Ecker, R. E., and Schaechter, M. (1963), *Ann. N. Y. Acad. Sci. 102*, 549.
Gordon, J. (1969), *J. Biol. Chem. 244*, 5680.
Gordon, J., Schweiger, M., Krisko, I., and Williams, C. (1969), *J. Bacteriol. 100*, 1.
Gottschalk, E. M., and Parmeggiani, A. (1969), *FEBS Abstracts*, 14.
Kjeldgaard, N. O., and Kurland, C. G. (1963), *J. Mol. Biol. 6*, 311.
Leder, P., Skogerson, L. E., and Nau, M. N. (1969), *Proc. Natl. Acad. Sci. U. S. 62*, 454.
Maaløe, O., and Kjeldgaard, N. O. (1966), Control of Macromolecular Biosynthesis, New York, N. Y., Benjamin, p 90.
Neidhart, F. C., and Magasanik, B. (1960), *Biochim. Biophys. Acta 42*, 99.
Nishizuka, Y., and Lipmann, F. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 212.
Ornstein, L., and Davis, B. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Parmeggiani, A. (1968), *Biochem. Biophys. Res. Commun. 30*, 613.
Schaechter, M., Maaløe, O., and Kjeldgaard, N. O. (1958), *J. Gen. Microbiol. 19*, 592.
Tissieres, A., Watson, J. D., Schlessinger, D., and Hollingworth, B. R. (1959), *J. Mol. Biol. 1*, 221.
Traut, R. R., Delius, H., Ahmad-Zadeh, C., Bickle, T. A., Pearson, P., and Tissieres, A. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 25.

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

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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.

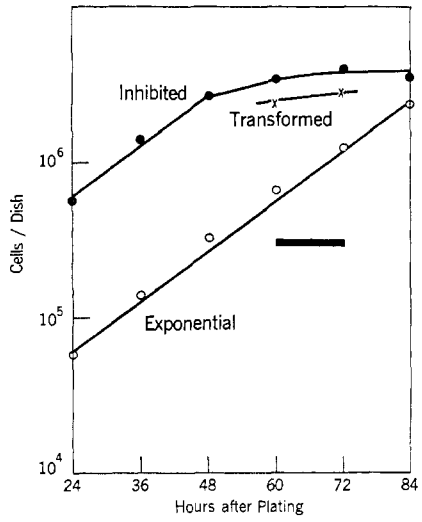

FIGURE 1: Growth kinetics of chick embryo fibroblast cells plated at different initial concentrations. The black bar shows the period during which cultures were labeled with $^{32}PO_4^{3-}$.

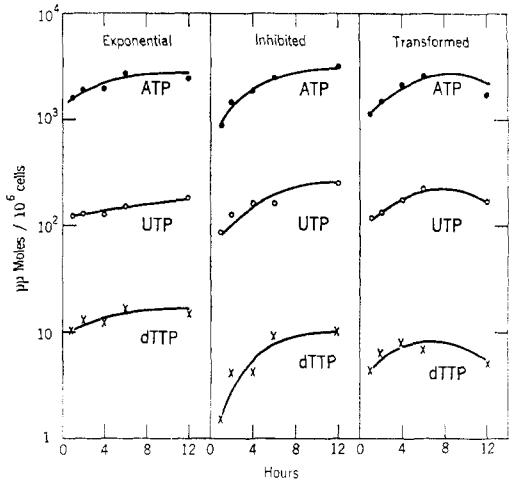

FIGURE 2: The kinetics of $^{32}PO_4^{3-}$ labeling of three nucleoside triphosphate pools in growing and growth-inhibited cultures of chick cells.

Methods and Materials

The preparation of chick embryo fibroblast cultures has been described in detail (Rein and Rubin, 1968). Cells were plated on 50 mm plastic petri dishes in Scherer's medium (Scherer, 1953) plus 2% tryptose phosphate broth, 1% calf serum, and 1% chicken serum. All experiments were done with primary cultures except for the RSV transformed cultures which were secondary cultures. Cell numbers were determined by resuspension with trypsin and enumeration in a Coulter Counter.

