synthesis to occur at the translational rather than the transcriptional level of gene expression.

There are several possible explanations for this apparent discrepancy. The most simple is that our procedures are not sensitive enough to reveal the presence of additional proteins synthesized in response to testosterone. However, it seems unlikely that a 50–70% qualitative change in the protein population could be missed by methods with such high-resolving power, and control experiments demonstrated that differences in other protein populations were readily detected by our procedures.

It is also possible that many additional RNA molecules are synthesized but are not translated into protein; the increase in protein synthesis could conceivably be an independent translational effect of testosterone. Our earlier studies (Breuer and Florini, 1965) did not clearly demonstrate a specific translational effect of testosterone; the observed differences in activity of ribosomes could be attributed to differences in mRNA content of the ribosome preparations. It is possible that there were also effects on the inherent activity of individual ribosomes, but the experiments were not designed to provide a sensitive assay of such effects.

In our opinion, the most likely explanation of our results is that most if not all of the additional DNA made available for transcription repeats sequences which are already transcribed in muscle of castrated rats. This redundancy might occur at the level of the integrator or producer genes postulated by Britten and Davidson (1969); in either case, increases in amount but not kinds of proteins synthesized would result.

Evaluation of these possibilities now requires detailed qualitative analysis of the RNA synthesized in muscle of testosterone-treated rats. Although we have been attempting such analyses for some time, thus far we have been unable to achieve sufficient labeling of muscle RNA to allow useful qualitative analyses. However, some progress is now being made, and our efforts are continuing; preliminary observations indicate that there are no major qualitative changes in muscle RNA synthesis following administration of testosterone propionate to castrated rats.

Acknowledgment

I am grateful to Mrs. Ruth Caverhill for her careful and patient technical assistance in these studies.

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Regulation of the *in Vivo* Synthesis of the Polypeptide Chain Elongation Factors in *Escherichia coli**

Julian Gordon

ABSTRACT: The rate of protein synthesis is constant per ribosome as the bacterial cell adjusts the relative concentrations of supernatant proteins to ribosomes at different growth rates. In this investigation, the regulation of the synthesis of the polypeptide chain elongation factors T and G was studied. *In vitro* activity measurements, polyacrylamide disc electrophoresis, and immunochemical assays were used to determine the relative levels of the polypeptide chain elongation factors in cells in different steady states of growth. It is concluded that the relative amount of factors to ribosomes stays constant at the different growth rates, and that the factors are present in the cell at *ca.* 1 mole of each/mole of ribosomes.

In a defined steady state of growth, bacterial cells have a unique macromolecular composition (Schaechter *et al.*, 1958; Neidhart and Magasanik, 1960; Kjeldgaard and Kurland, 1963; Ecker and Schaechter, 1963; Maaløe and Kjeldgaard, 1966). It seemed of interest to determine whether (1) the synthesis of the polypeptide chain elongation factors T and G (Nishizuka and Lipmann, 1966) was coordinated with that of the ribosomes in different steady states, since both form part of the protein synthetic machinery, or (2) whether they behaved as total cellular proteins (Maaløe and Kjeldgaard, 1966). The relative amount of total cellular protein to ribosomes increases with increasing generation times (Kjeldgaard and Kurland, 1963; Ecker and Schaechter, 1963). It is now found that under the same conditions, synthesis of the polypeptide chain elongation factors is coordinated with that of the ribosomes and not with the total cellular

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proteins. In addition, the cellular content of the factors is in approximately 1:1 stoichiometry with the amount of ribosomes. These findings are the subject of this communication.

