Biochemistry

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Volume 8, Number 7 July 1969

Metal Ion Complexes with Biotin and Biotin Derivatives. Participation of Sulfur in the Orientation of Divalent Cations*

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ABSTRACT: A probable function of the sulfur in *d*-biotin could be to coordinate in a specific way with the appropriate metal ions and thereby form an active enzyme–metal ion–substrate complex. Hence, the stability constants of the binary 1:1 complexes of Mn(II), Cu(II), and Zn(II) with *d*-biotin and its ring-altered derivatives, *viz.*, dethio, sulfoxide, and sulfone were measured by potentiometric titrations. Stabilities of the complexes in 50% aqueous dioxane (I = 0.1, T = 25°) are in the range: log $K_{MnL}^{Mn} \sim 2.0$, log $K_{CuL}^{Cu} \sim 3.4$, and log $K_{ZnL}^{Zn} \sim 2.4$. For *d*-biotin and water as solvent, the values are log $K_{CuL}^{Cu} = 1.63$ and log $K_{ZnL}^{Zn} = 0.82$. The values found (in 50% aqueous dioxane) for the complexes of chain-shortened biotin derivatives are of about the same order as for the other ligands in the same solvent. The investigation of the formation of ternary, *i.e.*, mixed, complexes between these ligands and the 1:1 complexes of Cu(II)-2,2′-bipyridyl and Zn(II)–2,2′-bipyridyl was also included in this study. The stability constants found for all these complexes are of the size expected, if basicity of the carboxylic acid group determines the complex stability. However, that the sulfur in biotin can also complex with metal ions, is demonstrated by the complexing between tetrahydrothiophene and Cu(II). Furthermore, nuclear magnetic resonance spectra of *d*-biotin with increasing amounts of Mn(II) or Cu(II) show, besides line broadening of the signal due to the methylene group neighbored to the carboxyl function, broadening of the quartet that is due to only one of the protons in the methylene group next to the sulfur. This suggests that these Me(II) complexes are formed in a stereospecific manner, *i.e.*, the orientation of these metal ions in the complex with *d*-biotin is analogous to that of oxygen in *d*-biotin *d*-sulfoxide. This structure-specific behavior is confirmed by comparison of the stability of the Cu(II)–chelate (1:1) of tetrahydrothiophene-2-carboxylic acid (log $K_{CuL}^{Cu} = 4.31$) with that of the Cu(II)–*d*-tetranorbiotin complex (1:1) (log $K_{CuL}^{Cu} = 2.89$); this difference in stability cannot be explained by the small differences of the acidity constants. In the last-mentioned complex, Cu(II) coordinated at the carboxylic acid group could only form a chelate by binding in an *l*-sulfoxide-like way; however, due to the steric hinderance of the ureido ring this seems not to occur, *i.e.*, at least not in a significant stability-increasing way.

Several papers have appeared dealing with the possible function of sulfur in *d*-biotin (Mildvan *et al.*, 1966; Bowen *et al.*, 1968). That the sulfur atom is essential for complete metabolism of this vitamin can be seen, for example, from investigations over the catabolism of *d*-biotin. The bacterial degradation of biotin is decreased by dethiobiotin (Brady *et al.*, 1966), since the side chain of dethiobiotin is degraded, but little or no degradation of the ureido ring in this compound occurs (Ruis *et al.*, 1968). There is also direct evidence that dethiobiotin can be converted into biotin in *Aspergillus niger* (Tepper *et al.*, 1966). During this process, the abstraction only of some hydrogen occurs, so that nearly the intact dethiobiotin molecule is converted into biotin (Li *et al.*, 1968b). Thus, it is certain that dethiobiotin can be a precursor for biotin, at least in this microorganism, but it cannot take over the coenzymatic role of biotin.

A possible function of the sulfur atom could be, at least suggested in the case of pyruvate carboxylase, that a transannular effect in biotin could conceivably facilitate removal of a proton from the substrate molecule by this enzyme (Mildvan

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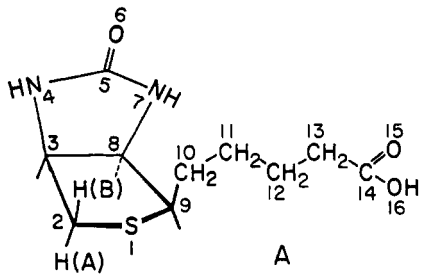
† Done during a leave of absence from the University of Basel to Cornell University.

