The Influence of Environment on the Specificity of Polynucleotide-dependent Amino Acid Incorporation into Polypeptide*

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Some of the environmental factors that alter the specificity of amino acid incorporation by synthetic polynucleotides in an *in vitro* system have been examined. Ammonium ion, magnesium ion, organic solvents, and urea have a marked effect on the specificity and quantity of various amino acids incorporated in the presence of synthetic polynucleotides. The ratio of phenylalanine to leucine to isoleucine incorporation in the presence of poly-U changes with increasing concentrations of ammonium ion. With increasing concentrations of magnesium ion, phenylalanine incorporation is inhibited while that of leucine and isoleucine is markedly stimulated by the addition of poly-U or poly-UA (5:1). These effects are similar to those found with increasing concentrations of organic solvents. The effects of magnesium ion and organic compounds are closely interrelated since ethanol lowers the optimum magnesium ion concentration while urea increases the magnesium ion requirement for incorporation of different amino acids. Streptomycin stimulates isoleucine incorporation in the presence of poly-U and its effect is additive with that of ethanol. The poly-U-dependent incorporation of isoleucine in the presence of alcohol and streptomycin is inhibited by chloramphenicol, while the incorporation of leucine in the presence of alcohol is not affected by chloramphenicol. These results emphasize the importance of environmental factors on the incorporation of amino acids in response to specific polyribonucleic acids in an *in vitro* system. Thus code assignments based upon *in vitro* experiments are valid only when environmental conditions reflect those found *in vivo*.

The effects of organic solvents on amino acid incorporation in a cell-free system from *Escherichia coli* were recently reported by So and Davie (1964). These agents were found to alter the specificity of amino acids incorporated in response to specific polynucleotides. Others have also observed that environmental factors can alter the coding properties of polynucleotides. Szer and Ochoa (1964) and Friedman and Weinstein (1964) observed that the leucine "ambiguity" increases with decreasing temperature and increasing magnesium ion concentration. Davies *et al.* (1964) have emphasized the role of the ribosomes in coding specificity. These investigators have observed that streptomycin stimulates poly-U-directed isoleucine incorporation only when the ribosomes are derived from a streptomycin-sensitive strain of *E. coli*.

In extending our previous studies, other variables in the composition of the incubation mixture have been examined and found to have a marked effect on the type and quantity of amino acids incorporated by synthetic polynucleotides. The current theory of amino acid coding is discussed in terms of these data.

MATERIALS

Crystalline ATP (disodium salt) and GTP were purchased from Pabst Laboratories, Milwaukee, Wis. Phosphoenolpyruvate (trisodium salt), pyruvate kinase, and L-[^{12}C]amino acids (A grade) were purchased from California Corp. for Biochemical Research, Los Angeles, Calif. L-[U-^{14}C]Phenylalanine, L-[U-^{14}C]leucine, L-[U-^{14}C]isoleucine, L-[U-^{14}C]tyrosine, and L-[U-^{14}C]-proline were purchased from New England Nuclear Corp., Boston, Mass. Polycytidylic acid, polyuridylic acid, and polyuridylic-adenylic acid copolymer (5:1) were purchased from Miles Chemical Co., Elkhart, Ind. A base analysis of these preparations has not been performed. According to the manufacturer, poly-U and poly-C have sedimentation coefficients ranging from 4 to 7. Crystalline deoxyribonuclease was purchased from Worthington Biochemical Corp., Freehold, N.J. Spermine and puromycin were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Erythromycin A, chloramphenicol, and streptomycin were purchased from Eli Lilly, Indianapolis, Ind., Parke, Davis and Co., Detroit, Mich., and Chas. Pfizer and Co., New York, N.Y., respectively. Polyproline was kindly provided by Dr. P. E. Wilcox. *E. coli* K_{12} F^- (streptomycin sensitive) was a gift of Mr. Don Brenner. All chemicals were the commercially available reagent grade.

METHODS

Most of the methods employed in these experiments were reported previously (So and Davie, 1964). *E. coli* cells were harvested in the early log phase of growth and stored (for no longer than 4 weeks) at −20°. An incubated S-30 fraction was prepared essentially by the method of Nirenberg and Matthaei (1961). Extracts were freshly prepared at least every 2 weeks.

