Biochemistry



Volume 9, Number 17 August 18, 1970

# Stability and Structure of Binary and Ternary Metal Ion Complexes with Biocytin, the Sulfoxide and Sulfone, *N*-Acetyl-L-lysine, and L-Alanine*

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ABSTRACT: The stability constants were determined by potentiometric titrations in aqueous solutions ($I$ = 0.1; $T$ = 25°) for the binary 1:1 complexes between Mn(II), Co(II), Zn(II), or Cu(II) and *d*-biocytin (ε-*N*-biotinyl-L-lysine), its sulfoxide and sulfone, $N^{\epsilon}$-acetyl-L-lysine, or L-alanine. Logs of the stability constants, log $K^{Me}_{MeL}$, with biocytin are Mn(II), 2.47; Co(II), 4.10; Zn(II), 4.38; and Cu(II), 7.73. The corresponding constants for the complexes with the other ligands are of the same order. For all five ligands, the stability of their metal ion complexes is dependent only on the amino acid group and the basicity of the latter. Recent investigations have shown that the sulfur atom of *d*-biotin interacts stereospecifically with metal ions. To learn whether or not a similar interaction can occur with biocytin, proton magnetic resonance spectroscopy was used. The spectra of biocytin with increasing amounts of Mn(II) or Cu(II) show a selective broadening of the signals due to only one of the two protons of the methylene group next to the sulfur. This suggests that these metal ions coordinate in a stereospecific manner to the sulfur, *i.e.*, the orientation is analogous to that of oxygen in *d*-biotin *d*-sulfoxide. The reason for this stereospecific interaction is assumed to be due to steric hindrance of the ureido ring in the biotinyl moiety. However, this stereospecific interaction between Mn(II) or Cu(II) and the sulfur atom in the biotinyl moiety of biocytin has no significant influence on the stability of the complexes. As mixed ligand–metal ion complexes are important in nature, the investigation of ternary 2,2′-bipyridyl–Cu(II)–amino acid complexes was included, using as amino acids, biocytin, $N^{\epsilon}$-acetyl-L-lysine, and L-alanine. For the equilibrium Cu(2,2′-bipyridyl)$_2$ + Cu(amino acid)$_2$ $\rightleftharpoons$ 2Cu(2,2′-bipyridyl)(amino acid), the corresponding constants are log $X$ = 3.13 with biocytin, 3.26 with $N^{\epsilon}$-acetyl-L-lysine, and 3.08 with L-alanine.

Obviously, the size of the amino acid molecule is without significant influence on the stability of the ternary complexes. However, of great interest is the surprisingly high stability of these ternary complexes which are at least 300 times more stable than one would expect from purely statistical reasons (log $X$ = 0.6).

The stereoselectivity of metal ion interaction in biological systems is surely important for the shape and reactivity of the resulting complexes (Sigel and McCormick, 1970). So far, not many examples of stereospecific interactions with biochemically important ligands are known. This is especially true if the ligand groups in question interact only weakly with metal ions as, for example, thioether groups do (Erlenmeyer *et al.*, 1968; Sigel *et al.*, 1969a; McCormick *et al.*, 1969).

Recently, by proton magnetic resonance spectroscopy, it was shown that Mn(II) or Cu(II) interacts stereospecifically with the thioether group of *d*-biotin (Sigel *et al.*, 1969b). The orientation of these metal ions in the complex with *d*-biotin is analogous to that of oxygen in *d*-biotin *d*-sulfoxide.

*d*-Biocytin was first isolated from autolyzed yeast extracts (Wright *et al.*, 1950) and shown to be ε-*N*-biotinyl-L-lysine (Peck *et al.*, 1952). Quite generally, the coenzyme form of *d*-biotin seems to be amide linked through the ε-amino group of L-lysine to the enzyme (Lane and Lynen, 1963). To

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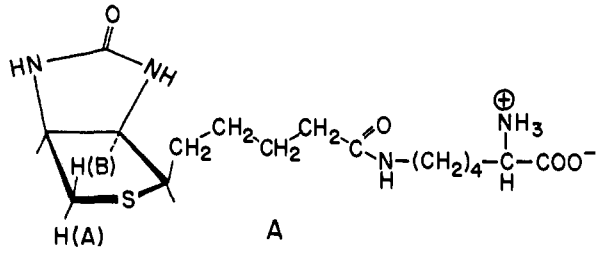
obtain activity, a metal ion, usually manganese, must be added to such biotin enzymes which usually catalyze carboxylations. A transcarboxylase which already contains tightly bound zinc and cobalt in a metal ion to biotin ratio of 1:1 has also been reported (Northrop and Wood, 1969a). As far as is known, all biotin enzymes need metal ions for activity.

Biocytin (structure A) offers several hypothetical sites for the binding of metal ions. Of these, the urea group (Sigel *et al.*, 1969b) and the amide linkage seem unlikely, as the steric conditions are too unfavorable for metal ion coordination in the physiological, *i.e.*, the neutral (and acid) range. No 5- or 6-membered chelate rings can be formed where these groups may be involved.[1] The metal ion binding sites to take into account are the thioether group of the biotinyl moiety and the $\alpha$-amino acid end of the ligand.




A

In view of the known and previously mentioned stereospecific interaction between metal ions and the sulfur of *d*-biotin (Sigel *et al.*, 1969b), it was of interest to see whether or not the sulfur in biocytin also shows such a stereospecific interaction. To answer this question, biocytin was investigated, in the absence and presence of Mn(II) or Cu(II), by proton magnetic resonance spectroscopy. The investigation of metal ion complexes of *d*-biotin and biotin derivatives (Sigel *et al.*, 1969b) had shown that the stability determining binding site of biotin is the carboxylate group. The thioether group has no significant influence on complex stability. As $\alpha$-amino acid chelates are much more stable than simple carboxylate complexes, similar relations could be expected for biocytin complexes. This means that the stability of biocytin–metal ion complexes would expectedly be determined by the qualities of the $\alpha$-amino acid group. However, the sulfoxide and sulfone derivatives of biocytin were also included in the present study.

The distance of the biotinyl moiety from the $\alpha$-amino acid end in biocytin can reach about 14 Å (Northrop and Wood, 1969b). Therefore, it was of interest to see if this long "tail" influences the stability of the binary 1:1 complexes of Mn(II), Co(II), Zn(II), and Cu(II) formed with the $\alpha$-amino acid group. For this reason, the 1:1 complexes of the same metal ions with the chain-shortened ligands, $N^{\epsilon}$-acetyl-L-lysine and L-alanine, were also studied.