et al., 1966; Mildvan and Scrutton, 1967). Apparently, though, such a transannular interaction in biotin has been excluded by the recent investigation of its basicity (Bowen *et al.*, 1968). Another possible function of the sulfur atom might be its coordination to a metal ion. This possibility has especially to be taken into account, since it is well known that for the biosynthesis of biotin, metal ions, especially Zn and Fe, are essential (Eisenberg, 1963); also it is known that biotin-containing enzymes are metal ion dependent. For example, Mn^{2+} is required for transcarboxylation in pyruvate carboxylase (Scrutton *et al.*, 1966). Moreover, it has been shown that thioether groups can coordinate to metal ions; at least for the case of Cu(II) this is considerable (Sigel *et al.*, 1969). The coordination of sulfur to a metal ion may thereby result in the right arrangement relating to space and reaction in an enzyme–metal ion–substrate complex. For these reasons, the present investigation was made on the structure and stability of metal ion complexes with biotin and certain of its derivatives.

In the present study, the stabilities of 1:1 complexes of Mn(II), Cu(II), and Zn(II) with *d*-biotin, lipoic (thioctic) acid, and biotin derivatives were investigated, in which the rings and/or the side chain were modified. Since enzyme–metal ion–substrate complexes are complexes of higher order, the stabilities of mixed, *i.e.*, ternary, 2,2′-bipyridyl–Me(II)–biotin-like complexes were also determined.

The structure (Traub, 1956; Bonnemere *et al.*, 1965) of *d*-biotin[1] is as seen in structure A. The terminology (Sternbach, 1963; Iwahara *et al.*, 1969) and numbering (Mistry and Dakshinamurti, 1964) used for the suffixes of several of the ligands investigated indicates the number of methylene functions in the aliphatic side chains as: homo, 5; bisnor, 2; and tetranor, O.



A

Experimental Section

Materials. *d*-Biotin was obtained from Hoffman-La Roche, Inc., Nutley, N. J., and *dl*-dethiobiotin was from Nutritional Biochemicals Corp., Cleveland, Ohio. Thioctic acid, valeric acid, tetrahydrothiophene, 2,2′-bipyridyl, and the metal ion perchlorates, used for the potentiometric titrations, were from Fluka AG., Buchs, Switzerland, and dioxane from Merck AG., Darmstadt, Germany. $Cu(ClO_4)_2 \cdot 6H_2O$ and $Mn(ClO_4)_2 \cdot 6H_2O$, used for the nuclear magnetic resonance spectra, were from Alfa Inorganics, Beverly, Mass. D_2O (99.9%) was purchased from Calbiochem, Los Angeles, Calif., and NaOD (30% in D_2O 99%) was from Volk Radiochemical Division of I. C. N., Burbank, Calif.

Compounds Chemically Synthesized. *d*-Biotin *d*-sulfoxide and *d*-biotin *l*-sulfoxide were made by treatment of biotin with equimolar H_2O_2, essentially following the method of Melville (1954), and purified by fractional crystallization from acetic acid–ethanol (Brady *et al.*, 1966; Ruis *et al.*, 1967). *d*-Biotin sulfone was made with excess H_2O_2, according to the method of Hofmann *et al.* (1941). *d*-Homobiotin was prepared according to a method suggested by Sternbach (*cf.* 1963; see also Ruis *et al.*, 1968). In the case of tetrahydrothiophene-2-carboxylic acid, the same sample was used as before (Sigel *et al.*, 1969).

Compounds Biosynthesized. The following compounds were isolated from cultures of several microorganisms that were grown on the corresponding biotin or dethiobiotin: *d*-bisnorbiotin from *Penicillium oxalicum* (Li, 1969), *d*-tetranorbiotin from *Pseudomonas* sp. (Iwahara *et al.*, 1969), and *dl*-bisnordethiobiotin and *d*- and *dl*-tetranordethiobiotin from *Aspergillus niger* (Li *et al.*, 1968a).

Apparatus.[2] Titrations were carried out with a Metrohm potentiograph E 336 and Metrohm UX glass electrode. The ultraviolet spectra were taken with a Beckman spectrophotometer Model DB (connected with recorder Model 43 from Photovolt Corp.) and a Bausch & Lomb Spectronic 600 spectrophotometer (Walz and Walz recorder). The nuclear magnetic resonance spectra were determined with a Varian analytical nuclear magnetic resonance spectrometer A-60A; for the adjustment of pH, a Corning research pH meter, Model 12, was used.

