0 ml of 0.2 N NaOH and kept at 23°C for about 18 h. Then, 0.5 ml of the alkaline extract was removed for the determination of protein (see below). Nucleotides from RNA hydrolysis in the remaining 1.5 ml were separated from alkali-resistant material after addition of 2.25 ml of 0.5 M perchloric acid at 0°C (we now routinely use 1.5 ml to increase the final absorbance 1.25-fold) by filtration through a nitrocellulose filter (0.45-μm pore size). The absorbance of the filtrate was measured at 260 nm (1-cm light path). From the mole fractions and the extinction coefficients (at acidic pH) of the different nucleotides in *E. coli* (ribosomal) RNA, it is calculated that, under our conditions, an A_{260} of 1.0 corresponds to 5.4×10^{16} RNA nucleotides or 3.26 μg of RNA per ml of culture.

Protein was measured by the method of Lowry et al. (19), adapted to our purposes: 0.5 ml of the alkaline cell extract (see above), 2.5 ml of alkaline copper reagent (50 volumes of 0.24 M Na_2CO_3 in 0.08 N NaOH plus 1 volume of 0.5% [wt/vol] $CuSO_4 \cdot 5\ H_2O$ and 1% [wt/vol] sodium potassium tartrate) and 0.5 ml (1 N) Folin phenol reagent (Fischer Scientific Co.) were mixed and, after 30 min at 23°C, filtered through a glass-fiber filter (same type as above). The A_{750} of the clear filtrate was determined. Calibration with bovine albumin (fraction V, Sigma Chemical Co.) indicated that an A_{750} of 1.0 corresponds to 300 μg of protein per assay or (about) 1.3×10^{18} amino acid residues or 240 μg of protein per ml of culture (109 daltons per amino acid residue [32]; 1.25 ml of culture per assay).

The dry weight of (washed) bacteria was determined to be 172 and 173 μg per A_{460} unit for bacteria grown in glucose plus amino acids or glycerol medium, respectively.

To find the absolute values for DNA, RNA, and protein from the relative (A) values of Table 1 and 2, these relative values have to be multiplied with the following conversion factors (see above). (i) For DNA: to obtain DNA nucleotides per A_{460} unit, multiply by 2.6×10^{16}; for micrograms of DNA per A_{460} unit: 14.3; for DNA as percentage of cellular dry weight, 8.29. (ii) For RNA: RNA nucleotides per A_{460} unit, multiply by 5.4×10^{16}; for micrograms of RNA per A_{460} unit, 32.6; for RNA as percentage of cellular dry weight, 18.9. (iii) For protein: amino acid residues per A_{460} unit, multiply by 1.3×10^{18}; for micrograms of protein per A_{460} unit, 240; for protein as percentage of cellular dry weight, 139.

RESULTS

Variability of the macromolecular composition during balanced growth. Before analyz-

ing a nutritional shift-up, the macromolecular composition of bacteria was determined during balanced growth in media supplemented with either succinate, or glucose, or glucose plus amino acids. The amounts of DNA, RNA, and protein were measured optically (Table 1), as described in Materials and Methods.

If the data of Table 1 are plotted as a function of growth rate, it is seen that different cultures, grown under what appear to be identical conditions, vary somewhat in their growth rate and macromolecular composition (Fig. 1). These variations are not inheritable but may depend on the treatment of the overnight culture, from which the experimental culture was started. (A colony from a culture growing in succinate medium with a 60-min doubling time may give rise to a culture growing with a 100-min doubling time in the same medium and vice versa. Also, a succinate culture started from a fresh overnight culture was often found to have a doubling time of 75 min; but when the same overnight culture was stored for several days at 4°C and a new culture was then started from it, the doubling time of this new culture was 85 to 95 min.)

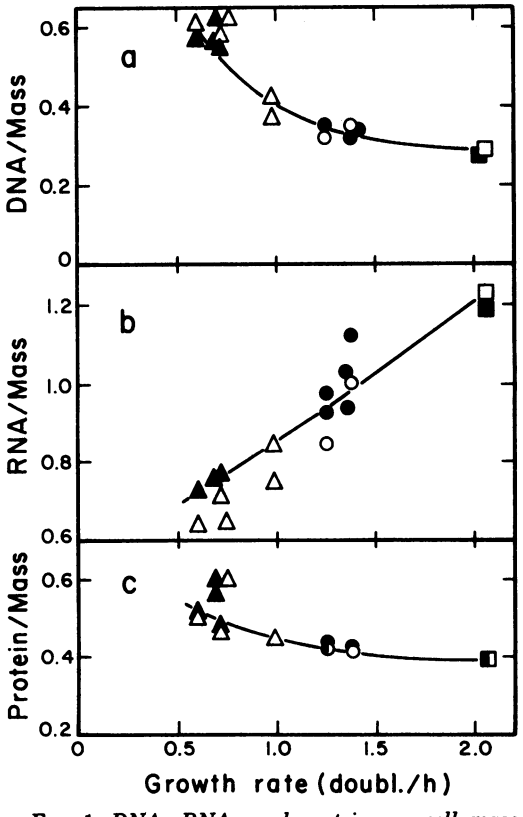

FIG. 1. *DNA, RNA, and protein per cell mass from eight bacterial cultures growing in succinate minimal medium (△, ▲), glucose minimal medium (○, ●), and glucose + amino acids medium (□, ■). Open symbols: Samples taken at an A_{460} of about 0.3; closed symbols: samples taken at an A_{460} of about 0.6. Each symbol represents the average from two duplicate samples; the actually observed values are shown in Table 1.*

Three types of variations observed. (i) Variations in growth rate. Different cultures in the same medium were found to vary in growth rate. This variation was maximal (60- to 120-min doubling time) for succinate-grown bacteria and correlated with changes in the macromolecular composition of the bacteria in a way to be expected from the general growth rate dependency of this composition (for example, in Fig. 1b, RNA/mass values for succinate cultures with different growth rates).

(ii) Changes in composition during growth of culture. In general, younger cultures ($A_{460} \approx 0.3$; Fig. 1, open symbols) had about 10% less RNA per mass than older cultures ($A_{460} \approx 0.6$; Fig. 1, closed symbols). This indicates that the cultures were not exactly in steady state at these cell densities, although mass increased exponentially. Protein and DNA per mass were not significantly affected by the culture age (Fig. 1a and c; see also Fig. 4).

(iii) Variations in macromolecular composition independent of growth rate and culture age. In one succinate culture (experiment 3 of Table 1) the protein/mass and DNA/mass values were 10 to 15% higher, and in one glucose culture (experiment 6 of Table 1) the RNA/mass value was about 15% higher than the assumed average given by the curves. Although these curves may be redrawn such that the deviations are less than 15%, the deviations are still greater than the measuring accuracy.

The causes of these different, apparently semistable physiological states of cell growth under identical genetic and environmental conditions are not understood (see Discussion).

Ratios of protein/DNA, RNA/protein, and RNA/DNA. The results of Fig. 1 can be replotted as macromolecular ratios (Fig. 2; Table 1, last three columns; the significance of these ratios is discussed in reference 13). The protein/DNA ratio increases about 60% between growth rates of 0.6 and 2.1 doublings per h and approaches a maximum value similar to previous observations (reference 13: 42% increase). The RNA/protein and RNA/DNA ratios also increase with growth rate, as has been described previously (12, 13, 20, 21, 24, 29-31). The RNA/protein ratio approaches proportionality with growth rate (dashed line in Fig. 2b), which implies that the ribosome efficiency approaches a constant value (see Discussion in reference 13).

