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Zachau, H. G., Dutting, D., Feldmann, H., Melchers, F., and Karau, W. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 417.

Transfer Ribonucleic Acids from *Escherichia coli* Treated with 5-Fluorouracil*

R. J. Lowrie and P. L. Bergquist

ABSTRACT: 5-Fluorouracil (FU) is readily incorporated into the transfer ribonucleic acids (tRNAs) of *Escherichia coli*. tRNA synthesized in the presence of the analog may have up to 100% replacement of uracil by FU. The relative amounts of the other three major bases are unchanged but the relative amounts of ribothymidylic and pseudouridylic acids are reduced. Incorporation of FU into tRNA does not affect its ability to accept amino acids or to transfer phenylalanine into polyphenylalanine or lysine into polylysine in an *E. coli* S-30 system. Transfer of lysine into polypeptides synthesized under the direction of bacteriophage R17 RNA is inhibited by tRNA from FU-treated cells. This inhibition does not appear to be due to errors in codon recognition by lysyl-tRNA containing FU. There was no difference in response to a series of A,G copolymers between lysyl-tRNA containing FU and control lysyl-tRNA in the ribosome binding assay. tRNA containing FU has been separated from contaminating unsubstituted tRNA by chromatography on columns of methylated albumin kieselguhr.

tRNA containing FU has been shown to possess an altered secondary structure as judged by its thermal denaturation profile.

5-Fluorouracil is readily incorporated into the ribonucleic acids of several strains of *Escherichia coli* (Horowitz and Chargaff, 1959; Horowitz *et al.*, 1960). One consequence of incorporation of FU[1] into RNA is the production of altered proteins (Bussard *et al.*, 1960; Gros and Naono, 1961; Nakada and Magasanik, 1964).

Champe and Benzer (1962) showed that growth in the presence of FU restored the wild-type phenotype to certain rII mutants of bacteriophage T_4. They suggested that the incorporation of the analog into mRNA caused occasional base-pairing errors during message translation, FU pairing sometimes with G instead of A. Other genetical evidence also supports faulty base pairing in translation as a site of action of FU *in vivo* (Rosen, 1965; Edlin, 1965a,b). Heidelberger (1965) suggested that FU may occasionally pair incorrectly during transcription of mRNA from DNA but an attempted experimental demonstration was unsuccessful (Bujard and Heidelberger, 1966).

It is possible that coding errors leading to the production of altered proteins may be introduced by tRNA containing FU. For example, errors may occur through altered codon–anticodon interaction during translation, or by mistakes in recognition by the aminoacyl synthetases of tRNA containing FU. However, the effects of FU on tRNA have received relatively little attention other than preliminary reports in the context of other experiments (Gros and Naono, 1961; Gros *et al.*, 1962; Sueoka and Yamane, 1963). In the work reported here, we will show that tRNA synthesized in the presence of the analog may have up to 100% replacement of uracil by FU and an altered content of certain minor bases. Furthermore, we have found that tRNA from FU-treated cells accepts amino acids to loading levels indistinguishable from normal tRNA. FU-tRNA can transfer phenylalanine into polyphenylalanine and lysine into polylysine in an *E. coli* S-30 system but cannot transfer lysine into polypeptides synthesized under the direction of bacteriophage R17 RNA. The tRNA containing FU has been separated from contaminating normal tRNA that does not contain FU and shown to possess a different secondary structure.

Materials and Methods

Bacterial Growth and Media. E. coli B was usually grown in C medium (Roberts *et al.*, 1955) with 0.2%

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: FU, 5-fluorouracil; FU-tRNA, tRNA from cells treated with FU; C-tRNA, tRNA from control cells; FU-enzyme, aminoacyl-tRNA synthetases prepared from cells treated with FU; C-enzyme, aminoacyl-tRNA synthetases from control cells; A_{260} unit, the quantity of material which dissolved in 1 ml of solvent has an absorbance at 260 mμ of one measured in a cell of 1-cm path length; MAK, methylated bovine serum albumin adsorbed to Kieselguhr.

TABLE I: Base Composition of tRNAs from *E. coli* B Treated with FU.[a]

Length of Exposure to FU[b]	C	A	G	U	FU	U + FU	Obsd % Replacement[c]	Predicted % Replacement[d]
0	21.8	16.0	29.3	19.2		19.2		
0.25	22.5	16.8	28.9	15.8	2.6	18.4	14.0	20.0
0.5	21.6	16.3	29.2	14.1	5.4	19.5	28.2	33.3
0.75	21.3	16.6	29.0	12.4	7.5	19.9	38.0	42.8
1.0	22.2	16.4	28.2	10.3	8.8	19.1	47.0	50.0
1.5	22.0	16.6	29.0	7.7	10.8	18.5	58.4	60.0
2.0	21.4	15.9	28.8	6.9	12.5	19.4	64.4	66.6
3.0	21.5	16.6	28.7	9.6	10.0	19.6	51.2	75.0
4.0	21.0	16.8	27.8	12.3	7.4	19.7	37.5	80.0

[a] A 14-l. culture of *E. coli* B was grown at 37° in C medium (generation time, 62 min) to an OD_{650} of 0.25 (2.2×10^8 cells/ml). Two liters were harvested to provide the zero-time sample. Thymidine (20 μg/ml) and FU (10 μg/ml) were added to the culture and the incubation was continued. Samples (2 l.) were harvested 0.25 and 0.5 generation times after FU addition. At 0.75, 1.0, and 1.5 generation times 1.5 l. was harvested and at two, three, and four generation times 1 l. was harvested. tRNA prepared as in Methods was hydrolyzed with 1 N KOH and neutralized, and the products were chromatographed by the method of Lane (1963). The spots and the appropriate blanks were cut out and eluted with 0.01 N HCl. Absorbance at 260 and 280 mμ was determined. The amounts of the nucleotides were calculated from the molar extinction coefficients (Beaven *et al.*, 1955; Dahl *et al.*, 1959), and are shown as nucleotides per tRNA molecule assuming a chain length of 86 nucleotides (Bergquist and Scott, 1964). [b] Generation times. [c] Observed % replacement = (FU/(U + FU)) × 100. [d] Predicted replacement calculated assuming a linear increase in the amount of tRNA in the culture and that all newly synthesized tRNA contains FU instead of uracil, using the equation given above.

glucose as the energy source. In some experiments the medium was enriched with Difco Bacto-Casitone. A low phosphate MGM medium (Lanni, 1961) was used in experiments with [^{32}P]orthophosphate.

Preparation of tRNA. tRNA was prepared by a modification of the method of Capecchi (1966) (Bergquist *et al.*, 1968).