Mixed ligand–metal ion complexes are surely important in biological fluids and as models for enzyme–metal ion–substrate complexes (Sigel and McCormick, 1970). To see whether or not the long "tail" of biocytin influences the stability of ternary complexes and especially to learn more generally about ternary complexes (Griesser and Sigel, 1970; Huber *et al.*, 1969) where amino acids are involved, these studies were extended. The stability of the ternary 2,2′-bipyridyl–Cu(II)–ligand complexes, containing biocytin, $N^{\epsilon}$-acetyl-L-lysine, or L-alanine, was determined.

## Experimental Section

*Materials.* *d*-Biotin was obtained from Hoffmann-La Roche, Inc., Nutley, N. J., and L-lysine (free base) from Sigma Chemical Co., St. Louis, Mo. $N^{\epsilon}$-Acetyl-L-lysine (grade I) was from Cyclo Chemical Corp., Los Angeles, Calif. L-Alanine, 2,2′-bipyridyl, and the metal ion perchlorates used for the potentiometric titrations were from Fluka AG, Buchs, Switzerland, $Mn(ClO_4)_2 \cdot 6H_2O$ and $Cu(ClO_4)_2 \cdot 6H_2O$, used for the nuclear magnetic resonance spectra, were from Alfa Inorganics, Beverly, Mass. $D_2O$ (99.9%) was purchased from Calbiochem, Los Angeles, Calif., NaOD (30% in 99% $D_2O$) and $D_2SO_4$ were from Volk Radiochemical Division of I.C.N., Burbank, Calif. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was obtained from Merck and Co., Inc., Teterboro, N. J.

Biocytin ($\epsilon$-*N*-*d*-biotinyl-L-lysine) was synthesized from *d*-biotin and L-lysine (Weijlard *et al.*, 1954) and purified by ion-exchange column chromatography (McCormick and Föry, 1970). A sample of biocytin also was obtained from D.E. Wolf of Merck Sharp & Dohme Research Lab., Rahway, N. J. The sulfoxide (mainly *d*) and sulfone of biocytin were obtained by oxidation with equimolar or excess, respectively, $H_2O_2$ in glacial acetic acid at room temperature as previously described (McCormick, 1969).

*Apparatus and Procedure.* The potentiometric titrations were carried out under $N_2$ in solutions with ionic strength, $I = 0.1$ ($NaClO_4$), at 25° with a Metrohm potentiograph E 336 and Metrohm UX combination glass electrodes. Standardization of NaOH for titration was effected with potassium hydrogen phthalate. The exact metal ion concentrations of the stock solutions were determined with ethylenediamine-*N*,*N*,*N*′*N*′-tetraacetic acid. The results given are from at least two, usually three or four, independent titrations, except those for the sulfoxide and sulfone of biocytin where only a little substance was available.

The nuclear magnetic resonance spectra were taken with a Varian analytical nuclear magnetic resonance spectrometer, A-60A; for the adjustment of pH, a Corning Model 12 research pH meter was used.

*Determination of the Acidity Constants, $K^{H}_{H_2L}$, of the Ligands.*[2] Aqueous solution (10 ml) containing $9.6 \times 10^{-3}$ M $HClO_4$ and $NaClO_4$ to 0.1 M in the presence and absence of the several ligands ($4 \times 10^{-3}$ M) was titrated with 0.1 M NaOH. Constants were calculated from the respective degree of neutralization for at least 10 points of the titration curves.

*Determination of the Acidity Constants, $K^{H}_{HL}$, of the Ligands and the Stability Constants, $K^{Me}_{MeL}$, of the 1:1 Complexes with Mn(II), Co(II), and Zn(II).* The constants, $K^{H}_{HL}$, were determined by titrating 50 ml of aqueous $1.6 \times 10^{-4}$ M $HClO_4$ and $NaClO_4$ to 0.1 M in the presence of the several ligands ($6 \times 10^{-4}$ M) with 0.05 M NaOH. The stability constants of the complexes, $K^{Me}_{MeL}$, were determined in solutions

[1] Under the conditions of this study, no hint was observed that would suggest the involvement of one of these groups.

[2] Abbreviations used are: L, ligand; Me, metal ion; Bipy, 2,2′-bipyridyl. In eq 1 through 15, charges are omitted.

of the same concentrations, but a part of $NaClO_4$ was replaced by $Me(ClO_4)_2$ which was between $6.8 \times 10^{-3}$ M and $3.3 \times 10^{-2}$ M. Thus, the excess in metal ion concentrations was about 11 to 55 with respect to the ligand concentration. These conditions eliminate formation of 1:2 complexes, $MeL_2$, and allow one to calculate the stability constants, with consideration of the hydrolysis curves, from the apparent acidity constants, $K_A'$, of the ligands in the presence of metal ions (*cf.* Sigel, 1967; Sigel *et al.*, 1969b).

*Determination of the Stability of the Binary and Ternary Cu(II) Complexes.* The conditions for measuring the stability constants, $K^{Cu}_{CuL}$, were the same as in the previous section for the acidity constants, $K^{H}_{HL}$, except that a part of $NaClO_4$ was replaced by sufficient $Cu(ClO_4)_2$ to obtain a ratio of Cu(II):L = 1:1. Again, titrations of solutions without ligand were used as a basis for the evaluation. The calculation of $K^{Cu}_{CuL}$ was done by taking into account the species $H_2L$, HL, L, Cu, CuL, and $CuL_2$ (*cf.* Griesser and Sigel, 1970). Previously, $K^{Cu}_{CuL_2}$ was determined from titrations where L was in excess with respect to Cu(II) (Bjerrum, 1941). In this case, 50 ml of aqueous $1.5 \times 10^{-4}$ M $HClO_4$, $3 \times 10^{-4}$ M $Cu(ClO_4)_2$, $NaClO_4$ to 0.1 M, and $9 \times 10^{-4}$ M ligand were titrated with 0.05 M NaOH. These experiments were repeated in the presence of $1.2 \times 10^{-3}$ M ligand.

The conditions for measuring the ternary complexes were the same as for the binary ones, but the solutions contained equivalent amounts of 2,2'-bipyridyl, Cu(II), and L. The evaluation of these titration curves was done using curves obtained after titrating the same solutions without L and taking into account the species $H_2L$, HL, L, H(Bipy), Bipy, $CuL_2$, CuL, $Cu(Bipy)_2$, Cu(Bipy), Cu, and Cu(Bipy)L. The calculated equilibrium constants was $\beta^{Cu}_{Cu(Bipy)L}$ (Griesser and Sigel, 1970). The evaluations were done with the computer, IBM 1620, made available by the Rechenzentrum der Universität Basel.

