The Effects of Organic Solvents on Protein Biosynthesis and Their Influence on the Amino Acid Code*

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The effects of organic solvents on amino acid incorporation in a cell-free system from *Escherichia coli* have been investigated. Evidence is presented showing that various organic solvents will either stimulate or inhibit the incorporation of a number of amino acids in the presence of specific polynucleotides. The effects are dependent on the concentrations of organic solvents, magnesium ions, and polyribonucleotides. With low concentrations of polyuridylic acid (5 μg/ml) which normally code for phenylalanine and to a lesser extent for leucine, 0.32 M ethanol markedly stimulates phenylalanine incorporation, while higher concentrations of ethanol inhibit. With the higher ethanol concentration, the incorporation of leucine is greatly stimulated and that of isoleucine is slightly stimulated. With high polyuridylic acid concentrations (100 μg/ml), inhibition of phenylalanine incorporation is seen with increasing ethanol concentrations, but both leucine and isoleucine incorporation are dramatically stimulated. Similar effects were observed with polycytidylic acid which normally directs the incorporation of proline. In the presence of this polymer, proline incorporation was increased approximately 20-fold by 0.32 M ethanol. Other amino acids such as leucine and threonine, which normally are not coded by polycytidylic acid, become incorporated in the presence of 0.64–0.96 M ethanol. It is 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 results cast doubt upon current methods used for code assignments.

The discovery by Nirenberg and his collaborators and Ochoa and his colleagues (see Nirenberg *et al.*, 1963, and Speyer *et al.*, 1963, for recent reviews) that synthetic polynucleotides stimulate the incorporation of amino acids in a cell-free system from microorganisms has provided a great deal of insight as to the nature of the genetic code for amino acids. From these studies it has been possible to assign various combinations of purines and pyrimidine nucleotides as code letters for each amino acid. In these calculations, a coding ratio of three has been assumed.

Considerable degeneracy has been observed since many amino acids can be coded by more than one triplet, *e.g.*, leucine can be coded by UUA, UUC, UUG, and UCC (Jones and Nirenberg, 1962; Crick, 1963).

In the present experiments it was found that organic solvents can stimulate or inhibit formation of specific polypeptides in the presence of messenger polyribonucleic acids in a cell-free system from *Escherichia coli*. Furthermore, these solvents stimulate amino acid incorporation which is normally not stimulated by polyuridylic and polycytidylic acid. The details of these experiments are reported in this communication.

MATERIALS

Crystalline ATP (disodium salt) and GTP were purchased from Pabst Laboratories, Milwaukee, Wisc. Phosphoenolpyruvate (trisodium salt) and pyruvic kinase were purchased from California Corp. for Biochemical Research, Los Angeles, Calif. L-[U-^{14}C]threonine, L-[U-^{14}C]leucine, L-[U-^{14}C]isoleucine, L-[U-^{14}C]-phenylalanine and L-[U-^{14}C]proline were purchased from New England Nuclear Corp., Boston, Mass. Polycytidylic acid and polyuridylic acid were purchased from Miles Chemical Co., Elkhart, Ind. According to the manufacturer, both preparations have sedimentation constants ranging from 4 to 7. A base analysis of these preparations has not been done. Crystalline deoxyribonuclease was purchased from Worthington Biochemical Corp. Spermine was purchased from Nutritional Biochemicals Corp. Polyproline was kindly provided by Dr. P. E. Wilcox. *E. coli* K_{12} F^- was a gift of Mr. Don Brenner. All chemicals were the commercially available reagent grade.

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METHODS

For all experiments, *E. coli* was harvested in the early log phase of growth and an incubated S-30 fraction was prepared essentially by the method of Nirenberg and Matthaei (1961). Protein was determined by the method of Gornall *et al.* (1949) or Lowry *et al.* (1951), with a fresh solution of crystalline bovine serum albumin used as the standard.

For polycytidylic-directed amino acid incorporation, 1-ml reaction mixtures were incubated at 25° and the reactions were terminated by the addition of 10 ml of a trichloroacetic acid–tungstate solution containing the appropriate nonradioactive amino acid. Polyproline (0.1 ml of a solution containing 20 mg/ml) was added after precipitation. After a 10-minute period at room temperature, the mixture was centrifuged for 15 minutes at 1600 $\times$ *g*. The precipitate was resuspended in 2.5 ml of the trichloroacetic acid–tungstate reagent, heated in a water bath at 95° for 15 minutes, chilled to 0°, and diluted to 10 ml by the addition of more trichloroacetic acid–tungstate reagent. The centrifugation and heating step was repeated, the precipitate was dissolved in 1 ml of 98% formic acid, and the radioactivity was counted as previously described (So and Davie, 1963). The trichloroacetic acid–tungstate reagent was prepared by stirring a solution of 0.5% sodium tungstate in 30% trichloroacetic acid for about 10 minutes at 40°. Removal of the precipitate was accomplished by centrifugation for 10 minutes at 6000 $\times$ *g* at 4°. Nonradioactive proline (2×10^{-4} M) was added after removal of the precipitate.