Preparation of Aminoacyl-tRNA Synthetase Fractions. Partially purified aminoacyl-tRNA synthetase fractions were prepared by the method of Bergquist (1966) from washed *E. coli* B cells harvested in midlogarithmic growth from C medium plus 0.2% Bacto-Casitone. Aminoacyl-tRNA synthetase was also prepared from FU-treated cells. FU (10 μg/ml) was added at an A_{650} of 0.30. The culture was harvested after one generation time (55 min).

Amino Acid Acceptance. The capacity of fractionated and unfractionated tRNA to accept amino acids was assayed by a membrane filtration technique (Scott, 1968). Aminoacylated tRNA was prepared by the method of Bergquist (1966).

Protein Synthesis in Vitro. The ability of FU-tRNA to transfer amino acids into protein *in vitro* was tested using a cell-free system from *E. coli* S26 (Capecchi, 1966) with the modifications of Bergquist *et al.* (1968). Bacteriophage R17 RNA was prepared by the method of Gesteland and Boedtker (1964).

Ribosomal Binding Assay. Assays of polynucleotide-directed binding of lysyl-tRNA to ribosomes were carried out according to Thach and Sundararajan (1965).

Base Composition Analysis of tRNA. Major nucleotides and 5-fluorouridylic acid in alkaline hydrolysates of tRNA were separated by one-dimensional paper chromatography (Lane, 1963). Nucleotides were eluted from the papers with 0.01 N HCl and their absorbance at 260 mμ was determined. The values of the appropriate blanks were subtracted. The amounts of the nucleotides were calculated from their extinction coefficients (Beaven *et al.*, 1955; Dahl *et al.*, 1959). Major and minor nucleotides in hydrolysates of ^{32}P-labeled tRNA samples were separated by the two-dimensional technique of Bergquist (1966). Minor nucleotides were located by radioautography. Radioactivity of nucleotides was determined by liquid scintillation counting in a Packard Model 3324.

MAK Column Chromatography. Samples (2–3 mg) of tRNA were fractionated on 9 × 1.5 cm MAK columns at 4°, as described in the appropriate figure legends. tRNA was recovered by combining fractions and adding 2.5 volumes of cold 95% ethanol. The precipitated RNA was collected by centrifugation, dissolved in water, dialyzed overnight against distilled water, and lyophilized. Dilute samples were dialyzed and concentrated by lyophilization before ethanol precipitation.

Temperature–Absorbance Profiles. tRNA samples were treated with 1 mg/ml of bentonite (Fraenkel-Conrat *et al.*, 1961) and changes in absorbance with increasing temperature were measured in a Cary Model 15 recording spectrophotometer.

TABLE II: Distribution of ^{32}P Radioactivity in the Major and Minor Nucleotide Constituents of tRNA from 5FU-Treated and Control *E. coli* B.[a]

Length of Exposure to FU and [^{32}P]-Orthophosphate[b]	0	0.25	0.5	0.75	1.0	1.5	2.0
Cytidylic acid	24.8	23.8	24.1	24.2	24.3	24.4	23.8
Adenylic acid	19.0	19.1	19.0	19.1	19.0	18.9	18.9
Guanylic acid	30.4	30.1	30.0	30.0	31.5	30.5	30.7
Uridylic and 5-fluorouridylic acids[c]	17.6	19.1	20.2	20.2	20.3	20.3	20.4
5-Fluorouridylic acid[d]		3.1	9.7	10.2	11.4	14.0	14.2
Pseudouridylic acid	1.65	0.95	0.27	0.18	0.14	0.13	0.11
Ribothymidylic acid	1.35	0.57	0.12	0.07	0.06	0.03	0.03
Inosinic acid	0.52	0.50	0.63	0.57	0.59	0.54	0.56
2-Methylinosinic acid	0.18	0.12	0.21	0.23	0.22	0.21	0.18
N^2-Methylguanylic acid and 1-methylguanylic acid	0.06	0.08	0.05	0.06	0.06	0.06	0.05
N^2-Dimethylguanylic acid	0.03	0.02	0.03	0.03	0.04	0.02	0.02
2-Methyladenylic acid	0.02	0.02	0.02	0.03	0.02	0.02	0.03
N^6-Methyladenylic acid	0.01	0.02	0.02	0.02	0.01	0.01	0.02
5-Methylcytidylic acid	1.61	1.63	1.71	1.76	1.68	1.72	1.69
Guanosine 3′,5′-diphosphate	3.29	3.45	2.53	3.38	3.34	2.93	2.90

[a] Six 30-ml cultures of *E. coli* B in MGM medium were grown at 37° (doubling time, 57 min) to an OD_{650} of 0.25. Thymidine (20 μg/ml) and 0.5 mCi of [^{32}P]orthophosphate were added to each, immediately followed by 10 μg/ml of FU (or 10 μg/ml of uracil in the control culture). At 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 generation times after FU addition one of the FU-treated cultures was harvested. The control was harvested after one generation time. tRNA was hydrolyzed with 1 N KOH, and the neutralized hydrolysates were subjected to paper electrophoresis in one dimension followed by chromatography in the second (Bergquist, 1966). Minor nucleotides were located by autoradiography. Regions of the paper corresponding to the spots on the X-ray film were cut out, dried, and counted in a liquid scintillation counter. Base compositions are expressed as percentage of the summed total of the radioactivity in all the nucleotide spots. [b] Generation times. [c] Uracil and FU are not separated in this system. [d] Substitution of uracil by FU in the [^{32}P]tRNA hydrolysates was determined following chromatography by the technique of Lane (1963).

Chemicals. 5-Fluorouracil was a gift of Hoffman La-Roche Inc., Nutley, N. J., to Professor R. E. F. Matthews. [^3H]FU (4.6 Ci/mmole), [^3H]lysine (0.48 Ci/mmole), and [^3H]phenylalanine (1.5 Ci/mmole) were from Schwarz BioResearch, Orangeburg, N. Y.; ^{14}C-labeled arginine, glycine, isoleucine, lysine, phenylalanine, proline, serine, threonine, and valine (80–351 mCi/momle), [^{14}C]asparagine (9.2 from mCi/mmole), and [^{14}C]tyrosine (26.6 mCi/mmole) from New England Nuclear Corp., Boston, Mass.; [^{14}C]uracil (40.6 mCi/mmole) and [^{32}P]orthophosphate (carrier-free) from Radiochemical Centre, Amersham, England; nonradioactive amino acids were obtained from Mann Research Laboratories, N. Y.; pyruvic kinase, ATP, CTP, phosphoenolpyruvate, 2-mercaptoethanol, glutathione, bovine serum albumin, and thymidine were supplied by Calbiochem, Los Angeles, Calif.; poly U and poly A were obtained from Miles Laboratories Inc., Elkhart, Ind.; adenylic-guanylic acid copolymers were a gift of Dr. R. Thach, Department of Chemistry, Harvard University.