The acid-soluble nucleotide pools were measured by addition of $^{32}PO_4^{3-}$ to a series of petri dishes containing cultures of chick fibroblast cells attached to the surface. When necessary the medium was readjusted to pH 7.4 with sodium bicarbonate. At various times after exposure to $^{32}PO_4^{3-}$ the radioactive medium was aspirated off and the cells were washed quickly with 5 ml of cold 0.15 M NaCl–0.1 M Tris (pH 7.4). The wash buffer was aspirated off and the cells were covered with 1.5 ml of iced 0.4 M $HClO_4$ and allowed to stand at 4° for 30 min. $HClO_4$ and suspended cellular debris (1 ml) were removed and neutralized with 0.5 ml of 0.72 M KOH–0.16 M $KHCO_3$ (Bagnara and Finch, 1968). The precipitate and cellular debris were removed by centrifugation (7000 rpm for 10 min) and the supernatant solution was collected. This solution (100–200 μl) was spotted along with about 10 mμmoles of each marker nucleotide on 1% polyethyleneimine-cellulose thin-layer plates (Randerath and Randerath, 1966) and the nucleotides were resolved by two-dimensional chromatography (Neuhard *et al.*, 1965). Separation of the ribo- and deoxyribonucleoside triphosphates was done in the following solvents: first dimension: 2 M LiCl–2 M CH_3COOH to 3 cm above origin; transfer plate to 2.5 M LiCl–2 M CH_3COOH to 13 cm above origin. The plate was air dried and washed in 800 ml of anhydrous methanol. After drying the plate was developed perpendicular to the first dimension in 2.5 M CH_3COONH_4–5% H_3BO_3 (adjusted to pH 7.0 with NH_3) to 5 cm above the origin and then transferred to 3.5 M CH_3COONH_4–5% H_3BO_3 (adjusted to pH 7.0 with NH_3) to 13 cm above the origin. Separation of the ribonucleoside mono-, di-, and triphosphates was in the following solvents: first dimension: 0.2 M LiCl, 2 min; 1.0 M LiCl, 6 min; 1.6 M LiCl to 13 cm above origin. The plate was dried and washed in methanol. It was then run perpendicular to the first dimension in 0.5 M HCOOH–0.5 M NaCOOH (pH 3.4) for 0.5 min; 2 M HCOOH–2 M NaCOOH (pH 3.4) for 2 min; 4 M HCOOH–4 M NaCOOH (pH 3.4) to 13 cm above the origin. This solvent system does not separate the deoxyribo- from the corresponding ribonucleotides but since the deoxynucleotide pools are only a few per cent of the total pools, the error in the ribonucleotide pool determinations is negligible.

After chromatography the marker nucleotides were localized under ultraviolet light and a Kodak Medical X-Ray film is placed over the plate for 15–20 hr. This was developed and the radioactive nucleotides were identified by correspondance with the marker nucleotides. The radioactive spots were cut out, glued to planchets, and counted in a Nuclear-Chicago gas-flow counter. The counts were converted into molar amounts by calculation of the phosphate concentration of the growth medium (1.13 mM) and by counting a dilution of the radioactive medium. The only assumption in this calculation is that the specific activity of inorganic phosphate inside the cell reflects the specific activity of the phosphate in the medium.

Results

Chick cells were seeded at two different concentrations such that the initial cell densities differed by a factor of ten. Figure 1 represents the growth kinetics of the two cultures. The cells seeded at low density grow exponentially for more than 3 days while the rate of growth of cells plated at high density decreases markedly after 48 hr. RSV-transformed cells were also seeded at high density and the cell number was measured before and after the labeling period. In addition a sample of the medium was removed from several dishes and assayed for RSV. It was shown that these cultures were actively producing virus.

TABLE I: Kinetics of Labeling Nucleotide Pools in Exponential, Inhibited, and Transformed Chick Embryo Fibroblast Cultures.

Nucleotide	Hours of Label 1	2	4	6	12	24
Exponential						
ATP[a]	1590[a]	1890	1910	2680	2390	1730
CTP	49	53	48	61	75	64
GTP	160	190	170	250	220	160
UTP	120	130	130	150	180	170
dATP[b]	11	12	12	17	19	14
dCTP	4.7	7.1	7.1	9.4	8.4	7.6
dGTP	5.7	5.3	6.0	8.7	6.1	4.2
dTTP	11	13	12	17	15	14
AMP		73	92	81	130	98
CMP		10	30	22		18
UMP		230	210	290	270	180
ADP		390	430	580	520	400
UDP		29	27	29	34	28
Inhibited						
ATP	880	1430	1840	2460	3090	3130
CTP	28	39	55	65	110	130
GTP	71	110	150	200	250	260
UTP	85	120	160	160	250	300
dATP	2.6	4.7	7.3	7.1	13	9.8
dCTP	1.0	1.9	2.8	5.1	5.8	4.8
dGTP	1.3	2.0	2.9	4.7	5.1	6.0
dTTP	1.5	4.2	4.3	8.9	9.8	5.8
AMP		16	61	55	87	
CMP		1.8	13	18	20	26
UMP		290	380	330	300	290
ADP		140	330	430	450	410
UDP		11	24	27	37	37
Transformed						
ATP	1120	1470	2190	2610	1410	
CTP	45	47	50	77	71	
GTP	120	140	190	100	120	
UTP	120	140	180	230	150	
dATP	15	22	23	35		
dCTP	3.4	6.0	5.8	7.7	3.5	
dGTP	3.3	7.0	7.1	5.8	3.4	
dTTP	4.7	6.7	8.0	7.0	3.4	
AMP		13	43	67	64	
CMP		3		6.2	10.2	
UMP		29	82	130	100	
ADP		150	240	400	280	
UDP		10	21	31	16	