The standard incubation mixture contained 0.100 M Tris buffer (*p*H 7.8), 0.011 M magnesium acetate, 0.006 M mercaptoethanol, 0.060 M NH_4Cl, 0.005 M phosphoenolpyruvate, 0.001 M ATP, 5×10^{-5} M GTP, and 100 μg of pyruvic kinase in a final volume of 1 ml. In all cases the amount of supernatant protein was approximately three times that of ribosomal protein. Tubes were routinely incubated for 30 minutes at 25°.

[^{14}C]Amino acids of high specific activity were routinely diluted to a specific activity of 25 μc/μmole with [^{12}C]amino acids. Sample counting was done by the method of So and Davie (1963) using a Packard scintillation spectrometer Model 314EX. One $\mu\mu$mole of isotopically diluted [^{14}C]amino acid produced approximately 25 cpm by this method. All experimental values reported have been corrected for the background count of the instrument (60 cpm) and incorporation in the absence of added synthetic polynucleotide, which generally did not exceed 50 cpm.

Absolute values (activity, magnesium optima, etc.) in amino acid incorporation varied somewhat from one

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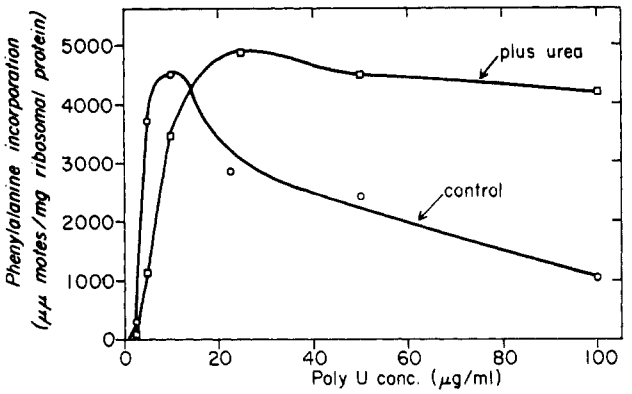

FIG. 1.—Effect of 0.5 M urea on phenylalanine incorporation. In addition to the standard reaction mixture each tube contained 1×10^{-5} M [^{14}C]phenylalanine, 3.2×10^{-4} M spermine, and 1.32 mg ribosomal protein.

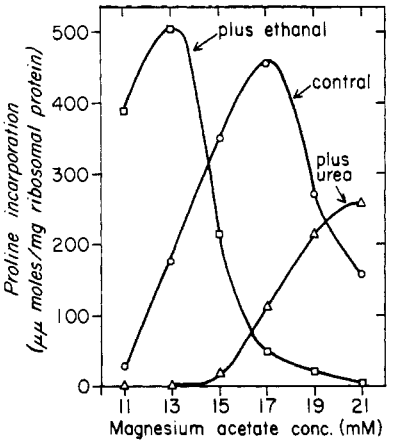

FIG. 2.—The magnesium optima for poly-C-directed proline incorporation in the presence and absence of 0.64 M ethanol and 0.5 M urea. In addition to the standard reaction mixture each tube contained 1.7 mg ribosomal protein, 2 mg poly-C, 1×10^{-1} M [^{14}C]proline, and 1×10^{-5} M each of the nineteen remaining [^{12}C]amino acids. The final concentrations of magnesium acetate are shown in the graph.

preparation to the next. The same general pattern, however, was observed with all preparations tested.

RESULTS

Effect of Urea on Amino Acid Incorporation.—In our previous study (So and Davie, 1964), the compounds tested were less polar than water. Thus it was of interest to examine the effect of a more polar compound, such as urea, on amino acid incorporation. In Figure 1 the effect of 0.5 M urea on phenylalanine incorporation is shown at varying levels of poly-U. In the control experiment, phenylalanine incorporation reaches an optimum at about 10 μg/ml poly-U and falls to about 25% of this value at 100 μg/ml. In the presence of urea, phenylalanine incorporation reaches a maximum at about 25 μg/ml of poly-U, and declines only slightly at 100 μg/ml. In contrast, less polar solvents such as ethanol stimulate phenylalanine incorporation at low poly-U and inhibit at higher poly-U concentrations. In general, urea inhibited leucine and isoleucine incorporation by poly-U.

A 1.5- to 2.0-fold stimulation of phenylalanine, leucine, and isoleucine incorporation by ethanol was also observed, employing nonpreincubated *E. coli* preparations. In these experiments no synthetic polynucleotide was added and amino acid incorporation was presumably due to endogenous natural messenger RNA. Other solvents such as dimethyl sulfoxide gave results similar to those with ethanol for phenylalanine, leucine, and isoleucine incorporation in the presence of poly-U (So and Davie, 1964).