Accumulation of DNA, RNA, and protein

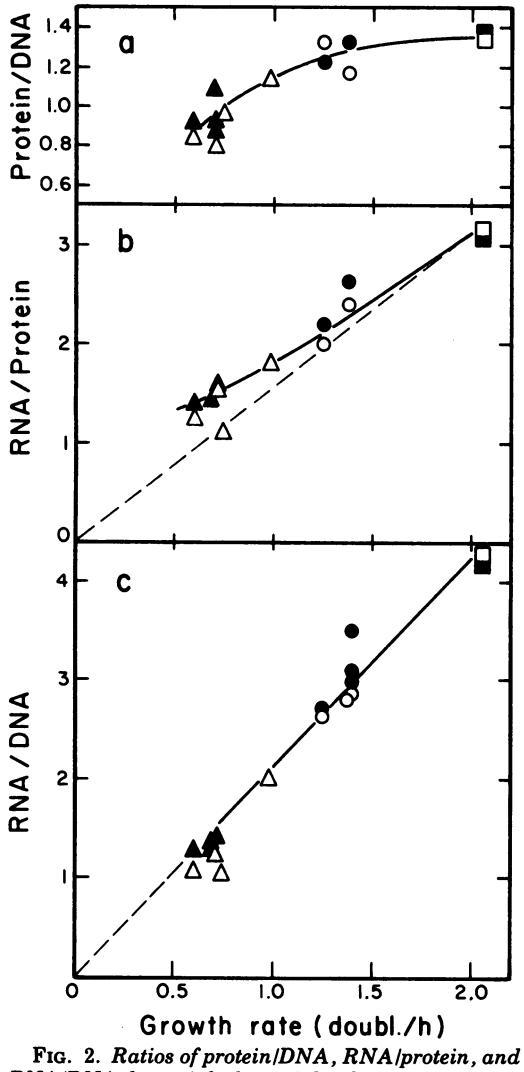

FIG. 2. *Ratios of protein/DNA, RNA/protein, and RNA/DNA from eight bacterial cultures growing in different media (same symbols as in Fig. 1). Data from Table 1; each point represents the average from two duplicate samples.*

after a nutritional shift-up. A previous analysis (4), based on observations with *E. coli* B/r during 30 min after a shift-up (11), suggested that the postshift transition period actually lasts for several hours. It was desirable, therefore, to extend the period of observation after the shift. This creates an experimental difficulty: to prevent the culture from approaching stationary phase during postshift growth, one could start with a very dilute culture. But then it would be difficult to follow the initial culture growth by turbidity measurements; besides, one would have to use radioactive labeling methods to measure synthesis rates, and the interpretation of such data is ambiguous, owing to pool and uptake effects, particularly after a shift-up. To circumvent these difficulties, we have used colorimetric assays at higher cell densities, which made it necessary to dilute the culture periodically during postshift growth. Control experiments had shown that macromolecular synthesis rates are not affected by this dilution.

The cell mass (A_{460}) and the amounts of DNA, RNA, and protein (per unit of culture volume) were measured for 2 h after a shift-up from succinate to glucose plus amino acids medium. The postshift kinetics of mass increase in an undiluted culture were constructed from the observed A_{460} in the diluted culture as described in Materials and Methods (Fig. 3). These kinetics were then used to determine the postshift accumulation of DNA, RNA, and protein (Table 2, Fig. 5) from the ratios DNA/mass, RNA/mass, and protein/mass (Fig. 4).

The postshift increments in the amounts of RNA and DNA (ΔR, ΔD) are proportional to the postshift increase in protein (ΔP), such that the ratios $\Delta R/\Delta P$ and $\Delta D/\Delta P$ are constants (k_1, k_2; Fig. 6). If the units for the amounts of protein, RNA, and DNA are normalized such that 1 unit corresponds to the amount of protein, RNA or DNA, respectively, per unit of culture volume present at the shift time (ordinate in Fig. 5), then the slopes (k_1, k_2) of the curves in Fig. 6a are (necessarily) equal to the final change in the RNA/protein or DNA/protein ratio, respectively (quotient of the postshift/preshift steady-state values). For example, if the DNA/protein (or protein/DNA) ratio does not change after a shift-up (dashed line, Fig. 6a), the slope would be equal to unity. Furthermore, the slopes k_1 and k_2 are equal to the change in the ratio of the accumulation rates $(dR/dt)/(dP/dt)$ and $(dD/dt)/(dP/dt)$ (see Appendix).

The postshift accumulation of RNA and protein was calculated (Fig. 7, two middle curves) using k_1 from reference 13 and the theoretical equations derived previously (4). From the relationship between DNA and protein synthesis in Fig. 6, the postshift accumulation of DNA can now also be calculated (Fig. 7, lower curve). Furthermore, by summing the amounts (weights) of protein, RNA, and DNA, a measure for the cell mass is obtained (Fig. 7, top curve; see also Fig. 2 of reference 13). When these calculated curves for RNA, protein, DNA, and mass are superimposed on the observed values (open circles) in Fig. 5b, it is evident that the calculated curves describe the observed data with no apparent deviation.

Synthesis rates of DNA, RNA, and protein

TABLE 1. *Amounts in relative units of RNA, protein, and DNA per cell mass in eight bacterial cultures grown in succinate minimal, glucose minimal, and glucose + amino acids media*[a]

Expt no.	Growth medium[b]	μ (doublings/h)	Mass (A_{460})	RNA/mass	Protein/mass	DNA/mass	RNA/DNA	RNA/protein	Protein/DNA
1	Succ.	0.59	0.30	0.62	0.51	0.60	1.06	1.24	0.85
		0.59	0.30	0.66	0.52	0.61			
		0.59	0.60	0.71	0.50	0.58	1.27	1.39	0.91
		0.59	0.60	0.74	0.54	0.56			
2	Succ.	0.71	0.30	0.70	0.44	0.56	1.22	1.53	0.79
		0.71	0.30	0.72	0.49	0.60			
		0.71	0.60	0.77	0.48	0.53	1.42	1.61	0.88
		0.71	0.60	0.76	0.47	0.55			
3	Succ.	0.74	0.27	0.64	0.58	0.63	1.04	1.08	0.96
		0.74	0.27	0.65	0.61	0.61			
		0.69	0.50	0.75	0.62	0.56	1.35	1.25	1.08
		0.69	0.50	0.76	0.59				
		0.69	0.74	0.74	0.55	0.59	1.20	1.31	0.92
		0.69	0.74	0.75	0.58	0.65			
4	Succ.	0.98	0.31	0.73	0.43	0.41	2.0	1.78	1.13
		0.98	0.31	0.76	0.46	0.43			
		0.98	0.31	0.88	0.47	0.37			
		0.98	0.31	0.79	0.42	0.37			
5	Gluc.	1.25	0.35	0.84	0.41	0.32	2.64	1.99	1.33
		1.25	0.35	0.85	0.44	0.32			
		1.25	0.71	0.90	0.42	0.34	2.68	2.18	1.23
		1.25	0.71	0.95	0.43	0.35			
		1.25	1.04	0.96	0.44	0.36	2.71	2.22	1.22
		1.25	1.04	0.99	0.44				
6	Gluc.	1.36	0.30	0.95	0.41	0.36	2.82	2.41	1.17
		1.36	0.30	1.06	0.42	0.35			
		1.36	0.61	1.12	0.43	0.32	3.5	2.64	1.33
		1.36	0.61	1.12	0.41				
7	Gluc.	1.40	0.50	0.94		0.33	2.85		
		1.40	0.54	1.03		0.33	3.12		
		1.40	0.64	1.02		0.34	3.00		
8	Gluc. + amino acids	2.06	0.30	1.25	0.42	0.31	4.26	3.17	1.34
		2.06	0.30	1.22	0.36	0.27			
		2.06	0.59	1.19	0.39	0.28	4.23	3.08	1.37
		2.06	0.59	1.18	0.38	0.28			

