Schmidt, G., and Levene, P. A. (1938), *J. Biol. Chem. 126*, 423.
Sober, H. A., Schlossman, S. F., Yaron, A., and Rushizky, G. W. (1965), *Science 150*, 3694.
Strauss, J. H., Jr., and Sinsheimer, R. L. (1963), *J. Mol. Biol. 7*, 43.
Tomlinson, R. V., and Tener, G. M. (1962), *J. Am. Chem. Soc. 84*, 2644.
Whitfield, P. R., and Witzel, H. (1963), *Biochim. Biophys. Acta 72*, 362.

Fidelity in the Translation of Messenger Ribonucleic Acids in Mammalian Subcellular Systems*

I. Bernard Weinstein,† Manuel Ochoa, Jr.,‡ and S. Marvin Friedman‡

ABSTRACT: Magnesium, low temperature, aminoglycoside antibiotics, and polyamines are known to produce mistakes in translation of the genetic code in subcellular bacterial systems. In the present study, the effects of these same environmental factors on fidelity in the translation of native messenger ribonucleic acids (m-RNA's) and of polyuridylic acid (poly-U) have been examined in a subcellular rat liver and a rabbit reticulocyte system. When protein synthesis was directed by native m-RNA's, high concentrations of magnesium, low temperature, or the addition of streptomycin, spermine, and spermidine inhibited protein synthesis and decreased the ratio of leucine to phenylalanine present in the product. When protein synthesis was directed by polyuridylic acid, these same factors did not appreciably enhance the ability of poly-U to code for leucine or isoleucine. In both mammalian systems, poly-U-directed leucine incorporation seldom exceeded 6% of that obtained with phenylalanine. There was no stimulation of isoleucine incorporation. The results suggest that the translation mechanism of mammalian cells functions with higher fidelity *in vitro* than that of bacteria.

Previous studies from this laboratory have indicated that specificity in the translation of synthetic messenger RNA's (m-RNA's) in a subcellular system derived from *Bacillus stearothermophilus* is influenced to a considerable extent by temperature, magnesium concentration, polyamines, and dihydrostreptomycin (Friedman and Weinstein, 1964, 1965a,b). It has also been demonstrated that with extracts of *Escherichia coli* a variety of factors can modify specificity in the translation of a synthetic m-RNA. These factors include: temperature (Szer and Ochoa, 1964), magnesium concentration (Szer and Ochoa, 1964; So and Davie, 1964; Grunberg-Manago and Dondon, 1965), streptomycin and related aminoglycoside antibiotics (Davies *et al.*, 1964, 1965; Pestka *et al.*, 1965), organic solvents (So and Davie, 1964, 1965), pH (Grunberg-Manago and Dondon, 1965), and the concentration of amino acids, or s-RNA (soluble ribonucleic acid) (So and Davie, 1965; Grunberg-Manago and Dondon, 1965; Pestka *et al.*, 1965; Davies *et al.*, 1965). We have chosen the term "fidelity" to refer to specificity in the translation of nucleotide sequences in m-RNA into amino acid sequences in protein (Friedman and Weinstein, 1964).

Previous studies with extracts of *Chlamydomonas* (Sager *et al.*, 1963) and mammalain cells (Weinstein and Schechter, 1962; Weinstein, 1963; Ochoa and Weinstein, 1964a) indicated that, in addition to directing the synthesis of polyphenylalanine, polyuridylic acid (poly-U) coded to a small extent for leucine. This suggested the existence of ambiguity for the UUU codon. It was of interest, therefore, to determine whether the factors which alter translation of RNA in bacterial systems have a similar effect in mammalian subcellular extracts.

In the present study we have examined the fidelity of protein synthesis in mammalian systems when directed by native m-RNA's ("endogenous reaction") and also when directed by poly-U. Preliminary reports of portions of this work have appeared in abstract form (Friedman *et al.*, 1966; Ochoa and Weinstein, 1966).

* Department of Medicine, Columbia University College of Physicians and Surgeons, and the Medical Service, Francis Delafield Hospital, New York, New York 10032. *Received May 5, 1966.* This research was supported by U. S. Public Health Service Research Grant No. R10 CA-02332 from the National Cancer Institute.

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Materials

Polyuridylic acid (poly-U) had a sedimentation coefficient of 8.5 S and was obtained from Miles Laboratories. Uniformly labeled L-[U-^{14}C]phenylalanine, L-[U-^{14}C]leucine, and L-[U-^{14}C]isoleucine were purchased from Schwarz BioResearch, Inc., and had specific activities of 177, 220, and 240 mc/mmoles, respectively. Spermine tetrahydrochloride, putrescine dihydrochloride, and streptomycin sulfate were purchased from Calbiochem; spermidine trihydrochloride and neomycin sulfate from Nutritional Biochemicals Corp. The sources of the remaining materials have been described previously (Ochoa and Weinstein, 1964b). "Standard buffer" contained: Tris, 0.01 M, pH 7.8, KCl, 0.06 M, magnesium acetate, 0.005 M, and 2-mercaptoethanol, 0.006 M. The "homogenizing buffer" was standard buffer in 0.25 M sucrose.

