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*THE SYNTHESIS OF MESSENGER RNA WITHOUT PROTEIN SYNTHESIS IN NORMAL AND PHAGE-INFECTED THYMINELESS STRAINS OF ESCHERICHIA COLI**

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Communicated by Britton Chance, March 28, 1963

During the study of strains of *E. coli* deficient in the ability to synthesize thymine, uracil, and an amino acid, we compared nucleic acid syntheses in variously deficient cultures. We observed a slight but significant incorporation of uracil-2-C^{14} into RNA in the absence of thymine and the essential amino acid. This incorporation, amounting to about 10% of that in the presence of the amino acid, was, in fact, markedly inhibited in the presence of thymine. We therefore supposed that the meager RNA synthesis was that of messenger RNA, which was affected by a competition between DNA and RNA synthesis for a common template, presumably that of DNA, at the bacterial chromosome. We have therefore continued to investigate this phenomenon in both normal and phage-infected bacteria and have demonstrated that messenger RNA can indeed be synthesized without concomitant protein synthesis. This is particularly clear with phage-infected bacteria.

Materials and Methods.—Uracil-2-C^{14} was obtained from the California Corporation for Biochemical Research. Formaldehyde-C^{14} was purchased from Isotope Specialties Company and was standardized by the procedure of Macfayden.[1] Tetrahydrofolic acid was synthesized.[2]

Two polyauxotrophic mutants of *E. coli* strain 15 were used in these experiments. Strain TAU which requires thymine, arginine, and uracil for its growth has been described in earlier papers from this laboratory.[3, 4] Strain THU is a newly-isolated mutant which is sensitive to many phages, including $T6r^{+5}$, and requires thymine, histidine, and uracil. Strain THU was isolated from a colicine-resistant (CR) strain of $15H^{-}T^{-}$,[6] which was furnished by Dr. T. Okada of Kanazawa University, Japan. Selection of the uracil-deficient strain was effected after four cycles of ultraviolet irradiation followed by penicillin screening according to the procedure of Lubin.[7]

Bacteria were grown in a synthetic medium[8] containing mineral salts and 1 gm of glucose per liter. For the growth of THU this medium was supplemented with 20 μg of L-histidine, 10 μg of uracil,

and 2 μg of thymine per ml. In the case of TAU, L-arginine (20 μg per ml) was added in place of histidine. The mass doubling time of THU and TAU in these media are 110 and 70 min respectively. The cells were cultivated aerobically at 37°, harvested at 5×10^8 cells per ml, and washed once. In most experiments, cells were starved for amino acid and/or uracil by incubating for 15 min prior to infection in the medium lacking the required compounds. Infection was performed in the medium supplemented with 3 mg of glucose and 50 μg of DL-tryptophan per ml.[9]

Incorporation of uracil-C^{14} was determined with the use of Millipore filters. The radioactivity of DNA was measured on samples incubated with 0.3 *N* KOH at 37°C for 20 hr and acidified prior to filtration. The radioactivity of RNA was estimated by subtracting the value for the alkali-treated sample from that for the untreated acid-precipitated sample.

Extracts, prepared from cells ground with alumina, were made in a buffer containing 0.05 *M* tris, pH 7.5, and 0.01 *M* mercaptoethanol (5 ml per 10^{11} cells). The dCMP hydroxymethylase was assayed by the method of Pizer and Cohen.[10] The assay for thymidylate synthetase was that of Wahba and Friedkin.[11, 12] The activity of lysozyme was determined by the method of Weidel and Katz.[13] The chloroform-treated cells used as substrate of lysozyme were derived from THU.

