STUDIES ON A MUTANT OF *ESCHERICHIA COLI* WITH UNBALANCED RIBONUCLEIC ACID SYNTHESIS

II. The Concomitance of Ribonucleic Acid Synthesis with Resumed Protein Synthesis[1]

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A role of an intermediary agent in protein synthesis has been postulated for ribonucleic acid (RNA) (Brachet, 1955). However, the evidence for this hypothesis is still largely circumstantial and the details of the mechanism are unknown at the present time. In our studies on the lysogenic strain, *Escherichia coli* strain K_{12}, we encountered an anomalous mutant which lends itself to a study of a possible relationship between RNA metabolism and protein synthesis.

The auxotroph *E. coli* (K_{12} W-6) accumulates RNA during starvation of an essential nutrient, methionine (Borek *et al.*, 1955). The RNA enriched organisms were found to be uniquely delayed in resuming protein synthesis after the restoration of the lacking amino acid (Borek *et al.*, 1956). Moreover, such organisms are incapable of supporting the replication of infectious phage (Borek and Rockenbach, 1956 *unpublished experiments*). It seemed of interest to study the metabolism of RNA in these organisms before and at the time of the resumption of protein synthesis to ascertain whether any correlation between RNA metabolism and protein synthesis could be detected.

It was found that the RNA accumulated on methionine starvation is stable after the restoration of the amino acid (recovery) in the sense that no extensive degradation of the polymer or excretion of RNA fragments could be detected. During the phase of recovery coinciding with the resumption of protein synthesis, significant net synthesis of RNA was observed. Such new synthesis in a bacterial cell which already contains more than its normal complement of RNA is of interest since it may point to the existence of different forms of the nucleic acid with different functional capacities.

Evidence for the nonhomogeneity of RNA in terms of metabolic potential in microorganisms infected by bacteriophage has been previously observed (Volkin and Astrachen, 1956).

While our studies were in progress two reports have appeared on the metabolism of RNA accumulated as a result of exposing bacteria to chloramphenicol (Gros and Gros, 1956; Hahn and Schaechter, 1957). Such nucleic acid enriched organisms also showed a delay in protein synthesis. However, there was a profound difference in the stability of the accumulated RNA in these organisms and in the methionine-starved auxotroph. Neidhart and Gros (1957), who permitted us to see this work prior to publication, have shown that a major portion of the RNA accumulated in presence of chloramphenicol is depolymerized and excreted from the bacterial cell during the period of recovery from the drug.

MATERIALS

The organism, the method of culture, and the method of starvation were described previously (Borek *et al.*, 1955).

P^{32} was used in the form of neutralized phosphate. Since the organisms used are lysogenic, the dosage of radioactivity to which they were exposed was carefully controlled. Organisms in logarithmic growth phase can tolerate 1 μc of P^{32} per ml of a culture containing 2×10^8 cells without showing any measurable induction. In starved cultures, which are inapt (Lwoff, 1953), a dose of 5 μc of P^{32} had no measureable effect on the viable cell count. The $P^{32}O_4^{\equiv}$ was added to the regular medium (Gray and Tatum, 1944) which is 0.0245 molar with respect to $PO_4^{\equiv}$.

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Adenine-8-C^{14}, obtained from the Schwartz Laboratories, Mount Vernon, N. Y., was used as a level of 50 μc per L of culture medium.

METHODS

We had found in our earlier experiments that the organisms enriched with RNA are unable to synthesize any protein during the first 45 min of recovery after the restoration of methionine (Borek *et al.*, 1956). During the second period, i. e., up to 90 min, protein synthesis commences, provided the organisms are receiving a carbon source to which they are adapted. Therefore, in these studies the metabolism of RNA was separately studied during the first and the second 45 min period of the recovery.

Two different types of RNA labeling were carried out. In one set of experiments the RNA accumulating during methionine starvation was labeled by the addition of P^{32} (5 mc) and adenine 8 C^{14} (100 μc) to 2 L of culture containing 3 to 5 $\times$ 10^8 cells/ml. After 3 hr of incubation in the absence of methionine, the culture was centrifuged, the cells were washed two times with medium lacking methionine and were resuspended to 2 L of medium at 37 C containing methionine. Perchloric acid (3.5 ml of 70 per cent per 100 ml of culture), was added to aliquots of the culture taken at zero time and after 45 and 90 min of incubation.

In another set of experiments the RNA synthesized during recovery was labeled. A 2-L culture of *E. coli* strain K_{12} W_6 containing about 3 $\times$ 10^8 cells per ml was starved of methionine for 3 hr, the culture was centrifuged, the cells were washed once with medium lacking methionine and were resuspended to the original volume in complete medium at 37 C and 5 mc of P^{32} were added. Perchloric acid (as above), was added to aliquots at zero time and after 45 and 90 min of incubation.