[a] Values are moles $\times 10^{-12}/10^6$ cells and are the average of two determinations of acid-soluble nucleotides from single petri dishes. [b] Abbreviations used are: dATP, dCTP, dGTP, dTTP are the deoxynucleotide triphosphates.

All three cultures were labeled with $^{32}PO_4^{3-}$ over the time interval indicated by the bar in Figure 1. The kinetics of labeling of ATP, UTP, and dTTP are shown in Figure 2.

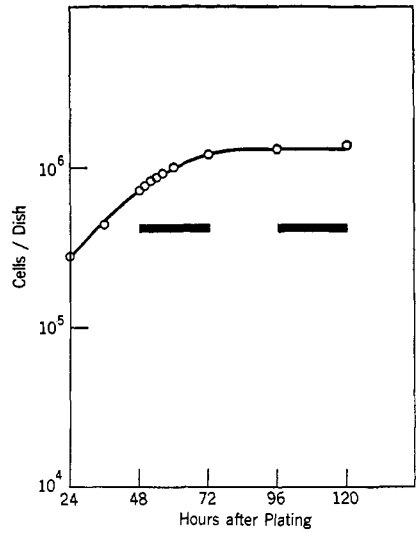

FIGURE 3: Growth kinetics of chick embryo fibroblast cells plated at a low cell density. The black bars show the two periods during which cultures were labeled with $^{32}PO_4^{3-}$.

The results show that the final pool levels are comparable in all three cultures. The decrease in pool levels in the RSV-transformed culture at later times may be due to cell lysis or to detachment of cells from the dish during removal of the medium. Table I gives the values for the other nucleotide pools measured in the same experiment described in Figure 2. It is evident from Table I that the ribonucleoside triphosphate pool levels are comparable in all cultures.

In general, the deoxynucleoside triphosphate pool levels seem to be lower in the growth inhibited culture as compared with the levels measured during exponential growth. However, some caution is warranted in the interpretation of these differences because of the variability in the deoxynucleoside triphosphate determinations. The total deoxynucleoside triphosphate pool is only about 1% of the ribonucleoside triphosphate pool which makes accurate quantitation difficult since all eight triphosphate pools are separated on the same chromatogram. However, from the kinetics of labeling it is clear that a short pulse of radioactivity (1 hr or less) would show a marked difference in incorporation between the exponential and growth-inhibited cultures and the difference would appear to be greatest for the deoxynucleoside triphosphates. It should be noted that these pool levels reflect the sum of both endogeneous synthesis and exogeneous uptake. Regardless of the path of nucleotide synthesis, it is apparent that the cessation of cell division in the growth-inhibited culture can not be due to a lack of available energy (ATP) or nucleotide precursors of RNA and DNA (UTP, dTTP). Thus, if in fact, the synthesis of RNA and DNA is reduced upon establishment of a confluent monolayer of cells, it would seem to result from a more specific regulation rather than to a general metabolic deficiency or from a lack of an essential growth factor other than nucleotides.

It might be argued that the plating of dense cell cultures to produce the growth inhibited state does not allow the usual changes in the membrane of the cells to occur, or that the cells do not recover from the trypsinization and seeding procedure, or that the medium is more rapidly depleted by

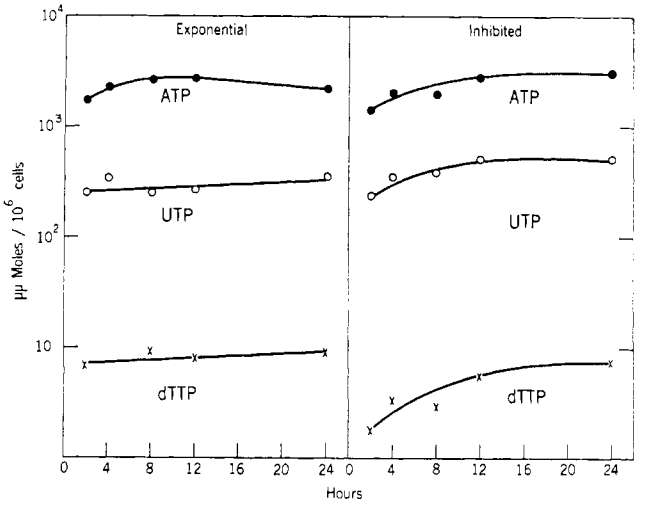

FIGURE 4: The kinetics of $^{32}PO_4^{3-}$ labeling of three nucleoside triphosphate pools in the same culture during exponential growth and after inhibition of growth.

dense cultures. To test these possibilities, cells were plated at low density and allowed to grow to a confluent monolayer.