Methods

Rat Liver Fractionation. Three male Sprague–Dawley rats, each weighing 250–300 g and fed *ad libitum*, were decaptitated. The livers (combined weight approximately 40 g) were quickly excised, rinsed with cold homogenizing buffer, minced with a scissors, and transferred to a Potter–Elvehjem homogenizer. The latter had a loosely fitting Teflon pestle (Kopp Scientific Co., New York City). All subsequent procedures were carried out at 0–4°. Homogenizing buffer (1 volume) was added, the tissue was disrupted with seven strokes of the pestle at 1500 rpm, and an additional volume of buffer was added. The homogenate was centrifuged at 8000*g* for 10 min in the SS-34 rotor of the Servall centrifuge. The turbid supernatant fluid (S-8) was filtered through four layers of coarse gauze, and then centrifuged at 30,000*g* for 60 min. The clear supernatant fluid (top two-thirds) was removed and centrifuged at 45,000 rpm (at R_{av} 122,250*g*) for 60 min in the 50 srotor of the Spinco Model L centrifuge. The resulting supernatant fluid (S-122) was used for the preparation of pH 5 fraction (see below). The surfaces of the pellets (P-122) were rinsed with standard buffer and then each pellet was suspended in 1 ml of buffer, with the aid of a Teflon pestle. An insoluble residue was removed by centrifugation at 10,000*g* for 10 min. The resulting supernatant fluid is referred to as "light microsomes." An aliquot was diluted 1/500 with standard buffer and used to determine D_{260} units/ml. The D_{260}/D_{280} ratio of this fraction was 1.3–1.4 and the RNA to protein ratio was 1/4. One D_{260} unit corresponded to approximately 12 μg of RNA.

The bulk of the ribosomes were contained in the 30,000*g* pellet ("heavy microsomes"). This fraction was not used in the present experiments since previous studies indicated that light microsomes give a better response to poly-U than heavy microsomes (Weinstein, 1964).

The pH 5 fraction was prepared by the following modification of the procedure of Hoagland *et al.* (1958). S-122 (1 volume, usually 18 ml) was diluted with 2 volumes of standard buffer and the solution was brought to pH 5.2 with 1 N acetic acid. After 10 min, the precipitate was harvested by centrifugation at 5000*g* for 10 min. The supernatant fluid was discarded and the precipitate was washed, by suspension and recentrifugation, in one-tenth the original volume of distilled water. The precipitate was then suspended in 5 ml of standard buffer and adjusted to pH 7.5 with 0.1 N NaOH. An insoluble residue was removed by centrifugation at 5000*g* for 10 min. The opalescent supernatant fluid is referred to as the "pH 5 fraction." An aliquot was diluted 1/100 and read at 260 and 280 mμ. Its D_{260}/D_{280} ratio was 1.2 and the RNA/protein ratio was approximately 1/9. One D_{260} unit corresponded to approximately 170 μg of protein and 15 μg of RNA. RNA and protein were assayed as described previously (Ochoa and Weinstein, 1964b).

Preparation of Reticulocyte Extracts. Rabbits were injected subcutaneously with 1.2% phenylhydrazine in 0.9% saline (5 mg/kg) on days 1–4, and on day 7 they were bled by cardiac puncture. Blood was collected in heparinized syringes and chilled. All subsequent procedures were done at 0–4°. The whole blood was centrifuged at 2000*g* for 15 min, and the plasma and buffy coat were aspirated and discarded. The pellet, consisting of more than 85% reticulocytes, was gently suspended in 3 volumes of 0.9% saline, recentrifuged, and the supernatant fluid together with any residual buffy coat was again aspirated and discarded. The pellets were then suspended in 2 volumes of standard buffer and transferred to plastic tubes. These tubes were placed in Dry Ice–acetone for 5 min and then thawed in a water bath at 40°. This freeze–thaw cycle was repeated and the lysate was centrifuged at 2000*g* for 10 min, and then at 10,000*g* for 10 min. The resulting supernatant fraction (S-10) was either used as such or further fractionated, by centrifugation at 122,000*g* for 60 min, into ribosomes (P-122) and a ribosomal supernatant fraction (S-122). After rinsing the surfaces of the ribosome pellets with standard buffer, the ribosomes were suspended in 0.5 ml of the standard buffer and clarified by centrifugation at 2000*g* for 10 min. One D_{260} unit of reticulocyte ribosomes is equivalent to approximately 445 μg of ribosomal protein and an equal amount of ribosomal RNA (Ts'o and Vinograd, 1961). The pH 5 fraction was prepared from the S-122 fraction of reticulocytes essentially as described above for rat liver.