[a] Duplicate samples were taken at different cell densities (A_{460}). For details and units, see Materials and Methods.
[b] Succ., succinate; Gluc., glucose.

after a shift-up. The rates of synthesis of DNA, "stable" RNA (rRNA, tRNA), and protein are nearly equal to their respective rates of accumulation (i.e., to the slopes of the accumulation curves in Fig. 5) if the turnover rates are small. DNA, rRNA, and tRNA are virtually stable during balanced growth ([34], although rRNA is unstable during chemostat growth [27]), as is protein (less than 5% loss of radioactive protein was observed in a culture during a "chase" in the presence of chloramphenicol to prevent recycling of labeled breakdown products; data not shown).

The rates of nucleic acid and protein accumulation after a shift-up were obtained by mathematical differentiation of the equations used to draw the calculated curves of Fig. 5 and 7 and by graphic differentiation of the linear plot of the accumulation kinetics in Fig. 5a.

(i) **Mathematical differentiation.** The equations describing nucleic acid and protein synthesis after a shift-up (Appendix equations 1, 3, and 5) contain algebraic parameters that were substituted by observed values to generate the curves shown in Fig. 5 or 7. These parameters are the doubling time, the RNA/protein and the protein/DNA ratios, measured during exponential growth in the pre- and postshift medium. If the kinetics are to be drawn with an absolute ordinate scale (e.g., Fig. 7, in micrograms per milliliter of culture), one additional absolute value must be measured, for example, RNA in

TABLE 2. *Accumulation of mass, RNA, DNA and protein in relative units after shift-up (at t = 0) from succinate minimal to glucose + amino acids medium*[a]

Time (min)	Mass	RNA/mass	DNA/mass	Protein/mass	RNA	DNA	Protein
0	1.00	0.66	0.61	0.59	1.00	1.00	1.00
10	1.15	0.72	0.58	0.58	1.25	1.09	1.13
20	1.35	0.82	0.54	0.57	1.68	1.19	1.30
30	1.60	0.90	0.49	0.55	2.18	1.29	1.49
40	1.98	0.96	0.44	0.53	2.88	1.43	1.80
50	2.33	1.00	0.40	0.52	3.53	1.53	2.05
60	2.90	1.04	0.38	0.51	4.57	1.81	2.51
80	4.40	1.11	0.34	0.51	7.4	2.45	3.80
100	6.80	1.17	0.33	0.50	12.0	3.68	5.76
125	11.70	1.23	0.32	0.49	21.8	6.13	9.71

[a] Mass: A_{460} values from Fig. 3 (triangular symbols) divided by 0.300 (= A_{460} at $t = 0$). RNA/mass, DNA/mass, and protein/mass: from Fig. 4 (curves through square symbols). RNA: product mass × RNA/mass, divided by 0.66 (RNA/mass at $t = 0$). DNA: product mass × DNA/mass, divided by 0.61. Protein: product mass × protein/mass, divided by 0.59.

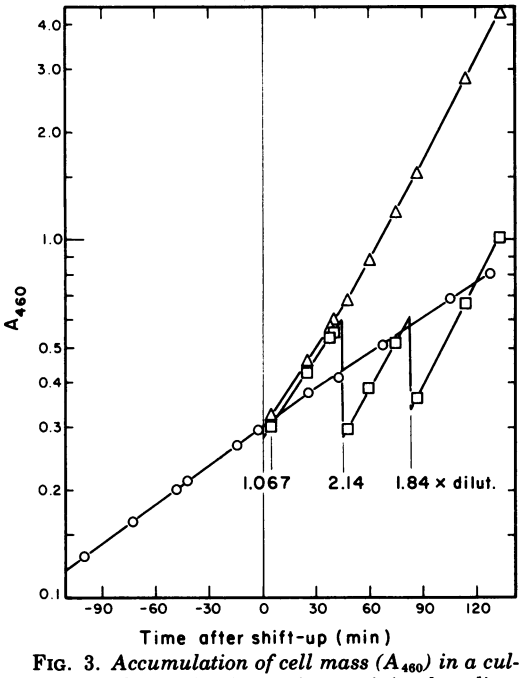

FIG. 3. *Accumulation of cell mass (A_{460}) in a culture (300 ml) growing in succinate minimal medium (○), and after the addition of glucose and 20 amino acids to part of the culture at zero time (□). At 45 and at 83 min, 100 ml of prewarmed, pre-aerated glucose + amino acids medium was added to an approximately equal volume of the remaining culture. The top curve (△) shows the accumulation of mass after correction for the dilution, i.e., after multiplication of the observed values with 1.067 (between 0 and 45 min), 1.067 × 2.14 (45 to 83 min), and 1.067 × 2.14 × 1.84 (after 83 min, see Materials and Methods). The succinate culture is experiment 3 of Table 1.*

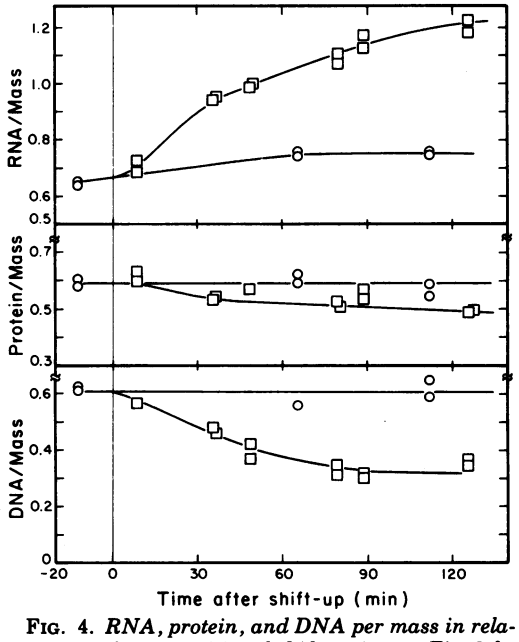

FIG. 4. *RNA, protein, and DNA per mass in relative units after a nutritional shift-up (□; see Fig. 3 for details); ○, unshifted control culture (same culture as in Fig. 3; experiment 3 of Table 1).*

micrograms per cell during exponential growth in the preshift medium.

Cultures grown under identical conditions usually show slight variations in the doubling time or in the proportions of RNA to DNA to protein or in both (Fig. 1 and 2). The theoretical accumulation curves shown in Fig. 7 were drawn, using average values of the growth parameters measured during exponential growth in succinate or glucose plus amino acids medium, respectively (from reference 13). Differentiation of the equations with these parameters gives the rate kinetics shown in Fig. 8a (see Appendix for details).