It was previously observed that the effects of alcohol and magnesium were closely interrelated (So and Davie, 1964). Figure 2 shows the effect of ethanol and urea on proline incorporation by high levels of poly-C (2 mg/ml) at various concentrations of magnesium. In the control, lacking ethanol or urea, proline incorporation was maximal at 17 mM magnesium. In the presence of ethanol this optimum was lowered to 13 mM. With urea, proline incorporation was completely abolished below 15 mM and was still increasing at 21 mM magnesium. Similar results were obtained with lower levels of polymer (100 μg/ml).

A similar pattern was observed for phenylalanine incorporation with poly-U, i.e., the optimal magnesium ion concentration was lowered by alcohol and increased by urea.

Effects of Varying Concentrations of Magnesium Ion on the Incorporation of Phenylalanine, Leucine, and Isoleucine by Poly-U.—Since alcohol decreases and urea increases the magnesium ion requirement for maximal amino acid incorporation, we reexamined the effects of a wider spectrum of magnesium ion concentration in the absence of polar and nonpolar compounds. The incorporation of phenylalanine, leucine, and isoleucine at a poly-U concentration of 10 μg/ml is shown in Figure 3A. With 16 mM magnesium, the incorporation of both phenylalanine and leucine is optimal. Higher concentrations of magnesium inhibit both phenylalanine and leucine incorporation but stimulate isoleucine incorporation. The incorporation of leucine or isoleucine at low poly-U concentrations never exceeded that of phenylalanine incorporation, although the leucine and isoleucine to phenylalanine ratios change with increasing magnesium concentrations.

With high levels of poly-U (100 μg/ml) the magnesium ion effects are quite different. As shown in Figure 3B, phenylalanine incorporation reaches a maximum at 8 mM magnesium, and with 16 mM magnesium marked inhibition is observed. Leucine incorporation reaches a peak at 24 mM magnesium while that of isoleucine is maximal at 32 mM magnesium. Thus the ratios of phenylalanine to leucine and isoleucine incorporation again change with increasing concentration of magnesium. Furthermore, at high poly-U and high magnesium concentrations, the absolute amount of incorporation of either leucine or isoleucine is greater than that of phenylalanine. The magnesium optimum for phenylalanine incorporation decreases from 16 to 8 mM when the poly-U concentration is increased from 10 to 100 μg/ml (Fig. 3).

Effects of Varying Magnesium Ion and Polymer Concentration on Poly-UA-directed Amino Acid Incorporation.—Most of the amino acid code assignments are based on experiments employing synthetic polynucleotides containing two different bases. The same parameters are important in determining the coding properties of copolymers as described for homopolymers. Figure 4A shows the amounts of phenylalanine, leucine, isoleucine, and tyrosine incorporated by increasing levels of poly-UA (5:1) employing 7 mM magnesium. Phenylalanine incorporation greatly exceeds that of the other three amino acids and the incorporation of all four increases gradually and then levels off at about 100 μg/ml of polymer. The incorporation of leucine, isoleucine, and tyrosine relative to that of phenylalanine is shown in Figure 4B. Isoleucine incorporation remains relatively con-

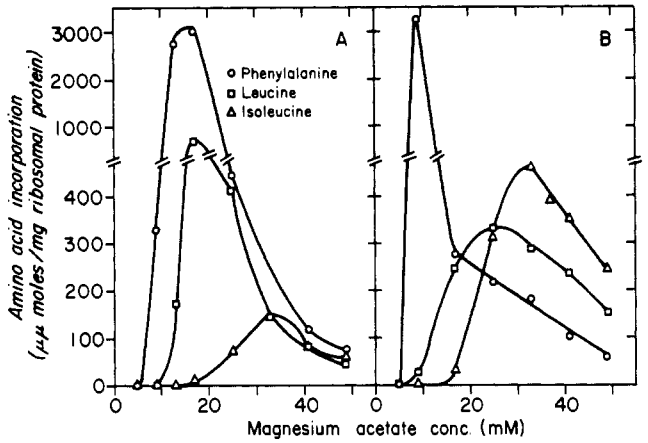

FIG. 3.—Effect of magnesium ion concentration on amino acid incorporation with 10 μg/ml (A) and 150 μg/ml (B) poly-U. In addition to the standard reaction mixture each tube contained 1.25 mg ribosomal protein and 1×10^{-5} M [^{14}C]amino acid (phenylalanine, leucine, or isoleucine, as indicated), and 1×10^{-5} M each of the nineteen remaining [^{12}C]amino acids. The final concentrations of magnesium ion are shown in the graph.

stant at about 20% that of phenylalanine. Leucine and tyrosine percentages increase with increasing polymer concentration from about 11 to 19%.