Ribosomes were extracted from alumina-ground cells by 0.05 *M* Tris, pH 7.5–0.005 *M* $MgCl_2$, and purified by differential centrifugation.[14] RNA was isolated from the ribosomal fraction by the Duponol-phenol method.[15] Aliquots containing about 0.5 mg of RNA were run in a starch bed[16] (30 cm × 3.8 cm) containing 0.03 *M* Tris buffer, pH 7.8, for 36 hr at 4° at 150 volts and 5 mA. After the run the starch was cut into 0.5 cm sections and each section was eluted with 3 ml of distilled water. Both absorbancy at 260 mμ and radioactivity were measured on each eluate. Protein was determined by the method of Lowry *et al.*[17]

Results.—RNA synthesis in the absence of the amino acid: When *E. coli* strain TAU, harvested during the exponential phase of growth, is incubated with arginine and uracil-C^{14}, a rapid incorporation of uracil into an acid-insoluble fraction is observed, as can be seen in Figure 1. In media lacking arginine, however, the

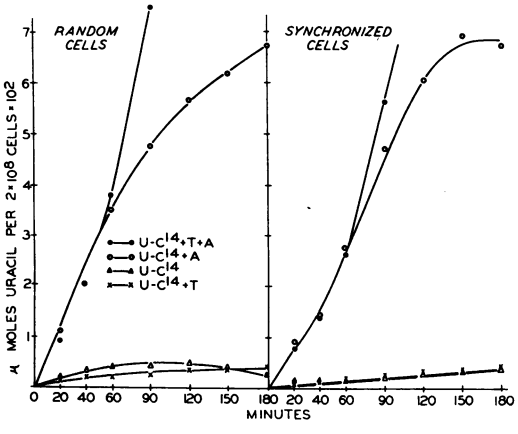

FIG. 1.—The incorporation of uracil-2-C^{14} in *E. coli* strain 15TAU. Random cells are those harvested in exponential growth. Synchronized cells have been previously exposed to a 90 min incubation period in mineral media plus glucose and thymine. At zero time the concentration of cells was 2×10^8 per ml and this concentration of cells per aliquot was maintained at this level. U-C^{14} = uracil-2-C^{14}. T = thymine. A = arginine.

initial rate of uracil incorporation is about 10 per cent of that in presence of the amino acid. A significant inhibition of even this low level of incorporation is effected by adding thymine to the medium. In cells which are synchronized by preincubation for 90 min in thymine alone, the presence of thymine did not inhibit uracil incorporation. In random cultures the presence of uracil does not depress thymine incorporation into DNA, and it appears that in the absence of exogenous uracil an adequate endogenous uracil source[4] supplies DNA-cytosine.

The uracil incorporated was present mainly (>75%) in the ribosomal fraction of TAU. Uracil incorporation into the separate nucleic acids, RNA and DNA, has also been studied in strains TAU and THU, as presented in Figure 2. The exten-

sive inhibition of RNA synthesis by thymine has been confirmed for both strains. Furthermore, the inhibition detectable in THU is even greater than that in TAU, since the presence of thymine has produced an absolute cessation of RNA synthesis for a short interval concomitant with a burst of DNA synthesis.

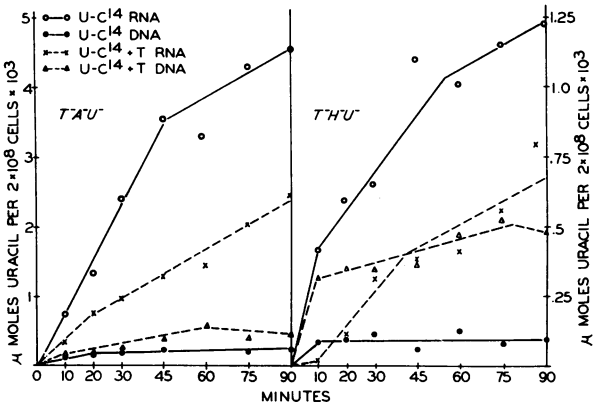

FIG. 2.—The effect of thymine on the incorporation of uracil into the nucleic acids of random *E. coli* in the absence of protein synthesis. U-C^{14} = uracil-2-C^{14}. T = thymine.

The presence of chloramphenicol produces at least a fourfold stimulation of RNA synthesis in the absence of amino acids in both random and synchronized cultures. This stimulated synthesis was not sensitive to the presence of thymine.