Results

The Incorporation of FU into tRNA. The base composition of *E. coli* B tRNA at a series of time points after addition of FU to the culture is shown in Table I. The replacement of uracil by FU occurs at a steady rate for two generation times. Cell death and the growth of FU-resistant cells may account for the decline in percentage replacement at the third and fourth generation time points. The value of 50% substitution at one generation time suggests that two components may be present in FU-tRNA preparations: (i) normal tRNA synthesized before addition of FU to the culture; and (ii) tRNA synthesized in the presence of the analog with 100% replacement of uracil by FU.

We can calculate the substitution at each time point if there is 100% replacement of uracil by FU in tRNA synthesized during FU treatment. We make the assumptions that tRNA synthesis continues at the same rate and that there is no lag between the time of FU addition and its incorporation into tRNA. Colony counts showed that *E. coli* B multiplied linearly and not exponentially after the addition of FU. Thus the predicted per cent replacement values (Table I) have been calculated on the assumption of a linear increase in tRNA content in the cultures that were treated with FU. Table I shows that for up to two generation times the experimental value approximates the predicted value for substitution at each time point.

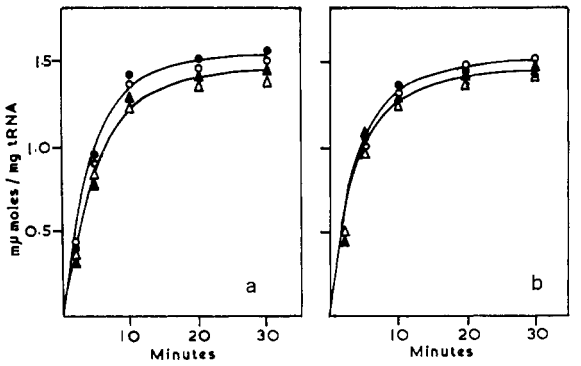

FIGURE 1: Time course of phenylalanine and lysine acceptance by C-tRNA and FU-tRNA. Phenylalanine (a) and lysine (b) acceptance assays were performed as in Table III but the amount of activating enzyme was reduced fivefold so that the rate of the reaction could be measured. Bovine serum albumin (25 μg) was added to each reaction mixture to ensure that the protein concentration was maintained at an adequate level. Portions (20 μl) were withdrawn and immediately precipitated with 5% TCA. Precipitates were processed by the method of Scott (1968) and radioactivity was determined by liquid scintillation counting. (●——●) C-tRNA and C-enzyme; (○——○) C-tRNA and FU-enzyme; (▲——▲), FU-tRNA and C-enzyme; (△——△) FU-tRNA and FU-enzyme.

The data of Table I show that there are no obvious changes in the major nucleotide composition of FU-tRNA other than the substitution of FU for uracil. We examined the possibility that FU brought about changes in the amounts of minor nucleotides as follows. [^{32}P]-Orthophosphate was added to cultures at the same time as FU and the base composition of the tRNA synthesized in the presence of the analog was calculated from the distribution of ^{32}P radioactivity among the nucleotides in tRNA hydrolysates.

Table II shows that the amounts of methylated purines and 5-methylcytosine were similar to the control values in all the samples containing FU. There was a steady decrease in the fraction of the total nucleotide radioactivity located in pseudouridylic and ribothymidylic acids as the time of exposure to FU and [^{32}P]orthophosphate increased. The percentage of the total radioactivity in the two uracil derivatives was less than one-tenth that of the control after one generation time of FU treatment. Thus, tRNA synthesized during exposure to FU has a lower content than normal of pseudouridylic and ribothymidylic acids. The presence of the fluorine atom in the 5 position of 5-fluorouridylic acid probably prevents methylation of FU to form ribothymidylic acid. Similarly the substitution with fluorine at the five position would prevent the attachment of the ribose moiety necessary to form pseudouridylic acid (Wagner and Heidelberger, 1962; Goldwasser and Heinrikson, 1966).

Amino Acid Acceptance. Table III shows that tRNA from cells exposed to FU for one generation time accepted all the amino acids tested to loading levels comparable to control tRNA when activating enzymes from control cells were used for charging. We then examined the ability of enzyme from FU-treated cells to charge control and FU-tRNA. Acceptance of five amino acids was tested with a synthetase preparation from a portion of the FU-treated culture from which the FU-tRNA had been prepared. Again there was no difference apparent in the acceptance of amino acids by control and FU-tRNA (Table III).

TABLE III: Amino Acid Acceptance by tRNA from Normal and 5FU-Treated *E. coli* B.[a]

	C-tRNA		FU-tRNA	
	C-enzyme	FU-enzyme	C-enzyme	FU-enzyme
Phenylalanine	1.47	1.44	1.47	1.48
Lysine	1.30	1.34	1.30	1.33
Glycine	1.64	1.68	1.64	1.69
Serine	1.51	1.49	1.47	1.45
Valine	2.03	2.01	1.98	2.03
Tyrosine	1.36	*b*	1.33	*b*
Proline	1.39		1.39	
Isoleucine	1.28		1.26	
Threonine	1.67		1.67	
Arginine	1.68		1.73	
Asparagine	1.20		1.23	
Alanine	1.15		1.20	
Glutamine	1.58		1.61	

[a] Control tRNA (C-tRNA) and tRNA from cells exposed to FU for one generation time (FU-tRNA) were assayed for amino acid acceptance by the method of Scott (1968). Aminoacyl-tRNA synthetase preparations (Bergquist, 1966) from control (C-enzyme) and FU-treated cells (FU-enzyme) were used. The specific activities of the [^{14}C]amino acids (counts per minute per millimicromole) were Phe, 8.04 × 10^3; Lys, 8.41 × 10^3; Gly, 5.72 × 10^3; Ser, 8.85 × 10^3; Val, 7.72 × 10^3; Tyr, 7.05 × 10^3; Pro, 9.32 × 10^3; Ile, 5.87 × 10^3; Thr, 8.29 × 10^3; Arg, 8.37 × 10^3; and Asp-N, 9.05 × 10^3. Reaction mixtures were incubated at 37° for 15 min and processed according to Scott (1968). Radioactivity was determined by liquid scintillation counting. Amino acid acceptance is expressed as millimicromoles of amino acid per milligram of tRNA. The appropriate RNA and activating enzyme blanks have been subtracted. [b] Not tested.

Figure 1 demonstrates that for lysine and phenylalanine acceptance there was no difference in the time course of the loading reaction. Furthermore, the time course of amino acid acceptance was identical whether activating enzymes from control or FU-treated cells were used in the assay.

Since our incorporation measurements (Table I) suggest that the FU-tRNA preparation used in the acceptance studies contained approximately 50% unsubstituted and 50% highly substituted tRNA chains, it would appear that high replacement of uracil by FU in the tRNA molecules neither affects their ability to accept amino acids nor inhibits the loading reaction.