For polyuridylic-directed amino acid incorporation incubation mixtures were washed in the presence of nonradioactive substrate essentially by the method of Siekevitz (1952). Radioactive samples were prepared and radioactivity was counted according to So and Davie (1963).

Each experiment reported was repeated at least once.

Results

Effect of Various Organic Solvents on Polyribonucleotide-dependent Incorporation of Amino Acids.—In the *in vitro* systems described to date, polyuridylic acid (poly-U) directs the incorporation of phenylalanine (Nirenberg and Matthaei, 1961) and to a lesser extent leucine (Bretscher and Grunberg-Manago, 1962). Polycytidylic acid (poly-C) directs the incorporation of proline into polyproline (Wahba *et al.*, 1963). Other amino acids have been reported not to be incorporated. Tables I and II show the effects of various organic solvents on the incorporation of phenylalanine and proline in the presence of poly-U and poly-C, respectively. Alcohols such as methanol, ethanol, or isopropanol stimulate the incorporation of phenylalanine from 1- to 4-fold over controls without solvent. A similar stimulation but of varying degree occurs with other organic solvents. When phenylalanine incorporation was studied as a function of time in the presence of 0.32 and 0.64 M ethanol, incorporation increased at a linear rate for at least 60 minutes.

Table I

Effects of Various Organic Solvents on Poly-U-directed Incorporation of Phenylalanine into Polypeptides[a]

Solvent	Final Concn (M)	Poly-U[b]	Phenylalanine Incorporated ($\mu\mu$moles/mg ribosomal protein)
		−	6.8
		+	149
Methanol	0.5	+	327
	1.0	+	170
Ethanol	0.32	+	216
	0.64	+	603
2-Propanol	0.26	+	371
	0.52	+	513
Acetone	0.26	+	124
	0.52	+	262
Dioxane	0.23	+	707
	0.46	+	147
Ethylene glycol	0.29	+	604
	0.58	+	339

[a] The complete reaction mixtures contained 0.100 M Tris buffer (*p*H 7.8), 0.011 M magnesium acetate, 0.006 M mercaptoethanol, 0.018 M KCl, 0.060 M NH_4Cl, 3.2×10^{-4} M spermine, 0.005 M phosphoenolpyruvate, 0.001 M ATP, and 5×10^{-5} M GTP. They also contained per final volume of 1 ml, poly-U (5 μg), L-[^{14}C]phenylalanine (0.011 μmole with 1×10^6 cpm), *E. coli* extract (1.35 mg of ribosomal protein and 4.20 mg of supernatant protein), and 100 μg of pyruvate kinase. Tubes were incubated for 30 minutes at 25°. Reactions were started by addition of poly-U or radioactive amino acid. [b] Incorporation in the presence of each solvent and in the absence of poly-U ranged from 6 to 11 $\mu\mu$moles (same as without solvent).

With proline in the presence of poly-C, the stimulation induced by organic solvents usually ranged from 10- to 15-fold, and under certain conditions approached 50-fold. Control experiments have shown that these effects were not due to more effective precipitation of the radioactive products by the addition of organic solvent.

Effect of Varying Concentrations of Poly-U and Magnesium Ions.—The stimulation of phenylalanine incorporation by 0.32 M ethanol was found to depend on the polyuridylic acid concentration. With increasing concentrations of poly-U the ethanol effect was reversed, with marked inhibition of incorporation occurring at high concentration of polynucleotide (Fig. 1). At poly-U concentrations of 5 μg/ml or below ethanol stimulation was observed, whereas at 10 μg/ml inhibition was observed.