RNA synthesis in normal and T6r$^+$-infected strain THU: Since it is known that only phage-induced messenger RNA but not ribosomal nor soluble RNA is made after phage infection,[18, 19] we studied how the depletion of amino acid affects RNA synthesis in phage-infected bacteria. These experiments were performed exclusively with *E. coli* THU, which is sensitive to infection by T6r$^+$.

The incorporation of uracil-C^{14} into RNA by normal and T6r$^+$-infected *E. coli* THU was determined during a 9 min interval. In the presence of histidine, uracil incorporation in infected cells is only about 35 per cent of that in normal cells. A marked reduction of the incorporation (about 85 per cent) is observed with normal cells incubated without histidine, in agreement with the previous observation with TAU. In contrast with this, the incorporation by infected cells is slightly reduced (about 30 per cent) in the absence of histidine as compared with that in the presence of the amino acid. In both normal and infected cells, chloramphenicol stimulates the incorporation to essentially the level established by histidine.

Figure 3 illustrates the turnover of the RNA made in the absence of the amino acid in infected cells. Bacteria were exposed to uracil-C^{14} shortly after infection in the absence of histidine and then transferred into the medium containing excess uracil-C^{14}. A rapid decrease in the radioactivity of RNA is observed even though histidine is absent during the entire period of incubation. Although the addition of chloramphenicol reduces the rate of decrease in the radioactivity in RNA as reported by Astrachan and Volkin,[20] even under this condition a significant release of this radioactivity is observed. It is evident that the RNA made in the absence of the amino acid is metabolically unstable, as is the normal phage-induced RNA, and that protein synthesis is not essential for the turnover of this RNA.

When histidine is added with uracil-C^{12} after prior incubation with uracil-C^{14}, the isotope initially found in the RNA can then be incorporated into DNA. Thus, phage-induced RNA made in the absence of protein synthesis possesses a complete turnover comparable to that made in normal virus multiplication.[21]

Properties of phage-induced RNA produced without protein synthesis: Some

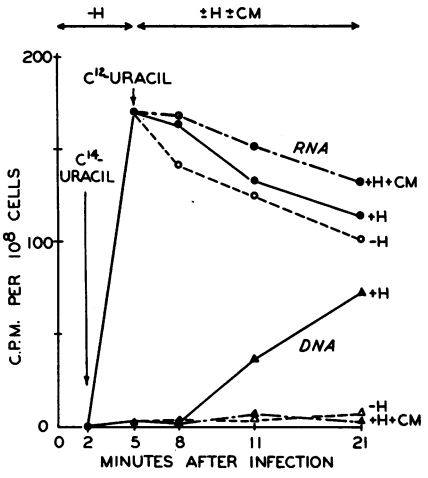

FIG. 3.—Turnover of phage-induced RNA synthesized in the absence of histidine. Cells (1×10^9 cells per ml) were suspended in a medium containing thymine alone, and infected by T6r+ phage at an average multiplicity of 8 phages per bacterium. At 2 min after infection C^{14}-uracil (10 μc per μmole) was added to give a final concentration of uracil of 0.1 μmole per ml. At 5 min after infection the culture was diluted by the addition of an equal volume of incubation media which contained: (−H) C^{12}-uracil, (+ H) C^{12}-uracil and histidine, (+H +CM) C^{12}-uracil, histidine, and chloramphenicol. Final concentrations of uracil, histidine, and chloramphenicol were 10 μg per ml, 20 μg per ml, and 40 μg per ml, respectively.

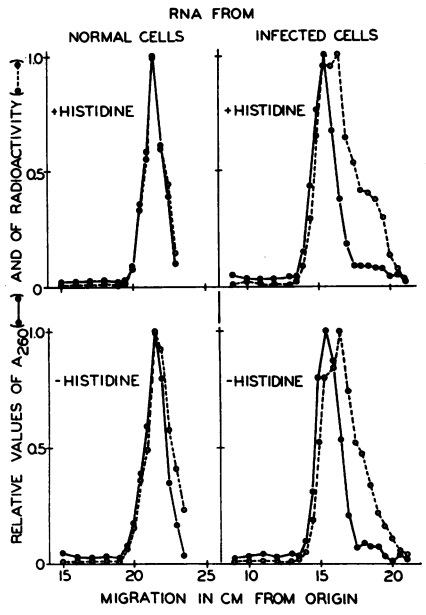

FIG. 4.—Starch gel electrophoresis of RNA from normal and phage-infected cells. Conditions and time of labeling of bacteria with C^{14}-uracil were the same as described in Table 1. RNA was isolated from ribosomal fractions by phenol treatment.

