centration of about 0.018 mM, with very low affinity for Na, the second at about 16 mM, with severe competition by Na. The latter but not the former mechanism is inhibited when SO_4 is the anion instead of Cl.

* This research was supported by grants from the National Science Foundation and the Office of Saline Water, U.S. Department of the Interior. Rb^{86} was furnished by Oak Ridge National Laboratory, Oak Ridge, Tennessee. K^{42} was obtained by neutron bombardment of K_2CO_3 in the LPTR facility of the University of California Lawrence Radiation Laboratory at Livermore, through the courtesy of the Director, Dr. John S. Foster, Jr., and Dr. Edward Teller. We appreciate the interest and cooperation of Mr. Ernest E. Hill, Reactor Supervisor.

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*SYNTHESIS OF TRANSFER RNA BY ISOLATED NUCLEI**

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Communicated by James Bonner, March 15, 1963

In the course of earlier experiments on the incorporation of labeled nucleosides into RNA by isolated nuclei[1] we observed that much of the newly synthesized RNA is soluble in 1 *M* NaCl, as is transfer RNA.[2-4] Sirlin has reported the incorporation of pseudo-uridine into nuclei, allegedly into transfer RNA,[5, 6] and the presence of amino-acyl RNA in thymus nuclei has been shown by Hopkins.[7] Since the pea nuclei with which we work are capable of protein synthesis,[8] they might therefore be suspected of containing transfer RNA. It will be shown below that isolated pea nuclei not only contain, but possess the ability to synthesize, transfer RNA.

Materials and Methods.—Analytical reagent grade chemicals were used throughout. ATP, CTP, GTP, UTP, UMP, uridine, phosphocreatine, and crystalline DNAse were obtained from Sigma. Creatine phosphokinase was obtained from the California Corp. for Biochemical Research. Sodium penicillin-G was a gift of Chas. Pfizer and Co., New York. 2-hydroxy-3-naphthoic

acid and tetrazotized o-dianisidine were supplied by Dajac Laboratories, Borden Chemical Co., Philadelphia. Uridine-H^3 and L-leucine-C^{14} (5 microcuries/micromole) were purchased from New England Nuclear Corp., Albany, and algal C^{14}-protein hydrolysate (30 microcuries/mg) from Nuclear Chicago Corp. Actinomycin D was a gift of Merck, Sharp and Dohme, Rahway, N.J.

Preparation of nuclei: 2 kg of 48 hr-old pea embryonic axes or the tips of 4 day-old pea seedlings, sterilized by treatment with 2% Purex for 3 min at room temperature, were soaked in 0.05 *M* $CaCl_2$ for 20 min and rinsed three times with ice-cold distilled water. They were then ground in the pea-popper[1] with the simultaneous addition of 25 ml grinding medium (2 *M* sucrose + 0.006 *M* $CaCl_2$) per 100 ml final volume of juice. The homogenate was then filtered through Miracloth and the nuclei sedimented by a 10 min centrifugation at 350 × *g* as previously described.[1] All procedures were carried out at 0–4°C unless otherwise specified.

Preparation of RNA: The nuclear suspension was stirred with an equal volume of water-saturated phenol in the cold for 45 min and the nuclear RNA prepared by a modification of the method of Kirby.[9] This procedure extracted 75–80% of the RNA. DNA contamination, measured by the diphenylamine reaction,[10] was found to be negligible (less than 3%).

If partial purification of the transfer RNA were required, the nuclear RNA was extracted at 0°C overnight with 1 *M* NaCl saturated with phenol, the insoluble material sedimented, and the RNA of the supernatant precipitated with 2.5 volumes of ethanol and washed three times with ethanol. Amounts of RNA were routinely determined by absorbancy at 260 mμ in distilled water. For counting, the acid-washed RNA samples[1] were dissolved in distilled water and 0.1 ml aliquots counted in 10 ml scintillation fluid[11] in a Packard automatic scintillation counter TRICARB Model No. 314-DC.

Preparation of pH 5 enzyme: 200 gm of pea embryonic axes were ground with 100 ml 0.4 *M* sucrose for 10–20 sec at full speed in a Waring blendor. After filtration through a double layer of Miracloth the juice was centrifuged for 4 hr at 104,000 × *g* and the supernatant was adjusted to pH 5 with acetic acid and the precipitate sedimented at 35,000 × *g* for 20 min. The pellet was homogenized in 0.01 *M* Tris at pH 7.4 and any insoluble material removed by centrifugation.