Protein Synthesis in Vitro. Table IV shows that tRNA from FU-treated cells was active *in vitro* in transfer of

phenylalanine and lysine into polyphenylalanine and polylysine in systems programmed either by poly U or by poly A. Stimulation was comparable to C-tRNA and to S26 tRNA (tRNA from the same strain of *E. coli* as the S-30 fraction). There was no difference in the time course of phenylalanine or lysine incorporation with control or FU-tRNA.

Addition of either FU-tRNA or C-tRNA increased phenylalanine incorporation in R17 RNA-directed *in vitro* protein synthesis, but the stimulation by FU-tRNA was only three-quarters that obtained with tRNA from control cells. In contrast, there was no stimulation by FU-tRNA of lysine incorporation into polypeptides coded by R17 RNA (Table IV).

It is possible that only the unsubstituted tRNA species in FU-tRNA were functional and that FU-containing tRNAs were inactive. If this were the case and if FU-tRNA was not inhibitory, a higher concentration of FU-tRNA than C-tRNA would be required for maximum stimulation of protein synthesis. We investigated the incorporation of phenylalanine and lysine into polyphenylalanine and polylysine at a series of C-tRNA and FU-tRNA concentrations. There was no difference in the concentrations of either tRNA required to give the greatest amount of incorporation. Similarly, in R17 RNA-directed *in vitro* systems there was no difference in the concentrations of control and FU-tRNA required to reach the plateau level of phenylalanine incorporation. At none of the concentrations of FU-tRNA tested was there any stimulation of lysine incorporation into polypeptides coded by R17 RNA. We also investigated the stimulation of phenylalanine and lysine incorporation by control and FU-tRNA with increasing amounts of poly U, poly A, and R17 RNA. Again there was no obvious difference in the amount of mRNA required for maximum incorporation of these amino acids.

It is apparent from these results that FU-tRNA is able to transfer phenylalanine and lysine into polyphenylalanine and polylysine as efficiently as unsubstituted tRNA. However, FU-tRNA appears to inhibit strongly incorporation of lysine into polypeptides in R17 RNA-directed *in vitro* protein synthesis. Stimulation of phenylalanine incorporation is also lower than that obtained with control tRNA. We attempted to increase the incorporation of phenylalanine and lysine into polypeptides coded by R17 RNA in the presence of FU-tRNA by adding up to twice the concentration of control tRNA together with the FU-tRNA. Phenylalanine incorporation was unchanged. There was a slight increase in lysine incorporation but the total was still substantially below that obtained with C-tRNA alone. Hence, FU-containing tRNAs in addition to having reduced activity in protein synthesis *in vitro* appear to inhibit transfer of amino acids by unsubstituted tRNAs.

Coding Response of Unfractionated Lysyl-tRNA Containing FU. Lack of lysine transfer may be caused by the inability of FU-tRNA^{Lys} to participate in the ribosome–mRNA–tRNA complex. We examined this possibility using the ribosome binding technique of Nirenberg and Leder (1964). Control tRNA was charged with [^{3}H]lysine and FU-tRNA with [^{14}C]lysine and their response to poly A and a series of A,G random copolymers tested. Table V shows that there was no marked difference in polymer-dependent binding to ribosomes. Although the binding of lysyl-FU-tRNA was consistently lower than the control, a reduction of at least 50% would be expected if only the normal unsubstituted spe-

TABLE IV: Stimulation of Protein Synthesis *in Vitro* by Unfractionated Control and FU-Containing tRNA.[a]

mRNA	tRNA	Amino Acid Incorpd[b] ($\mu\mu$moles)	% Stimuln[c]
		Phenylalanine	
Poly U		172	0
Poly U	Control	540	214
Poly U	FU	527	206
Poly U	S26	550	220
R17		37	0
R17	Control	59	60
R17	FU	49	32
R17	S26	71	92
		Lysine	
Poly A		50	0
Poly A	Control	105	110
Poly A	FU	96	92
Poly A	S26	112	124
R17		32	0
R17	Control	57	81
R17	FU	29	0
R17	S26	65	104

[a] An *in vitro* synthesizing system was prepared from *E. coli* S26 by a method slightly modified from Capecchi (1966) (Bergquist *et al.*, 1968). Each reaction mixture contained: 25 μl of preincubated S-30; 1 μmole of ATP; 0.2 μmole of GTP; 5 μg of pyruvic kinase; 3 μmoles of magnesium acetate; 2.1 μmoles of NH_4Cl; 1.25 μmoles of Tris-Cl (pH 7.6); 2.5 μmoles of reduced glutathione; 3 mμmoles of asparagine; 95 μg of FU-tRNA, 97 μg of C-tRNA, or 100 μg of S26 tRNA; 35 μg of R17 RNA or 1.0 A_{260} units of poly A or 1.0 A_{260} units of poly U; 2.2 mμmoles of [^{14}C]lysine (specific activity 1.09×10^5 cpm/mμmole) or 2.1 mμmoles of [^{14}C]-phenylalanine (specific activity 1.22×10^5 cpm/mμmole); and 1 mμmole each of 19 nonradioactive amino acids (minus phenylalanine or lysine), in a total volume of 100 μl. Reaction mixtures were incubated at 37° for 20 min and radioactivity of TCA-insoluble material was determined by liquid scintillation counting. Reaction mixtures containing [^{14}C]lysine were precipitated by the TCA–tungstate method (Kaziro *et al.*, 1963). [b] Amino acid incorporation is expressed as $\mu\mu$moles of amino acid incorporated/100 μg of ribosomes per 20 min. [c] Percentage increase in amino acid incorporation above the value for assays which received no additional tRNA.

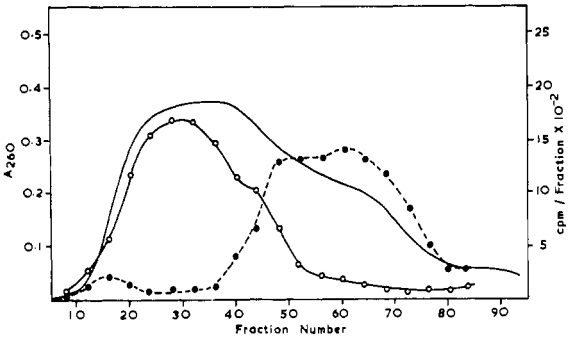

FIGURE 2: MAK column chromatography of tRNA from FU-treated *E. coli* B. RNA (2.5 mg) from cells exposed to FU for one generation time was fractionated on a 9.0 × 1.5 cm MAK column at 4° together with [^{3}H]FU-labeled FU-tRNA (0.2 mg) and [^{14}C]uracil-labeled C-tRNA (0.2 mg). After washing with 100 ml of 0.1 M NaCl in 0.05 M phosphate buffer (pH 6.8), the RNA was eluted with a 240-ml linear gradient of 0.3–0.7 M NaCl in the same buffer. Fractions (2 ml) were collected. Absorbance at 260 mμ of each fraction was measured. An equal volume of 10% TCA was added to every fourth fraction. The precipitated RNA was collected on membrane filters. Radioactivity was determined by liquid scintillation counting. (——) Absorbance at 260 mμ; (○——○) [^{14}C]uracil radioactivity; (●- - - ●) [^{3}H]FU radioactivity.

cies present in FU-tRNA were able to bind to ribosomes.