investigators have reported that chloramphenicol, when added before or at infection, interferes in the metabolism of RNA in infected bacteria, and it has been suggested that the synthesis of phage-induced RNA requires protein synthesis.[20] If protein synthesis required a prior production of specific RNA, and if synthesis of phage-induced RNA required protein synthesis, evidently phage synthesis could not begin. In fact, the latter requirement does not exist in these systems, as will be seen below. Existence of a fraction of phage-induced RNA in chloramphenicol-treated cells has recently been demonstrated.[22, 23]

The intracellular distribution and base ratio[14] of the RNA labeled under various conditions are presented in Table 1. The depletion of amino acid or the addition of chloramphenicol does not cause a significant change in the distribution of the RNA. About 75 per cent of the RNA formed after phage infection is found in the ribosomal fraction. In addition, the ratio of radioactivity of uracil to cytosine in the RNA from infected cells in the presence and absence of protein synthesis is about twice as high as this ratio for the RNA from normal cells.

Incubation of ribosomes with inorganic phosphate results in a selective degradation of the phage-induced RNA.[14] The stabilities of the labeled RNA's on ribosomes from cells prepared in a variety of conditions were similarly determined. The labeled RNA's from infected cells whose protein synthesis is prevented by the

TABLE 1

DISTRIBUTION AND BASE-RATIO OF LABELED RNA IN NORMAL AND T6R$^+$-INFECTED CELLS

Cells	Conditions	Radioactivity in ribosomes (% of total)	C^{14}-RNA on Ribosomes: Specific activity (cpm per μmole of pyrimidine)	C^{14}-RNA on Ribosomes: Amount of labeled RNA (% total RNA)	UMP/CMP
Normal	+histidine	89	6.5×10^4	10	1.3
	−histidine	86	8.8×10^3	1.4	1.6
	+histidine +chloramphenicol	72	4.5×10^4	6.9	1.4
T6r$^+$-infected	+histidine	79	1.4×10^4	2.1	2.5
	−histidine	75	6.8×10^3	1.0	3.2
	+histidine +chloramphenicol	77	5.7×10^3	0.9	3.4

Bacteria (1×10^9 cells per ml) were incubated in 100 ml each of following media: basal medium containing thymine alone; basal plus histidine (20 μg per ml); basal plus histidine plus chloramphenicol (40 μg per ml). T6r$^+$ phage was added to three samples at 5 min of incubation (multiplicity of 8 phages per bacterium). C^{14}-uracil (0.5 μc per μmole) was added to all samples at 7 min of incubation (final concentration of uracil, 0.1 μmole per ml). The reaction was stopped at 20 min of incubation.

depletion of amino acid or the addition of chloramphenicol is degraded as rapidly and as selectively as the RNA from normal infected cells.

Figure 4 illustrates the distribution of radioactivity and ultraviolet absorbancy when the RNA's isolated from ribosomes by the Duponol-phenol method are subjected to starch gel electrophoresis. The phage-induced RNA can be distinguished as a fraction which migrates faster than does the bulk of ribosomal RNA.[18] A considerable fraction corresponding to the phage-induced RNA is found in the RNA from infected cells incubated without histidine. The above demonstrate the presence on ribosomes of a fraction of RNA of characteristic base composition, high sensitivity to P_i-stimulated degradation, and unusual electrophoretic mobility. Thus, although protein synthesis is prevented, infected cells can produce RNA which is indistinguishable from normal phage-induced RNA.