The supernatant contains a considerable amount of RNA as well as the pH 5 enzymes. The preparation was partially freed of RNA by precipitation with protamine sulfate added as solution (10 mg/ml) in the ratio 1.5 mg protamine sulfate to 1 mg RNA.[12] After 5 min at 0°C the pellet was centrifuged at 20,000 × *g* in the Spinco for 15 min and the supernatant withdrawn. Such a treatment removed 70% of the RNA from the enzyme preparation.

Formation of dye-bound RNA: This procedure, originated by Zamecnik and co-workers,[13] has been slightly modified for the present material. Nuclei were incubated with uridine-H^3 (2020 microcuries/micromole) for 20 min under the conditions described earlier.[1] The RNA was then extracted[9] and, in one set of experiments, purified by 1 *M* NaCl extraction. It was next incubated with pH 5 enzyme as described in Table 1 except for the C^{14}-L-leucine which was replaced by a mixture of all the L-amino acids, 20 micrograms each/ml incubation mixture. Incubation was for 15 min at 37°C. The reaction was stopped by the addition of an equal volume of phenol. The amino-acyl RNA was extracted and precipitated with $^1/_{10}$ volume of 2 *M* potassium acetate and 2.5 volumes of ethanol, and kept at −12°C overnight. The precipitate was then dissolved in a small volume of distilled water and stirred with acid-washed charcoal. The RNA was next reprecipitated with $^1/_{10}$ volume of 20% sodium acetate and 2.5 volumes of ethanol for at least 4 hr. The RNA pellet was then redissolved in distilled water.

Oxidation: The oxidation with periodate was carried out as described by Zamecnik *et al.*[13] After precipitation of the periodate, the supernatant was dialyzed against a large volume of distilled water to remove the remaining periodate and any contaminating oligonucleotides and the RNA then precipitated with sodium acetate and ethanol as before.

Addition of 2-hydroxy-3-naphthoic acid: The oxidized RNA pellet was dissolved in distilled water and the addition of 2-hydroxy-3-naphthoic acid carried out as described by Zamecnik *et al.*[13] Some precipitation of brownish material occurred and this could not again be brought into solution. In a preliminary study with C^{14}-leucyl-RNA, it was found that the insoluble material contains no radioactivity and therefore does not represent a loss of transfer RNA.

Formation of dye-bound RNA and fractionation: Reaction of tetrazotized-o-dianisidine with the hydrazono-RNA was carried out as described by Zamecnik *et al.*[13] The mixture of dye-bound and free RNA was alcohol washed, then dissolved in distilled water and fractionated in a propanol-

phosphate system.[13] Alternatively, an ammonium sulfate fractionation was used. Nine volumes of ice-cold 2.5 *M* ammonium sulfate, pH 5, were added to the aqueous solution to yield a final concentration of 2.25 *M*.[14] This solution was kept at 0°C for 10 min and the fine precipitate then centrifuged down at 35,000 × *g* for 15 min. This procedure precipitates approximately 90% of the dye-bound RNA[14] leaving the dye-non-bound amino-acyl RNA in solution. The precipitate of dye-bound RNA was washed once with 5% TCA, and then twice with 70% ethanol +0.5% sodium acetate pH 5 and once with absolute ethanol. The dye-non-bound RNA was precipitated from the dialyzed sulfate solution by the addition of TCA to give a final concentration of 5%, the mixture kept at 0°C for 10 min, and the precipitate sedimented at 35,000 × *g* in the Servall for 15 min. The precipitate was washed as above. Each precipitate was dissolved in distilled water, 560 mμ and 260 mμ absorptions determined, and an aliquot counted.