Materials and Methods

Growth Media and Conditions. The basal salts mixture of Schaechter *et al.* (1958) was used. It was supplemented with various nutrients and carbon sources to give different growth rates. The generation times given in Table I were obtained reproducibly for the following growth media: (1) broth–yeast extract: 0.8% Difco nutrient broth, 0.5% Difco yeast extract, and 1% glucose; (2) broth: 0.8% Difco nutrient broth and 1% glucose; (3) high glucose: 1% glucose; (4) low glucose: 0.02% glucose; (5) glycerol: 0.06% glycerol; and (6) glutamate: 0.06% glutamate. The carbon sources were sterilized separately and the cells were grown in 15-l. fermenters under conditions of vortex aeration, to a final density of 1.0 A_{450} unit, measured on the Zeiss PMQ II spectrophotometer. The growth proceeded logarithmically to this density in the above media. The strain was *E. coli* S/6. The generation times, reported in Table I, probably differ from the literature values because of the high cell densities used here.

The growth was stopped by pouring the cultures on to ice precooled to −10°, and the cells were harvested in the Szent-Györgi Blum continuous-flow rotor of the Sorvall RC2B refrigerated centrifuge. The cells were resuspended in three volumes of 10 mM Tris-HCl–10 mM $MgCl_2$–0.12 M NH_4Cl (pH 7.8) and were recentrifuged. They were stored frozen as pellets at liquid nitrogen temperature until the preparation of the extracts.

Preparation of Extracts. The cells were thawed and resuspended in two volumes of 10 mM Tris-HCl–10 mM $MgCl_2$–1 mM dithiothreitol (pH 7.8), and 20 μg of DNase was added. The suspension was passed through the French press under a pressure of 10,000 psi, and was centrifuged at 30,000*g* for 30 min to pellet the cell debris. The yield of cellular proteins and ribosomes was made quantitative by a second extraction with one volume of the same buffer. The pooled supernatant fluids from these two centrifugations were completely clarified by an additional 30-min centrifugation at 30,000*g*. The ribosomes were pelleted by centrifugation for 2 hr at 200,000*g*. The supernatant fluid (S-200 fraction) was then refrozen in liquid nitrogen, and stored there until the assays were carried out. The pelleted ribosomes were resuspended in the same buffer and clarified by centrifugation at 30,000*g* before refreezing with the S-200 fraction.

Assay Procedures. The preparation of the antisera directed against the polypeptide chain elongation factors is described in detail elsewhere (Gordon *et al.*, 1969). The levels of the elongation factors were determined in terms of antibody equivalence. The equivalence points were determined from titration curves, using the initial slope of turbidity formation (as A_{380} units per minute) as a measure of antigen–antibody reaction. This procedure gave sharper peaks and consequently higher precision in the determination of the equivalence point than measuring the amount of precipitate after a fixed time. The assay system was calibrated with the use of the electrophoretically homogeneous preparations of T and G factors described elsewhere (Gordon, 1969).

Ribosomes were estimated from the A_{260} units of the pellets, using a factor of 14.4 A_{260} units/mg. Protein determinations were made on the material from the S-200 fractions that was insoluble after 10 min at 90° in 5% trichloroacetic acid, by the method of Lowry. Crystalline egg white lysozyme (Worthington) was used as the standard.

Disc electrophoresis (Ornstein and Davis, 1964) was carried out with 7.5% polyacrylamide running gels (5.5 cm) and at pH 8.7, and 0.7-cm stacking gels. Electrophoresis was for 1 hr at 3 mA/tube. Densitometry was carried out with the linear transport attachment of the Gilford spectrophotometer.

TABLE I: Generation Times Achieved in Different Media.[a]

Medium	Generation Time (min)
1. Broth-yeast extract	24
2. Nutrient broth	49
3. High glucose	77
4. Low glucose	89
5. Glycerol	65
6. Glutamate	80

[a] The media were prepared as described under Growth Conditions and Media, and the cells were grown from density of 0.1–1.0 A_{450} unit/ml.

Results

Specific Activity Measurements. As the cell adjusts to its macromolecular composition in different growth media, the relative amount of cellular protein to ribosomes increases with increasing generation time (Maaløe and Kjeldgaard, 1966). We have investigated whether the control of the synthesis of the T and G factors is coordinated with ribosomes or with other cellular proteins by measurements of the specific activity of the factors in cells grown with different generation times.