For assessing the extent of RNA labeling specific activities were determined on chromatographically separated, eluted nucleotides obtained by the hydrolysis of the isolated RNA. For the isolation of the RNA, its hydrolysis and subsequent treatment, the procedure described by Price *et al.* (1954)[3] was followed. The perchloric acid treated cultures were kept in the icebox overnight, and were centrifuged. The precipitate was washed copiously with absolute alcohol and finally with ether. The dry powder (1.2 g from 600 ml of culture) was extracted with 10 vol of 10 per cent LiCl solution at 95 C for 1 hr. The filtered extract was mixed with twice its volume of absolute ethanol and the mixture was kept in an icebox overnight. The precipitated nucleic acids were centrifuged and the precipitate was washed once with 95 per cent alcohol. To the residue 2 ml (40 mg) of 0.3 N LiOH was added and the solution was kept at 37 C for 17 hr. Saturated K_3PO_4 equivalent to 90 per cent of the Li^+ present was added to the alkaline solution and the Li_3PO_4 was allowed to precipitate during 1 hr at room temperature. The precipitate was centrifuged, the supernatant fluid was decanted and its pH was adjusted to 7.0 with 4.0 N $HClO_4$. After a few minutes the precipitated $KClO_4$ was removed by centrifugation. The clear fluid was acidified to pH 3 with $HClO_4$ and the solution was chilled in an ice bath and mixed with twice its volume of cold absolute ethanol. After a few minutes the precipitated deoxyribonucleic acid (DNA) was centrifuged and the supernatant fluid was neutralized with excess solid NH_4HCO_3.

The chromatography of the RNA nucleotides was performed in two different sets of solvents. For the resolution of adenylic acid the mixed nucleotides were chromatographed in a mixture of 5 parts of isobutyric acid and 3 parts of 0.5 N NH_4OH (Magasanik *et al.*, 1950).

For the separation of guanylic acid, the mixed nucleotides were chromatographed in 70 per cent aqueous isopropanol with 0.35 ml of conc NH_4OH added for each L of gas space (Markham and Smith, 1952). The effectiveness of the chromatographic separation was kindly analyzed for us by Dr. Price who determined the coincidence of radioactivity with the ultraviolet absorbing material on some of our chromatograms by the scanning technique described by him (Price and Hudson, 1955). The appropriate areas containing the various nucleotides were cut out and extracted, and specific activity was determined by measuring absorption at 260 mμ and determining radioactivity on aliquots evaporated on planchets.

Specific activities with respect to C^{14} and P^{32} were determined on the same planchet by first obtaining total counts, then P^{32} counts by shielding the window of the Geiger tube with a

[3] We are indebted to Dr. Price for giving us the details of his analytical procedure prior to publication.

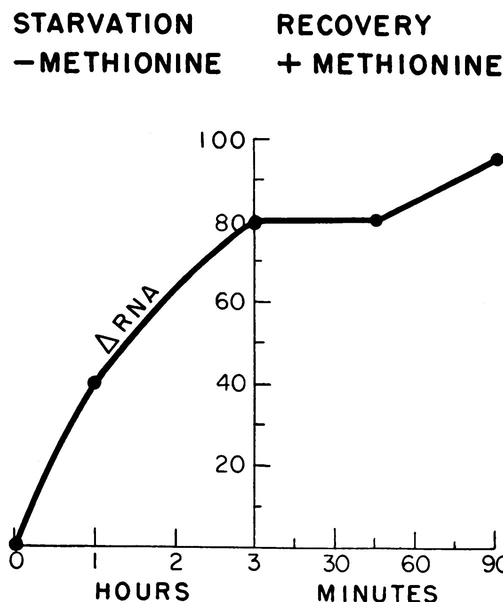

Figure 1. Ribonucleic acid (RNA) levels in *Escherichia coli* strain K_{12} W-6 during starvation of methionine and during subsequent recovery after the restoration of the amino acid. The values represent per cent changes from the level of RNA phosphorus of the organisms in logarithmic growth phase, which is 130 $\mu g/10^{10}$ cells.