*Nuclear Magnetic Resonance Experiments.* The proton magnetic resonance spectra of *d*-biotin (pD ~8.5), $N^{\epsilon}$-acetyl-L-lysine (pD ~5.5), and biocytin (pD ~5.5) were taken in 0.2 M solutions in $D_2O$ at 37° (adjusted with $D_2SO_4$ and NaOD) in the presence of the standard, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (Bhacca *et al.*, 1963). The assignments of the signals of *d*-biotin were done according to Glasel (1966), and those for $N^{\epsilon}$-acetyl-L-lysine by integration of the number of protons and the chemical shifts (for L-lysine, see Mandel, 1965). Assignment of the peaks in the spectrum of biocytin was done on the basis of its composition of biotin and lysine.

The experiments in the presence of metal ions were carried out in more dilute solutions (0.09 M) of biocytin in $D_2O$ (pD ~5.5), because only small amounts of the ligand were available. In these cases, the HOD signal was used as internal standard; the value of 4.70 ppm used is the average of the values given by Bhacca *et al.* (1962).

In all cases, the pD of solutions was calculated from the relationship pD = pH + 0.4 (Lumry *et al.*, 1951).

## Results

*Stability of the Binary 1:1 Complexes.* The constants for acidity (eq 1 and 2) of the ligands which were biocytin, its sulfoxide and sulfone, $N^{\epsilon}$-acetyl-L-lysine and L-alanine, and for stability of the binary 1:1 complexes (eq 3) with Mn(II), Co(II), Zn(II), and Cu(II) were determined in aqueous solution ($I$ = 0.1; $T$ = 25°) by potentiometric titration. For the case of biocytin and the mentioned metal ions, the results of such titrations are shown in Figure 1, where the pH of the solution is plotted against the neutralization degree. The experimental data obtained for the other ligands were very similar to those shown here. The results of the calculations are given in Table I.

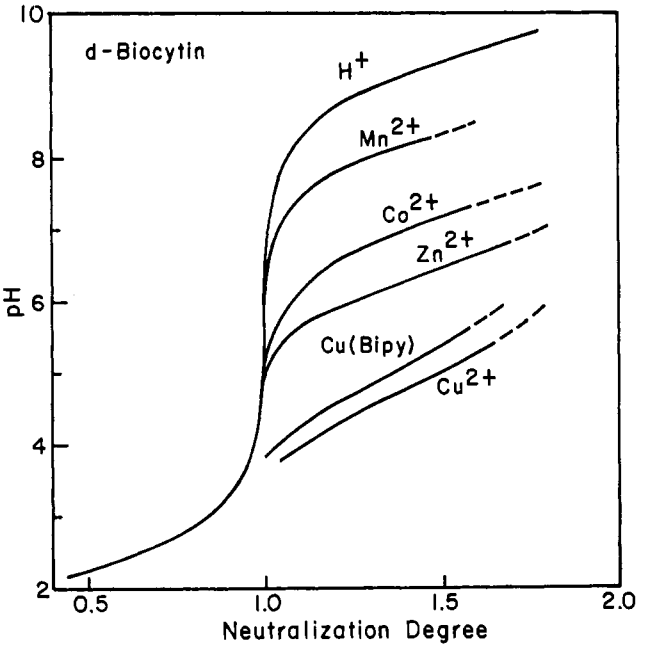



FIGURE 1: Dependence of neutralization degree upon pH during potentiometric titration of biocytin with and without metal ions in aqueous solution ($I$ = 0.1; $T$ = 25°). The broken-line portions extended toward higher degrees of neutralization indicate uncertainty due to hydrolysis. Biocytin was $6 \times 10^{-4}$ M ($4 \times 10^{-3}$ M at low pH values); Mn(II) was $3.3 \times 10^{-2}$ M; Co(II), $1.2 \times 10^{-2}$ M; Zn(II), $3.1 \times 10^{-2}$ M; and Cu(II) or Cu(II) and 2,2'-bipyridyl, $6 \times 10^{-4}$ M when added.

$$H_2L \rightleftharpoons HL + H \quad K^{H}_{H_2L} = [H][HL]/[H_2L] \qquad (1)$$

$$HL \rightleftharpoons L + H \quad K^{H}_{HL} = [H][L]/[HL] \qquad (2)$$

$$Me + L \rightleftharpoons MeL \quad K^{Me}_{MeL} = [MeL]/([Me][L]) \qquad (3)$$

The acidity constants, $K^{H}_{H_2L}$ (eq 1), which are due to the carboxylic acid group of biocytin, $N^{\epsilon}$-acetyl-L-lysine, and L-alanine are, within experimental error, the same for all three amino acids (Table I). But, the acidity constants, $K^{H}_{HL}$ (eq 2), which characterize the basicity of the amino groups are slightly different for the three ligands. The basicity decreases, with increasing chain length, in the order L-alanine > $N^{\epsilon}$-acetyl-L-lysine > biocytin. However, the oxidation of the sulfur of biocytin is without any significant influence; the values obtained for biocytin and its sulfoxide and sulfone are practically the same.

Since there was not enough substance available to determine $K^{H}_{H_2L}$ for the sulfoxide and sulfone of biocytin, the assumption was made, based on the findings described in the last paragraph, that $K^{H}_{H_2L}$ is the same as with biocytin.