T and G factors were measured in terms of the phenylalanine polymerizing activity. G activity was measured by titration of the fraction in the presence of an excess of purified T factor. T factor seemed to be the limiting one in the assays of the crude S-200 fraction, and the same values were obtained in the presence or absence of added G factor. We have not measured T_u and T_s separately as we have not yet developed reliable assays for these in crude extracts. The results shown in Table II (line 1) show that the specific activities of T and G in the S-200 fraction were both lower in the cells with longer generation times. These results may have been a result of the selective inhibition by nucleases or proteases in the slower growing cells. The material was therefore analyzed through several steps of the purification, up to the step where the T and G factors were separated. It can also be seen in Table II that the relatively lower specific activities of T and G in the cells with longer generation times was preserved through these early steps of the purification. In fact, the relatively differences between two cultures became greater for the G factor. It is concluded that just as for ribosomes, the amount of T and G factors relative to other proteins decreases with increasing generation time.

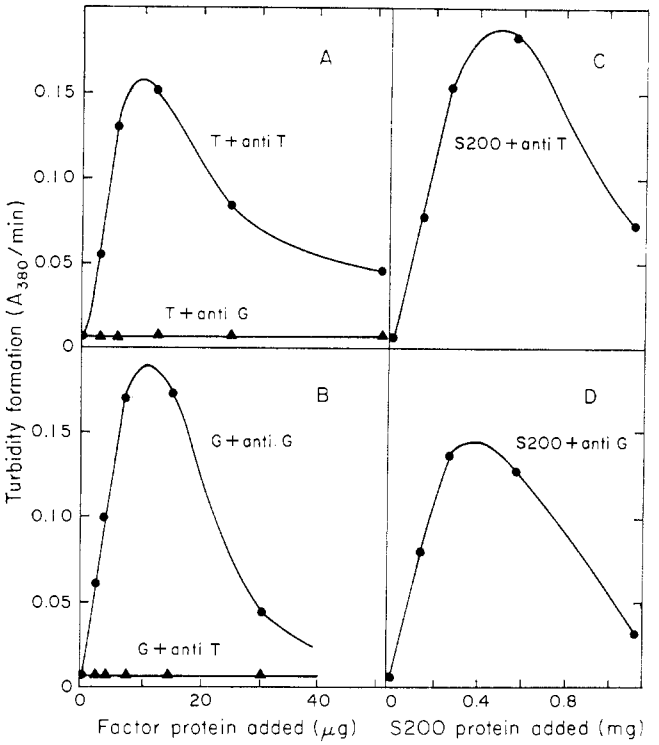

FIGURE 1: Immunochemical assays of the levels of T and G factors, A and B with purified factors, and C and D with S-200 fractions (cells grown in medium 5, Table II). Each assay contained either 100 μl of antiserum prepared against the T factor (A and C) or 200 μl of the anti-G factor serum (B and D). Each reaction mixture contained, in a total volume of 1 ml, the amount of protein indicated on the abscissae, and 0.15 M NaCl–0.01 M potassium phosphate buffer (pH 8.0) at 25°. At zero time the antiserum was added, and the absorbance increased was followed at 380 mμ in a Gilford recording spectrophotometer. Ordinates are the slope of the tangent at the origin of the traces obtained.

TABLE II: Specific Activity of T and G Factors from Cells Grown in Different Media.[a]

Fraction	Sp Act. (nmoles/10 min mg)			
	T (1)	T (3)	G (1)	G (3)
S-200	0.72	0.44	1.20	0.97
Polyethylene glycol phase	0.94	0.57	1.91	1.00
Ammonium sulfate fractions				
24.0% eluate	2.29	1.10		
19.6% eluate			6.65	2.98

[a] The S-200 fractions were from cells grown in the media indicated by numbers in parentheses (see Table I) and Preparation of Extracts. Further fractionation was carried out, scaled down 50 times from that described in detail elsewhere (Gordon, 1969). The steps were polyethylene glycol aqueous two-phase fractionation, followed by ammonium sulfate fractionation to separate the factors. The T factor was obtained free of G in the 24.0% eluate (w/w) ammonium sulfate eluate, and most of the G in the 19.6% eluate. The fractions indicated above were assayed for T and G activity in the presence of excess G or T factor, respectively, in the polyphenylalanine system, using standard assay methods (Gordon, 1969). The specific activities were then derived after measurement of the protein concentrations (see Assay Procedures).