Table II

Effect of Various Organic Solvents on Poly-C-directed Incorporation of Proline into Polypeptides[a]

Solvent	Final Concn (M)	Poly-C[b]	Proline Incorporated ($\mu\mu$moles/mg ribosomal protein)
		−	4.3
		+	50
Methanol	0.5	+	476
	1.0	+	941
Ethanol	0.32	+	410
	0.64	+	1000
2-Propanol	0.26	+	193
	0.52	+	843
Acetone	0.26	+	75
	0.52	+	170
Dioxane	0.23	+	75
	0.46	+	316

[a] Incubation conditions were the same as those in Table I except that poly-C (800 μg) was substituted for poly-U and L-[^{14}C]proline (0.01265 μmole containing 1×10^6 cpm) replaced L-[^{14}C]phenylalanine. [b] Incorporation in the presence of each solvent and in the absence of poly-C ranged from 2 to 5 $\mu\mu$moles (same as without solvent).

The degree of stimulation by 0.32 M ethanol at low poly-U concentrations was dependent on the magnesium ion concentration, optimal stimulation occurring at about 11 μmoles/ml as shown in Figure 2. At higher levels of poly-U the ethanol inhibition could be readily reversed by lowering the magnesium ion concentration. As shown in Figure 3, considerable inhibition by 0.32 M ethanol occurs at a magnesium ion concentration of 11 mM, but at 7 mM a stimulation of about 3-fold is observed.

Influence of Ethanol on the Incorporation of Various Amino Acids by Polyuridylic Acid.—The effects of increasing alcohol concentrations on the incorporation of phenylalanine, leucine, and isoleucine at a polyuridylic acid concentration of 5 μg/ml are shown in Figure 4. With 0.32 M ethanol the usual phenylalanine stimulation is observed. With 0.64 M ethanol, phenylalanine incorporation begins to fall off while leucine incorporation is greatly increased. Thus the ratio of incorporation of phenylalanine to leucine changes with increasing alcohol concentration. With low poly-U concentrations a small stimulation of isoleucine occurs at high levels of alcohol.

With high levels of polyuridylic acid (100 μg/ml) the ethanol effects are different. As seen in Figure 5, phenylalanine incorporation is inhibited by 0.32 M ethanol whereas leucine incorporation is stimulated. Higher levels of ethanol (1.6 M) inhibit leucine but stimulate isoleucine incorporation. Thus the ratio of phenylalanine to leucine to isoleucine incorporation again changes with increasing concentrations of ethanol. In addition, the isoleucine incorporated is considerably greater with the higher concentrations of polyuridvlic acid.

Influence of Ethanol on the Incorporation of Various Amino Acids by Polycytidylic Acid.—The stimulation of proline incorporation by poly-C requires far greater concentrations of polyribonucleotide than the phenylalanine incorporation in the presence of poly-U. In Figure 6 the effect of increasing concentrations of poly-C

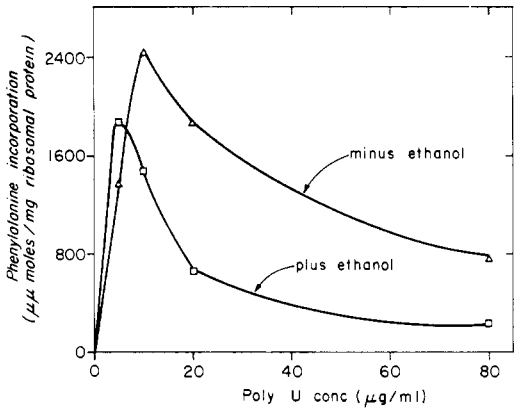

FIG. 1.—Effect of poly-U concentration on the incorporation of phenylalanine in the presence and absence of ethanol. Incubation conditions were similar to those in Table I except (1) the quantity of *E. coli* supernatant protein was 2.8 mg and *E. coli* ribosomal protein was 0.9 mg, and (2) the concentration of ethanol was 0.32 M.

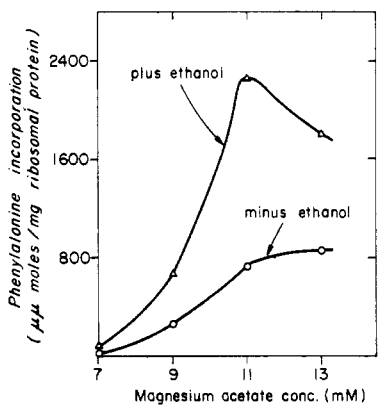

FIG. 2.—Effect of magnesium-ion concentration on [^{14}C]phenylalanine incorporation at 5 μg of poly-U per ml in the presence and absence of alcohol. Ethanol concentration was 0.32 M. See Table I for incubation conditions.