Assay for Amino Acid Incorporation. The total volume of the *in vitro* incorporation system was 0.4 ml. The standard assay system contained the following components (in micromoles per milliliter unless otherwise specified): Tris–HCl buffer, pH 7.8, 10; magnesium acetate, 5; potassium chloride, 60; 2-mercaptoethanol, 3; ATP,[1] 0.63; GTP, 0.03; phosphoenolpyruvate, potassium salt, 3.12; phosphoenolpyruvate kinase, 30 μg; a mixture of 20 L-[^{12}C]amino acids (Nirenberg

[1] Abbreviations used: ATP and GTP, adenosine and guanosine triphosphates.

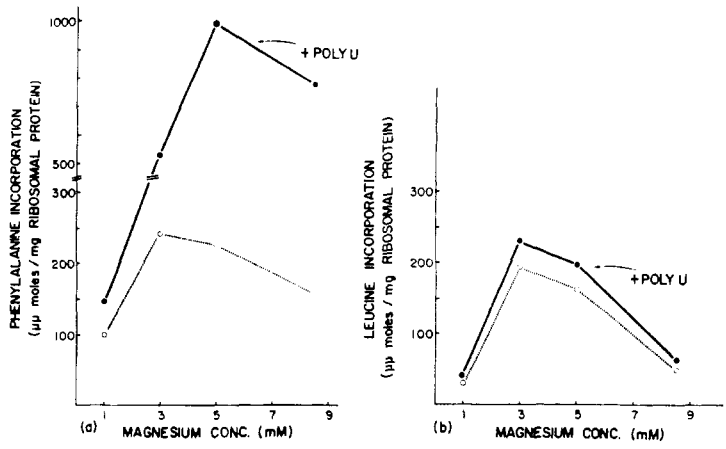

FIGURE 1: The effect of magnesium concentration on leucine and phenylalanine incorporation in a rat liver system in the absence and presence of poly-U (100 μg). Assay systems contained rat liver pH 5 fraction (1.5 D_{260} units) and light microsomes (3.0 D_{260} units) and were incubated at 30° for 45 min. The remaining conditions are as described under *Methods*.

and Matthaei, 1961), excluding the radioactive amino acid, 0.03 of each; an L-[^{14}C]amino acid, approximately 2 mμmoles, and cell fractions from either rat liver or reticulocytes. Assays were performed in the absence of added poly-U ("endogenous reaction") and in the presence of 100 μg of poly-U. Additional details or modifications are described in the legends. After incubation under the specified conditions (see legends), reactions were stopped by the addition of 0.7 ml of a solution containing 8% trichloroacetic acid and 3% casamino acids. After 10 min at room temperature, samples were heated at 85° for 30 min. The precipitates were then dispersed by vigorous mixing, deposited on Whatman No. 1 filter paper disks, washed under suction with three 2-ml volumes of a 1% solution of trichloroacetic acid containing 1% casamino acids, and then finally washed twice with 2 ml of 70% ethanol. The filters were mounted on planchets, dried, and counted with an error of less than ±3% in a Nuclear-Chicago low-background gas-flow counter with a Micromil end window (efficiency of approximately 25%). A reagent blank, containing all of the components of a given reaction system except the cell extract, was always incubated and processed with test samples. It contained 40 ± 10 cpm and these counts were subtracted from the value obtained for each test sample. "Net poly-U stimulation" refers to incorporation of a given amino acid in the presence of poly-U minus incorporation of the same amino acid in the absence of poly-U.

Results

Effect of Magnesium Concentration in the Rat Liver System. The effects of magnesium concentration on the incorporation of phenylalanine and leucine in a rat liver system are indicated in Figure 1. The endogenous incorporation (no poly-U) of both amino acids was maximal at 3 × 10^{-3} M magnesium. The optimal magnesium concentration for poly-U-directed phenylalanine incorporation was 5 × 10^{-3} M, and under the latter condition phenylalanine incorporation was approximately five times that obtained in the absence of poly-U. The addition of poly-U resulted in a small stimulation of leucine incorporation. Maximal leucine stimulation occurred at 3–5 × 10^{-3} M magnesium and incorporation in the presence of poly-U was only about 1.2 times the incorporation obtained in the absence of poly-U. A higher concentration of magnesium, 8.5 × 10^{-3} M, resulted in marked inhibition of total leucine incorporation as well as inhibition of the stimulation due to poly-U. Table I summarizes the differential effect of increasing magnesium concentration on the incorporation of leucine and phenylalanine

TABLE I: Effect of Magnesium Concentration on Leucine and Phenylalanine Incorporation in a Rat Liver System.[a]

Mg^{2+} (× 10^{-3} M)	Leu/Phe Ratio (%)	
	−Poly-U	+Poly-U (net stim)[b]
1	29	24
3	80	12
5	71	5
8.5	33	2

[a] Figures are in per cent and were calculated from the amino acid incorporation data presented in Figure 1. [b] Net poly-U stimulation incorporation of a given amino acid in presence of poly-U minus incorporation in the absence of poly-U.