Function of phage-induced RNA: The effect of uracil on the appearance of phage-induced enzymes in T6r$^+$-infected THU is shown in Figure 5. When cells are incubated in a complete medium, the activities of dCMP hydroxymethylase, thymidylate synthetase, and lysozyme increase rapidly and consecutively after infection. However, if uracil is not added to the medium, the increase of these activities is considerably delayed and their maximum levels become low compared with the normal level of enzyme attained. This delayed appearance of enzymes reflects the fact that in the uracil-starved cells uracil, which is essential for the synthesis of RNA, must be furnished from the turnover of pre-existing nucleic acid. From these experiments it seems evident that the synthesis of phage-induced RNA is essential for the appearance of phage-induced enzymes.[24] However, the RNA essential for a very early enzyme such as the hydroxymethylase evidently obtains the uracil more readily than that necessary for a late protein such as lysozyme.

We then tested the functional capability of the phage-induced RNA formed without protein synthesis. Cells were incubated with uracil (without histidine) for 6 min after infection, and then washed and resuspended in a fresh medium. As shown (Fig. 6), during the incubation in the absence of histidine, there is no increase of hydroxymethylase activity, and the addition of histidine induced a very rapid increase of the activity.[25] Essentially the same curves are obtained in the presence and the absence of uracil with the cells which are preincubated with uracil before the

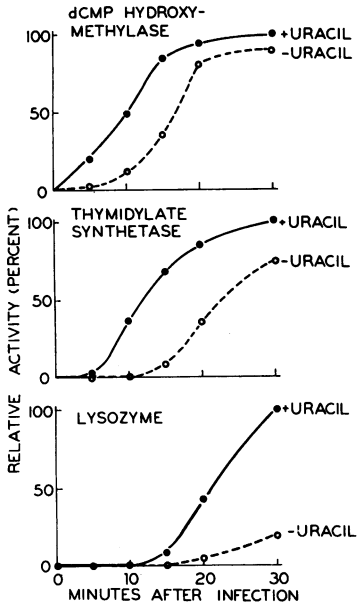

FIG. 5.—Effect of uracil depletion on the formation of phage-induced enzymes in *E. coli* THU. Bacteria were incubated in a medium lacking uracil for 15 min and then infected with T6r$^+$ at a multiplicity of 4. A half portion of the suspension received uracil (20 μg per ml) at the time of infection, and the other half was incubated in the absence of uracil.

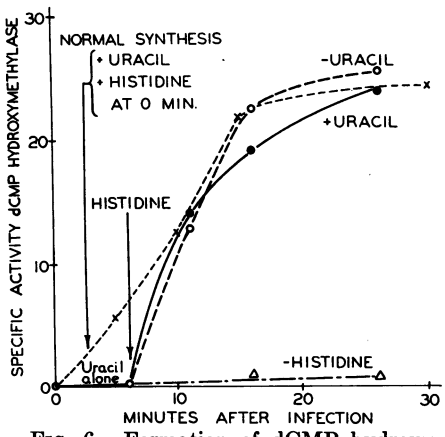

FIG. 6.—Formation of dCMP hydroxymethylase in *E. coli* THU preincubated with uracil after infection. Bacteria were starved for uracil and histidine for 15 min and infected with T6r$^+$ (4 phages per bacterium). Uracil was added at the time of infection. After incubation for 6 min with uracil (without histidine), the bacteria were collected, washed in cold, and resuspended in three different media: + histidine + uracil; + histidine − uracil; − histidine + uracil. A curve for normal production of the enzyme (+ histidine + uracil at the time of infection) was obtained in the course of the experiment shown in Fig. 4.

addition of histidine (Fig. 6). However, the initial rate of increase of activity in the preincubated cells is twice as high as that of the normal infected cells which received both histidine and uracil at the time of infection. From these results, it appears that the RNA necessary for the synthesis of dCMP hydroxymethylase is produced during the preincubation with uracil alone and then functions as histidine is supplied.