Experimental Results.—That nuclei contain an active transfer RNA is shown by the following experiment: isolated pea stem nuclei were incubated and extracted as described in the legend of Figure 1. The data of Figure 1 show that labeled

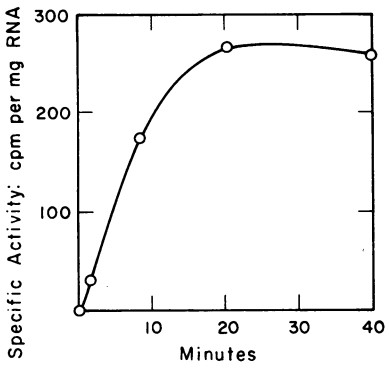

FIG. 1.—Formation of amino-acyl RNA in isolated nuclei. Incubation mixture: ATP, CTP, GTP, and UTP, .0001 *M* each; tris .02 *M;* phosphocreatine .02 *M;* creatine phosphokinase 100 μg/ml; $CaCl_2$ .003 *M;* $MgCl_2$ .0001 *M;* C^{14}-protein hydrolysate 2 μc/ml; final pH 7.0, incubation at 37°C. Aliquots were precipitated at intervals by addition of an equal volume of phenol. RNA was extracted[9] and washed as described in Table 1.

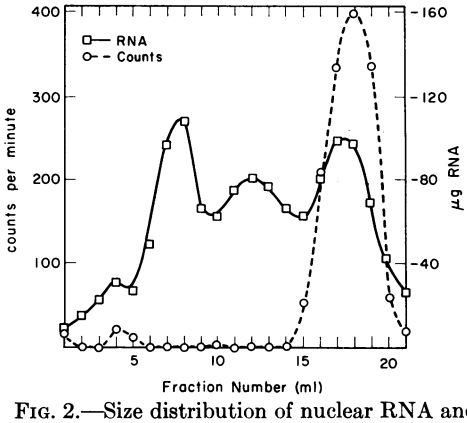

FIG. 2.—Size distribution of nuclear RNA and localization of transfer RNA on a sucrose density gradient. Sucrose density gradient (20 ml) of 20–5% sucrose in .01 *M* tris (pH 7.1) and 1% phenol was set up according to Bolton *et al.*[17] and overlayered with an inverse gradient of approximately 1 mg RNA in a sucrose gradient of 4–0%. A covering layer of paraffin oil prevented tubes from collapsing and rendered gradients more stable for handling. The tubes were centrifuged 12–14 hr in the Spinco rotor No. 25 at 24,000 rpm, then pierced and 1 ml fractions collected. Each fraction was precipitated with ethanol/acetate (66/2%), thoroughly washed to remove the phenol, and then tested for C^{14}-leucine incorporation as described in Table 1.

amino acids are incorporated into nuclear amino-acyl RNA.

That nuclear RNA can also bind amino acids *in vitro* is shown by the results of experiments in which phenol-extracted nuclear RNA was incubated with L-leucine-C^{14} and pH 5 enzyme prepared from whole pea tissue. The data of Table 1*a* show that under these conditions nuclear RNA incorporates labeled amino acid. The amino acid incorporated is completely liberated by hot 5% TCA treatment. There is no such incorporation in the absence of pH 5 enzyme. These facts indicate that the amino acid is incorporated into amino-acyl RNA. The active transfer RNA present in the nuclear preparation could not have derived from contamination of the preparation by cytoplasmic transfer RNA, or even from transfer RNA released

TABLE 1

INCORPORATION OF L-LEUCINE-C^{14} INTO RNA

Experiment	RNA source	pH 5 enzyme source	Incorporation (cpm/mg RNA)‡
(*a*)	Nuclei	Cytoplasm	147
	Purified* nuclei	Cytoplasm	136
	Cytoplasm	Cytoplasm	276
(*b*)	Cytoplasm	Nuclei†	265
	Nuclei	Nuclei†	86
	Nuclei	Cytoplasm	83

(*a*) 300 μg RNA incubated with 2 mg enzyme protein.
(*b*) 300 μg RNA incubated with 250 μg enzyme protein.
* These nuclei were purified by resuspension in 4% ficoll, containing 0.003 *M* $CaCl_2$; 0.35 *M* sucrose, tris 0.001 *M*; pH 7.2 and sedimented at 350 × *g* for 10 min.
† The pH 5 enzymes were prepared from the 104,000 × *g* nonparticulate supernatant of the nuclear homogenate[15] as described in *Methods*.
‡ The incubation mixture contained: ATP 10^{-3} *M*; CTP 10^{-3} *M*; $MgCl_2$ 10^{-3} *M*; phosphocreatine 0.01 *M*; tris-HCl 0.01 *M*; creatine phosphokinase 100 μg/ml; L-leucine-C^{14} 2 μC/ml; pH 5 enzymes and RNA as specified above. Incubation at 37°C for 10 min. The RNA was precipitated together with 500 μg carrier RNA and 0.5% unlabeled D,L-leucine in 66% ethanol + 0.5% sodium acetate pH 5.0. Zero time samples were precipitated immediately after addition of pH 5 enzyme to the otherwise complete incubation mixture. Samples were washed and counted according to Hoagland *et al.*[16] Control incubation without added RNA showed only negligible incorporation.