Using the parameters from the particular shift-up in Fig. 5 (k_1 and k_2 from Fig. 6, preshift doubling time from mass increase in Fig. 3, and

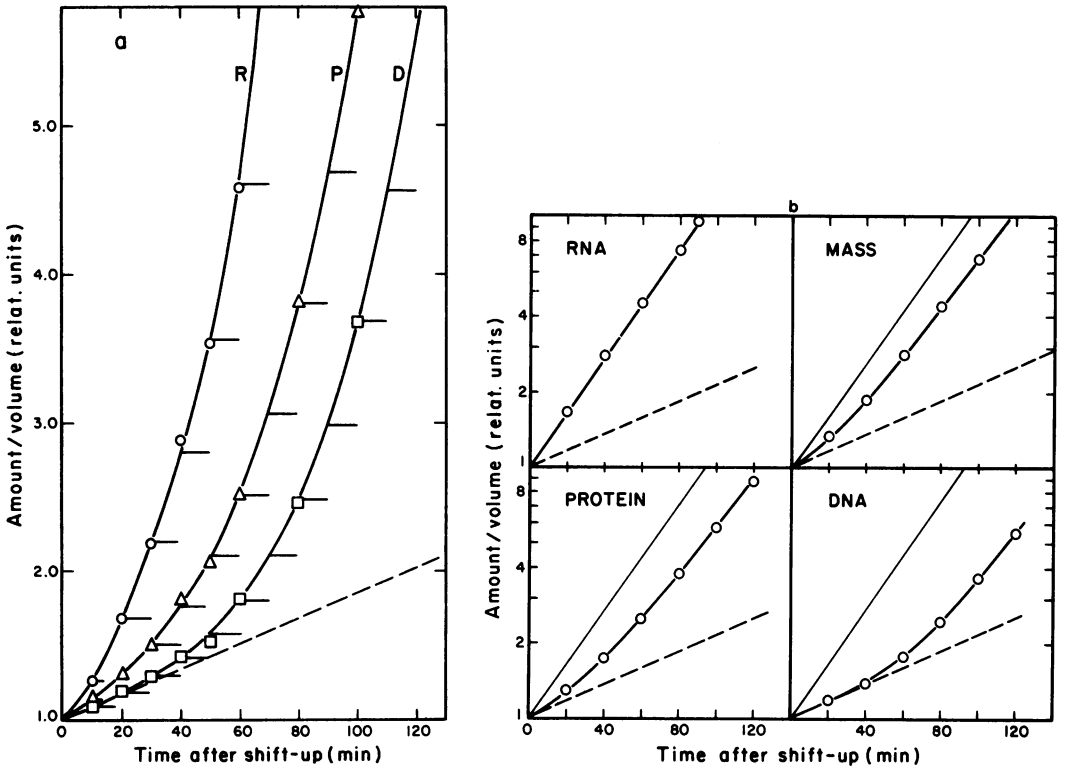

FIG. 5. *Accumulation of RNA, protein, DNA, and mass after a shift-up. Symbols:* (○, △, □) *Values plotted from Table 2, based on the data in Fig. 3 and 4. (a) Linear plot: horizontal marks in 10-min intervals for determination of the rates in Fig. 8b;* (-----) *preshift slope at t = 0. (b) Semilog plot:* (——) *postshift steady-state slope (= RNA curve);* (-----) *preshift steady-state slope;* (——) *theoretical curves, obtained as in Fig. 7, but normalized by setting the zero-time values equal to unity (see Appendix for details).*

postshift doubling time from RNA curve in Fig. 5b), one obtains the rate kinetics shown in Fig. 8b (solid lines).

(ii) Graphic differentiation. In Fig. 5a, the curves for the accumulation of nucleic acids and protein after a shift-up were marked in 10-min intervals. The vertical distances between these marks are a measure for the rate of accumulation at the time of the interval midpoints. These rate values were normalized by setting the preshift rate equal to unity (slope of dashed line in Fig. 5a = ln2/81 min = 0.0086/min; preshift doubling time was 81 min). In Fig. 8b (dashed curves), these normalized rate values are plotted (in semilog manner) together with the theoretical curves. The main differences are found for the DNA curve. The integrals over the theoretical and the graphic rate curves are so similar (less than 5% deviation) that they cannot be easily distinguished experimentally (Fig. 5b: no apparent deviation).

Postshift changes in genome activity. When the rates of RNA and protein accumulation (per milliliter of culture volume) from Fig. 8 are divided by the amount of DNA (in genome equivalents per milliliter of culture volume), one obtains the "rates per genome" (Fig. 9). These rates approach the new steady-state values in a biphasic manner during a transition period, which lasts for several hours. The RNA kinetics show that, on the average, the genome (template) activity of the bacterial DNA increases during the transition period. This also indicates that the apparently simple postshift RNA curve of Fig. 5b is actually more complex. We have previously (3, 4) inferred that the increasing genome activity during the postshift transition reflects an increasing number of functioning RNA polymerase molecules per genome. Presumably, this increase involves a regulation of RNA polymerase synthesis induced by the shift-up (see Discussion).

Changes in ribosome function after a shift-up. The kinetics of the rate of protein synthesis (from Fig. 8b) and the kinetics of the accumulation of RNA (from Fig. 5) were plotted together in semilog manner (Fig. 10). The vertical distance between these curves corresponds to the

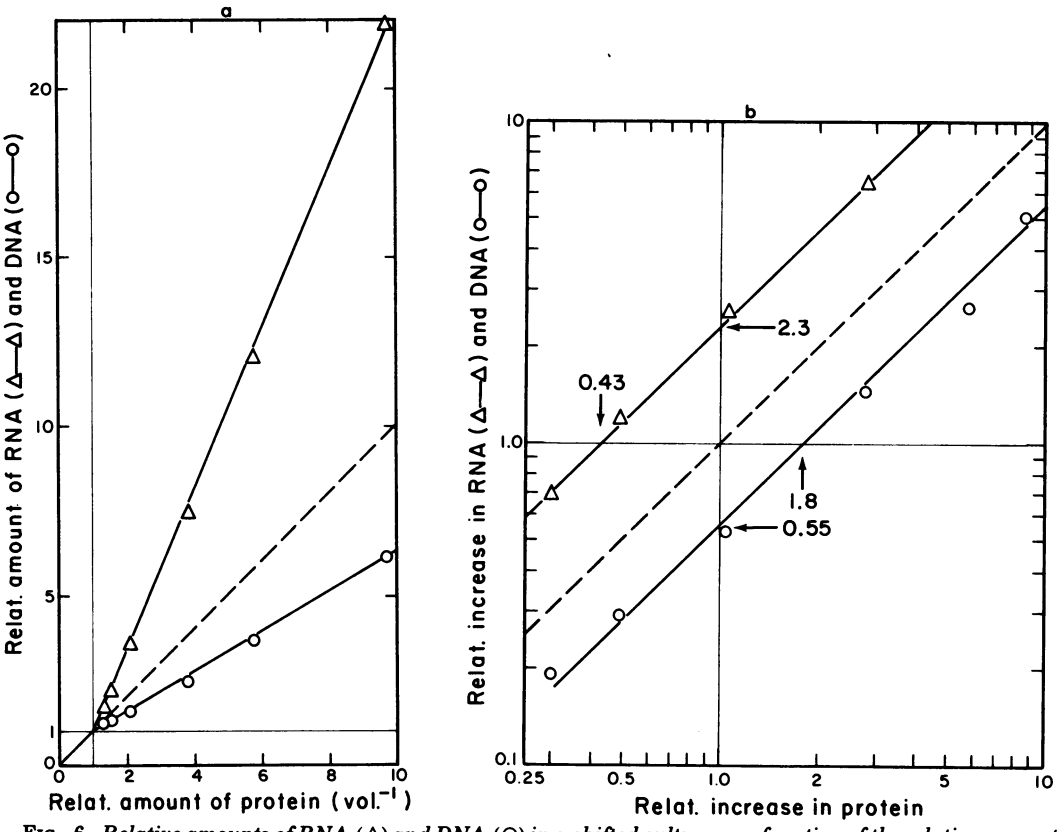

FIG. 6. *Relative amounts of RNA* (Δ) *and DNA* (O) *in a shifted culture as a function of the relative amount of protein (from Table 2);* (-----) *preshift or unshifted culture. (a) Linear plot; (b) log-log plot to give more weight to the early points. The 45° slope implies a linear relationship among protein, RNA, and DNA. The horizontal arrows indicate the changes in the slope at zero time; the vertical arrows indicate the reciprocals used for equations 3d and 5d (Appendix).*