TABLE I: Negative Log Acidity Constants of Biocytin and of Analogous Ligands and Log Stability Constants of the Binary Mn(II), Co(II), Zn(II), and Cu(II) 1:1 Complexes ($I = 0.1$; $T = 25°$).[a]

| Ligand | $pK^{H}_{H_2L}$ | $pK^{H}_{HL}$ | $\log K^{Mn}_{MnL}$ | $\log K^{Co}_{CoL}$ | $\log K^{Zn}_{ZnL}$ | $\log K^{Cu}_{CuL}$ |
|---|---|---|---|---|---|---|
| Biocytin | 2.26 ± 0.03 | 9.29 ± 0.02 | 2.47 | 4.10 | 4.38 | 7.73 ± 0.03 |
| Biocytin sulfoxide | | 9.30 ± 0.02 | 2.45 | | 4.33 | 7.87 ± 0.05[b] |
| Biocytin sulfone | | 9.31 ± 0.03 | 2.44 | | 4.32 | 7.83 ± 0.02[b] |
| $N^{\epsilon}$-Acetyl-L-lysine | 2.24 ± 0.01 | 9.63 ± 0.02 | 2.50 | 4.27 | 4.51 | 8.09 ± 0.06 |
| L-Alanine | 2.26 ± 0.03 | 9.83 ± 0.03 | 2.67 | 4.44 | 4.51 | 8.25 ± 0.07 |

[a] The reproducibility of the apparent acidity constants, $pK_A'$ (*cf.* Sigel, 1967), in the presence of an excess of Mn(II), Co(II), or Zn(II) was at least ±0.05 log unit. [b] These values were calculated taking into account $K^{H}_{H_2L}$ and $K^{CuL}_{CuL_2}$ of biocytin; an evaluation of the titration data not considering these constants resulted in $\log K^{Cu}_{CuL} = 7.92 \pm 0.06$ for biocytin sulfoxide and $\log K^{Cu}_{CuL} = 7.88 \pm 0.03$ for biocytin sulfone.

Therefore, the value of $K^{H}_{H_2L}$ obtained for biocytin was also used in the calculations of the Cu(II) 1:1 complexes with the sulfoxide and sulfone of biocytin. Aside from this, the $K^{CuL}_{CuL_2}$ of biocytin was also taken into account in the mentioned calculation. These assumptions are completely reasonable. In addition, the concentrations of $H_2L$ and $CuL_2$ are very small under the conditions of these experiments. If these two species are neglected in the calculations, the results obtained are still reasonable (*cf.* footnote *b* in Table I). In the case of the other metal ions, $H_2L$ (*cf.* Figure 1) and $MeL_2$ ($[Me]_{tot}:[L]_{tot} \geq 11$) are also insignificant under the conditions of the experiments.

The stability constants that relate to eq 4 and 5 of the binary Cu(II)–amino acid systems were determined by titrations in the presence of an excess of ligand. The constants obtained are given in Table II and were used in the evaluation of the mixed ligand systems.

$$MeL + L \rightleftharpoons MeL_2 \quad K^{CuL}_{CuL_2} = [CuL_2]/([CuL][L]) \quad (4)$$

$$Me + 2L \rightleftharpoons MeL_2 \quad \beta^{Cu}_{CuL_2} = [CuL_2]/([Cu][L]^2) \quad (5)$$

TABLE II: Log Stability Constants of the Binary Cu(II) Complexes of Biocytin, $N^{\epsilon}$-Acetyl-L-lysine, L-Alanine, and Glycine Used for Calculation[a] of and Comparison with the Corresponding Ternary 2,2′-Bipyridyl–Cu(II)–Amino Acid Complexes ($I = 0.1$; $T = 25°$).

| Ligand | $\log K^{Cu}_{CuL}$ | $\log K^{CuL}_{CuL_2}$ | $\log \beta^{Cu}_{CuL_2}$ | $\log K^{CuL}_{CuL_2} - \log K^{Cu}_{CuL}$ |
|---|---|---|---|---|
| Biocytin | 7.73 | 6.56 | 14.29 | −1.17 |
| $N^{\epsilon}$-Acetyl-L-lysine | 8.09 | 6.83 | 14.92 | −1.26 |
| L-Alanine | 8.25 | 7.05 | 15.30 | −1.20 |
| Glycine[b] | 8.27 | 6.92 | 15.19 | −1.35 |
| 2,2′-Bipyridyl[c] | 8.00 | 5.60 | 13.60 | |

[a] The acidity constants used for evaluation of the data for the ternary complexes were those of Table I. [b] Griesser and Sigel (1970). [c] $pK^{H}_{HL} = 4.49$; Anderegg (1963).

*Stability of the Ternary Cu(II) Complexes.* The stability of the mixed ligand complexes, 2,2′-bipyridyl–Cu(II)–amino acid, was determined by potentiometric titration of solutions containing the two ligands and Cu(II) in a ratio 1:1:1 (*cf.* Figure 1). The stability constants calculated (*cf.* eq 6) were $\beta^{Cu}_{Cu(Bipy)L}$; the results are given in Table III.

$$Cu + Bipy + L \rightleftharpoons Cu(Bipy)L$$
$$\beta^{Cu}_{Cu(Bipy)L} = [Cu(Bipy)L]/([Cu][Bipy][L]) \quad (6)$$

The stability constants of eq 7 and 8 were calculated according to eq 9 and 10 and are also given in Table III.

$$Cu(Bipy) + L \rightleftharpoons Cu(Bipy)L$$
$$K^{Cu(Bipy)}_{Cu(Bipy)L} = [Cu(Bipy)L]/([Cu(Bipy)][L]) \quad (7)$$

$$CuL + Bipy \rightleftharpoons Cu(Bipy)L$$
$$K^{CuL}_{CuL(Bipy)} = [Cu(Bipy)L]/([CuL][Bipy]) \quad (8)$$

$$\log K^{Cu(Bipy)}_{Cu(Bipy)L} = \log \beta^{Cu}_{Cu(Bipy)L} - \log K^{Cu}_{Cu(Bipy)} \quad (9)$$

$$\log K^{CuL}_{CuL(Bipy)} = \log \beta^{Cu}_{Cu(Bipy)L} - \log K^{Cu}_{CuL} \quad (10)$$

Under the conditions of measurements, in a Cu(II)–2,2′-bipyridyl 1:1 mixture, the Cu(II)–2,2′-bipyridyl 1:1 complex is nearly completely formed. This means that the equilibrium depicted by eq 11 is far to the right.

$$Cu + Bipy \rightleftharpoons Cu(Bipy)$$
$$K^{Cu}_{Cu(Bipy)} = [Cu(Bipy)]/([Cu][Bipy]) \quad (11)$$

The assumption of complete formation of the Cu(II)–2,2′-bipyridyl 1:1 complex (Griesser and Sigel, 1970) also allows one to calculate the stability constants, $K^{Cu(Bipy)}_{Cu(Bipy)L}$ (*cf.* eq 7), of the mixed ligand systems in a way usual for binary complexes. The results of Table III (and footnote *a* therein) show that the agreement between the two methods of calculation is very good; hence, the assumption of complete formation of the Cu(II)–2,2′-bipyridyl 1:1 complex under these conditions is reasonable. This finding may be of importance

TABLE III: Log Stability Constants of Some Ternary 2,2′-Bipyridyl Cu(II) Ligand Complexes Containing Biocytin, $N^{\epsilon}$-Acetyl-L-lysine, and L-Alanine ($I = 0.1$; $T = 25°$).