It has been suggested that FU may occasionally base pair with G during mRNA translation (Champe and Benzer, 1962) or mRNA transcription from DNA (Heidelberger, 1965). The presumed anticodon sequence of lysine tRNA (UUU) has three residues of which any one, or all, may be replaced by FU. Hence, if FU were able to pair like C, that is with G, lysyl-FU-tRNA may show a difference from control lysyl-tRNA in response to A,G copolymers in the binding assay. Table V shows that there was no change in the relative amounts of control lysyl-tRNA and lysyl-FU-tRNA bound to ribosomes as the proportion of guanylic acid in the A,G copolymers increased.

Fractionation of tRNA from Cells Treated with FU. Incorporation measurements (Table I) indicated that tRNA from FU-treated cells was a mixture of FU-containing species synthesized in the presence of an analog and normal unsubstituted tRNAs present in the cells before FU was added to the culture.

Of the fractionation methods tested, only MAK column chromatography (Sueoka and Yamane, 1962) gave a separation of the FU-containing and unsubstituted tRNAs. Other methods employing heated DEAE-cellulose and DEAE Sephadex columns (Bergquist *et al.*, 1968) were unsuccessful. Figure 2 shows that FU-tRNA labeled with [^{3}H]FU eluted from the MAK column after the bulk of the [^{14}C]uracil-labeled controlled tRNA.

Other low molecular weight RNA species elute from MAK columns in a position close to that of tRNA (Ishihama *et al.*, 1962; Monier *et al.*, 1962; Rosset and Monier, 1963; Rosset *et al.*, 1964). We confirmed that the RNA labeled with [^{3}H]FU was tRNA by sucrose density gradient centrifugation and gel filtration on columns of Sephadex G-100 (Schleich and Goldstein, 1966).

TABLE V: Binding to Ribosomes of Unfractionated Control and FU-Containing Lysyl-tRNA.[a]

Polymer	tRNA	% Input Bound	FU-tRNA Bound: C-tRNA Bound
	Control	1.9	
	FU	1.7	
Poly A	Control	27.2	
			0.71
Poly A	FU	19.3	
Poly (A_9G_1)	Control	38.4	
			0.72
Poly (A_9G_1)	FU	28.1	
Poly (A_8G_2)	Control	38.4	
			0.71
Poly (A_8G_2)	FU	27.9	
Poly (A_7G_3)	Control	21.1	
			0.71
Poly (A_7G_3)	FU	14.8	

[a] Reaction mixtures (0.05 ml) contained: 0.1 M Tris-ammonium acetate buffer (pH 7.2), 0.02 M magnesium acetate, 2 A_{260} units of ribosomes, 0.18–0.21 A_{260} unit of polymer, and 35 μg of control-tRNA labeled with [^{3}H]lysine (1.61×10^4 cpm/mg) or 36 μg of FU-tRNA labeled with [^{14}C]lysine (1.44×10^4 cpm/mg). After incubation at 30° for 15 min, reaction mixtures were diluted tenfold with buffer (0.05 M Tris, 0.1 M ammonium acetate, and 0.02 M magnesium acetate, pH 7.2) and filtered as described by Leder and Nirenberg (1964). Filters were counted in a liquid scintillation counter.

Aminoacylated tRNAs from control and FU-treated cells were observed to elute from MAK columns at different salt molarities. Control and FU-tRNA preparations aminoacylated *in vitro* with [^{3}H]phenylalanine and [^{14}C]phenylalanine, respectively, were combined and fractionated on a MAK column (Figure 3). The tritium counts showed that control phenylalanyl-tRNA eluted as a single peak. FU-tRNA charged with [^{14}C]phenylalanine was resolved as two peaks. The first coincided with the [^{3}H]phenylalanine peak and probably represents the unsubstituted $tRNA^{Phe}$ in the FU-tRNA preparation. It is likely that the second peak of [^{14}C]phenylalanine is the corresponding $tRNA^{Phe}$ that contains FU. In an analogous experiment with C-tRNA and FU-tRNA charged with radioactive lysine a similar result was obtained (Figure 4).

Partially Purified tRNA Containing FU. The amount of tRNA that could be fractionated at one time under our conditions was limited to 2–3 mg. Nevertheless,

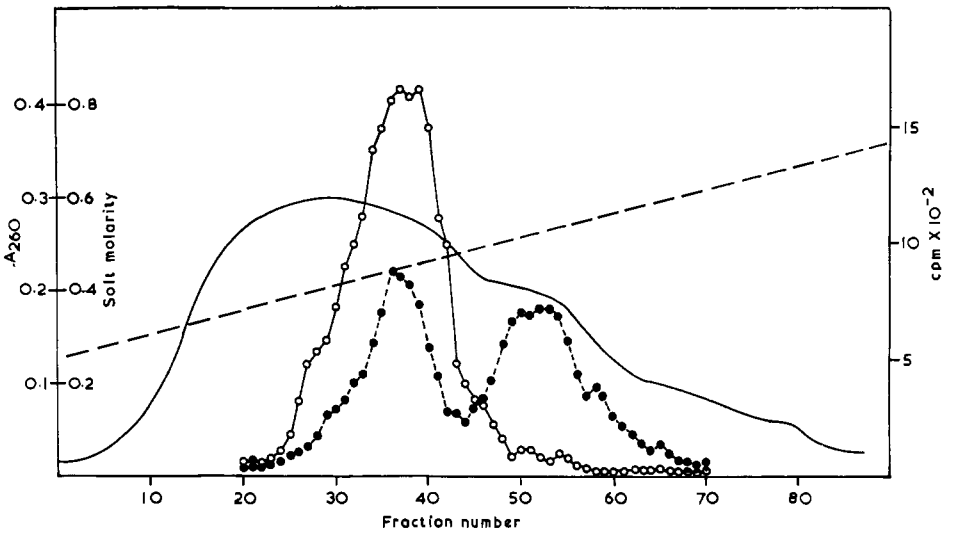

FIGURE 3: MAK column chromatography of [^{3}H]phenylalanine C-tRNA and [^{14}C]phenylalanine of FU-tRNA. Unfractionated C-tRNA and FU-tRNA were separately charged with [^{3}H]phenylalanine (5.9 × 10^4 cpm/mμmole) and [^{14}C]phenylalanine (6.6 × 10^4 cpm/mμmole), respectively, by the procedure of Bergquist (1966) up to the phenol-extraction step. The aqueous phase was extracted with ether (six times) to remove phenol and residual ether was removed by passing a stream of air over the solution. The solutions containing RNA were combined, run onto a 9 × 1.5 cm MAK column at 4° washed with 100 ml of 0.18 M NaCl in 0.05 M phosphate buffer (pH 6.8), and eluted with a 240-ml linear gradient of 0.25–0.72 M NaCl in the same buffer. Fractions (2.5 ml) were collected and their absorbance at 260 mμ was measured. Each fraction (2 ml) was added to 2 ml of 10% TCA and the precipitated RNA was collected on membrane filters and counted in a liquid scintillation counter. (——) Absorbance at 260 mμ; (O——O) [^{3}H]phenylalanine radioactivity; (●- - -●) [^{14}C]phenylalanine radioactivity; (- - - -) salt gradient.