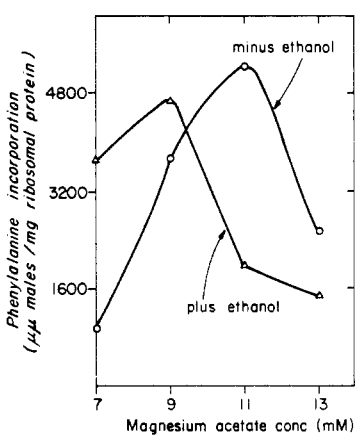

FIG. 3.—Effect of magnesium-ion concentration on [^{14}C]phenylalanine incorporation at 20 μg of poly-U per ml in the presence and absence of alcohol. Ethanol concentration was 0.32 M. Incubation conditions were the same as those in Table I.

on the incorporation of proline in the presence and absence of 0.64 M ethanol is shown. The control experiments are similar to those of Wahba *et al.* (1963), but the incorporation is lower. This is owing in part to our using a shorter incubation period (30 versus 60

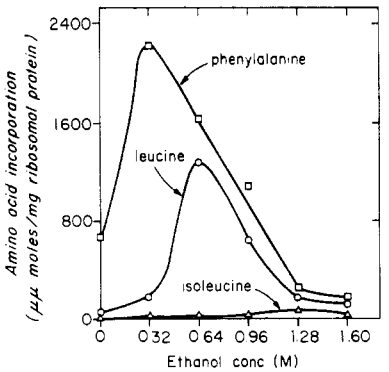

FIG. 4.—Effect of increasing concentrations of ethanol on the specificity of incorporation of amino acids at a low poly-U concentration. Incubation conditions were the same as those in Table I except that the reaction mixture contained either L-[^{14}C]leucine (0.0127 μmole with 1 × 10^6 cpm), L-[^{14}C]phenylalanine (0.011 μmole with 1 × 10^6 cpm), or L-[^{14}C]isoleucine (0.0120 μmole with 1 × 10^6 cpm).

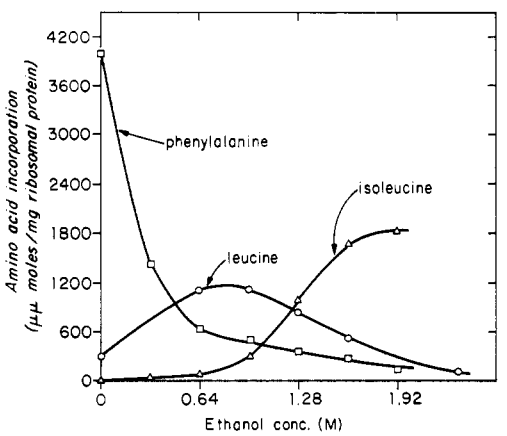

FIG. 5.—Effect of increasing concentrations of ethanol on the specificity of incorporation of amino acids at a high poly-U concentration. Incubation conditions were the same as those in Table I except (1) poly-U was 100 μg, and (2) *E. coli* supernatant protein w s 3.25 mg and *E. coli* ribosomal protein was 1.08 mg.

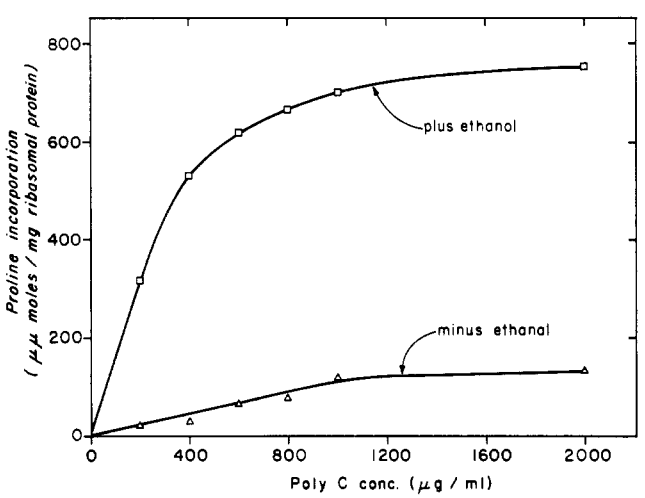

FIG. 6.—Effect of poly-C concentrations on the incorporation of proline into polypeptide in the presence and absence of alcohol. Incubation conditions were the same as those shown in Table I except that the *E. coli* extract contained 2.7 mg supernatant protein and 0.9 mg ribosomal protein. Ethanol concentration was 0.64 M.