from contaminating cytoplasmic ribosomes, since nuclei which had been washed free of lighter contaminants in 4% ficoll solution also yielded active transfer RNA (Table 1*a*).

Since intact nuclei possess the ability to form amino-acyl RNA, they should also contain amino acid activating enzymes as well as transfer RNA. Active pH 5 enzymes may in fact be prepared from nuclei as described in Table 1. Nuclear pH 5 enzyme and RNA are as reactive with the corresponding cytoplasmic fractions as with each other. This is shown by the cross-incubation experiment of Table 1*b*. Similarly, cytoplasmic RNA is reactive with both nuclear and cytoplasmic activating enzymes.

The apparent differences in activity between nuclear and cytoplasmic RNA (Table 1*b*) are unimportant. Since total extractable RNA was used for these experiments, the differences probably reflect no more than the different amounts of active transfer RNA in the two preparations.

To distinguish transfer RNA from other RNA species in our nuclei we have used the sucrose density gradient technique described in the legend of Figure 2. The results show clearly that the ability to accept C^{14}-L-leucine is confined to a peak of small molecule RNA. Labeled uridine can also be incorporated into the material of this peak. Thus, in a further experiment RNA was extracted with phenol from embryo nuclei previously incubated with uridine-H^3 (25 microcuries/ml; 1200 microcuries/μM) and subsequently fractionated by the density gradient procedure (Fig. 3). Control nuclei were incubated with uridine-H^3 at 0°C for the duration of the experiment and fractionated in an identical manner.

After 8 min incubation with uridine-H^3 the extractable RNA contains approximately 20% of the total activity (the remainder being nonextractable messenger RNA which is retained in the phenol phase[18]) of which half is present in the transfer RNA peak. The data of Figure 3 show that specific activity reaches a high level in the peak previously identified as transfer RNA by amino acid labeling. It is clear therefore that RNA of the same size as the previously characterized transfer RNA is synthesized within the nucleus. The very high specific activity observed in the last fraction is probably due to newly synthesized oligonucleotides since it was not observed in samples which had been dialyzed before fractionation.

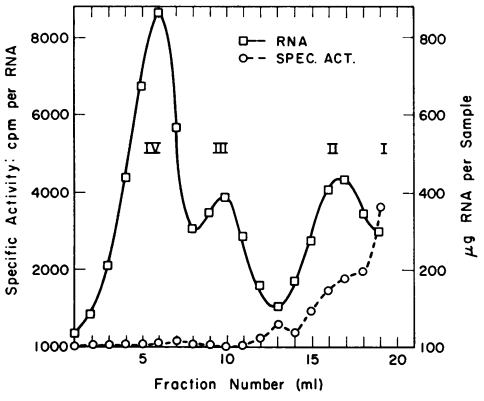

FIG. 3.—Incorporation of uridine-H³ into RNA of different molecular size by nuclei. An incubation mixture optimal for incorporation of tritiated nucleosides into RNA[1] was used. It was supplemented by penicillin-G (500 units/ml). Zero degree controls were kept in ice for the duration of the experiment. The reaction was stopped by the addition of unlabeled uridine and UMP (1000-fold excess) and an equal amount of phenol. The sucrose density gradient fractionation was carried out as described in Fig. 2, and the individual fractions washed (3 times) with 80% alcohol +2% perchloric acid, containing excess uridine, UMP, and UTP, twice with ethanol, and assayed for radioactivity and 260 mμ absorption.