| Ligand | $\log \beta^{Cu}_{Cu(Bipy)L}$ | $\log K^{Cu(Bipy)\ a}_{Cu(Bipy)L}$ | $\log K^{CuL}_{CuL(Bipy)}$ | $\Delta \log K$ | $\log X$ |
|---|---|---|---|---|---|
| Biocytin | 15.51 ± 0.05 | 7.51 | 7.78 | −0.22 | 3.13 |
| $N^{\epsilon}$-Acetyl-L-lysine | 15.89 ± 0.03 | 7.89 | 7.80 | −0.20 | 3.26 |
| L-Alanine | 15.99 ± 0.07 | 7.99 | 7.74 | −0.26 | 3.08 |
| Glycine[b] | 15.92 | 7.92 | 7.65 | −0.35 | 3.05 |

[a] Log $K^{Cu(Bipy)}_{Cu(Bipy)L}$ calculated by taking into account only the species, Cu(Bipy), Cu(Bipy)L, $H_2L$, HL, and L, is for L = biocytin, 7.49 ± 0.04; $N^{\epsilon}$-acetyl-L-lysine, 7.87 ± 0.03; L-alanine, 7.97 ± 0.07. The values given in the table were calculated taking into account Cu(Bipy), $Cu(Bipy)_2$, Cu(Bipy)L, CuL, $CuL_2$, Cu, H(Bipy), Bipy, $H_2L$, HL, and L. [b] Griesser and Sigel (1970).

for cases where not all species can be taken into account (*e.g.*, Sigel *et al.*, 1967).

*Nuclear Magnetic Resonance Spectra.* A direct method for investigating a possible interaction between metal ions and a specific binding site in a ligand is offered by nuclear magnetic resonance spectroscopy. This method is especially valuable, as even weak interactions can be characterized. Because of proton relaxation effects, complexation with paramagnetic metal ions, *e.g.*, Mn(II) and Cu(II), results in a selective broadening of the signals from protons close to the binding sites. Due to the rapid exchange between the complexes and free ligand, a trace of the paramagnetic ion is sufficient to broaden such signals (Cohn and Hughes, 1962; Swift and Connick, 1962; Mathur and Li, 1964).

The assignments of the proton magnetic resonance signals of biocytin were carried out with the aid of the spectra taken from *d*-biotin and $N^{\epsilon}$-acetyl-L-lysine (Figure 2). In fact, the spectrum of biocytin can be arrived at from the two latter substances if the signal due to the methyl group of the $N^{\epsilon}$-acetyl end of $N^{\epsilon}$-acetyl-L-lysine is omitted.

The nuclear magnetic resonance spectra of biocytin with increasing amounts of Mn(II) or Cu(II) were taken; the measured line widths of the proton signals due to H(A) and H(B) are given in Table IV. The signals due to the proton of the α-carbon in the amino acid group (*cf.* Figure 2) are broadened very strongly even in the presence of only $2.5 \times 10^{-4}$ M Mn(II). This behavior is well known from α-L-alanine, α-L-amino-*n*-butyric acid, and other amino acids (McCormick *et al.*, 1969). As with *d*-biotin (Sigel *et al.*, 1969b), the pattern due to the methylene groups in the side chain remains nearly uninfluenced by the addition of Mn(II) or Cu(II).

The strong broadening of the signals due to the proton of the α carbon indicates that the α-amino acid group of biocytin is involved with the complex formation. This is not surprising, as this group can form 5-membered chelates, which from studies on usual α-amino acids (Sillén and Martell, 1964), are known to be very stable. However, the hydrogens of the methylene group next to the sulfur atom are also sig-

TABLE IV: Line Widths[a] of Proton Magnetic Resonance Spectra of the Methylene Group Neighbored to Sulfur in Biocytin with Increasing Amounts of Mn(II) or Cu(II) (Biocytin was 0.09 M in $D_2O$; pD ~5.5; $T = 37°$).

| $Me^{2+}$ | $[Me^{2+}]$ | H(A) | H(B) |
|---|---|---|---|
| | | 1.5 | 2.4 |
| Mn | $2.5 \times 10^{-4}$ | 2.2 | 2.4 |
| | $5 \times 10^{-4}$ | 3.3 | 2.4 |
| | $10^{-3}$ | 6 | 3 |
| Cu | $10^{-3}$ | 4.2 | 2.8 |

[a] Given in cycles per second; measured at the half-height of the peaks from spectra taken at expanded sweep width. Only the main peaks of the protons H(A) and H(B) were taken into account, since the second-order splitting of the ABXY pattern (*cf.* Glasel, 1966) is poorly resolved under the given conditions.

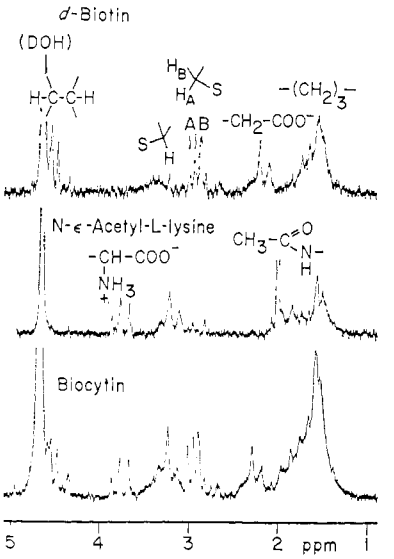



FIGURE 2: Proton magnetic resonance spectra in $D_2O$ at 37° of 0.2 M solutions of *d*-biotin (pD ~8.5), $N^{\epsilon}$-acetyl-L-lysine (pD ~5.5), and biocytin (pD ~5.5). The unlabeled peaks in the spectrum of $N^{\epsilon}$-acetyl-L-lysine are due to the methylene groups ($CON(H)CH_2$, 3.2 ppm; $(CH_2)_3$, ~1.6 ppm). The assignments to the spectrum of *d*-biocytin can be arrived at from the spectra of *d*-biotin and $N^{\epsilon}$-acetyl-L-lysine. The signals due to protons bound to atoms other than carbon are not visible in $D_2O$ as solvent, due to exchange. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was used as a standard (*cf.* Bhacca *et al.*, 1963).