With high magnesium concentration (27 mM) the pattern of incorporation is greatly altered. Phenylalanine incorporation no longer exceeds that of leucine or isoleucine except at very low polymer concentration (Fig. 5A). The incorporation of tyrosine relative to phenylalanine shown in Figure 5B is somewhat higher (32%) than at lower magnesium concentrations. The leucine and isoleucine, however, reach 205 and 336%, respectively. At intermediate magnesium concentrations, percentages between those observed at 7 and 27 mM were found.

In general it was observed that low levels of magnesium or polynucleotide led to lower percentages of leucine, isoleucine, and tyrosine incorporation relative to phenylalanine. The sum of the four amino acids incorporated at high magnesium, however, was about 40% of the sum of that incorporated at low magnesium concentrations.

Effects of Varying Concentration of Ammonium Ion on Incorporation of Various Amino Acids by Poly-U.—Most of the cell-free systems from microbial sources currently being used in studying protein biosynthesis contain ammonium ion. The ammonium ion has been found to be more effective than potassium ion in stimulating amino acid incorporation in *E. coli* (Lubin and Ennis, 1963). More recently, ammonium ion was reported to be important in the binding of phenylalanyl-s-RNA to ribosomes in the presence of poly-U (Spyrides, 1964).

Table I shows the effects of various concentrations of ammonium ion on the incorporation of phenylalanine, leucine, and isoleucine at three different levels of magnesium ion in the presence of 100 μg/ml of poly-U.

Phenylalanine incorporation was stimulated about 33% by ammonium ion at the different magnesium concentrations studied. With 8 mM magnesium, where the greatest incorporation of phenylalanine occurs, an optimal effect of ammonium ion is seen at 60 mM. At this level of magnesium only a very small amount of leucine, and no isoleucine, is incorporated.

With 16 mM magnesium the incorporation of leucine is stimulated 2.5-fold by 150 mM ammonium ion. At this concentration of ammonium ion the absolute

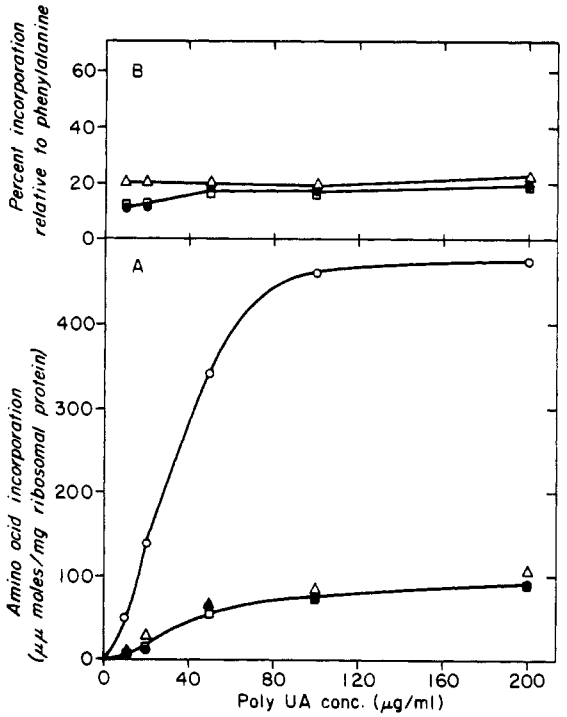

FIG. 4.—Effect of varying poly-UA (5:1) concentration on amino acid incorporation with 7 mM magnesium ion. In addition to the standard reaction mixture each tube contained 1.74 mg ribosomal protein, 3.2×10^{-4} M spermine, and 1×10^{-5} M [^{14}C]amino acid (phenylalanine, O—O; leucine, □—□; isoleucine, Δ—Δ; tyrosine, ●—●) and 1×10^{-5} M each of the nineteen remaining [^{12}C]amino acids.