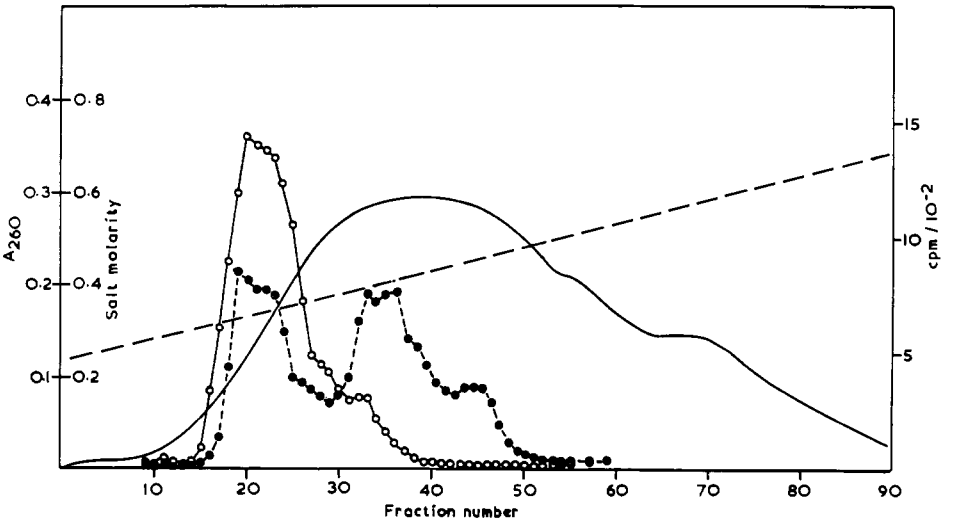

FIGURE 4: MAK column chromatography of [^{3}H]lysine C-tRNA and [^{14}C]lysine FU-tRNA. Unfractionated C-tRNA and FU-tRNA were separately charged with [^{3}H]lysine and [^{14}C]lysine, respectively, as in Figure 3 except that reaction mixtures contained 100 mμmoles of lysine ([^{3}H]lysine, 7.2 × 10^4 cpm/mμmole or [^{14}C]lysine, 8.4 × 10^4 cpm/mμmole) and 100 mμmoles of 19 nonradioactive amino acids minus lysine. The deproteinized RNA solutions were run onto a 9 × 1.5 cm MAK column at 4° washed with 100 ml of 0.18 M NaCl in 0.05 M phosphate buffer (pH 6.8) and eluted with a 240-ml linear gradient of 0.22–0.68 M NaCl in the same buffer. Fractions (2.5 ml) were collected and assayed as in Figure 3. (——) Absorbance at 260 mμ; (O——O) [^{3}H]lysine radioactivity; (●- - -●) [^{14}C]lysine radioactivity; (- - - -) salt gradient.

sufficient quantities of tRNA enriched in species containing FU were separated from the contaminating unsubstituted tRNA present in unfractionated FU-tRNA to conduct a preliminary investigation of amino acid acceptance and to prepare temperature-absorbance profiles.

Base composition analysis of the unfractionated FU-tRNA and the substituted and unsubstituted components isolated by MAK column chromatography was carried out. The unfractionated FU-tRNA had 48.6% replacement of uracil by the analog, the substituted fraction 82%, and the predominantly unsubstituted component, 15.9% replacement. There was no significant alteration in the content of other major nucleotides. There was insufficient material to examine the content of pseudouridylic and ribothymidylic acids.

Acceptance of phenylalanine, lysine, and serine by unfractionated FU-tRNA and the substituted and un-

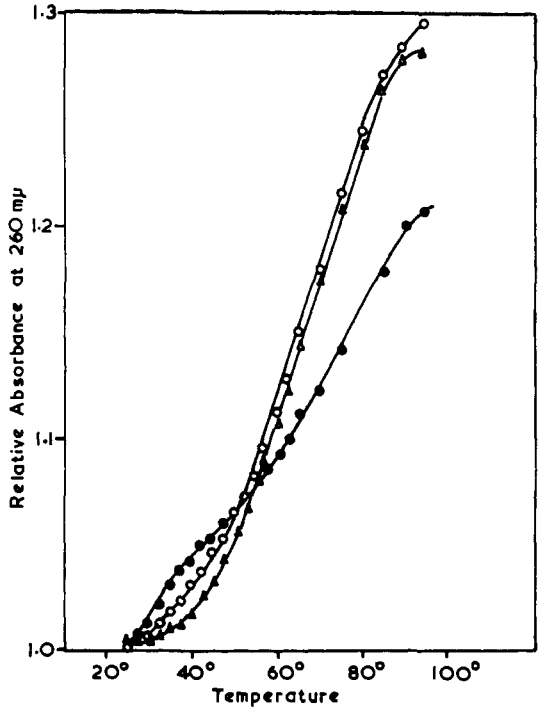

FIGURE 5: Temperature–absorbance profiles of tRNAs. The change in absorbancy at 260 mμ with increasing temperature was measured as described in Methods. tRNA samples were adjusted to an A_{260} of 0.520 at 20° in 0.14 M NaCl and 0.01 M trisodium citrate (pH 7.0). Absorbance was measured at temperature intervals of 2.5–70° and at intervals of 5–95°. After renaturation by slow cooling overnight the absorbancies at 260 mμ at 20° were: control tRNA, 0.535; unsubstituted FU-tRNA 0.540; and substituted FU-tRNA, 0.542. (○——○) Control tRNA; (△——△) unsubstituted FU-tRNA; (●——●) substituted FU-tRNA.

substituted fractions is shown in Table VI. In all cases amino acid acceptance by the fractionated samples was lower than unfractionated FU-tRNA, possibly as a result of degradation during the prolonged isolation procedure and loss in fractions that were not recovered because they contained both substituted and unsubstituted species. Since lysine tRNA elutes early from MAK columns (Figure 4), the absence of lysine-acceptance activity in the sample enriched in FU-containing tRNA is undoubtedly due to the elution of the analog-substituted lysine tRNA with predominantly unsubstituted tRNA.