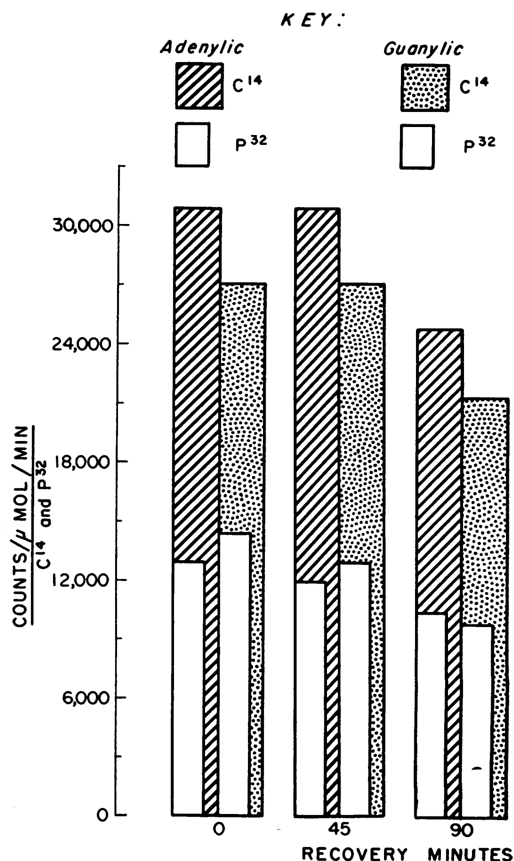

Figure 2. The stability during recovery of ribonucleic acid accumulated during starvation of methionine.

The bar graphs represent specific activities of adenylic and guanylic acids—with respect to both C^{14} and P^{32}—at the end of the starvation and 45 and 90 min after the start of the recovery.

single layer of "Parafilm." It was found that such a shield eliminates virtually all the counts produced by C^{14} and reduces the counts originating from P^{32} by 20 per cent. Appropriate corrections were applied for this screening as well as for the known rate of disintegration of P^{32}.

Total RNA per bacterial cell was measured as previously described (Borek *et al.*, 1955).

RESULTS

In figure 1 the changes in RNA level per bacterial cell during the course of starvation of methionine and during the course of recovery, after the restoration of methionine, are presented. It should be emphasized that no cell division takes place either during the period of starvation or during the first 90 min of recovery.

The RNA level per cell increases during the 3 hr period of starvation about 80 per cent over the normal RNA content of these organisms when they are in exponential phase of growth. During the first 45 min of recovery in presence of methionine there is no net change in the RNA level; however, during the next 45 min there is a further increase of 15 per cent, yielding organisms with nearly double their complement of the nucleic acid. It is during this phase of the recovery that new protein synthesis is measurable.

Confirmation of the extent of new RNA synthesis during recovery was obtained from experiments in which the RNA was labeled from the start of the recovery. Typical experimental findings are as follows: In one experiment the counts from P^{32} added at the start of the recovery were 9.5×10^4 per μmole of $PO_4^{\equiv}$ per min. The counts per μmole of adenylic acid phosphate found were 1×10^3 and 1.2×10^4 after 45 and 90 min of recovery respectively, corresponding to 1 per cent and 15 per cent increase in the RNA.

In figure 2 data are presented which describe the fate, during the recovery phase, of previously labeled, accumulated RNA. The increase in RNA during starvation, calculated from the specific activity with respect to P^{32} in the isolated nucleotides was, in this case, 65 per cent. The data given in the bar graphs are specific counts from C^{14} and P^{32} in RNA adenylic and guanylic acids. It is apparent that there is no significant decrease in specific activity during the first 45 min of recovery. The decrease in specific activity of the two nucleotides at the 90 min period represents not a loss in total counts—and of RNA—but merely a dilution produced by the RNA newly formed from unlabeled precursors.

DISCUSSION

The accumulation of RNA in *E. coli* strain K_{12} W-6 is an anomalous phenomenon. It is the only auxotroph of several investigated (Pardee and Prestidge, 1956) which behaves in this manner upon the starvation of its requisite amino acid.[4]

One must be cautious in drawing conclusions from so restricted a phenomenon. However, since, in the past, abnormal metabolism has often proved to be a mere exaggeration of the normal, it may be hoped that a study of the RNA metabolism in this mutant might yield some more generalized information on the relationship between RNA metabolism and protein synthesis.

It is apparent both from figures 1 and 2 that the RNA accumulated during the methionine starvation is stable even after the organisms are returned to their complete medium. The stability is evident both from the analytical data of the level of RNA during recovery and from the lack of loss of labeled RNA nucleotides which had polymerized during starvation.

Such stability is in complete contrast to the behavior of the RNA accumulated in microorganisms as a result of exposure to the drug chloramphenicol. In the latter case the RNA is depolymerized and largely excreted when the drug is removed from the culture medium. A variety of causes could account for this striking difference in stability. For example, one fundamental difference in the condition of the two organisms is the amount of DNA in each. The organisms exposed to chloramphenicol apparently accumulate both RNA and DNA whereas in the methionine auxotroph DNA accumulation is negligible. Moreover, the extent of unbalanced synthesis is much greater in the organisms treated with the drug: Increases of as much as 300 per cent in RNA have been reported. However, an understanding of the critical difference which yields the RNAs with such unequal stabilities must await the elucidation of the mechanism of action of the drug.