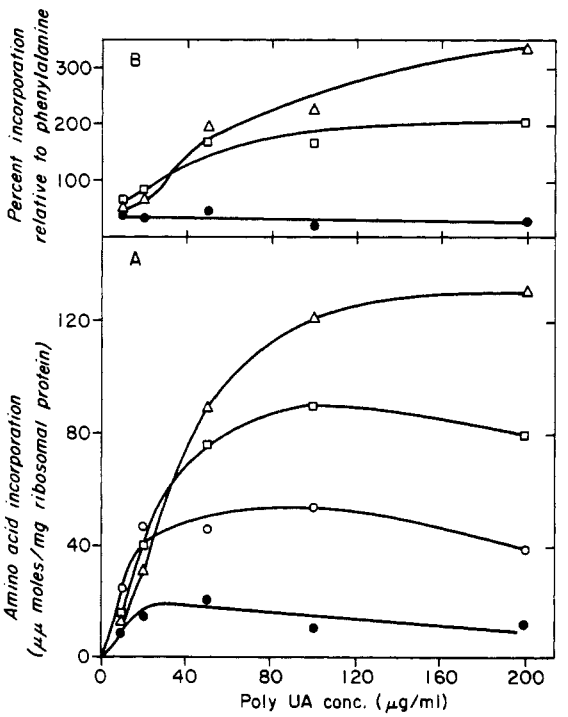

FIG. 5.—Effect of varying poly-UA (5:1) concentration on amino acid incorporation with 27 mM magnesium ion. With the exception of the magnesium ion concentration the conditions and symbols are identical to those of Fig. 4.

amount of leucine incorporation is greater than that of phenylalanine (611 versus 475).

With 32 mM magnesium concentration, phenylalanine incorporation is only slightly stimulated, while leucine incorporation is moderately stimulated and iso-

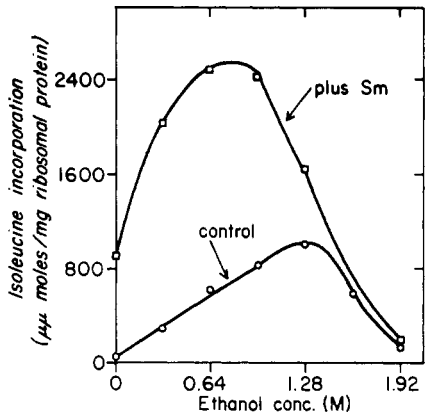

FIG. 6.—The effect of ethanol concentration on the incorporation of isoleucine by poly-U (100 μg/ml) in the presence and absence of streptomycin (20 μg/ml). In addition to the standard reaction mixture each tube contained 1×10^{-5} M [^{14}C]isoleucine, 3.2×10^{-4} M spermine, and 1.5 mg ribosomal protein.

TABLE I
INFLUENCE OF AMMONIUM ION ON INCORPORATION OF VARIOUS AMINO ACIDS BY POLY-U[a]

[^{14}C]Amino Acid	Ammonium Chloride Concn (mM)	Amino Acid Incorporation[b] at Various Magnesium Acetate Concentrations (mM)		
		8	16	32
Phenylalanine	0	1060	380	102
	60	1590	432	153
	90	1431	443	148
	150	1060	475	148
Leucine	0	15	242	149
	60	20	350	261
	90	18	432	329
	150	15	611	318
Isoleucine	0	<1	31	135
	60	<1	46	408
	90	<1	64	418
	150	<1	58	477

[a] Incubation mixture was the same as the standard incubation mixture under Methods except (1) concentrations of magnesium and ammonium ions as indicated in the table, (2) 1×10^{-5} M [^{14}C]amino acid (phenylalanine, leucine, or isoleucine) and each of the nineteen remaining [^{12}C]amino acids were added, and (3) *E. coli* extract contained 0.75 mg ribosomal protein. [b] Amino acid incorporation is expressed as μμmoles/mg ribosomal protein. The concentration of poly-U was 100 μg/ml.

leucine incorporation is markedly stimulated by increasing concentrations of ammonium ion. In the absence of ammonium ion, the ratio of phenylalanine to leucine to isoleucine incorporated is 1.0:1.5:1.35, as seen in the third column of Table I. In the presence of 150 mM ammonium ion, this ratio changes to 1.0:2.0:3.0.