Use of the polymerization assay of the G factor is preferable for these crude preparations since the high levels of nonspecific GTPase means that small increments must be measured. However, similar results were obtained with the use of the ribosome-dependent GTPase assays. The relatively smaller purification obtained through these steps (Table II) compared with data being published elsewhere (Gordon, 1969) is probably due to greater selective losses associated with the much smaller scale here. The data of Table I are based on measurements of cells in different media but harvested at a standard density. The same specific activities of factors were found in extracts from cells harvested at one-half and one-fourth of the standard density (data not shown). It is concluded that the important parameter is the rate of growth, and not the cell density, provided the cells are in logarithmic phase of growth.

Immunochemical Analysis. The above activity measurements indicated that, as for ribosomes, the proportion of T and G factors relative to S-200 proteins decreased with increasing generation time. Conclusions based on activities alone are unreliable due to unknown effects of inhibitors that may or may not be present in the crude extracts. In addition, the linearity of the assays with respect to extract concentration was consistently poor, so the values in Table II are only estimates. Leder *et al.* (1969) used antibody precipitation measurements as an alternative estimate of the abundance of G factor in crude extracts. We have independently followed similar lines. The principle of the assay used here is to find the amount of extract that contains a quantity of T or G equivalent to a standard volume of specific antiserum. The antisera were prepared as described in detail elsewhere (Gordon *et al.*, 1969) with highly purified preparations of T and G factors (homogeneous on analytical disc electrophoresis). The same preparation of factors was used to calibrate the assay system. The validity of the assay method was tested for crude material, and was verified by addition of increasing amounts of the purified T or G factor to a constant amount of S-200 fraction. The same values as in the absence of the S-200 fraction were obtained, after subtracting the contribution of the factor in the S-200 itself. The curves obtained with the pure factors are given in Figure 1C,D. The data of Figure 1A,B confirm that the T and G are free of cross-reacting antigens, as already shown elsewhere by immunodiffusion (Gordon *et al.*, 1969).

S-200 preparations were prepared from cells grown in different media, and each was analyzed for T and G factor content by the above method. It can be seen from the data in Figure 2 that the relative abundance of T and G factors decreased with increasing generation time of the culture, thus confirming the activity measurements. It can also be seen from Figure 2 that both factors represent a relatively high proportion of the supernatant protein. This is to be compared with the values of 2–3% of Leder *et al.* (1969) for the G factor, and of 2% for both T and G factors by Parmeggiani (1968). The slight differences between their data and those in Figure

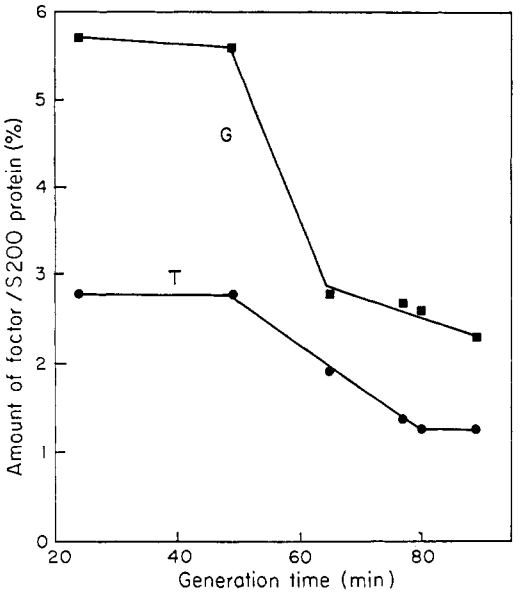

FIGURE 2: Relative amounts of T and G factors in the S-200 fraction of cells grown in different media. These were determined as described under Assay Procedures and in Figure 1, from the cells grown in the media in Table I. The ordinates represent the fraction of the total S-200 protein as T factor (●—●) or G factor (■—■).