This implication is supported by an experiment in which the effects of the preincubation on the increase of enzyme were examined more closely. If uracil is present in the preincubation medium, a higher rate of increase is obtained in the first 5 min with the cells incubated with uracil after infection than with the cells incubated with uracil before infection. However, when the preincubation is performed in the absence of uracil, there is no significant difference between the cells incubated before and after infection. The low rate of hydroxymethylase production in infected cells preincubated with uracil prior to infection may even conceivably reflect the saturation of the ribosomes with bacterial messenger RNA which must be eliminated before a high rate of hydroxymethylase can be achieved.

Incubation of infected cells with uracil alone for 6 min, followed by addition of histidine, did not significantly affect the rate of synthesis, nor even the time of appearance of the late enzyme, lysozyme. We tentatively infer that the RNA made in the first 6 min of infection relates to early enzymes alone.

Synthesis of β-galactosidase in strain THU: Since it appeared possible to charge ribosomes in infected cells with phage-induced RNA capable of translation into dCMP hydroxymethylase, it was asked if evidence for a similar phenomenon could not be detected in uninfected cells. Strain THU, starved for uracil and histidine, was incubated for 10 min in a medium containing uracil, a mixture of amino acids lacking histidine, and an inducer, thiomethyl galactoside (TMG). Glucose was replaced by succinate to minimize catabolite repression.[26] In each of four experiments, cultures preincubated in uracil and TMG rapidly developed a short burst of enzyme synthesis (to twice the basal level) on supply of histidine in the absence of TMG. Cultures preincubated with uracil but without TMG did not increase their enzyme content on addition of histidine.

Discussion.—Ribosomes charged *in vivo* with messenger RNA in the absence of protein synthesis appear to be functionally ready for protein synthesis, as the bursts of the dCMP hydroxymethylase in infected cells, and of β-galactosidase in normal induced cells seem to indicate. Such ribosomes charged with RNA evidently provide a new type of experimental material for the study of numerous problems of protein synthesis. Concerning the general availability of charged ribosomes, it seems possible that a similar synthesis of messenger RNA without protein synthesis can occur in other bacterial strains. However, the synthesis would tend to be minimized in all nonthymineless cells, i.e., cells simultaneously engaged in DNA synthesis.

The synthesis of the messenger RNA in these systems is clearly competitive with DNA synthesis, a process which seems to take precedence over RNA synthesis. The competitive quality of this relation probably stems from the dual roles of DNA in supplying templates for both DNA synthesis and for messenger RNA. The mechanism by which DNA synthesis wins in such a competition may arise from the possible requirement for the double-stranded molecule as a template for synthesis of messenger RNA and that of single strands for DNA synthesis. If this is true, it is implied by our data that the very filling in of a complementary DNA chain separates the double strands on which RNA is being synthesized and in so doing eliminates much of the synthesis of messenger RNA in these bacteria. In the autoradiographic study of Prescott[27] in Euplotes, DNA synthesis proceeds in a narrow band from the ends of the elongated macronucleus to the middle and in so doing eliminates RNA from the region of DNA replication.

We have been attempting for some years to approach the problem of the mechanism of the sequential synthesis of phage-induced proteins. One of the obvious hypotheses to explain this phenomenon, which is so characteristic of differentiation in general, supposes a sequential reading or transcription from the phage chromosomes into classes of messenger RNA. Among the difficulties in exploring this problem is the phenomenon of RNA turnover, caused in large part by polynucleotide phosphorylase and phosphodiesterase.[14] In extending the study of the effect of withholding uracil on later enzyme synthesis as presented in Figure 5, it became evident that the RNA turnover cannot make uracil sufficiently available for a really late enzyme such as lysozyme. The uracil deficiency was significant also for thymidylate synthetase, which seems to fall into a somewhat intermediate temporal position in the sequence. Since the RNA turnover is almost adequate in providing uracil in producing messenger RNA for a very early enzyme, the developing depletion of uracil suggests that the chromosome is read later in transcrib-

ing RNA for the thymidylate synthetase and still later in providing RNA for lysozyme.