Relationship between Streptomycin and Ethanol.—Davies *et al.* (1964) recently have reported that under certain conditions streptomycin alters the coding properties of poly-U. The relationship of the code changes brought about by streptomycin to those provoked by ethanol is shown in Figure 6. In the absence of streptomycin and ethanol, isoleucine incorporation is low (58 μμmoles). Streptomycin (20 μg/ml) and ethanol (1.28 M) raised this value to 915 and 1010 μμmoles, respectively. When ethanol and streptomycin were both present, isoleucine incorporation approached 2600 μμmoles. In all of our experiments the effects of

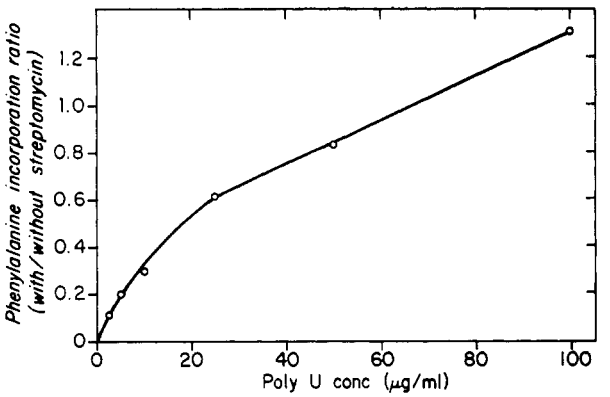

FIG. 7.—The effect of poly-U concentration on the incorporation of phenylalanine in the presence and absence of streptomycin (20 μg/ml). In addition to the standard reaction mixture each tube contained 1×10^{-5} M [^{14}C]-phenylalanine, 3.2×10^{-4} M spermine, and 1.5 mg ribosomal protein.

these two agents were more than additive under optimal conditions. In fact with some preparations of *E. coli* extract, the level of isoleucine incorporated by poly-U (in the presence of streptomycin and ethanol) equaled or exceeded the optimal phenylalanine incorporation in the absence of these agents. Dialysis experiments (4 hours against 100 volumes of buffer) with incubated S-30 fractions to which streptomycin or ethanol had been added indicated that streptomycin-induced code changes were irreversible while those of ethanol were reversible.

During the course of our experiments, it was observed that streptomycin invariably stimulated phenylalanine incorporation in the presence of 100 μg/ml poly-U. At low polymer concentrations, incorporation is inhibited up to 90% (Fig. 7). In the range of optimal poly-U concentration, phenylalanine incorporation is reduced 50–75% by streptomycin. Similar inhibitions have been reported by investigators (Speyer *et al.*, 1962; Flaks *et al.*, 1962; Van Knippenberg *et al.*, 1964). With a further increase in the level of poly-U a point (45–80 μg/ml) is reached where streptomycin has no effect on phenylalanine incorporation. In some experiments a 3-fold stimulation by streptomycin has been observed with 100 μg/ml or more of poly-U.

It should be noted, however, that the incorporation of phenylalanine in the presence of streptomycin never exceeds 50% of its optimal incorporation in the absence of streptomycin.

Effects of Various Antibiotics on Alcohol- and Streptomycin-stimulated Incorporation of Isoleucine in the Presence of Poly-U.—The effects of chloramphenicol on polynucleotide-directed incorporation of various amino acids in a cell-free system from *E. coli* have been shown to depend on the nucleotide compositions of the synthetic polynucleotides used (Kucan and Lipmann, 1964). Poly-U- and poly-UA-promoted amino acid polymerization is only slightly inhibited by chloramphenicol. Poly-UC- and poly-UG-dependent incorporation is markedly inhibited and the degree of inhibition is dependent on the G and C content of polynucleotides.

Alcohol-promoted incorporation of leucine and of isoleucine in the presence of poly-U shows a different response to chloramphenicol. The isoleucine incorporation stimulated by alcohol is markedly inhibited by both chloramphenicol and erythromycin (Table II). Leucine incorporation, however, is rather resistant to either antibiotic. The streptomycin-promoted isoleucine incorporation is similarly inhibited by chloram-

TABLE II
EFFECT OF VARIOUS ANTIBIOTICS ON ISOLEUCINE INCORPORATION STIMULATED BY ETHANOL AND STREPTOMYCIN[a]

Additions	Isoleucine Incorporation[b]			
	Control	Plus Ethanol	Plus Streptomycin	Plus Ethanol and Streptomycin
None	40	1360	1144	2666
+ Chloramphenicol (1×10^{-4} M)	28	453	280	1200
+ Erythromycin (3×10^{-5} M)	22	312	411	1066
+ Puromycin (1×10^{-4} M)	1	4	8	79

[a] In addition to standard incubation mixture each tube contained 1.45 mg ribosomal protein, 3.2×10^{-4} M spermine, and 1×10^{-5} M [^{14}C]isoleucine. The experiments contained 50 μg/ml poly-U and 1.28 M ethanol. [b] $\mu\mu$Moles/mg ribosomal protein.

phenicol. Both alcohol- and streptomycin-directed incorporation of isoleucine is extremely sensitive to puromycin.