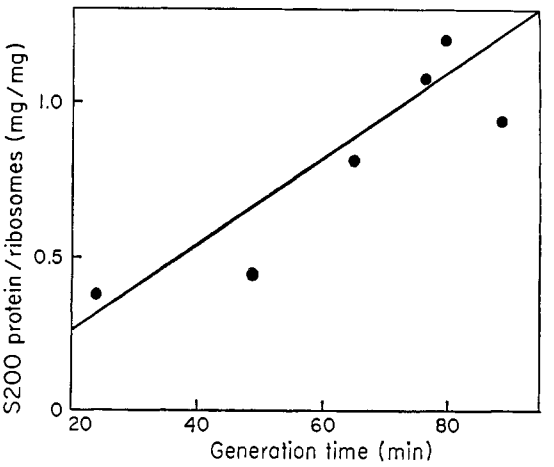

FIGURE 3: Relative amounts of S-200 protein and ribosomes in cells grown with different generation times. These amounts were determined from the same cells used in the analysis given in Figure 2. The protein was determined as hot trichloroacetic acid insoluble material in the supernatant of the 200,000*g* centrifugation, and the ribosomes were determined from 260-mμ absorbance of the sedimentable material (see Assay Methods).

2 may be due to different growth conditions, assay methods, and methods of preparation of the extracts.

The activity measurements of Table II, suggested that control of the synthesis of the T and G factors is coordinated with that of the ribosomes, since both decrease in abundance relative to the cellular proteins with increasing generation times. This possibility can be tested directly using the data of Figure 2. The ribosome contents of the cells analyzed in Figure 2 were estimated from the 260-mμ absorbancy of the material sedimenting at 200,000*g*. These data are given in Figure 3. The relative amount of S-200 protein to ribosomes increased with increasing generation time, which is consistent with the data of Kjeldgaard and Kurland (1963) and of Ecker and Schaechter (1963). It has been possible to estimate molar ratios of factors to ribosomes using values of 2.6×10^6 Daltons for the molecular weight of the 70S ribosome (Tissieres *et al.*, 1959) and 70,000 and 80,000 as approximate values for the T and G factors, respectively (Gottschalk and Parmeggiani, 1969; Leder *et al.*, 1969). The results are plotted in Figure 4. It can be seen that there was no systematic trend, and with the accuracy of the method, the ratio of both T and G factors to ribosomes was constant (±25%). The pelleted ribosomes were also analyzed for their T and G factor content by the same method. The relative contribution of the ribosome-associated material is shown (hatched area) in Figure 4. Although G factor is the most difficult to remove during washing, the amounts left in the crude ribosomal pellet were the same for T and G factors. The data in Figure 4 show that when the ribosomal contribution was taken into account, the amount of both T and G factors approximated to 1 mole/mole of ribosomes.

Electrophoretic Analysis. From the data of Figure 2 it is clear that both T and G factors should represent a sizeable fraction of the total S-200 proteins. This was seen by Leder *et al.* (1969) for the G factor, where it was observed that the factor corresponded to a major band in the polyacrylamide disc electrophoresis of the S-200 proteins. We have performed