Determinations of Constants for Acidity of Ligands and for Stability of Complexes. For the determination of acidity constants, K_{HL}^{H}, 50 ml of water solutions containing 50% dioxane, 3×10^{-4} M $HClO_4$, and $NaClO_4$ to 0.1 M were titrated in the presence and absence of the ligands (6×10^{-4} M) under N_2 with 5×10^{-2} M NaOH ($I = 0.1$; $T = 25°$). The stability constants of the complexes, K_{MeL}^{Me}, were determined in solutions of the same concentrations, but a part of $NaClO_4$ was replaced by $Me(ClO_4)_2$ (2.4×10^{-2} M). The concentrations of the $Me(ClO_4)_2$ stock solutions were standardized by titration with EDTA. The stability constants, with consideration of the hydrolysis curves, were calculated according to eq 1, where K_A' is the apparent acidity constant of the ligand in the presence of metal ions (*cf.* Sigel, 1967).

$$K_{MeL}^{Me} = (K_A' - K_{HL}^{H})/(K_{HL}^{H} \cdot [Me_{total}^{2+}]) \qquad (1)$$

The constants for the *d*-biotin complexes in water were determined under the same conditions and in the same way.

The stability constants of the ternary complexes, $K_{Me(Bipy)L}^{Me(Bipy)}$, were also determined in water solutions containing 50% dioxane. The conditions were as given before; instead of $Me(ClO_4)_2$, however, 1:1 mixtures of $Cu(ClO_4)_2$ or $Zn(ClO_4)_2$ and 2,2′-bipyridyl (2.4×10^{-2} M each) were used. For the calculations of stability constants, again eq 1 could be used, since the Cu(II)–2,2′-bipyridyl (1:1) complex is practically

[1] *d*, for example in *d*-biotin, means that the side chain is above the tetrahydrothiophene ring plane in the structural formula, A. *d*-Sulfoxide in *d*-biotin *d*-sulfoxide means that the oxygen bond to the sulfur atom (sulfur is tetrahedral) is below this plane. An *l* indicates the opposite structures.

[2] Abbreviations used are: L, ligand; Me, metal ion; Bipy, 2,2′-bipyridyl.

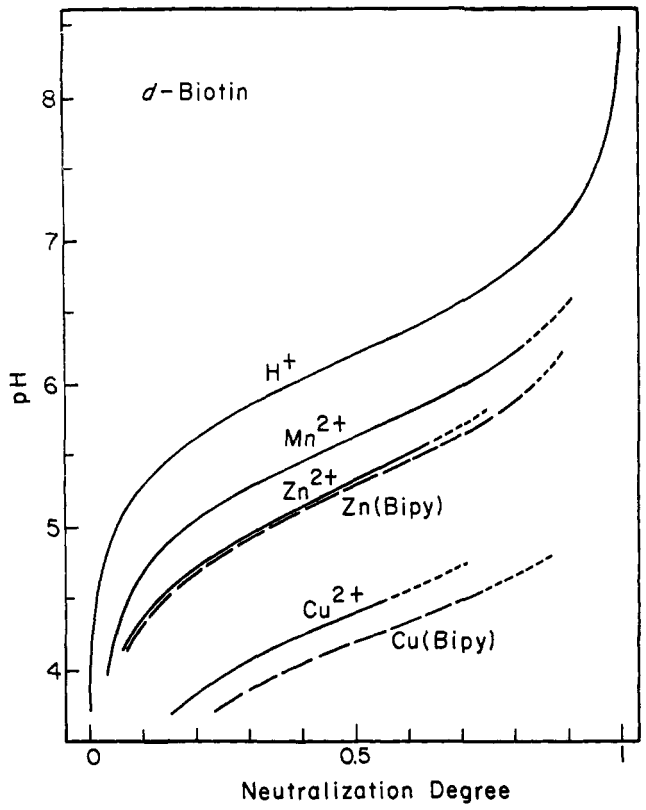


FIGURE 1: Neutralization degree of dependence upon pH during potentiometric titration of *d*-biotin with and without metal ion (—) or metal ion and 2,2′-bipyridyl (— · —) in 50% aqueous dioxane. Portions of lines (– –) extended toward higher degrees of neutralization indicate uncertainty due to hydrolysis. *d*-Biotin was 6×10^{-4} M; Mn(II), Cu(II), Zn(II), and 2,2′-bipyridyl, when added, were 2.4×10^{-2} M.

100% formed under these conditions (*cf.* also L'Heureux and Martell, 1966). The same is still a very reasonable assumption for the case of $Zn(Bipy)^{2+}$, so that again eq 1 could be used. In both cases, the release of protons due to the complexing between Cu(II) and Zn(II) with 2,2′-bipyridyl is completely finished before the reaction between $Cu(Bipy)^{2+}$ or $Zn(Bipy)^{2+}$ with L occurs (*cf.* also Griesser *et al.*, 1968). This means the curves that resulted from the titration of a solution containing only $HClO_4$ and those containing also Cu(II) or Zn(II) and 2,2′-bipyridyl are superimposable. This is true at lower pH values and in the pH region used for the calculation of stability of complexes (*cf.* Figure 1); at higher pH values the curves are different, of course, due to hydrolysis of the Me(II)–2,2′-bipyridyl (1:1) complexes.