DISCUSSION

The present experiments show clearly that environmental factors in addition to base sequence greatly influence the specificity and quantity of amino acids incorporated in response to synthetic polynucleotides. Such factors as magnesium ion and ammonium ion concentration, as well as the presence of polar and nonpolar compounds and streptomycin, all have marked effects on the reading of the amino acid code in the presence of specific polynucleotides in an *in vitro* system.

Depending on the relationships of varying concentrations of polar and nonpolar agents with that of magnesium, a stimulation or inhibition of phenylalanine incorporation in the presence of poly-U or proline incorporation in the presence of poly-C is observed. The effects of magnesium ion mimic those of alcohol in that the ratio of phenylalanine incorporation to that of leucine or isoleucine changes with increasing concentration of magnesium in the presence of poly-U. Furthermore, a significant incorporation of isoleucine occurs only with high concentrations of poly-U.

A similar pattern is seen with poly-UA-directed incorporation of various amino acids. According to the present method of code assignment (Speyer *et al.*, 1963), the sum of calculated triplet frequencies for poly-UA (5:1) would be 100, 20, 24, and 20 for phenylalanine, leucine, isoleucine, and tyrosine, respectively. With low magnesium, these theoretical ratios are approximated experimentally. However, by raising the magnesium and polymer concentrations, the observed ratios change to 100, 200, 300, and 30, respectively, for phenylalanine, leucine, isoleucine, and tyrosine. These experiments clearly show the importance of environmental conditions on code assignment.

As previously stated (So and Davie, 1964), the effects of organic solvents are probably multiple. It was suggested that organic solvents bring about structural changes in nucleic acids or ribosomal particles and thus alter the usual *in vitro* interaction of components necessary for translation of the genetic code. The suggestion is consistent with preliminary results which have shown that effects of organic solvents are not associated with the esterification of s-RNA by various amino acids (A. G. So and E. W. Davie, unpublished results). Similarly, the effects of magnesium ions are probably also multiple and, likewise, alter the interaction of s-RNA, messenger RNA, and ribosomes.

The recent findings of Davies *et al.* (1964) that streptomycin alters the coding properties of synthetic polynucleotides only if the ribosomal fraction from a sensitive strain of *E. coli* is used indicates the importance of a ribosomal component in influencing the reading of the code. It is possible that both organic solvents and magnesium somehow change the properties of some ribosomal component, thus resulting in the binding of a different aminoacyl-s-RNA with messenger RNA bound to ribosomes. This possibility is consistent with the observations of Gado and Horvath (1963) that different alcohols and acetone can replace streptomycin in promoting growth of a streptomycin-dependent strain of *E. coli*. This may indicate that the effects of organic solvents are similar to those of streptomycin in this strain of *E. coli*. In the present experiments with streptomycin-sensitive extracts, alcohol and streptomycin effects are additive. Furthermore, the alcohol effect is also observed with a streptomycin-resistant strain (J. W. Bodley, unpublished results). Thus the effects of alcohol and streptomycin on amino acid incorporation in an *in vitro* system are independent of each other.

The experiments of Grunberg-Manago and Michelson (1964) show that chemical modification of poly-U can also alter the coding specificity. These investigators observed a marked increase in leucine and isoleucine incorporation in the presence of polybromo-U as compared to poly-U. Szer and Ochoa (1964) recently have studied the effects of magnesium ions and temperature on the coding properties of poly-U and polyribothymidylic acid in a cell-free system from *E. coli*. Their results showed that, by either lowering the temperature or increasing the magnesium ion concentration, the degree of "ambiguity" increases. They stressed the importance of the "complexing ability" of the polymer. Similar results were obtained by Friedman and Weinstein (1964) in studying a cell-free system from *Bacillus stearothermophilus*. The latter investigators questioned the accuracy of codon assignments obtained with *in vitro* systems.