Kano-Sueoka and Spiegelman[28] have presented evidence that different classes of RNA molecules are made at different periods of infection but they have not provided evidence of the functionality, much less of differences in functionality, of such different RNA molecules. Although it is possible that differences in functionality of RNA can be established in appropriate systems of intact infected cells, the phenomenon of RNA turnover would seem to minimize the possibility of obtaining the most rigorous answer on this question in such systems. Evidently the most rigorous answer would require that in an *in vitro* system ribosomes containing RNA made early produce an early protein and those containing RNA made late produce a late protein. The phenomenon of a selective charging of ribosomes without protein synthesis will perhaps become useful in the *in vitro* testing of differences between RNA made in different periods of phage multiplication.

Summary.—Escherichia coli deficient in the ability to synthesize thymine, uracil, and an amino acid synthesize a small fraction of their normal RNA from uracil in the absence of thymine and the amino acid. The synthesis of even this fraction of RNA is markedly inhibited by concomitant DNA synthesis. The RNA made appears on the ribosomes. When synthesized in the presence of an inducer of β-galactosidase, the RNA made permits the rapid synthesis of a small amount of this enzyme in the apparent absence of the inducer.

T6r$^+$-infected cells make characteristic phage-induced RNA in the absence of protein synthesis. Such RNA stimulates a rapid synthesis of the early enzyme, dCMP hydroxymethylase, in the infected cells but not of the late enzyme, lysozyme. Evidence is presented to support the hypothesis that the sequential production of phage proteins may be determined by the sequential transcription of the phage genome, i.e., the sequential production of phage-induced messenger RNA.

* This research was supported by grant No. E-3063 from the National Institute of Arthritis and Metabolic Diseases of the U.S. Public Health Service.

† 1961 Fulbright Travel Grantee. On leave from Kanazawa University Medical School, Japan.

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*THE SECONDARY STRUCTURE OF REOVIRUS RNA**

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Communicated by Walther F. Goebel, March 18, 1963

Reoviruses occur widely in the respiratory and enteric tracts of man and animals, but little is known about their relation to disease.[1] Reovirus particles measure 700–750 A in diameter[2, 3] and contain RNA.[2, 4] The protein coat of the virus is made up of 5- and 6-sided prismatic subunits and appears to be free of lipid.[2] The reproductive process of reovirus type 3 is characterized by three features which distinguish it from other RNA viruses.[2] The multiplication of reovirus is relatively slow compared to many RNA viruses; the inclusion body which develops in reovirus-infected cells stains orthochromatically greenish-yellow with acridine orange, as if it contained DNA, yet it contains RNA; and finally, the reproduction of reovirus is inhibited by actinomycin D. In its effect on cells, reovirus does not behave as do other cytoplasmic viruses. Infection of cells results in a specific inhibition of cellular DNA synthesis, with no apparent inhibition of RNA or protein synthesis.[5]

These findings have provided a strong stimulus to us to undertake an investigation of the chemical structure of reovirus RNA, and to ascertain whether this component possesses distinguishing features which are responsible for the unusual behavior of the virus. The anomalous staining of the viral inclusion with acridine orange, indicating an unexpectedly low binding of the dye by reovirus RNA, suggested that the RNA of this virus might be double-stranded.[2] The experimental results about to be described provide strong evidence that this is indeed the case.

Materials and Methods.—Cell culture: A continuous cell line derived from mouse fibroblasts was used. These cells, designated as L cells, strain 929, were grown either in monolayers or in suspension.[2, 6] Monolayer cultures were used for the preparation of the virus stock and for plaque assays. Suspension cultures were used for the preparation of large quantities of virus for physical-chemical examination. The cells in suspension culture were collected and inoculated with virus when the cell density reached 4–5 $\times$ 10^5 cells per ml or 6–7.5 $\times$ 10^7 cells per bottle.

Virus: The Dearing strain of reovirus 3[2] was cloned five times in succession and a stock prepared. The latter was stored at -55°C. Batches of virus were prepared as follows: L cells from suspension cultures were collected by centrifugation, and resuspended in 3.5 ml of reovirus stock containing 2–3 $\times$ 10^8 PFU/ml. The ratio of virus to cell was approximately 10:1. After an adsorption period of 2 hr at room temperature, during which the cells were frequently resuspended,