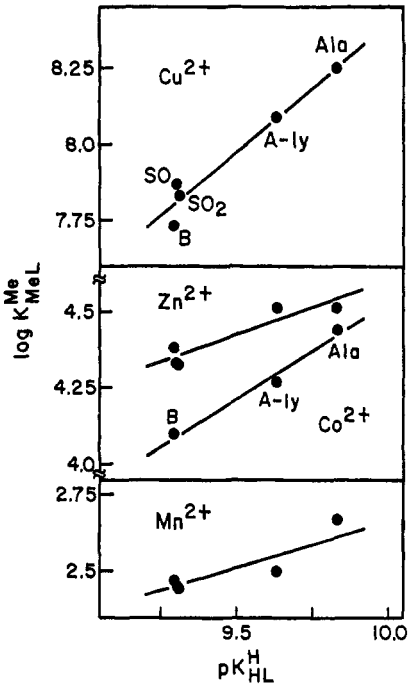



FIGURE 3: Relation between log $K^{Me}_{MeL}$ and $pK^{H}_{HL}$ for the binary Me(II) 1:1 complexes (eq 3) of biocytin (B), the sulfoxide (SO) and sulfone ($SO_2$), $N^{\epsilon}$-acetyl-L-lysine (A-ly), and L-alanine (Ala). The data are from Table I.

nificantly influenced. The most interesting fact in this case is that the influence is asymmetric, *i.e.*, the signals of proton H(A) are more strongly broadened than those of H(B). Since the influence on the lifetime of the excited state of the proton, *i.e.*, the line width, is dependent upon the distance of the metal ion (Sternlicht *et al.*, 1965), this experimental result means that Mn(II) or Cu(II) is closer to H(A) than to H(B). This indicates that these metal ions are bonded to sulfur from below the plane (*cf.* structure A), *i.e.*, *d*-sulfoxide-like complexes are formed.

An attempt to observe a chemical shift in the proton magnetic resonance spectrum (*cf.* Cohn and Hughes, 1962) of biocytin (0.15 M) in the presence of Zn(II) (0.15 M) was without result. Biocytin–$Zn(ClO_4)_2$ (1:1) mixtures in $D_2O$ precipitate at pD ~4.5, and in the pD range 2 to 4.5, no chemical shift could be observed. This is not surprising, as Zn(II)–thioether complexes are not very stable (Sigel *et al.*, 1969a), and for the observation of a chemical shift, most of the ligand should be bound to the diamagnetic metal ion. This case is different from the observed line broadening by the paramagnetic metal ions, Mn(II) and Cu(II), which depends upon rapid ligand exchange, and therefore, the concentration of the complex may be lower.

## Discussion

*Binary Complexes.* As one would expect for all the present ligands, the stability of the binary complexes, MeL, follows the series: Mn(II) < Co(II) < Cu(II) > Zn(II) (*cf.* Table I). This is in accord with the Irving–Williams (1953) sequence. The stability of the Co(II) and Zn(II) complexes is nearly the same. From this standpoint, it is not too surprising that Co(II) can be substituted for Zn(II) in many biological systems. This even seems to occur *in vivo* as suggested from the example of transcarboxylase (Northrop and Wood, 1969a).

A superficial glance at the data of Table I reveals that the constants are of the same order within a series for all five amino acids. For a closer examination of the stability of the investigated binary complexes, MeL, from which we may be able to learn something about the influence of the biotinyl moiety and the long tail of some of the ligands, it is necessary to consider the somewhat different basicity of the amino group of the ligands. This can be done by plotting the stability constants against the acidity constants. For a series of structurally related ligands, a straight line should result (Martell and Calvin, 1952; Sigel *et al.*, 1969b). The data of Table I are so plotted in Figure 3. For all four metal ions, Mn(II), Co(II), Cu(II), and Zn(II), lines which are straight within the experimental errors result. This demonstrates that the small differences in stability of the binary complexes, MeL, are due to the small differences in basicity of the ligands. The long tails up to 14 Å (Northrop and Wood, 1969b), of some of the ligands have no great influence on the stability of the complexes. Aside from this, the interaction between the thioether group of the biotinyl moiety and metal ions shown by the nuclear magnetic resonance measurements does not manifest itself in the global stability constant. This is in agreement with the results obtained for *d*-biotin (Sigel *et al.*, 1969b). In addition, there are no differences in stability between the complexes of biocytin and sulfur-oxidized derivatives, the sulfoxide and sulfone. Therefore, the stability of the biocytin–metal ion complexes is completely determined by the basicity of the $\alpha$-amino acid group, which can participate in formation of 5-membered chelates. However, this does not mean that no interaction between the metal ions and the thioether group occurs. It means only that these interactions are weak. Such a behavior is well known for many biochemically important ligands such as nucleotides (Schneider *et al.*, 1964; Sigel, 1968), amino acids (McCormick *et al.*, 1969), and others (Sigel *et al.*, 1969b).

That metal ions can interact with the thioether group of biocytin is shown by the nuclear magnetic resonance measurements. It is of interest to note that the metal ion–sulfur interaction is obviously largely independent of the distance of the stability-determining ligand group, which in *d*-biotin is the carboxylate group (Sigel *et al.*, 1969b) and in biocytin, the $\alpha$-amino acid moiety. Therefore, in both ligands, *d*-biotin and biocytin, the stability-determining factor for the metal ion complexes is not the thioether group.

The nuclear magnetic resonance results can be due to (a) a simple metal ion–sulfur complex, (b) an intramolecular macrochelate formed through binding of the metal ion to the thioether and the $\alpha$-amino acid group at the same time, (c) an intermolecular complex species, or (d) a mixture of the three possibilities a–c. A decision among these possibilities cannot be made based on the present results. However, with respect to biotin enzymes, this is not of importance, as the $\alpha$-amino acid end of biocytin is amide linked to other amino acids of the protein, and hence, no longer available for metal ion coordination. The important thing to note is that the thioether group can interact with metal ions and that this interaction occurs in a stereospecific way, *i.e.*, from below the plane of the tetrahydrothiophene ring (*cf.* structure A). The reason for this stereospecific interaction is probably due to steric hin-

drance of the ureido ring in the biotinyl moiety (Sigel *et al.*, 1969b).