In view of the stability of the accumulated RNA in the organisms starved of methionine the synthesis of more RNA during the second 45 min period of recovery is of particular interest. Apparently the RNA accumulated during starvation is inadequate for some reason. In composition the accumulated RNA differs neither qualitatively nor quantitatively from the RNA extracted from the organism in exponential growth phase (Borek *et al.*, 1957). The inadequacy of the accumulated RNA may stem from a variety of causes. It is possible that for RNA to be functional for protein synthesis—if, indeed, it does play such a role—it must be synthesized *de novo* for a specific task. Part of the RNA found in any cell at a given time may be the spent, inactive form, the components of which serve merely as the precursors of fresh, newly formed, functional RNA, for it is apparent from these studies that the controlling factor for protein synthesis cannot be the absolute amount of RNA in a cell but rather, the cell's ability to synthesize new RNA. The inadequacy of the accumulated RNA may also possibly be due to chemical or physical differences from functional RNA. Finally, the underlying cause may be morphological; the accumulated RNA may be deposited in areas beyond the periphery of functional sites.

[4] The only other mutant which approximates this phenomenon is a leucine auxotroph of *Escherichia coli* strain K_{12} isolated by Goldstein *et al.* (1957), which upon starvation of leucine accumulates large amounts of nucleotides but not the polymer. It may be significant that both of these mutants have been derived from the same strain of *E. coli*, i. e., K_{12}, which is lysogenic.

SUMMARY

Escherichia coli strain K_{12} W-6 accumulates ribonucleic acid (RNA) during starvation of methionine and then shows a long lag before protein synthesis is resumed after the restoration of the lacking amino acid. The RNA in these

cells, unlike RNA accumulated as a result of exposure to the drug chloramphenicol is not degraded during the early stages of recovery. The RNA enriched organisms synthesize new RNA during the phase of recovery which coincides with the synthesis of new protein.

REFERENCES

BOREK, E., RYAN, A., AND ROCKENBACH, J. 1955 Nucleic acid metabolism in relation to the lysogenic phenomenon. J. Bacteriol., **69,** 460–467.

BOREK, E., ROCKENBACH, J., AND RYAN, A. 1956 Studies on a mutant of *Escherichia coli* with unbalanced ribonucleic acid synthesis. J. Bacteriol., **71,** 318–323.

BOREK, E., RYAN, A., AND PRICE, T. D. 1957 Relation of RNA metabolism to protein synthesis. Federation Proc., **16,** 1561.

BRACHET, J. 1955 The biological role of the pentose nucleic acids. In *The nucleic acids*, Vol. II. Academic Press, Inc., New York.

GOLDSTEIN, D. B., GOLDSTEIN, A., AND BROWN, B. J. 1957 Accumulation of acid-soluble nucleotide compounds in an *E. coli* mutant deprived of an essential amino acid. Federation Proc., **16,** 300.

GRAY, C. H. AND TATUM, E. L. 1944 X-Ray induced growth factor requirements in bacteria. Proc. Natl. Acad. Sci. U. S., **30,** 404–410.

GROS, F. AND GROS, F. 1956 Role des aminoacides dans la synthese des acides nucleiques chez *Escherichia coli*. Biochim. et Biophys. Acta, **22,** 200–201.

HAHN, F. E. AND SCHAECHTER, M. 1957 Studies on the interrelations between nucleic acid and protein biosynthesis in *Escherichia coli*. Bacteriol. Proc., **1957,** 138–139.

LWOFF, A. 1953 Lysogeny. Bacteriol. Revs., **17,** 269–337.

MAGASANIK, B., VISCHER, E., DONIGER, R., ELSON, D., AND CHARGAFF, E. 1950 The separation and estimation of ribonucleotides in minute quantities. J. Biol. Chem., **186,** 37–50.

MARKHAM, R. AND SMITH, J. D. 1952 The structure of ribonucleic acids. 2. The smaller products of ribonuclease digestion. Biochem. J. (London), **52,** 558–565.

NEIDHART, F. AND GROS, F. 1957 Metabolic instability of the ribonucleic acid synthesized by *Escherichia coli* in the presence of chloromycetin. Biochim. et Biophys. Acta, **25,** 513–520.

PARDEE, A. B. AND PRESTIDGE, L. S. 1956 The dependence of nucleic acid syntheses on the presence of amino acids in *Escherichia coli*. J. Bacteriol., **71,** 677–683.

PRICE, T. D., HUDSON, P. B., EISLER, L., AND FUTTER, I. 1954 Incorporation of P^{32} into mononucleotide and polynucleotide components of pentose nucleic acid. Federation Proc., **13,** 276.

PRICE, T. D. AND HUDSON, P. B. 1955 Specific-activity determination with beta and UV-photon counter. Nucleonics, **13,** 54–58.

VOLKIN, E. AND ASTRACHEN, L. 1956 Intracellular distribution of labelled ribonucleic acid after phage infection of *Escherichia coli*. Virology, **2,** 433–437.