The small molecule RNA (4S) does not exhibit rapid turnover as does the messenger RNA which several workers have reported in higher organisms.[18–20] This is evident in the pulse chase experiment of Figure 4. The addition of unlabeled uridine (as uridine, UMP, and UTP) to whole nuclei quenches the incorporation of tritiated uridine almost immediately. Changes in the activity of RNA components after quenching are therefore due to redistribution of the previously incorporated label. The effect of quenching upon the redistribution of label is shown in Table 2. It is clear that all fractions but one increase in specific activity after quenching. Ribosomal fractions (III and IV) exhibit a more than 2-3-fold increase in specific activity. Transfer RNA shows a smaller increase in specific activity. Only the oligonucleotides exhibit a dramatic decrease in specific activity after the quenching, showing that the quench has in fact taken effect on the extractable RNA. Since the oligonucleotides are probably precursors of the larger RNA molecules, one would expect them to be sensitive to the quenching.

TABLE 2

CHANGES IN DISTRIBUTION OF URIDINE-H³ LABEL IN PULSE-CHASE EXPERIMENT

Region*	Sedimentation constant $S_{20,w}$	Type of RNA	Specific activity† (cpm/mg RNA) After 7 min pulse	After 23 min chase
I	—	Oligonucleotide	39,300	11,500
II	4	Transfer RNA	8,620	12,400
III	18	Ribosomal	1,260	2,660
IV	28	Ribosomal	714	2,160

* Compare with Figure 3.

† PVP-purified nuclei were incubated for 7 min with uridine-H³ (40 μc/ml; 6740 μc/μM) as described in Figure 3. One half was precipitated and extracted with phenol, and the other half quenched by the addition of unlabeled uridine (1000-fold), UMP (1000-fold), and UTP (500-fold), and incubated further for 23 min at 37°C. The RNA from the chased preparation was isolated likewise. Each RNA preparation was fractionated on a sucrose density gradient, as described in Figure 2, and the individual peak fractions examined in an analytical Spinco Model E.

The synthesis of small molecule RNA appears to be DNA-dependent. Thus, treatment of pea nuclei with DNAse depresses the incorporation of uridine-H³ and cytidine-H³ into RNA by 90–100%. Actinomycin D, a specific inhibitor of DNA-primed RNA synthesis,[22–24] also inhibits incorporation of uridine-H³ by nuclei (Fig. 5). The highest concentration of actinomycin D employed (1.6 μM/ml) suppresses all but 18% of the incorporation into RNA. In an experiment in which the RNA, extracted from nuclei after incubation with 1.2 μM/ml actinomycin, was fractionated by 1 *M* NaCl extraction, it was found that incorporation of uridine-H³

into the nucleus as a whole was inhibited by 76% and that the actinomycin suppressed incorporation into both soluble and insoluble RNA fractions by about 75%. Thus, the synthesis of all nuclear RNA species is inhibited by actinomycin D. This confirms the results of Tamaoki[25] and is in agreement with work on the formation of DNA-sRNA hybrids[26] which indicates that DNA is the template for sRNA synthesis.

Rigorous evidence that the small molecule RNA synthesized by isolated nuclei is in fact transfer RNA is provided by application of the fractionation procedure of Zamecnik *et al.*[13] The basis of this fractionation is that the terminal nucleotide of amino-acyl RNA cannot be oxidized by periodate treatment whereas that of non-amino-acyl RNA may be so oxidized. It is then possible to attach a dye molecule to such oxidized RNA. The dye-bound RNA may then be differentiated from the

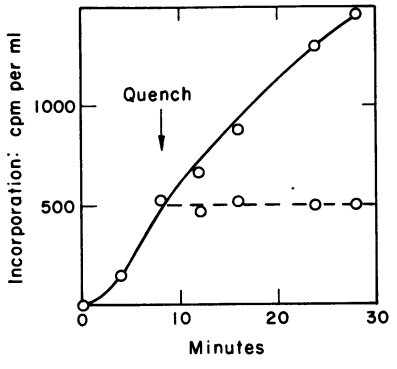

FIG. 4.—Quenching of uridine-H^3 incorporation by addition of uridine compounds. Incubation as described earlier.[1] Unlabeled uridine, UMP, and UTP in 1000-fold, 1000-fold, and 500-fold excess, respectively, were added to one half the nuclear suspension after 8 min incubation. Aliquots were precipitated in 3% perchloric acid and washed as described.[1]