protein synthesis rate per amount of RNA. Since the amount of RNA in a culture is a measure for the number of ribosomes (about 85% of the stable RNA is rRNA; see Discussion in reference 13), the vertical distance between the curves in Fig. 10 is also a measure of the protein synthesis rate per average ribosome (= ribosome efficiency; see equation 2, Appendix). Figure 10 indicates that in the experiment of Fig. 5 the ribosomal efficiency increased 25% immediately after the shift-up and then remained constant at the higher level. On the average, this increase is 40% (Fig. 8a, initial step in protein curve).

The ribosome efficiency is (by definition) the product of two factors: the peptide chain elongation rate (= rate of protein synthesis per functioning ribosome) and the fraction of total ribosomes that is functioning. Measurements of the synthesis time of β-galactosidase in *E. coli* B/r growing in succinate or glucose + amino acids medium (9) suggest that the increase in the ribosome efficiency observed here after a shift-up results mostly from an increase in the peptide chain elongation rate. Although this increase is small (i.e., 20 to 40% for a threefold increase in the growth rate), it may be very significant for the regulation of the bacterial growth rate; it implies a reduced "idling" of ribosomes on mRNA and, thus, a reduced synthesis of guanosine tetraphosphate (14); this nucleotide is assumed to regulate the synthesis of rRNA and tRNA (18, 28, 33). A small increase in aminoacylation of tRNA (e.g., from 80 to 90%) can mean a large change in the concentration of uncharged tRNA (e.g., a twofold reduction from 20 to 10%) and, thus, a large effect on the (uncharged tRNA-dependent) synthesis of guanosine tetraphosphate.

DISCUSSION

Composition of *E. coli* B/r. The ratios of the amounts, RNA/DNA, RNA/protein, and protein/DNA for cultures of *E. coli* B/r determined here nearly agree with those estimated previously (Table 3), although entirely different

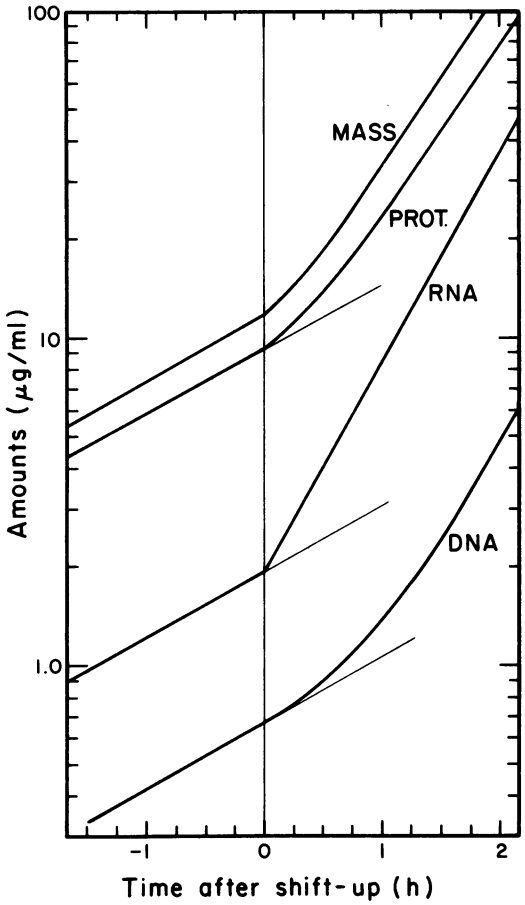

FIG. 7. *Calculated accumulation of RNA, protein, DNA, and mass (sum of the weights of RNA, protein, and DNA) in micrograms per milliliter of culture after a nutritional shift-up from succinate minimal (90-min doubling time) to glucose + amino acids medium (28-min doubling time), assuming 10^8 cells per ml at zero time (data from reference 13; see Appendix for details).*

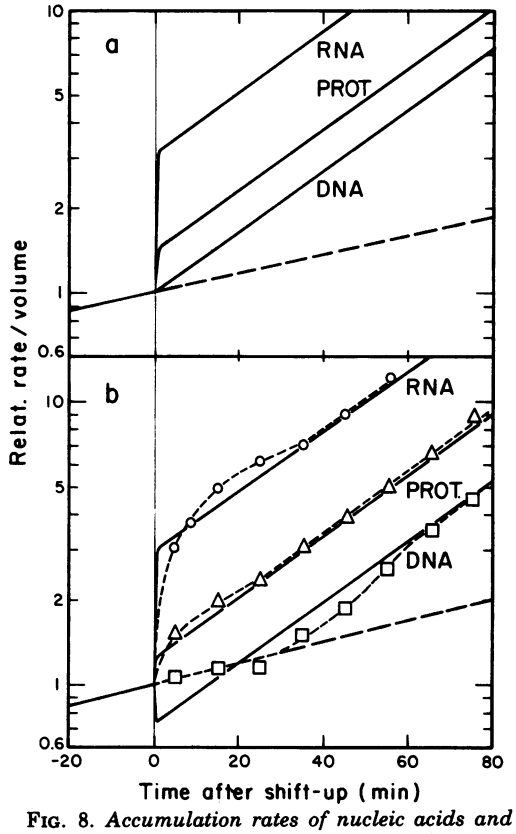

FIG. 8. *Accumulation rates of nucleic acids and protein after a shift-up from succinate to glucose + amino acids medium. (a) Average shift-up; calculated curves, obtained by mathematical differentiation of the kinetics shown in Fig. 7 (see Appendix, equations 7a, 8a, and 9a). (b) Experiment of Fig. 5;* (——) *calculated curves obtained by mathematical differentiation (see Appendix, equations 7b, 8b, and 9b).* (-----) *Curves obtained by graphic differentiation of the kinetics shown in Fig. 5a;* (- - -) *preshift slope.*

methods were used (see reference 13: measurement of the differential rate of synthesis of ribosomal protein for the RNA/protein ratio and [^{14}C]uracil labeling for the RNA/DNA ratio). Therefore, these ratios are assumed to be essentially correct. However, the absolute amounts of protein and nucleic acids per mass unit estimated here (Table 3) are 14 to 23% higher than the corresponding values estimated previously (4, 13). These absolute amounts were here measured directly, but previously they were obtained indirectly from the ratios above and from the calculated value of DNA/mass. That calculation was based on the theoretical number of genome equivalents per cell as a function of growth rate (8), the number of DNA nucleotides per *E. coli* chromosome (*E. coli* chromosome = 1,100 to 1,400 μm [7]; number of base pairs per micrometer of DNA = 2,960 [1]: 3.3×10^6 to 4.1×10^6 base pairs = 6.6×10^6 to 8.2×10^6 nucleotides/genome), and the number of cells per mass unit (Coulter counts per A_{460} unit from reference 13). With an uncertainty of the chromosome length of ±12%, the uncertainty of the calculated DNA/mass estimate may account for the differences in the amounts of nucleic acids and protein observed here and those estimated previously.