With high concentrations of magnesium, the ratio of leucine to phenylalanine incorporation fell, both in the absence and presence of poly-U. Therefore in this system, in contrast to bacterial systems (Friedman and Weinstein, 1964; Szer and Ochoa, 1964; So and Davie, 1964), high magnesium concentrations do not favor leucine-phenylalanine ambiguity.

Effect of Streptomycin in the Rat Liver System. It is well known that mammalian cells *in vivo* are quite resistant to growth inhibition by streptomycin and dihydrostreptomycin. With sensitive strains of bacteria, 10^{-5} M streptomycin causes complete growth inhibition (Hurwitz, 1963; Brock, 1964), but concentrations of 10^{-2}–10^{-3} M are required to inhibit the growth of mammalian cells in tissue culture (Moskowitz and Keller, 1963). With extracts of sensitive strains of *E. coli*, concentrations of streptomycin in the range of 10^{-5}–10^{-6} M produce greater than 50% inhibition of poly-U-directed phenylalanine incorporation (Speyer *et al.*, 1962; Flaks *et al.*, 1962). With extracts of rat liver, concentrations of the drug in the range of 1–5 × 10^{-4} M produced greater than 50% inhibition of endogenous or poly-U-directed phenylalanine incorporation (Table II). This inhibition suggests that the resistance of mammalian cells *in vivo* to even higher concentrations of streptomycin is due, at least in part, to impermeability (Brock, 1964; Ekzemplyarov, 1965). We have repeatedly found that with the endogenous reaction leucine incorporation was inhibited more than phenylalanine incorporation. As a consequence of this differential sensitivity, high concentrations of the drug produced a fall in the leucine to phenylalanine ratio. In contrast to the results obtained with bacterial systems (Davies *et al.*, 1964, 1965; Friedman and Weinstein, 1964), streptomycin caused no, or at most, a very small enhancement of poly-U-directed leucine incorporation (Table II).

TABLE II: Effect of Streptomycin on Leucine and Phenylalanine Incorporation in a Rat Liver System.[a]

	Streptomycin (× 10^{-5} M)	−Poly-U Leu	Phe	Leu/Phe (%)	+Poly-U (net stim) Leu	Phe	Leu/Phe (%)
A	0	230	270	85	20	2600	<1.0
	5	210	240	88	30	2090	1.0
	10	180	210	86	10	1590	<1.0
	50	30	130	23	20	360	6.0
B	0	390	410	95	0	2850	—
	25	120	170	71	60	960	6.0
	50	40	120	33	10	360	3.0
	75	20	100	20	0	100	—

[a] Assay systems contained: rat liver pH 5 fraction (1.5 D_{260} units) and light microsomes (3.0 D_{260} units) and streptomycin at the final concentrations indicated. Incubation was at 30° for 45 min. The remaining conditions were as described under *Methods*. Values for Leu and Phe represent micromicromoles of each amino acid incorporated per milligram of ribosomal protein, and Leu/Phe is the ratio of the two incorporations expressed as per cent. Experiments A and B were done with two different preparations of extracts.

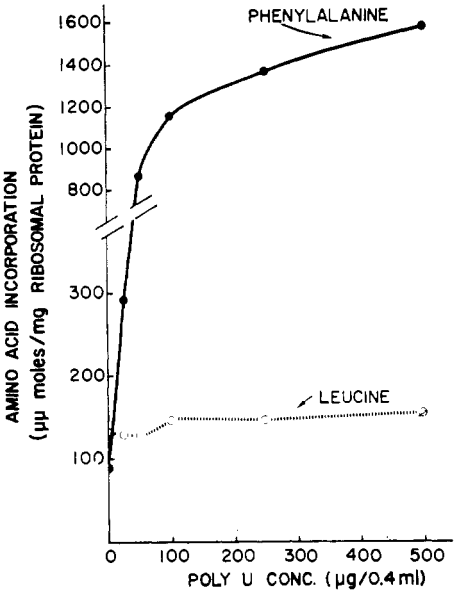

FIGURE 2: The effect of poly-U concentration on phenylalanine and leucine incorporation in a rabbit reticulocyte system. Assay systems contained 0.1 ml of reticulocyte S-10 fraction, increasing amounts of poly-U, and the indicated [^{14}C]amino acid. In this experiment no [^{12}C]amino acids were added and the magnesium concentration was 0.008 M. The reaction mixture was incubated at 37° for 60 min. The remaining conditions are as described under *Methods*.