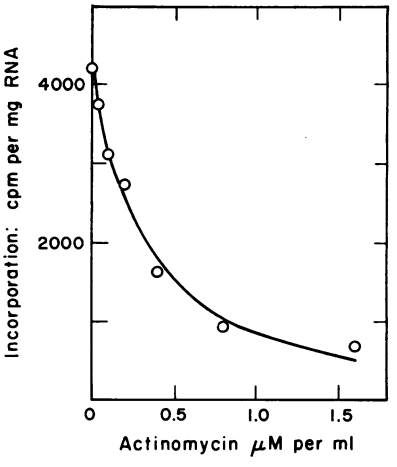

FIG. 5.—Inhibition of uridine-H^3 incorporation by actinomycin D. Incubation mixture: ATP, GTP, CTP, each 10^{-4} *M*; $CaCl_2$ 2.5×10^{-3} *M*; Tris:HCl 10^{-2} *M*; creatine phosphokinase 100 μg/ml; uridine-H^3 (sp. act. 2770 μC/μ*M*, 25 μC/ml); final pH 7.6; DNA 178 μg/ml. Incubation for 10 min at 37°C. Aliquots counted by the method of Mans and Novelli.[21]

dye-non-bound RNA by their different solubilities in a propanol-phosphate system.

This procedure yields amino-acyl RNA of high purity but in rather poor yield, due largely to coprecipitation of dye-non-bound amino-acyl RNA with the dye-bound RNA (Zamecnik *et al.*[13]). For this reason the procedure does not provide a quantitative measure of amino-acyl RNA. The presence of radioactivity in the thus purified amino-acyl RNA fraction does however provide good evidence that the labeled RNA is transfer RNA.

The results of such a fractionation are shown in Table 3. The virtually pure amino-acyl RNA, obtained by the propanol-phosphate fractionation of RNA obtained from nuclei previously incubated with uridine-H^3, was found to contain radioactivity and to possess a specific activity three times that of the dye-bound RNA. The recovery of amino-acyl RNA is rather low, probably due to coprecipitation of

TABLE 3

DYE FRACTIONATION OF NUCLEAR RNA

	μg RNA	Uridine-H^3 incorporated (cpm)	Specific activity (cpm/mg RNA)	% dye
Propanol-phosphate fractionation[13] of total phenol extractable nuclear RNA*:				
Amino-acyl RNA (dye-non-bound)	140	142*	1,020	<0.5
Dye-bound RNA	3,920	1,160*	298	>99.5
Ammonium sulfate fractionation[14] of partially purified nuclear soluble RNA				
Nonprecipitable amino-acyl RNA (dye-non-bound)	760	1,340†	1,765	14.6
$(NH_4)_2SO_4$ precipitable dye-bound RNA	585	810†	1,385	85.4

* Incubation 15 min (25 μc/ml; 2020 μc/μ*M*).
† Incubation 20 min (25 μc/ml; 2020 μc/μ*M*).

amino-acyl RNA with the dye-RNA or to dye-binding of transfer RNA lacking the amino-acyl or terminal nucleotide groups.

When nuclear transfer RNA is first partially purified by 1 *M* NaCl extraction and then fractionated by the ammonium sulfate procedure, a much higher yield of amino-acyl RNA is obtained. In this case the specific activities of amino-acyl and dye-bound RNA are more nearly the same than are those obtained by fractionation of whole nuclear RNA in propanol-phosphate. This is consistent with the view that some of the dye-bound RNA consists of incomplete transfer RNA molecules.

The results clearly show that the uridine-labeled RNA was bound to amino acid during the fractionation procedure. Isolated nuclei are therefore able to synthesize transfer RNA.

Summary.—We have shown that isolated pea nuclei contain an active transfer RNA which will accept amino acids from both cytoplasmic and nuclear amino acid activating enzymes.

Isolated pea nuclei synthesize *in vitro* a small molecule RNA of the physical characteristics of amino acid transfer RNA. This synthesis is DNA-dependent. It has been shown that this RNA is in fact transfer RNA and that the *in vitro* synthesized molecules are capable of accepting amino acids.

We wish to thank Professors James Bonner and Paul O. P. Ts'o for their helpful suggestions and criticisms. We are grateful to Phil Martin and Abbas Soleimani for their technical assistance.

* This research was supported in part by grants RG-5143, GM-03977, AM-03102 from the National Institutes of Health, U.S. Public Health Service, and by grant G-7129 of the National Science Foundation.

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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,