Two parameters determine the macromolecular composition of bacteria. During the 2- to 3-h transition period between two steady states of balanced growth, the composition of the bacteria changes continuously. For any given postshift time, the composition can be

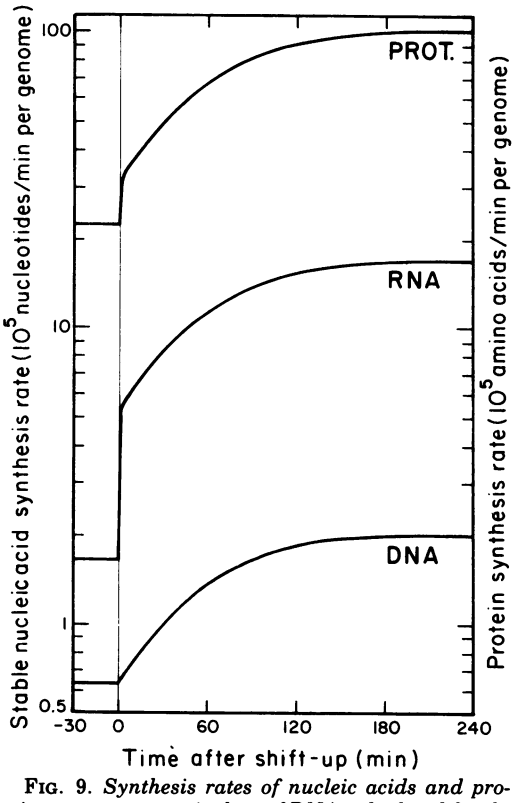

FIG. 9. *Synthesis rates of nucleic acids and protein per genome equivalent of DNA calculated for the average shift-up from succinate to glucose + amino acids medium shown in Fig. 7. RNA, protein, and DNA curves were obtained as quotients of equations 7a and 5c, 8a and 5c, or 9a and 5c, respectively, and converted to absolute values as determined in the Appendix.*

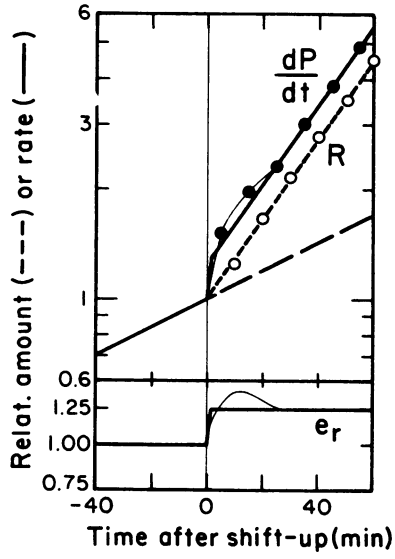

FIG. 10. *Postshift changes in the protein synthesis rate per average ribosome (= e_r). Calculated kinetics of the accumulation of RNA (R, -----) and of the protein synthesis rate (dP/dt, ——), using the parameters from the experiment in Fig. 5 (equations 1c and 8b, Appendix). The quotient of these equations (= vertical distance between the curves) is assumed to be a measure for the protein synthesis rate per average ribosome (= ribosome efficiency, e_r; bottom curve). Symbols: (○) Amount of RNA from Table 2, (●) protein synthesis rate from Fig. 8b. The results suggest an initial "overshoot" in the protein synthesis rate per average ribosome during the first 20 min after the shift* (——).

predicted from the changes observed immediately after the shift-up, i.e., from the parameters k_1 and k_2 (= slopes of the curves in Fig. 6a). This means the final steady-state composition is determined many generations before it is reached. The reason for this predictable behavior is the constancy during postshift growth of k_1 and k_2. The constancy of k_1 indicates a constant protein synthesis rate per average ribosome (Results and Fig. 10). Formally, the parameter k_2 implies a relation between initiation of DNA replication and protein synthesis (see Discussion in reference 13); we suppose that it expresses the differential rate of synthesis of a protein required for initiation of replication. Thus, the ribosome efficiency and the synthesis of the (hypothetical) replication initiation protein are assumed to determine the macromolecular composition of bacteria during steady-state growth and during the transition between steady states. These two metabolic rates, related to k_1 and k_2, change in a stepwise fashion shortly after the shift.

Postshift changes in the macromolecular synthesis rates. In the semilog plot of Fig. 5b, the kinetics describing the postshift accumulation of RNA are linear, whereas the kinetics of accumulation of protein and DNA are curves. It is surprising, therefore, that a plot of one relative to the other, e.g., RNA versus protein or DNA versus protein (as in Fig. 6a), gives linear relationships. The reason for this latter linearity is a property of the postshift protein and DNA accumulation curves that is not obvious from Fig. 5b: although these curves are nonexponential, they share with exponential functions (i.e., the RNA curve) an exponentially increasing slope (Fig. 8, same rate constant); this means that the curves are the sum of a constant plus an exponential term, which generates the linear relationships in Fig. 6.

The constant (and parallel) slopes in the rate kinetics of Fig. 8 do not mean that the rates of accumulation of nucleic acids and proteins become constant immediately after the shift-up.

TABLE 3. *Comparison of the macromolecular composition of E. coli B/r growing in glucose minimal medium (μ = 1.36)*[a]

Ratio	Colorimetric method		Previous	Ratio of colorimetric/previous
	Relative units[b]	Absolute units[c]	Absolute units[d]	
Protein/mass	0.41	5.3×10^{17}	4.5×10^{17}	1.18
RNA/mass	0.98	5.3×10^{16}	4.3×10^{16}	1.23
DNA/mass	0.33	8.6×10^{15}	7.5×10^{15}	1.14
Protein/DNA	1.24 (0.41/0.33)	62	60	1.03
RNA/DNA	2.97 (0.98/0.33)	6.2	5.7	1.08
RNA/protein	2.39 (0.98/0.41)	0.10	0.096	1.04

[a] As determined here by colorimetric methods (Fig. 1) and previously by other methods involving radioactive labeling (13).

[b] Protein, RNA, and DNA per mass in relative units from Fig. 1. The ratios in the lower three rows were formed from the per-mass values; they are also illustrated in Fig. 2.

[c] Absolute units; protein/mass in amino acid residues per A_{460} unit of culture = relative units $\times 1.3 \times 10^{18}$ amino acids (calibration factor, see Materials and Methods section). RNA/mass in RNA nucleotides per A_{460} unit of culture = relative units $\times 5.4 \times 10^{16}$ nucleotides. DNA/mass in DNA nucleotides per A_{460} unit of culture = relative units $\times 2.6 \times 10^{16}$ nucleotides. Ratios in amino acid residues per DNA nucleotide, RNA nucleotides per DNA nucleotide, and RNA nucleotides per amino acid residue, respectively, formed from the per-mass values.