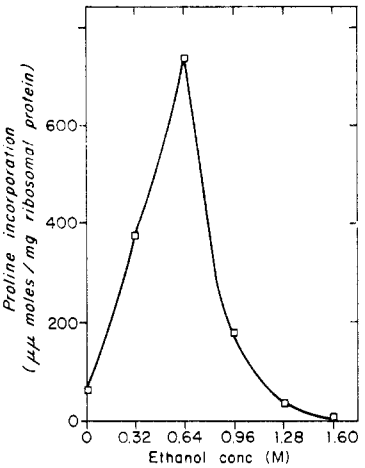

FIG. 7.—Effect of increasing concentrations of ethanol on the poly-C-directed incorporation of proline. Incubation conditions were the same as those in Table I except (1) *E. coli* supernatant protein was 0.9 mg and *E. coli* ribosomal protein was 2.7 mg, and (2) L-[^{14}C]proline was added (0.01265 μmole containing 1 × 10^6 cpm).

minutes) and a lower incubation temperature (25 versus 37°). An ethanol stimulation of approximately 10-fold is observed at all poly-C concentrations. With increasing ethanol concentrations the incorporation of proline is stimulated, whereas higher levels inhibit (Fig. 7). Similar effects are seen with leucine and threonine (Fig. 8). In the absence of either poly-C or ethanol, incorporation of leucine and threonine is negligible.

DISCUSSION

The present experiments with a cell-free system from *E. coli* show dramatic effects of organic solvents on the incorporation of amino acids in the presence of specific polynucleotides. These solvents affect both the quantity and the specificity of amino acid incorporation into polypeptide. The effects appear to be fairly general in that different organic solvents such as alcohols, acetone, and dioxane cause similar results. Depending on the relationship of varying concentrations of organic solvent, magnesium ions, and polynucleotides, a stimulation or inhibition occurs. Preliminary results have also shown that these effects are dependent upon ribosome concentration.

It is of interest that organic solvent start to stimulate the incorporation of leucine and isoleucine under conditions where phenylalanine stimulation is falling off (Fig. 5). These results are particularly important in regard to amino acid code assignments based upon experiments with synthetic polynucleotides in an *in vitro* system. In such experiments, the incorporation of proline or phenylalanine is arbitrarily set at 100 in the presence of poly-C- and poly-U-containing polymers, respectively. The incorporation of other amino acids is expressed as per cent of proline or phenylalanine incorporation. Code letters are then assigned on the basis of the frequency of the various triplets and the per cent of incorporation of various amino acids. Since the incorporation ratio of phenylalanine to leucine to isoleucine changes with alcohol, magnesium ions, and polymer concentrations, the validity of the method used in assigning code triplets to various amino acids is open to serious question.

The extent to which the code is altered by organic solvents is being investigated with a complete spectrum of amino acids. For example, in the poly-U system it would be of particular interest to demonstrate incorporation of an amino acid which, according to current concepts, does not include U as one of its code letters.

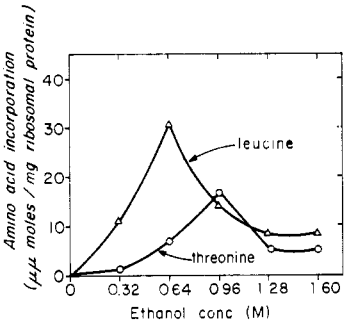

FIG. 8.—Effect of increasing concentrations of alcohol on poly-C-directed incorporation of leucine and threonine. Incubation conditions were the same as those shown in Table I except (1) *E. coli* extract contained 0.9 mg supernatant protein and 2.7 mg ribosomal protein. Mixtures were incubated with either L-[^{14}C]leucine (0.0127 μmole containing 1 × 10^6 cpm) or L-[^{14}C]threonine (0.0126 μmole containing 1 × 10^6 cpm).

In the present complex *in vitro* systems the effects of organic solvents probably are multiple. It appears unlikely that the present results with ethanol are explained by an effect on an enzyme(s) associated with protein biosynthesis, which is common to all amino acids. Such an enzyme(s) would have to be associated with specific amino acids since increasing alcohol concentrations increase the incorporation of certain amino acids while decreasing the incorporation of others.

The recent results of Davies *et al.* (1964) emphasize the role of ribosomes in influencing the coding properties of synthetic polynucleotides. They have shown that streptomycin causes alterations in the coding properties of synthetic polynucleotides when ribosomes from streptomycin-sensitive cells are employed. A shift in the code by streptomycin was not observed with ribosomes from streptomycin-resistant cells. In the present experiments, it is possible that ethanol may also affect the ribosomes in an analogous manner resulting in a shift in the code. It is of interest to note that in the presence of poly-U the amino acids shifted by streptomycin are the same as those affected by alcohol (phenylalanine, leucine, and isoleucine).