*$CuL_2$ and Cu (Bipy)L Complexes.* If a part of the coordination sphere of Cu(II) is occupied by one of the investigated amino acids or by 2,2′-bipyridyl, that is if eq 4 and 7 are considered, the same reasoning as given the last paragraph is still true. Plots of the stability constants, $K^{CuL}_{CuL_2}$ (Table II) or $K^{Cu(Bipy)}_{Cu(Bipy)L}$ (Table III), against the acidity constants, $K^{H}_{HL}$ (Table I), also result in straight lines for the ligands (*cf.* Figure 4). Again, the large tail and the biotinyl moiety are without any great influence on the complex stability, *i.e.*, the stability is still completely determined by the basicity of the amino acid group, and there is no major steric hindrance by the large tail. The same conclusions can be drawn by considering the differences, $\log K^{CuL}_{CuL_2} - \log K^{Cu}_{CuL}$, in stability between the 1:2 and 1:1 Cu(II)–amino acid complexes; all differences are of the same order (*cf.* Table II).

*Ternary Cu(II) Complexes.* One way to characterize the stability of ternary complexes is by comparing the stability constant of the binary complex derived from eq 3 with that of the ternary complex according to eq 7 (Sigel, 1967; Griesser and Sigel, 1970). Hence, the difference in stability can be defined by eq 12.

$$\Delta \log K = \log K^{Cu(Bipy)}_{Cu(Bipy)L} - \log K^{Cu}_{CuL} = \log K^{CuL}_{CuL(Bipy)} - \log K^{Cu}_{Cu(Bipy)} \quad (12)$$

Usually, it holds that $K^{Me}_{MeL} > K^{MeL}_{MeL_2}$. For the Cu(II)–amino acid complexes studied here, the difference, $\log K^{CuL}_{CuL_2} - \log K^{Cu}_{CuL}$, is about −1.2 log units (*cf.* Table II). Surprisingly, the values of $\Delta \log K$ are only about −0.2 to −0.3 log unit; in other words, the ternary complexes appear to be very stable.

It has been shown for several cases that ligands containing O as donor atoms form more stable complexes with the Cu(II)–2,2′-bipyridyl 1:1 complex (eq 7) than with the free (hydrated) Cu(II) ion (eq 3), *i.e.*, positive $\Delta \log K$ values (about +0.5) were observed. With ligands containing N as donor atoms, the values for $\Delta \log K$ were negative (about −1.2), and with "mixed" ligands containing O and N as donors, intermediate values were observed (Sigel, 1967; Griesser and Sigel, 1970). Hence, the Cu(II)–2,2′-bipyridyl 1:1 complex shows discriminating qualities toward the coordination of a second ligand. From this point of view, the present results fit exactly in the described picture; the values observed for $\Delta \log K$ (Table III) are in the order expected for amino acids which contain O and N as donors.

Another way of characterizing the stability of ternary complexes is according to eq 13 through 15.

$$Cu(Bipy)_2 + CuL_2 \rightleftharpoons 2Cu(Bipy)L$$

$$X = [Cu(Bipy)L]^2/([Cu(Bipy)_2][CuL_2]) \quad (13)$$

$$\log X = 2 \log \beta^{Cu}_{Cu(Bipy)L} - (\log \beta^{Cu}_{Cu(Bipy)_2} + \log \beta^{Cu}_{CuL_2}) \quad (14)$$

$$\log X = (\log K^{Cu(Bipy)}_{Cu(Bipy)L} - \log K^{CuL}_{CuL_2}) + (\log K^{CuL}_{CuL(Bipy)} - \log K^{Cu(Bipy)}_{Cu(Bipy)_2}) \quad (15)$$

The value of the constant, $X$, of eq 13 expected on statistical grounds, is 4, *i.e.*, $\log X = 0.6$ (DeWitt and Watters, 1954; Kida, 1956). The values determined for $\log X$ in the present 2,2′-bipyridyl–Cu(II)–amino acid systems are all in the order of about 3.1 log units (*cf.* Table III). Hence, these ternary complexes are more stable by a factor of about 300 than expected on the basis of a purely statistical argument. The reasons for the large stability of such ternary complexes were recently discussed (Sigel, 1967; Griesser and Sigel, 1970; R. F. Pasternack and H. Sigel, manuscript in preparation, 1970).

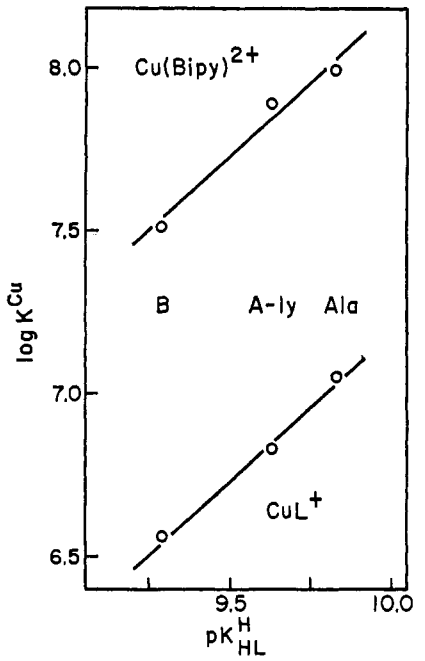



FIGURE 4: Relation between log $K^{Cu}$ ($\widehat{=}$ log $K^{CuL}_{CuL_2}$ or log $K^{Cu(Bipy)}_{Cu(Bipy)L}$) and $pK^{H}_{LH}$ for the binary Cu(II) 1:2 complexes ($CuL_2$; eq 4) is shown in the bottom line and that for the ternary 2,2′-bipyridyl–Cu(II)–ligand complexes (Cu(Bipy)L; eq 7) in the top line for biocytin (B), $N^{\epsilon}$-acetyl-L-lysine (A-ly), and L-alanine (Ala). The data are those of Tables I through III.

It is of interest to compare the formation of a ternary complex with the corresponding binary one and to note the influence of pH. For this reason, the distributions of the several complex species as dependent upon pH were calculated for the binary Cu(II)–biocytin 1:1 system using the constants from Tables I and II. The same was done for the ternary 2,2′-bipyridyl–Cu(II)–biocytin (1:1:1) system with the constants given in Tables I through III. The percentage of the total concentration of Cu(II) in the several complex species as influenced by pH is shown in Figure 5.[3] All concentrations tend to approach limiting values with increasing pH. The concentration of the ternary complex, 2,2′-bipyridyl–Cu(II)–biocytin, goes up to 93% of the total Cu(II) present in solution, while the binary complex in the corresponding system reaches only about 66%. Thus, in the binary system, the concentrations of Cu(II) and the 1:2 complex are still reasonably high (each about 17%), while in the mixed ligand system the ternary complex dominates very strongly.

[3] At higher pH values, hydrolysis (*cf.*, *e.g.*, Perrin and Sharma, 1966) will affect somewhat the distribution of the several complex species; this was neglected in the calculations for Figure 5.