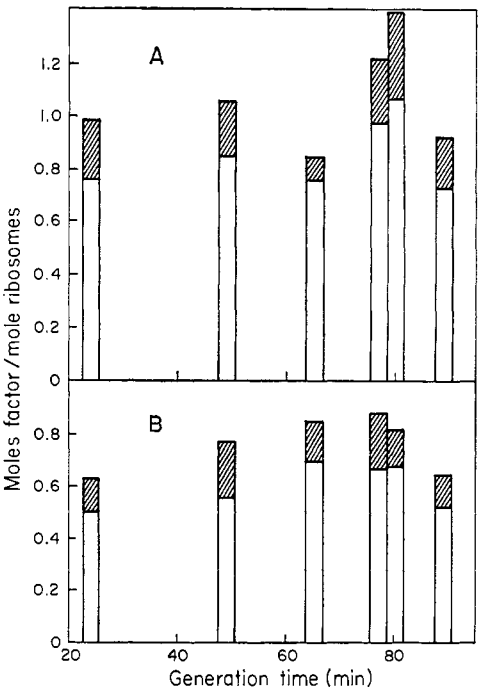

FIGURE 4: The relative amounts of (A) G and (B) T factors to ribosomes from cells grown in different media. The contribution of the factors in the S-200 fraction were compiled from the data of Figures 2 and 3, assuming the molecular weights given in the text (lower boxes). The ribosomal contributions were also determined with the immunochemical assay on the ribosomal pellets (hatched boxes).

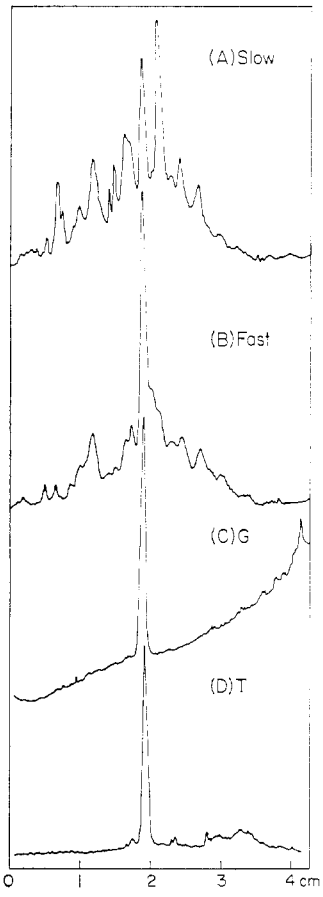

FIGURE 5: Electropherograms of S-200 proteins from cells grown with different generation times. Gels were run as described in Materials and Methods with (A) 20 μg of protein from cells grown in medium 4 of Table II and (B) 16 μg from cells grown in medium 1. Purified factors were run as reference: (C) 10 μg of purified G and (D) 6 μg of purified T factor. After running, the gels were stained with Amido-Schwartz, destained electrophoretically, and densitometered. The ordinate represents the densitometer trace. The abscissa represents distance from the top of the running gel.

a similar analysis of the S-200 fraction derived from cells with different generation times. The densitometer traces from the stained gels are shown in Figure 5. A major band was coincident with the positions of T and G factors (which were not resolved in this system, Figure 5C,D). This band was greatly decreased in intensity relative to the other S-200 proteins in the preparation from the cells with the longer generation time (Figure 5A), compared with the rapidly growing cells (Figure 5B). The strong protein band in the S-200 fraction was confirmed as overlapping T and G factors by slicing the gel and eluting protein from the slices; both activities coincided with this major protein band. This gives a third determination of the relative amounts of factors in bacterial cells growing in different steady states, and supports the data obtained by activity and the immunochemical estimations. Again, the conclusion is that the regulation of the synthesis of the polypeptide chain elongation factors is coordinated with that of the ribosomes and not with the other S-200 proteins.

Discussion

These studies were prompted by the observations by Parmeggiani (1968) that the T and G factors were obtained in homogeneous crystalline form after only 50-fold purification. Leder *et al.* (1969) also found the G factor to represent approximately 3% of the proteins by immunochemical methods. We have now found that the T and G factor content of the cell approximates to 1 mole/mole of ribosomes (Figure 4). Further, the ratio of T and G factor to ribosomes remained constant over the range of steady states of growth tested, whereas the relative amount of supernatant proteins varied over a threefold range (Figures 3 and 4).

The measurements in this work have been based on the ribosomal RNA content of the cells. Implicit in this treatment is the assumption that the ribosome behaves as a homogeneous entity whose composition is invariant with growth rate. It has been suggested recently that *E. coli* ribosomes are heterogeneous (Craven *et al.*, 1969; Traut *et al.*, 1969). However, recent experiments have failed to detect any variation in ribosomal proteins with growth rate (C. G. Kurland, personal communication).