Figure 3 represents the kinetics of growth of chick embryo fibroblast cells seeded at a low cell density. It shows that after a period of exponential growth cell number ceases to increase once a confluent monolayer of cells has been established. As shown in a previous report (Colby and Rubin, 1969), the incorporation of radioactive uridine and thymidine into RNA and DNA, respectively, decreases during the onset of growth inhibition. Figure 4 shows the kinetics of $^{32}PO_4^{3-}$ labeling of the pools of ATP, UTP, and dTTP in the same culture during exponential growth and after inhibition of growth. The periods of labeling were from 48 to 72 hr and from 96 to 120 hr after plating of the cells. During exponential growth the nucleotide pools become fully labeled within a few hours. In the growth-inhibited cultures the pools appear to be labeled more slowly but the final levels are as high or higher than in the exponential culture.

The pool of ATP is 10 times larger than the pool of UTP and more than 100 times larger than the pool of dTTP. Considering the quantity of DNA that must be synthesized in these cells per generation it is somewhat surprising to find such low levels of precursor deoxynucleoside triphosphates. The lack of a large DNA precursor pool may indicate that the synthesis of both DNA and of the precursor nucleotides are localized within the cell, perhaps on the membrane.

Discussion

The data reported here show that the pool levels of nucleoside triphosphates are essentially the same in cultures of chick embryo fibroblasts during exponential growth, contact inhibition of growth, or after transformation by RSV infection. The similarity in the levels of ATP argue that under all these conditions growth is not limited by the availability of energy. The availability of ribo- and deoxyribonucleoside triphosphates implies that, whatever the differences in the rates of RNA and DNA synthesis between the various cultures, the differences are not caused by a lack of nucleotide precursors although the lower levels of deoxynucleoside triphosphates in the growth-inhibited culture may be related to an actual reduction in DNA synthesis. This interpretation depends on the assumption that the $^{32}PO_4^{3-}$ in the medium equilibrates readily with the inorganic phosphate in the cell. This assumption seems justified in light of the plateau values reached by the pools after a few hours of labeling. Experiments in which cells have been labeled for as long as 5 days show no increase over the values obtained by labeling for 4–6 hr.

The kinetics of $^{32}PO_4^{3-}$ labeling suggest that the rate of nucleotide synthesis or uptake of phosphate from the medium might be slower in growth inhibited as compared with exponentially growing cultures. However, due to the paucity of points at the early incorporation times and the variability of the data at these low values, additional experiments (in progress) are necessary to substantiate the differences in kinetics.

It seems unlikely, however, that changes in cell permeability which affect the synthesis or uptake of nutrients involved in nucleotide biosynthesis can account for the regulation of growth by normal cells or the lack of such regulation exhibited by RSV-transformed cells. A similar conclusion concerning protein synthesis was reached by Foster and Pardee (1969) based on measurements of uptake of amino acids into growing and contact-inhibited cultures of mouse 3T3 cells. These results do not exclude the possibility that there may be growth-related permeability changes which do affect, perhaps, ion transport or uptake of other growth essential nutrients.

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References

Abercrombie, M., Heaysman, J. E. M., and Karthauser, H. M. (1957), *Exp. Cell Res. 13*, 276.

Bagnara, A. S., and Finch, L. R. (1968), *Biochem. Biophys. Res. Commun. 33*, 15.

Colby, C., and Rubin, H. (1969), *J. Nat. Cancer Inst. 43*, 437.

Foster, D. O., and Pardee, A. B. (1969), *J. Biol. Chem. 244*, 2675.

Gurney, T. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 906.

Neuhard, J., Randerath, E., and Randerath, K. (1965), *Anal. Biochem. 13*, 211.

Pardee, A. B. (1964), *Nat. Cancer Inst. Monogr. 14*, 7.

Randerath, K., and Randerath, E. (1966), *J. Chromatog. 22*, 110.

Rein, A., and Rubin, H. (1968), *Exp. Cell Res. 49*, 666.

Sanford, K. K., Barker, B. E., Woods, M. W., Parshad, R., and Law, L. W. (1967), *J. Nat. Cancer Inst. 39*, 705.

Scherer, W. (1953), *Amer. J. Pathol. 29*, 113.

Todaro, G. T., Lazar, G. K., and Green, H. (1966), *J. Cell Comp. Physiol. 66*, 325.