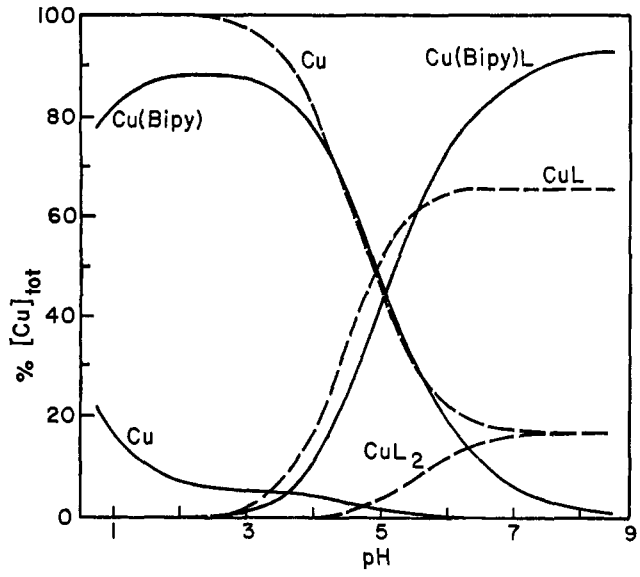



FIGURE 5: Influence of pH on the concentrations (given as the percentage of the total Cu(II) present) of the several species present in an aqueous solution of the binary system (interrupted lines), Cu(II) and biocytin (each $10^{-3}$ M), and of the ternary system (solid lines), Cu(II), 2,2′-bipyridyl, and biocytin (each $10^{-3}$ M); computed with the constants of Tables I through III. The sum of the concentrations of $Cu(Bipy)_2$, CuL, and $CuL_2$ in the ternary system was always less than 7%. Hydrolysis was omitted in these calculations.[3]

## Conclusion

The present results demonstrate the stability-determining qualities of the α-amino acid group for metal ion complexes with biocytin and derivatives. With respect to their stability in binary and ternary complexes, these ligands behave as simple amino acids. The large stability of ternary complexes is not only interesting, but at the same time this fact makes it more understandable why mixed ligand complexes are important in nature. The study of relatively simple mixed ligand–metal ion systems as models should improve our understanding for the driving forces which lead, for example, to the right enzyme–metal ion–substrate complexes. This is especially true, as not only the Cu(II)–2,2′-bipyridyl (1:1) complex but also Cu(II)–complexes containing the imidazole group (Huber *et al.*, 1969; Sigel, 1969) show discriminating behavior toward the coordination of the second ligand.

Although the complexes formed by coordination of metal ions to thioether groups are not very stable (Sigel *et al.*, 1969a), they may be stable enough to originate special structures as discussed elsewhere (Sigel *et al.*, 1969b; McCormick *et al.*, 1969). Moreover, these metal ion–thioether complexes seem to be labile enough to allow rapid rearrangement of the complexes necessary for an enzyme to act as a catalyst. So far it is not known whether or not the function of the sulfur atom is to bind to a metal ion during one of the biochemical reactions where a biotinyl group is involved. At least this investigation demonstrates that this might be a possibility which is as probable as others suggested previously (Mildvan *et al.*, 1966; Bowen *et al.*, 1968; Caplow, 1969). Recently, an interesting postulate has been made (Northrop and Wood, 1969b): "Biotin is attached to the protein of transcarboxylase through an amide bond between the carboxyl group of the side chain of biotin and an ε-amino group of lysine of the protein. The chain linkage between the ring structure of biotin can reach a distance of 14 Å when fully extended, and therefore a structural basis is provided for the possibility of migration between physically distinct portions of the enzyme protein." If the biotin moiety is in such a fairly exposed and movable state, a weak but stereospecific metal ion interaction could be, in fact, the right means to create the active enzyme–substrate complex.

## Acknowledgments

The measurements of complex stabilities were performed with the skillful technical assistance of Miss R. Baumbusch and Miss M. Nicholson.

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# Studies of Self-Association and Conformation of Peptides by Thin-Film Dialysis*

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ABSTRACT: The tyrocidine antibiotic polypeptides are known to exhibit self-association. They were therefore used as test substances to document the behavior to be expected by the method of thin-film dialysis. Self-association with these peptides is indicated by concentration dependence, a reverse curvature of the escape plot, and a retardation of the rate of diffusion by addition of salt but an acceleration by the addition of ethanol or other hydrophobic bond breaking solvent additives. A survey of dipeptides by thin-film dialysis has revealed that L-lysyl-L-lysine, L-lysyl-L-arginine, and L-histidyl-L-histidine exhibit a behavior characteristic of self-association in a pH region where all the acidic and basic groups are charged. L-Glutamyl-L-glutamic acid does likewise at a pH in the range where the carboxyl groups are fully charged. L-Arginyl-L-arginine does not appear to show this behavior.

Since thin-film dialysis rates are particularly sensitive to conformational changes, comparative data for a number of di-, tri-, and tetrapeptides in various solvent environments are presented. The results in many cases are those expected from the molecular size and shapes of Corey–Pauling–Koltun molecular models. In other cases the results suggest whether a compact conformation or an extended conformation is preferred.

The so-called quaternary structures of proteins and nucleotides are thought to result from the interplay of various binding forces such as hydrophobic bonds, coulombic forces, $\pi$ bonds, and hydrogen bonds. The contribution of each individual interaction or small section of the molecule is considered to be relatively weak as compared with most covalent bonds but their sum in concerted effect can be strong. Such a theory agrees well with experience but leaves much to be understood about the detailed nature of each type of interaction and its contribution to the whole. A truly clear understanding is made uncertain by the many variables involved in the study of the larger molecules. There is also a corresponding vagueness about the relative contribution each of these forces makes in the determination of tertiary structure.

In the attempt to simplify the experimental approach to the development of more definite concepts it would seem logical to turn to the more simple problem presented by smaller molecules. Here, on the basis of present theory, it is to be expected that specificity would be much reduced and that the interactions would be much weaker. For example, no enzyme or antibody containing less than 100 or more amino acid residues has been discovered. Nonetheless, it could be worthwhile to study smaller molecules provided meaningful techniques could be found and applied to selected models of well-known structure.

Techniques available at present for this type of study include among others ultracentrifugation, nuclear magnetic

* From The Laboratories of The Rockefeller University, New York, New York 10021. *Received April 23, 1970.* This work was supported in part by Grant AM 02493 from the National Institutes of Health.

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