Effect of Poly-U on Leucine and Phenylalanine Incorporation in the Reticulocyte System. Initial studies with reticulocytes were done with crude extracts, the S-10 fraction, since it was thought that this fraction might approximate the conditions of the intact cell. Poly-U, in the range of 25–500 μg/system, gave a concentration-dependent stimulation of [^{14}C]phenylalanine incorporation (Figure 2). At 100 μg of poly-U, phenylalanine incorporation was approximately 14 times and at 500 μg 19 times that obtained in the absence of polymer. Poly-U gave a very small stimulation of leucine incorporation, which was at most 5% of that with phenylalanine, and was maximal at 100 μg. This small stimulation appears to be a coding function of the polymer rather than nonspecific enhancement of protein synthesis, since poly-U (25–500 μg/ml) did not stimulate, and actually inhibited, the incorporation of lysine and arginine (unpublished studies).

Nair and Arnstein (1965) have also found that the stimulation of leucine incorporation by poly-U in the reticulocyte system is extremely low compared with phenylalanine.

Time-course studies indicated that maximal poly-U stimulation of leucine incorporation occurred by 30 min, whereas poly-U stimulation of phenylalanine incorporation continued to rise for at least 120 min. Previous studies with extracts of leukemia cells (Ochoa and Weinstein, 1964b) and extracts of *Chlamydomonas* (Sager *et al.*, 1963) also indicated that the poly-U leucine reaction reached a plateau earlier than the poly-U phenylalanine reaction.

Effects of Temperature and Magnesium in the Reticulocyte System. Table III summarizes the effects of temperature and magnesium concentration on leucine and phenylalanine incorporation by the S-10 fraction of reticulocytes. When the endogenous reaction (which presumably reflects hemoglobin synthesis) was studied at 37° and 0.005 M magnesium, leucine incorporation was greater than phenylalanine incorporation. This is consistent with the amino acid composition of rabbit hemoglobin (Naughton and Dintzis, 1962). Raising the magnesium concentration to 0.008 M inhibited the endogenous incorporation of both amino acids to about the same extent. When the temperature was lowered to 20° and the magnesium concentration kept at 0.005 M, the endogenous incorporation of leucine, but not of phenylalanine, was inhibited. At 20° and 0.008 M magnesium, the endogenous incorporation of both amino acids was inhibited, but leucine incorporation was inhibited to a greater extent than phenylalanine incorporation. Therefore, lowering the temperature from 37 to 20° and raising the magnesium concentration from 0.005 to 0.008 M produced a fall in the ratio of leucine to phenylalanine incorporated into the product from 136 to 65% (Table III).

Poly-U-directed phenylalanine incorporation at 37° was inhibited slightly by raising the magnesium concentration from 0.005 to 0.008 M, and there was a very slight enhancement of poly-U-directed leucine incorporation. Higher concentrations of magnesium markedly inhibited the incorporation of both amino acids. Lowering the temperature to 20° failed to enhance poly-U-directed leucine incorporation, either at 0.005 or 0.008 M (Table III).

Rabbit hemoglobin contains only a few isoleucine residues per molecule in the α chain and none in the β chain (Naughton and Dintzis, 1962). Miscoding for this amino acid might be more easily detected, therefore, than miscoding for leucine. Table IV indicates the effects of increasing concentrations of magnesium on isoleucine and phenylalanine incorporation when the reticulocyte system was incubated at 30°. In the absence of poly-U, the ratio of isoleucine to phenylalanine remained at approximately 15% across a threefold magnesium concentration. In the presence of poly-U, high concentrations of magnesium also failed to produce detectable poly-U stimulation of isoleucine incorporation.

Effect of Neomycin in the Reticulocyte System. Davies *et al.* (1964, 1965) found that neomycin markedly enhances poly-U-directed isoleucine incorporation in the *E. coli* system. In the reticulocyte system, in-

TABLE III: Effect of Temperature and Magnesium Concentration on Leucine and Phenylalanine Incorporation in a Reticulocyte System.[a]

Temp (°C)	Mg^{2+} ($\times 10^{-3}$ M)	−Poly-U Leu	Phe	Leu/Phe (%)	+Poly-U (net stim) Leu	Phe	Leu/Phe (%)
37	5	121	89	136	−32	1367	—
37	8	74	57	130	24	1250	2
20	5	112	94	119	−9	1186	—
20	8	30	46	65	0	166	—

[a] Assay systems contained 0.1 ml of the rabbit reticulocyte S-10 fraction. The magnesium concentration and incubation temperature were as indicated. All samples were incubated for 60 min. The remaining conditions were as described under *Methods*. Values for Leu and Phe represent micromicromoles of each amino acid incorporated per milligram of ribosomal protein, and Leu/Phe is the ratio of the two incorporations expressed as per cent.