Magnesium ions are known to be effective in shielding the negative charges of phosphate groups in ribonucleic acid thus stabilizing its helical structure (Littauer and Eisenberg, 1959). The interaction between magnesium ions and phosphate groups also depends upon the polarity of the solvent. It is known that the degree of solvation of ions decreases when the dielectric constant is lowered by compounds such as ethanol. This would increase the interaction of oppositely charged magnesium ions and phosphate groups in nucleic acids. This may result in a change in the conformation of polyribonucleotides and/or s-RNA and thus affect the interaction of polyribonucleotide with s-RNA and ribosomes. Such conformational changes may account for alterations in the specificity of amino acid incorporation by synthetic polynucleotides. The concentration of magnesium also influences the association of ribosomal particles (Tissières *et al.*, 1959). Magnesium of 3–10 mM causes aggregation to the 100 S component while magnesium levels above 20 mM result in the breakdown of the ribosomal particles. Whether these findings are related to code changes observed in high magnesium in the presence of poly-U will require further study.

Recent investigations emphasize the importance of hydrophobic bonds in stabilizing such polymers as poly-C (Fasman *et al.*, 1964) and DNA (Crothers and Zimm, 1964). The adaptor hypothesis, however

(Crick, 1958; Hoagland, 1960), suggests that base pairing by hydrogen bonds between aminoacyl-s-RNA and complementary base sequences in messenger RNA are responsible for specifying amino acid sequences in proteins. Changes in the environmental conditions such as decreasing temperature (Szer and Ochoa, 1964; Friedman and Weinstein, 1964) or low concentrations of organic solvents would tend to weaken hydrophobic bonds and strengthen hydrogen bonds (Singer, 1962). It has been suggested (see Bennett and Dreyer, 1964) that conditions which strengthen or stabilize hydrogen bonds may lead to mistakes in protein synthesis (i.e., the leucine "ambiguity"). In this regard, it has been pointed out that several of the leucine code words (and one of the isoleucine code words as well) contain two U's. It is possible that by strengthening base-base interactions, two-base (in addition to the assumed three-base) binding becomes sufficient to hold s-RNA's to the template which contains only two complementary bases. Merely allowing two-base pairing would not appear to exclude the three-base type, except perhaps by competition. Even if three-base pairing were for some reason excluded, there is no reason to assume that phenylalanine-s-RNA could not also bind by two bases. Thus it would seem unlikely that two-base pairing could account for the incorporation of more leucine or isoleucine than phenylalanine by poly-U. However, it is possible that the above conditions might alter the type (see Steiner and Beers, 1961) of pairing which occurs between aminoacyl-s-RNA and messenger RNA and thus result in changes in the specificity of coding.

Spyrides (1964) and Conway (1964) recently reported that ammonium ions are more effective than sodium ions in binding phenylalanyl-s-RNA to the poly-U-ribosome complex. Cannon *et al.* (1963) showed that the binding of s-RNA by *E. coli* ribosomes occurred at high magnesium ion concentration. The present findings with high magnesium concentrations have shown that increasing concentration of ammonium ions increases the incorporation of isoleucine and leucine relative to that of phenylalanine. It is likely that the binding of isoleucyl and leucyl-s-RNA to the poly-U-ribosome complex requires a higher concentration of ammonium ions than that required for the binding of phenylalanyl-s-RNA.

The reasons for the dependence of incorporation of various amino acids on polynucleotide concentration are not obvious at present. The inhibition of phenylalanine incorporation at high poly-U concentration is not owing to a greater requirement for magnesium since the magnesium optimum decreases with increasing concentrations of poly-U. Indeed, the degree of inhibition by high poly-U concentrations is greater with increasing concentrations of magnesium. Recently Jones *et al.* (1964) studied the effect of chain length on template activity of poly-U with phenylalanine. They observed that long-chain poly-U was more effective than short-chain polymers in directing the incorporation of phenylalanine. Furthermore, phenylalanine incorporation in the presence of long-chain polymer is inhibited by addition of short-chain poly-U. Sager (1963) has found that leucine incorporation was insensitive to polymer size, whereas phenylalanine incorporation diminished when the chain length of poly-U was reduced. It is possible that the participation of short-chain poly-U in the polymerization of amino acids requires a higher level of magnesium and ammonium ions. This might explain the changes in the ratio of incorporation of various amino acids with increasing concentration of magnesium and ammonium ions.

In conclusion, it seems apparent that changes in the composition of the cell-free system may alter the expression of the genetic code through effects on any of several components in the system.

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