The conclusion that the factors T and G are present in the cell in amounts in 1:1 stoichiometry with respect to the amount of ribosomes is tentative. The calculation depends on the following: the measurements of the amounts of the T and G factors, the calibration of the immunochemical assays with pure T and G factors, the molecular weights of the T and G factors, the assumption of homogeneity of the purified factors, the assumption that the ribosomes were the only contribution to the 260-mμ-absorbing material in the material pelleted at 200,000*g*, and the molecular weight of the ribosomes. Error in some of these would lead to systematic error in the determination. However, it is safe to conclude that the stoichiometry is 1:1 by order of magnitude.

The apparent coordinate control and 1:1 stoichiometry suggest the T and G factors as possible candidates for ribosomal proteins. This may seem especially likely for the G factor, which is well known to be difficult to remove quantitatively. The following points tend to argue against this: the large proportion of both T and G factors was found in the S-200 fraction (Figure 4); no protein band corresponding to G factor was seen in disc electrophoresis of ribosomal proteins (J. Gordon and C. G. Kurland, unpublished experiments); the G factor activity that remains on the ribosomes cannot be associated with any one subunit as it is quantitatively released when the ribosomes are dissociated into subunits at low Mg^{2+} concentration (J. Gordon and C. G. Kurland, unpublished experiments). It remains to be seen whether the T and G factors map at genetic loci associated with the ribosomal determinants.

Acknowledgments

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Nucleotide Pool Levels in Growing, Inhibited, and Transformed Chick Fibroblast Cells*

C. Colby† and G. Edlin‡

ABSTRACT: Nucleotide pool levels were measured in cultures of chick fibroblast cells during exponential growth, contact inhibition of growth, and after transformation by Rous sarcoma virus by labeling with $^{32}PO_4^{3-}$ of known specific activity. The final level of each ribonucleoside and deoxynucleoside triphosphate pool measured was similar under the different growth conditions. These results suggest that differences in the regulation of growth exhibited by these cultures cannot be explained by differences in nucleotide biosynthesis.

A variety of animal cells cultured *in vitro* has been shown to grow exponentially until a confluent monolayer is established whereupon growth slows dramatically or ceases. The phenomenon has been referred to as contact inhibition of growth (Abercrombie *et al.*, 1957; Todaro *et al.*, 1966), and seems to be the direct or indirect consequence of the establishment of intercellular contact. One explanation advanced to account for this inhibition of growth in dense cultures of animal cells is that the capacity to take up and incorporate essential nutrients from the medium is impaired once cells establish physical contact with one another. More specifically, it has been proposed that changes in the membrane structure of animal cells may be involved in the regulation of growth (Pardee, 1964; Sanford *et al.*, 1967).

Chick embryo fibroblast cells cultured in plastic petri dishes exhibit the characteristic inhibition of growth on establishment of a confluent monolayer of cells (Colby and Rubin, 1969; Gurney, 1969). In addition, transformation of such cells by Rous sarcoma virus infection releases the growth inhibition and allows the cells to continue to multiply even after a confluent monolayer has been established. In an earlier report (Colby and Rubin, 1969), the rate of nucleic acid synthesis was measured in dense monolayer and RSV[1] transformed cultures of chick cells by determining the incorporation of either radioactive thymidine or uridine into acid-precipitable material. The incorporation of these radioactive precursors was shown to decrease markedly during the course of the experiment, as did the acid-soluble pool of radioactive uridine. From these results it was suggested that RNA synthesis might be partially regulated by the levels of available precursors. However, it is not possible to draw conclusions concerning the actual rates of RNA or DNA synthesis from the incorporation of radioactive uridine or thymidine since the specific activities of the precursor pools are unknown. As a first step around these difficulties, chick embryo fibroblast cells were labeled with $^{32}PO_4$ in medium of known specific activity. This report presents the kinetics of labeling and actual levels of ribo- and deoxyribonucleotide pools under the different conditions of growth and in RSV transformed cells.

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[1] Abbreviation used is: RSV, Rous sarcoma virus.