TABLE IV: Effect of Magnesium Concentration on Isoleucine and Phenylalanine Incorporation in a Reticulocyte System.[a]

Mg^{2+} ($\times 10^{-3}$ M)	−Poly-U Ile	Phe	Ile/Phe (%)	+Poly-U (net stim) Ile	Phe	Ile/Phe
3	50	336	15	−19	1930	—
5	36	273	13	−7	2128	—
7	23	166	14	−3	1049	—
10	8	59	14	0	352	—

[a] Assay systems contained rabbit reticulocyte pH 5 fraction (3 D_{260} units) and ribosomes (4 D_{260} units.) The magnesium concentration was as indicated. Samples were incubated at 30° for 45 min. The remaining conditions were as described under *Methods*. Values for Ile and Phe represent micromicromoles of each amino acid incorporated per milligram of ribosomal protein and Ile/Phe is the ratio of the two incorporations expressed as per cent.

TABLE V: Effect of Neomycin on Isoleucine and Phenylalanine Incorporation in a Reticulocyte System.[a]

Neomycin (μg/ml)	−Poly-U			+Poly-U (net stim)		
	Ile	Phe	Ile/Phe (%)	Ile	Phe	Ile/Phe (%)
0	34	199	17	−6	2043	—
25	24	162	15	6	1692	<1
50	11	55	20	6	995	<1
100	0	6	—	0	82	—
250	0	0	—	0	0	—

[a] Assay systems contained reticulocyte pH 5 fraction (3 D_{260} units) and ribosomes (4 D_{260} units), and neomycin sulfate at the concentrations indicated. Samples were incubated at 37° for 30 min. The remaining conditions were as described under *Methods*. Values for Ile and Phe represent micromicromoles of each amino acid incorporated per milligram of ribosomal protein, and Ile/Phe is the ratio of the two incorporations expressed as per cent.

creasing concentrations of neomycin brought about a progressive inhibition of protein synthesis (Table V). With the endogenous reaction, the incorporations of isoleucine and phenylalanine were inhibited to approximately the same extent and, with the poly-U reaction, the drug failed to significantly enhance poly-U-directed isoleucine incorporation.

Effect of Polyamines in the Reticulocyte System. Since aliphatic polyamines are among the polybasic substances which enhance ambiguity in bacterial systems (Friedman and Weinstein, 1964), spermine, spermidine, and putrescine were also tested in the reticulocyte system (Table VI). Spermine at 1×10^{-5} M and spermidine at 1×10^{-4} M inhibited the endogenous incorporation of leucine and phenylalanine. Leucine was inhibited more than phenylalanine and, therefore, both agents produced a fall in the leucine to phenylalanine ratio. Poly-U-directed phenylalanine incorporation was strongly inhibited by spermine and spermidine, but spermine at 1×10^{-5} M and spermidine at 1×10^{-4} M caused a very small poly-U stimulation of leucine incorporation. Putrescine, in the range of 1×10^{-6}–1×10^{-4} M, was without significant effect on amino acid incorporation, both in the absence and presence of poly-U.

Discussion

These results indicate that factors which greatly enhance the ability of poly-U to code for leucine and isoleucine in bacterial systems produced either no effect, or a very small effect, on the ability of poly-U to "miscode" for these two amino acids in a rat liver and a rabbit reticulocyte system. Poly-U-directed leucine incorporation seldom exceeded 6% of that obtained with phenylalanine, and there was no detectable stimulation of isoleucine incorporation. This is in contrast to results obtained with the *B. stearothermophilus* (Friedman and Weinstein, 1964) and *E. coli* (Davies *et al.*, 1964, 1965) subcellular systems in which poly-U-directed leucine or isoleucine incorporation can be forced to exceed poly-U-directed phenylalanine incorporation. The fidelity of mammalian systems in translation of other synthetic m-RNA's remains to be determined.

Since mammalian cells are resistant to growth inhibition by streptomycin (Moskowitz and Keller, 1963), resistance to the miscoding effects of this drug is not

TABLE VI: Effect of Polyamines on Leucine and Phenylalanine Incorporation in a Reticulocyte System.[a]

Polyamine (M)		−Poly-U			+ Poly-U (net stim)		
		Leu	Phe	Leu/Phe (%)	Leu	Phe	Leu/Phe (%)
None		133	66	202	−23	1188	—
Spermine	1×10^{-6}	140	71	197	−34	1110	—
	1×10^{-5}	60	36	167	8	142	6
	1×10^{-4}	9	14	64	0	0	—
Spermidine	1×10^{-6}	160	73	219	−45	1215	—
	1×10^{-5}	149	64	233	−35	1094	—
	1×10^{-4}	54	36	150	10	263	4
Putrescine	1×10^{-6}	144	67	215	−28	1090	—
	1×10^{-5}	145	66	220	−28	1075	—
	1×10^{-4}	159	66	241	−42	1048	—

[a] Assay systems contained reticulocyte pH 5 fraction (3 D_{260} units) and ribosomes (4 D_{260} units), and polyamines at the final concentrations indicated. Samples were incubated at 37° for 30 min. The remaining conditions were as described under *Methods*. Values for Leu and Phe represent micromicromoles of each amino acid incorporated per milligram of ribosomal protein, and Leu/Phe is the ratio of the two incorporations expressed as per cent.