The effects of alcohol may also be due to structural changes in the ribonucleic acid. Poly-U apparently has little or no secondary structure at 25° under conditions similar to those employed in the present system (Lipsett, 1960; Takanami and Okamoto, 1963). Thus any effects of alcohol on ribonucleic acid would probably be associated with s-RNA and would alter its interaction with messenger polyribonucleic acid bound to the ribosomes. Such an interaction might well be influenced by changes in magnesium-ion concentrations. With poly-C, considerable secondary structure exists at 25° (Takanami and Okamoto, 1963). In experiments employing this polymer as a messenger, the effects of alcohol could be associated with either s-RNA or messenger polyribonucleic acid.

Grunberg-Manago and Michelson (1964) recently have studied amino acid incorporation in the presence of various poly-U and poly-C analogs. Their results show a marked increase in incorporation of leucine and isoleucine in the presence of polybromouridylic acid as compared to polyuridylic acid, and a similar increase in incorporation of threonine in the presence of polybromocytidylic acid as compared to polycytidylic acid. These workers pointed out the importance of keto-enol tautomerization in the polynucleotides and how the halogens can influence tautomerism. It is well known

that the enol-keto ratio for a given pair of tautomers at equilibrium in solution depends markedly on the polarity of the solvent, and that this ratio tends to be greatest in the least polar solvents (Gould, 1959). Thus it is feasible that an enol-keto shift in the s-RNA or messenger RNA or both may account for a shift in the code for poly-U from phenylalanine to leucine and isoleucine as the alcohol concentration is increased.

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Copper(II) Complexes of Glycylglycine*

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The study of copper(II)-glycylglycine complexes in aqueous solutions by infrared and visible spectrophotometric methods, as well as by potentiometric *p*H measurements, provides evidence for the structures of all the metal complex species present. The frequency changes of the infrared-absorption bands of the carboxyl and peptide carbonyl groups that occur in the course of complex formation gives the first direct proof of the displacement of protons from the peptide nitrogen atoms by reaction with metal ions in solution. The molar absorptivities of each species in the visible spectral region are reported. All the equilibrium constants are calculated for a medium of 1.0 M KCl at 24.9°.

The copper complexes of polypeptides have been studied extensively by many workers mainly because of their biological significance in enzyme reactions. General features of the reactions occurring in aqueous copper-peptide solutions had been worked out by Dobbie and Kermack (1955) from potentiometric and visible-spectrophotometric measurements. By crystallization of certain species (Manyak *et al.*, 1955), or by varying the functional groups of the ligands (Datta and Rabin, 1956), some of the structures of these complexes were inferred. More recently, Koltun and co-workers (Koltun and Gurd, 1959; Koltun *et al.*, 1960, 1963) reported the reactions and species present in solution by potentiometric, kinetic, ultraviolet, and visible-spectral methods. Although all these workers agree that copper-complex formation involves displacement of the peptide hydrogen, there remains disagreement in the number of species present and their structures.

As has been shown for the infrared spectra of glycylglycine (Kim and Martell, 1963), the frequency changes of peptide carbonyl groups at various *p*D values in aqueous solution suggest that it might be possible to find evidence for the binding sites of the corresponding metal complexes by infrared spectrophotometric measurements. The results of such infrared measurements of complexes in solution are of further interest for comparison with the solid-state spectra of the same compounds, the only known example of which was reported by Rosenberg (1957).

Since copper complexes show interesting visible-color changes depending on the number of peptide linkages in the ligand, and on the *p*H of the solution, the visible-spectra and potentiometric measurements are studied simultaneously in the same sample of solution in order to provide further evidence for the identities of all the species formed in solution.

EXPERIMENTAL

Infrared Spectral Measurements.—The method employed is the same as the one described previously (Kim and Martell, 1963) except that exact amounts of metal ion were added to the sample solutions to make the metal-to-ligand ratio 1:1 and 1:2. In both cases, the ligand concentration was −0.20 M. The infrared spectrum of a crystalline sample of aquoglycylglycino-Cu(II) prepared by the method previously described (Manyak *et al.*, 1955) was measured in D_2O as a solvent to check the spectra obtained by the combination of metal ion and ligand in solution.

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