[d] Protein and RNA per-mass values calculated from revised (see below) per-genome values of Table 2 in reference 13, assuming 2.0 genomes per cell and 4.6×10^8 cells per mass unit (also from Table 2, reference 13); protein: 4.86×10^8 amino acids/genome $\times 2.0 \times (4.6 \times 10^8) = 4.5 \times 10^{17}$ amino acids/A_{460}; RNA: 4.68×10^7 nucleotides/genome $\times 2.0 \times (4.6 \times 10^8) = 4.3 \times 10^{16}$ RNA nucleotides/A_{460}; DNA: 8.2×10^6 DNA nucleotides/genome $\times 2.0 \times (4.6 \times 10^8) = 7.5 \times 10^{15}$ DNA nucleotides/A_{460}. The per-genome values were recalculated using the equations provided in Table 2 of reference 13, but using a value of 109 (32) for the molecular weight of the average *E. coli* amino acid residue (instead of 118) and a value of 8.2×10^6 DNA nucleotides per genome (2) instead of 7.6×10^6.

Rather, the relative increments in the rates per unit of culture volume per unit of time (second derivatives of the amount kinetics in Fig. 5) become immediately constant. The reasons for this apparent coupling of the three rate kinetics are discussed in the following sections.

Exponential increase in RNA after a shift-up has complex causes. Whereas the macromolecular composition of bacteria, expressed as ratios of the amounts, or as amounts per genome, is determined by the parameters k_1 and k_2, the absolute amounts of nucleic acids and protein (per unit of culture volume) at a given postshift time depend on one additional parameter. Formally, this additional parameter could be the amount of either RNA, DNA, or protein. Most likely, the amount of RNA is the biologically significant parameter, because only the RNA accumulation responds immediately to the shift-up and immediately assumes the doubling time characteristic of postshift growth. Once the amount of RNA is given, the amounts of protein and DNA follow from k_1 and k_2: RNA determines the ribosome number and thus protein synthesis (k_1) and protein; synthesis of protein, including replication initiation protein (k_2), determines DNA synthesis and thus DNA.

Although the accumulation of RNA follows a sample exponential function (in contrast to the more complex protein and DNA curves), it must have complex causes. The initial stepwise increase in the rate of RNA synthesis (see RNA curves in Fig. 8 and 9) determines the new postshift growth rate; the increase is equal to the ratio μ_2/μ_1 (see Appendix). This step reflects mainly a redistribution of RNA polymerase molecules over the genes for rRNA, tRNA, and mRNA (11). The redistribution of polymerase is assumed to be brought about by a guanosine tetraphosphate-mediated differential change in the affinity of RNA polymerase for the promoters of the genes for rRNA, tRNA, and mRNA (28).

The continued exponential increase in the rate of RNA synthesis cannot be explained by these two effects alone; further regulation must occur, for example (3, 4):

(i) Ribosomal protein (r protein) synthesis is induced. This induction is less than the induction of stable RNA genes (5, 10); e.g., for a 3-fold increase in rRNA synthesis, a 1.5- to 2-fold increase in the synthesis of mRNA for r protein

is sufficient to match r protein to rRNA synthesis, since mRNA is translated more frequently after the shift (10, 13).

(ii) RNA polymerase synthesis is induced at the same time, and to the same extent, as r protein induction. Earlier studies indicated that RNA polymerase synthesis increases only slowly over a period of 60 min after a shift-up (22). In this case, a temporary drop in the rate constant of RNA accumulation is expected to occur during the first 60 min after a shift-up (4). A more recent study indicates that RNA polymerase synthesis after a shift-up increases much faster, like r protein synthesis (16). Consistent with this recent result is the observation here that the doubling time of RNA is constant during postshift growth.

As long as little is known about the regulation of ribosomal protein and RNA polymerase synthesis, we will not understand the exponential increase in RNA (per unit of culture volume) after a shift-up. Formally, this exponential increase is characterized by the change in growth rate, given by the ratio μ_2/μ_1. This parameter, together with k_1 and k_2, determines the postshift growth and composition of the culture. Just as k_1 and k_2 were interpreted as an expression of the ribosome function and of the synthesis of a replication initiation protein, the formal parameter (μ_2/μ_1) is interpreted as a complex expression of RNA polymerase function and synthesis, and ribosome synthesis.

Relation that restricts the values of the growth parameters. On the average, the ratio of the steady-state amounts of RNA and DNA (RNA/DNA) is proportional to the bacterial growth rate μ (Fig. 2). This implies that the ratio $\Delta R/\Delta D$ observed during postshift growth is equal to μ_2/μ_1. Hence (since $\Delta R/\Delta D = k_1/k_2$), the three parameters k_1, k_2, and (μ_2/μ_1) are restricted in their values by the relationship $(k_1/k_2) = (\mu_2/\mu_1)$.

A relationship between k_1, k_2, and (μ_2/μ_1), reflecting a general interdependency of metabolic rates, is not surprising. However, the particular type of this relation (i.e., the fact that the RNA/DNA ratio is proportional to μ) does not follow from an obvious principle. According to this relationship, the (steady-state) rate of stable RNA synthesis per genome is proportional to μ^2 (4, 21), which implies an increased activity (transcription frequency) of the rRNA and tRNA genes at higher growth rates (5). Similarly, the rate of mRNA synthesis per genome increases with growth rate, although less than with μ^2 (13). Less drastic changes in genome activity with μ are conceivable: for example, the initiation of DNA replication might be regulated in a way that the genome activity is approximately constant and optimal. However, the observed growth rate dependency of the genome activity is such that, in slow-growing bacteria, the DNA is greatly "underemployed," suggesting that, generally in bacteria, the amount of DNA is not limiting transcription. Although this appears to be uneconomical, it has the advantage that these cells can rapidly adapt to improved growth conditions: any section of the genome can be quickly activated. This is illustrated by the initial step in the RNA curve in Fig. 8 and 9, which shows a sudden increase in the activity of the stable RNA genes at the time of the shift-up.

Variability of growth parameters. In different shift-up experiments, the parameters k_1 and k_2 (i.e., presumably the pre- and postshift values of the ribosome efficiency and of the differential rate of synthesis of replication initiation protein) vary to some extent. This variability also affects the restricting relationship mentioned above, which is true only on the average. For any particular shift-up the relation becomes $(k_1/k_2) = x(\mu_2/\mu_1)$, where $x = 1.0 \pm 0.3$. The factor x is equal to the extrapolation value for $t \rightarrow 0$ of the DNA rate kinetics: for the average shift-up, the postshift DNA rate kinetics extrapolates to 1.0 for $t \rightarrow 0$ (Fig. 8a). In a particular shift-up, the extrapolation value may be greater or smaller than 1.0; in the experiment of Fig. 5, the extrapolation value was 0.7 (Fig. 8b).

A value of $x = 1.0$ means that the rate of DNA synthesis shortly after the shift-up is equal to the rate shortly before the shift-up. This suggests that the velocity of traveling replication forks does not immediately change, i.e., that the C period (= time to replicate the chromosome [8]) is not rapidly affected by the shift-up. In a shift from succinate to glucose + amino acids medium, one might expect an increase in this velocity (8), but not a sudden decrease, as seen in Fig. 8b (solid-line DNA curve). This consideration makes the curve obtained by graphic differentiation (Fig. 8b, dashed-line DNA curve) more likely; i.e., the DNA accumulation curve in Fig. 5 shows no visible break (neither decrease nor increase in the slope) at $t = 0$, suggesting an at least initially constant C period.