surprising and is reminiscent of the resistance demonstrated by certain strains of *E. coli* (Davies *et al.*, 1964). With mammalian cells, however, resistance to streptomycin is associated with resistance to the miscoding effects of other agents, which is not the case in *E. coli* (Davies *et al.*, 1964, 1965). The component of the mammalian systems which accounts for their higher fidelity in translation of poly-U remains to be identified. In the rat liver system, an effect of the endoplasmic reticulum might be postulated, but this seems unlikely for reticulocytes since the ribosomes isolated from them are not associated with an endoplasmic reticulum (Rifkind *et al.*, 1964). Nor is it likely that our findings are restricted to highly differentiated animal cells, since recent studies by Dr. Ruy Soeiro (personal communication) indicate that streptomycin, guanidine, neomycin, spermine, and spermidine fail to enhance the ability of poly-U to code for leucine or isoleucine in subcellular systems derived from chick embryos or chick fibroblasts grown in tissue culture. In addition it appears, from *in vitro* studies of *Chlamydomonas* by R. Sager and F. G. Toback (personal communication) and *in vivo* studies of *Euglena* by Scher (1966), that algae are resistant to the miscoding effects of magnesium and aminoglycoside antibiotics. The possibility that the ribosomes of higher organisms have a structure which is inherently different from that of bacteria, and permits them to perform with higher fidelity than bacterial ribosomes, is being tested in current studies on the coding properties of *in vitro* systems containing mixtures of bacterial and animal cell components.

Despite the high specificity in translation of poly-U in mammalian subcellular systems, specificity in translation of native m-RNA's was influenced by changes in magnesium concentration and incubation temperature, or the addition of spermine, spermidine, or high concentrations of streptomycin. These effects were observed only when amino acid incorporation was inhibited and, in general, were a consequence of the fact that leucine incorporation was inhibited to a greater extent than phenylalanine. We have observed a similar effect with proflavine and related basic dyes (I. B. Weinstein, M. Ochoa, and S. M. Friedman, unpublished studies). Additional experiments are required to determine whether this effect is exerted during the synthesis of aminoacyl s-RNA's or at the ribosomal level. Since the reticulocyte system synthesizes mainly hemoglobin (Schweet *et al.*, 1958), it is possible that the change in leucine to phenylalanine ratio observed in this system reflects an actual modification of hemoglobin composition. Alternatively, the change in ratio may reflect the effects of partial *vs.* complete chain synthesis. Peptide analyses are required, therefore, to determine whether or not environmental factors can actually produce amino acid substitutions in the hemoglobin molecule. In this regard studies on the amino acid composition of rabbit hemoglobin peptides are of interest, since it has been found that a specific peptide (α T6) is apparently synthesized in more than one form *in vivo* (Weisblum *et al.*, 1965; G. Von Ehrenstein, personal communication). A similar situation has been recently described with mouse hemoglobin (Rifkin *et al.*, 1966). Though alternative explanations have not been excluded, the latter studies raise the possibility that ambiguity in the translation of hemoglobin m-RNA exists *in vivo*.

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References

Brock, T. D. (1964), *Federation Proc. 23*, 965.
Davies, J., Gilbert, W., and Gorini, L. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 883.
Davies, J., Gorini, L., and Davis, B. D. (1965), *Mol. Pharmacol. 1*, 93.
Ekzemplyarov, O. N. (1965), *Antibiotiki 10*, 425.
Flaks, J. G., Cox, E. C., and White, J. R. (1962), *Biochem. Biophys. Res. Commun. 7*, 385.
Friedman, S. M., Ochoa, M., Jr., and Weinstein, I. B. (1966), *Federation Proc. 25*, 775.
Friedman, S. M., and Weinstein, I. B. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 988.
Friedman, S. M., and Weinstein, I. B. (1965a), *Biochem. Biophys. Res. Commun. 21*, 339.
Friedman, S. M., and Weinstein, I. B. (1965b), *Biochim. Biophys. Acta 114*, 593.
Grunberg-Manago, M., and Dondon, J. (1965), *Biochem. Biophys. Res. Commun. 18*, 517.
Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. I., and Zamecnik, P. C. (1958), *J. Biol. Chem. 231*, 241.
Hurwitz, C. (1963), *in* Antimicrobial Agents and Chemotherapy, Sylvester, J. C., Ed., Ann Arbor, Mich., Braun-Bromfield, p 371.
Moskowitz, M., and Keller, N. E. (1963), *Science 141*, 647.
Nair, K. G., and Arnstein, H. R. V. (1965), *Biochem. J. 97*, 595.
Naughton, M. A., and Dintzis, H. M. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1822.
Nirenberg, M. W., and Matthaei, J. H. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1588.
Ochoa, M., Jr., and Weinstein, I. B. (1964a), *Proc. Natl. Acad. Sci. U. S. 52*, 470.
Ochoa, M., Jr., and Weinstein, I. B. (1964b), *J. Biol. Chem. 239*, 3834.
Ochoa, M., Jr., and Weinstein, I. B. (1966), *Clin. Res. 14*, 323.
Pestka, S., Marshall, R., and Nirenberg, M. W. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 639.
Rifkind, R. A., Luzzato, L., and Marks, P. A. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1227.