We examined the time course of phenylalanine and serine acceptance by the substituted tRNA fractions. There was no difference between the fractions in the rate of the loading reaction.

Temperature–absorbance profiles of control tRNA and the substituted and unsubstituted fractions from FU-tRNA were prepared to investigate the possibility that the presence of FU in tRNA altered the secondary structure. FU-substituted tRNA possesses a less sharply defined thermal transition by comparison with the unsubstituted and control samples (Figure 5). The magnitude of the hyperchromic effect was only two-thirds that of the unsubstituted tRNA. Levin and Litt (1965) observed a similar broadening of the thermal transition and reduced hyperchromicity in tRNA containing the

TABLE VI: Amino Acid Acceptance by Unfractionated FU-tRNA and Its Substituted and Unsubstituted Component tRNAs.[a]

	FU-tRNA	Unsubstituted FU-tRNA	Substituted FU-tRNA
Phenylalanine	1.42	0.58	0.49
Lysine	1.33	0.72	0.09
Serine	1.53	0.48	0.59

[a] FU-substituted and unsubstituted tRNA prepared from FU-tRNA by MAK column chromatography were assayed for amino acid acceptance as in Table III. Amino acid acceptance is expressed as millimicromoles of amino acid accepted per milligram of tRNA.

unnatural base 8-azaguanine. The reduced hyperchromic effect suggests that the total number of base pairs in the tRNA that contains FU may be lower than in normal tRNA or that the strength of the individual base pairs involving FU has decreased. Hence, it is likely that FU-substituted tRNA, like tRNA containing 8-azaguanine, has a less ordered secondary structure than control tRNA.

Discussion

Our data show that there are two distinct components in tRNA from FU-treated cells: (i) normal unsubstituted tRNAs that were present in the cells before the addition of FU to the culture; and (ii) tRNA with a high replacement of uracil by FU synthesized during exposure to the analog. The separation by MAK column chromatography of FU-tRNA with 48.6% replacement of uracil by FU into two fractions with 15.9% and 82% substitution supports this conclusion.

There are no significant changes in major nucleotide composition other than the substitution of uracil by FU. Among the minor nucleotides the content of pseudouridylic and ribothymidylic acids is markedly reduced (Table II). It is difficult to assess the consequences of the absence of these two uracil derivatives in the FU-containing tRNA, since the role of the minor bases is virtually unknown and may be obscured by the effects of replacement of uracil by FU. However, replacement by FU of a pseudouridine residue in an anticodon sequence may prove to be an important site of action of the analog. It is of interest that pseudouridylic acid has been shown to be part of the anticodon sequence of a tyrosine tRNA from yeast (Madison *et al.*, 1966).

Studies on the biological activity *in vitro* of unfractionated FU-tRNA from cells exposed to the analog for one generation time have shown that it accepts all the amino acids tested with yields and rates indistinguishable from control tRNA (Table III, Figure 1). A reduction of about 50% would have been anticipated had the

FU-containing tRNA been inactive in amino acid acceptance. Gray and Rachmeler (1967) have claimed that tRNA with a low replacement of uracil by FU has a reduced capacity to accept some amino acids. However, the acceptance values that they have reported for both control and FU-containing tRNA are so low that the differences may not be significant. We have been unable to show any significant difference from control tRNA in acceptance of 13 amino acids by tRNA from FU-treated cells.

The MAK column fractionation patterns of FU-tRNA labeled with either radioactive phenylalanine or lysine confirm that tRNAs containing FU accept amino acids. FU-tRNA gave two peaks of phenylalanine and lysine that were present in approximately the same proportions. This observation is in contrast to the single peaks observed in control tRNA (Figures 3 and 4). The first peak coincided in elution position with radioactively labeled phenylalanyl- or lysyl-tRNA from control cells. The additional peak of phenylalanine or lysine in FU-tRNA probably represents the FU-containing phenylalanine or lysine tRNAs, since the substituted molecules are retarded by the MAK column (Figure 2).

Direct evidence that tRNAs containing FU can accept amino acids is provided by the preliminary studies of acceptance by the FU-tRNA fraction enriched in FU-containing tRNA species (average replacement of uracil by FU of 82%). The acceptance values were lower than unfractionated FU-tRNA (Table VI) but it is likely that this was caused by recovery losses or degradation during isolation.

Unfractionated FU-tRNA was active in transfer of phenylalanine into polyphenylalanine and lysine into polylysine coded by poly U and poly A in protein synthesis *in vitro* (Table IV). There was no difference in the concentrations of FU-tRNA and C-tRNA required for maximum stimulation of amino acid incorporation, suggesting that the FU containing tRNA was active and that stimulation was not due solely to unsubstituted species in the preparation.

In vitro systems programmed by R17 RNA showed differences in response to added control and FU-tRNA (Table IV). Stimulation of phenylalanine incorporation was reduced by about 25%. There was no stimulation of lysine incorporation. Furthermore, the effect of added FU-tRNA could not be reversed by addition of up to twice as much control tRNA. Hence, as well as being unable to bring about incorporation of lysine into polypeptides coded by R17 RNA, tRNA containing FU also appears to inhibit amino acid transfer by normal tRNAs.

The reduced stimulation of phenylalanine into polypeptides by FU-tRNA in R17RNA-directed *in vitro* protein synthesis may be related to the inhibition of lysine incorporation. If translation of the R17 message is stopped before the first lysine residue by a charged tRNA that contained FU, phenylalanine residues occurring after the first lysine will not be incorporated. Coat protein accounts for about 90% of the products of R17 RNA-directed *in vitro* protein synthesis (Capecchi and Gussin, 1965). The R17 coat is very similar to that of bacteriophage f_2 (Weber and Konigsberg, 1967). Three of the four phenylalanine residues in f_2 coat protein occur before the first lysine residue (Weber and Konigsberg, 1967). If translation of the coat protein cistron is halted before the first lysine codon by an FU-tRNA, incorporation of phenylalanine would be only 75% that obtained with control tRNA. The data in Table IV are in good agreement with this prediction.

The site of inhibition of lysine transfer into R17 coat protein is unknown. Since lysyl-tRNAs that contain FU accept amino acids, bind to ribosomes, and transfer lysine into polylysine, it would appear likely that some other amino acid specific tRNA is affected by FU incorporation. We have performed some preliminary experiments with the RNA from an amber mutant of R17 that chain terminates at position 50 in the coat protein (Tooze and Weber, 1967). Double-label experiments using pairs of amino acids show isoleucine and arginine to be incorporated to identical levels irrespective of whether C- or FU-tRNA is used in the reaction mixture. Tyrosine and lysine are not incorporated when FU-tRNA is used, but are incorporated to the expected levels when C-tRNA is employed in the reaction. These experiments suggest that the incorporation of FU into one or more of the tRNAs specific for serine, glutamine, alanine, or tyrosine (Weber and Konigsberg, 1967) may be the cause of the exclusion of lysine from protein synthesized *in vitro* using R17 RNA as message. This observation may be attributed to the incorporation of FU into an anticodon in place of U and in some way causing premature chain termination or the FU-tRNA might bind so tightly to a ribosomal tRNA site (or sites) that further translation of the message is impossible.