Further, an extrapolation value of 1.0 generates the proportionality of the steady-state amount of RNA per DNA with the bacterial growth rate seen in Fig. 2c (see above). This proportionality was previously thought to be important for the regulation of the bacterial growth rate (e.g., references 17 and 21). How-

ever, the variability of x observed here indicates that the proportionality of RNA/DNA with μ has no absolute validity and supports the idea that this relation is not the direct expression of a regulatory mechanism, but rather a fortuitous result of the values of k_1 and k_2 (13).

APPENDIX

Kinetic equations for RNA, protein, and DNA accumulation. The postshift accumulation of RNA (R) per unit of culture volume can be described by the exponential function (Fig. 5):

$$R = R_0 e^{k\mu_2 t} \tag{1}$$

where R_0 is the amount of RNA at zero time (= shift time), k = ln2/60 (hours per minute), and μ_2 = postshift steady-state growth rate (doublings per hour).

The postshift accumulation of protein (P) was previously calculated (4) from the number (N_r) of ribosomes and the ribosome efficiency (e_r), defined (20, 31) by

$$e_r = \frac{dP/dt}{N_r} \tag{2}$$

The number of ribosomes was assumed to be proportional to R (from equation 1; the factor of proportionality is included in the value of P_0 below). Resolving equation 2 for dP/dt and integration (after substitution of equation 1) gives

$$P = P_0 [1 + \Delta P] \tag{3a}$$

where P_0 is the amount of protein at zero time and ΔP is the relative (dimensionless) increase in protein after the shift:

$$\Delta P = \frac{e_{r2}}{e_{r1}} \cdot \frac{\mu_1}{\mu_2} [e^{k\mu_2 t} - 1] \tag{3b}$$

(e_{r1}, e_{r2}, μ_1, and μ_2 are the preshift [subscript 1] and postshift [subscript 2] values of the ribosome efficiency or growth rate, respectively; for details of this derivation, see reference 4). The product $(e_{r2}/e_{r1}) \cdot (\mu_1/\mu_2)$ in equation 3b is experimentally obtained as the quotient of the pre- and postshift (steady-state) RNA/protein ratios (13, 31) equal to k_1^{-1} (Results, iii; Fig. 6):

$$\frac{e_{r2}}{e_{r1}} \cdot \frac{\mu_1}{\mu_2} = \frac{(R/P)_1}{(R/P)_2} = \frac{1}{k_1} \tag{4}$$

This follows from equation 2 by setting $dP/dt = Pk\mu$ and $N_r \sim R$. The ratio (e_{r2}/e_{r1}) is equal to the zero-time step in the kinetics of the protein synthesis rate (Fig. 8–10).

The postshift accumulation of DNA:

$$D = D_0 [1 + \Delta D] \tag{5a}$$

is obtained from Fig. 6:

$$\Delta D = k_2 \Delta P \qquad k_2 = (P/D)_1/(P/D)_2 \tag{5b}$$

Calculation of the kinetic curves for the average shift-up in Fig. 7 and 8a. The accumulation kinetics in Fig. 7 were calculated using the following parameters (from Table 2 of reference 13): $\mu_1 = 0.67$ doublings per h; $\mu_2 = 2.14$ doublings per h; $R_1 = 2.14 \times 10^7$ nucleotides per genome; $R_2 = 6.83 \times 10^7$ nucleotides per genome; $P_1 = 2.91 \times 10^8$ amino acids per genome; $P_2 = 4.11 \times 10^8$ amino acids per genome. This gives the quotients in equation 4:

$$\frac{(R/P)_1}{(R/P)_2} = \frac{2.14 \cdot 10^7 / 2.91 \cdot 10^8}{6.83 \cdot 10^7 / 4.11 \cdot 10^8} = 0.44$$

and in equation (5b):

$$\frac{(P/D)_1}{(P/D)_2} = \frac{2.91 \cdot 10^8}{4.11 \cdot 10^8} = 0.71$$

Using these values, the postshift accumulation kinetics (setting $R_0 = 1$; $P_0 = 1$; $D_0 = 1$; and k = ln2/60) are:

$$R = e^{k\mu_2 t} = e^{0.0247t} \tag{1b}$$

$$P = 1 + 0.44\,(e^{0.0247t} - 1) \tag{3c}$$

$$D = 1 + (0.71 \cdot 0.44)\,(e^{0.0247t} - 1) \tag{5c}$$

The (normalized) preshift kinetics (for $t < 0$) are identical:

$$R = P = D = e^{k\mu_1 t} = e^{0.00774t} \tag{6}$$

The absolute values for 10^8 cells per ml at zero time were found by assuming 1.61 genome equivalents per cell for succinate-grown bacteria (i.e., at $t = 0$) and 7.6×10^6 DNA nucleotides per genome (7, 8); the values for R_1 and P_1 were multiplied with 1.61 genomes per cell $\times$ 10^8 cells per ml, and then converted to micrograms.

For the rate kinetics in Fig. 8a, equations 6 (for $t < 0$), 1b, 3c, and 5c (for $t > 0$) were differentiated and normalized (to 1.0 at $-t \rightarrow 0$; normalization factor = 1/0.00774 = 1/rate constant of equation 6):

$$\frac{dR}{dt} = \frac{0.0247}{0.00774} \cdot e^{0.0247t} = 3.2 \cdot e^{0.0247t} \tag{7a}$$

The factor (3.2) in equation 7a is equal to the change in the steady-state growth rates, μ_2/μ_1.

$$\frac{dP}{dt} = \frac{0.44 \cdot 0.0247}{0.00774} \cdot e^{0.0247t} = 1.4 \cdot e^{0.0247t} \tag{8a}$$

The factor (1.4) in equation 8a is equal to the change in the ribosome efficiency, e_{r2}/e_{r1}.

$$\frac{dD}{dt} = \frac{0.71 \cdot 0.44 \cdot 0.0247}{0.00774} \cdot e^{0.0247t} = 1.0 \cdot e^{0.0247t} \tag{9a}$$

The factor (1.0) in equation 9a implies that the amount of RNA per DNA is proportional to the growth rate μ, which is true on the average (Fig. 1b of reference 13; Fig. 2 of this paper), but not for any particular pair of cultures (Fig. 2).

Calculation of the kinetic curves for the particular shift-up in Fig. 5b and 8b. The curves in Fig. 5b were calculated using the following parameters: $\mu_1 = 0.74$ doublings per h (experiment 3 of Table 1 at $A_{460} = 0.27$; after the shift, the growth rate of the unshifted portion of the culture slowed down slightly to 0.69 doubling/h); $\mu_2 = 2.14$ doublings/h

(RNA curve of Fig. 5); $(R/P)_1/(R/P)_2 = 0.43$ (Fig. 6b); $(P/D)_1/(P/D)_2 = 0.55$ (Fig. 6b). These parameters give the following accumulation kinetics (for $t > 0$) of Fig. 5:

$$R = e^{0.0247t} \quad (1c)$$
$$P = 1 + 0.43\,(e^{0.0247t} - 1) \quad (3d)$$
$$D = 1 + (0.55 \cdot 0.43)\,(e^{0.0247t} - 1) \quad (5d)$$

and the rate kinetics of Fig. 8b:

$$\frac{dR}{dt} = 2.9 \cdot e^{0.0247t} \quad (7b)$$

$$\frac{dP}{dt} = 0.43 \cdot 2.9 \cdot e^{0.0247t} = 1.25 \cdot e^{0.0247t} \quad (8b)$$

$$\frac{dD}{dt} = 0.55 \cdot 0.43 \cdot 2.9 \cdot e^{0.0247t} = 0.69 \cdot e^{0.0247t} \quad (9b)$$

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