Rifkin, D. B., Rifkin, M. R., and Konigsberg, W. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 586.
Sager, R., Weinstein, I. B., and Ashkenazi, Y. (1963), *Science 140*, 304.
Scher, S. (1966), *Biochem. Biophys. Res. Commun. 22*, 572.
Schweet, R. S., Lamfrom, H., and Allen, E. (1958), *Proc. Natl. Acad. Med. Sci. U. S. 44*, 1029.
So, A. G., and Davie, E. W. (1964), *Biochemistry 3*, 1165.
So, A. G., and Davie, E. W. (1965), *Biochemistry 4*, 1973.
Speyer, J. F., Lengyel, P., Basilio, C. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 684.
Szer, W., and Ochoa, S. (1964), *J. Mol. Biol. 8*, 823.
Ts'o, P., and Vinograd, J. (1961), *Biochim. Biophys. Acta 49*, 113.
Weinstein, I. B. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 579.
Weinstein, I. B. (1964), *Bull. N. Y. Acad. Med. 40*, 89.
Weinstein, I. B., and Schechter, A. N. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1686.
Weisblum, B., Gonano, F., Von Ehrenstein, G., and Benzer, S. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 328.

Fluorinated Pyrimidines. XXVII. Attempts to Determine Transcription Errors during the Formation of Fluorouracil-Containing Messenger Ribonucleic Acid*

Hermann Bujard† and Charles Heidelberger‡

ABSTRACT: The hypothesis is presented that the inhibition of β-galactosidase production in *Escherichia coli* and the mutants of tobacco mosaic and polio viruses produced by 5-fluorouracil are a consequence of the incorporation of 5-fluorouracil into ribonucleic acid (RNA) in place of cytosine during the process of transcription or viral replication, and that the 5-fluorouracil so incorporated would be read in translation as if it were uracil instead of cytosine. This was tested using a synthetic deoxyribonucleic acid (DNA), with one strand consisting of alternating thymine and guanine sequences and the other strand consisting of alternating adenine and cytosine sequences, as the primer for purified *E. coli* RNA polymerase. In this system, the incorporation of isotope from [α-^{32}P]5-fluorouridine triphosphate and from [α-^{32}P]uridine triphosphate into the 2′-(3′)-adenosine monophosphate, obtained on alkaline hydrolysis of the polyribonucleotides, was measured. However, no difference could be detected between the two labeled precursors in this system, which were both incorporated into polyribonucleotides at a frequency of replacement of less than one uracil or 5-fluorouracil per 3000 cytosines. This low degree of incorporation can be explained by the presence of a very small amount of deoxyribonucleic acid in the purified ribonucleic acid polymerase preparation, which also had a little polyadenylate polymerase activity.

The primary mechanism whereby 5-fluorouracil[1] (Duschinsky *et al.*, 1957) inhibits the growth of bacterial, mammalian, and neoplastic cells involves the inhibition of the enzyme, thymidylate synthetase, by 5-fluoro-2′-deoxyuridine 5′-monophosphate (Cohen *et al.*, 1958; Hartmann and Heidelberger, 1961; Reyes and Heidelberger, 1965). On the other hand, the incorporation of FU into ribonucleic acid (RNA) has been reported in mouse tumors (Chaudhuri *et al.*, 1958), *Escherichia coli* (Horowitz and Chargaff, 1959), tobacco mosaic virus (TMV) (Gordon and Staehelin, 1959), polio virus (Munyon and Salzman, 1962), etc. The consequences of such incorporation have not always been clear. Polyfluorouridylic acid (poly-FU) has been

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† Holder of National Institutes of Health International Postdoctoral Fellowship 1-F05-TW-789-01. Present address: Southwest Center for Advanced Studies, Dallas, Texas.

‡ American Cancer Society Professor of Oncology.

[1] Abbreviations used: FU, 5-fluorouracil; FUDRP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; U, uracil; C, cytosine; T, thymine; G, guanine; A, adenine; ATP, adenosine triphosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate; FUTP, 5-fluorouridine triphosphate; 2′,3′-AMP, adenosine 2′-(3′)-monophosphate; poly-dAT, polydeoxyadenylate–polythymidylate C (alternating sequence); [^{3}H]GTP, tritiated guanosine triphosphate; 2′,3′-UMP, uridine 2′-(3′)monophosphate; TCA, trichloroacetic acid.