$FU\text{-}tRNA^{Lys}$ may have up to three residues in the presumed anticodon sequence replaced by FU. FU may occasionally base pair incorrectly, for example, with G instead of A (Champe and Benzer, 1962). Thus, an increased level of binding to ribosomes in the presence of A,G copolymers with an increasing G content would be expected of lysyl-FU-tRNA compared to control lysyl-tRNA. Our data showed little difference between the two RNAs in binding levels. However, the magnesium-ion concentration used was 0.02 M, a concentration at which there is little coding ambiguity in the binding assay (Kellog *et al.*, 1966). At other magnesium concentrations lysyl-tRNA containing FU may exhibit greater coding ambiguity than normal lysyl-tRNA.

It would appear that tRNA containing FU can accept amino acids, bind to ribosomes, and transfer some amino acids into polypeptides. The temperature–absorbance profiles indicated that tRNA containing FU has a more open secondary structure than normal tRNA. A well-defined secondary and tertiary structure is required for the function of tRNA (Gartland and Sueoka, 1966; Lindahl *et al.*, 1966, 1967; Sueoka *et al.*, 1966). Acceptance and transfer of amino acids by tRNA that contains FU suggest that, *in vitro* at least, a unique macromolecular structure may not be necessary for the function of tRNA.

References

Beaven, G. H., Holiday, E. R., and Johnson, E. A.

(1955), The Nucleic Acids, Vol., I, Chargaff, E., and Davidson, J. N., Ed., New York, N. Y., Academic, p 493.
Bergquist, P. L. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 435.
Bergquist, P. L., Baguley, B. C., and Ralph, R. K. (1967), *Methods Enzymol. 12A*, 660.
Bergquist, P. L., Burns, D. J. W., and Plinston, C. A. (1968), *Biochemistry 7*, 1751 (this issue; preceding paper).
Bergquist, P. L., and Scott, J. F. (1964), *Biochim. Biophys. Acta 87*, 199.
Bujard, H., and Heidelberger, C. (1966), *Biochemistry 5*, 3339.
Bussard, A., Naono, S., Gros, F., and Monod, J. (1960), *Compt. Rend. 250*, 4049.
Capecchi, M. R. (1966), *J. Mol. Biol. 21*, 173.
Capecchi, M. R., and Gussin, G. (1965), *Science 149*, 417.
Champe, S. P., and Benzer, S. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 532.
Dahl, J. L., Way, J. L., and Parks, R. E. (1959), *J. Biol. Chem. 234*, 2998.
Edlin, G. (1965a), *J. Mol. Biol. 12*, 356.
Edlin, G. (1965b), *J. Mol. Biol. 12*, 363.
Fraenkel-Conrat, H., Singer, B., and Tsugita, A. (1961), *Virology 14*, 54.
Gartland, W. J., and Sueoka, N. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 948.
Gesteland, R. F., and Boedtker, H. (1964), *J. Mol. Biol. 8*, 496.
Goldwasser, E., and Heinrikson, R. L. (1966), *Progr. Nucleic Acid Res. Mol. Biol. 5*, 399.
Gray, P. N., and Rachmeler, M. (1967), *Biochim. Biophys. Acta 138*, 432.
Gros, F., and Naono, S. (1961), Protein Biosynthesis, Harris, R. J. C., Ed., London, Academic, p 195.
Gros, F., Naono, S., Hayes, D., Hayes, F., and Watson, J. D. (1962), *Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 106*, 437.
Heidelberger, C. (1965), *Progr. Nucleic Acid Res. Mol. Biol. 4*, 1.
Horowitz, J., and Chargaff, E. (1959), *Nature 184*, 1213.
Horowitz, J., Saukkonen, J. J., and Chargaff, E. (1960), *J. Biol. Chem. 235*, 3266.
Ishihama, A., Mizuno, N., Takai, M., Otaka, E., and Osawa, S. (1962), *J. Mol. Biol. 5*, 251.
Kaziro, Y., Gossman, A., and Ochoa, S. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 54.
Kellog, D. A., Doctor, B. P., Loebel, J. E., and Nirenberg, M. W. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 912.
Lane, B. G. (1963), *Biochim. Biophys. Acta 72*, 110.
Lanni, Y. T. (1961), *Virology 15*, 127.
Leder, P., and Nirenberg, M. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 420.
Levin, D. H., and Litt, M. (1965), *J. Mol. Biol. 14*, 506.
Lindahl, T., Adams, A., and Fresco, J. R. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 941.
Lindahl, T., Adams, A., Geroch, M., and Fresco, J. R. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 178.
Madison, J. T., Everett, G. A., and Kung, H. (1966), *Science 153*, 531.
Monier, R., Naono, S., Hayes, D., Hayes, F., and Gros, F. (1962), *J. Mol. Biol. 5*, 311.
Nakada, D., and Magasanik, B. (1964), *J. Mol. Biol. 8*, 105.
Nirenberg, M., and Leder, P. (1964), *Science 145*, 1399.
Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T., and Britten, R. J. (1955), Studies of Biosynthesis in *Escherichia coli*, Washington, D. C., Carnegie Institute of Washington, Publication 607.
Rosen, B. (1965), *J. Mol. Biol. 11*, 845.
Rosset, R., and Monier, R. (1963), *Biochim. Biophys. Acta 68*, 653.
Rosset, R., Monier, R., and Julien, J. (1964), *Bull. Soc. Chim. Biol. 46*, 87.
Schleich, T., and Goldstein, J. (1966), *J. Mol. Biol. 15*, 136.
Scott, J. F. (1968), *Methods Enzymology* (in press).
Sueoka, N., Kano-Sueoka, T., and Gartland, W. J. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 571.
Sueoka, N., and Yamane, T. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1454.
Sueoka, N., and Yamane, T. (1963), Informational Macromolecules, Vogel, H. J., Bryson, V., and Lampen, J. O., Ed., New York, N. Y., Academic, p 205.
Thach, R. E., and Sundararajan, T. A. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1021.
Tooze, J., and Weber, K. (1967), *J. Mol. Biol. 28*, 311.
Wagner, N. J., and Heidelberger, C. (1962), *Biochim. Biophys. Acta 61*, 373.
Weber, K., and Konigsberg, W. (1967), *J. Biol. Chem. 242*, 3563.