The results given in Tables I–III are usually the averages of two or three independent titrations. In those cases where only very little substance was available and therefore only one titration could be carried out, this is mentioned.[3]

Estimation of the Stability Constant of the Cu(II)–Tetrahydrothiophene (1:1) Complex. Difference spectra were taken in water solutions containing dioxane (*cf.* Table II) in 1-cm quartz cuvets. In the reference beam were two cuvets, one containing $Cu(ClO_4)_2$ and $NaClO_4$ and the other tetrahydrothiophene (THT); in the sample beam also were two cuvets, one containing $Cu(ClO_4)_2$, tetrahydrothiophene, and $NaClO_4$, and the other plain solvent. The rest of the conditions were similar to those used for the determination of the stability constant of the Cu(II)–thiophene (1:1) complex. The stability constant was determined again graphically by plotting $1/[THT]$ against $1/\Delta E_{265}$ (*cf.* Kahmann *et al.*, 1964). Since it was difficult to get all the compounds into solution and the values for ΔE were small, the constants given in Table II can only be considered as estimations.

[3] The deviations of the average values of pK_A' were of about the same magnitude as those for pK_{HL}^{H} (*cf.* Tables I–III).

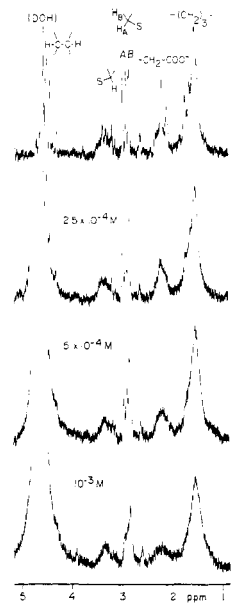


FIGURE 2: Proton magnetic resonance spectra of 0.12 M *d*-biotin alone (top) and with increasing concentrations of $Mn(ClO_4)_2$ in D_2O at 37° and pD ~ 7.8.

Nuclear Magnetic Resonance Experiments. The nuclear magnetic resonance spectra were taken in 0.12 M solutions (about half-saturated under the conditions) of *d*-biotin in D_2O at pD ~ 7.8 (adjusted with NaOD). 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). The assignments of the signals were done according to Glasel (1966). The concentrations of the metal ions in the solutions, when added, are given in Figure 2. The increasing HOD signal (*cf.* Figure 2) is due to the increasing amounts of H_2O which is brought into the solution by adding $Mn(ClO_4)_2 \cdot 6H_2O$; besides this the broadening of the signal increases with increasing concentration of Mn(II).

Results

Stability of Complexes. The stability constants of the binary complexes, ML, and the ternary complexes, Me(Bipy)L, according to equilibria eq 2 and 3 have been determined in water solutions containing 50% dioxane ($I = 0.1$; $T = 25°$). The mixed solvent was used for solubility reasons. The determination of the constants was done by means of potentiometric titrations in the presence of an excess of metal ions, or of an excess of metal ions and 2,2′-bipyridyl in a 1:1 ratio, with respect to the concentration of the ligand.

TABLE I: Negative Log Acidity Constants, pK_{HL}^{H}, and Log Stability Constants of Binary, Log K_{MeL}^{Me}, and Ternary, Log $K_{Me(Bipy)L}^{Me(Bipy)}$, Mn(II), Cu(II), and Zn(II) Complexes with Biotin and Biotin Derivatives Which Are Modified in the Rings, and with Lipoic Acid, Also As with Derivatives of Biotin and Dethiobiotin Which Are Modified in the Side Chain, Determined in Water Containing 50% Dioxane (I = 0.1; T = 25°).

		Binary Complexes			Ternary Complexes			
Ligand	pK_{HL}^{H}	Log K_{MnL}^{Mn}	Log K_{CuL}^{Cu}	Log K_{ZnL}^{Zn}	Log $K_{Cu(Bipy)L}^{Cu(Bipy)}$	Log $K_{Zn(Bipy)L}^{Zn(Bipy)}$	ΔLog K_{Cu}	ΔLog K_{Zn}
d-Biotin	6.22 ± 0.01	2.07	3.39	2.45	3.64	2.49	0.25	0.04
dl-Dethiobiotin	6.35 ± 0.02	1.96	3.37	2.45	3.70	2.48	0.33	0.03
d-Biotin *d*-sulfoxide	6.04 ± 0.01	1.98	3.39	2.38	3.51	2.34	0.12	−0.04
d-Biotin *l*-sulfoxide	6.05 ± 0.02	1.97	3.34	2.37	3.53	2.36	0.19	−0.01
d-Biotin sulfone	6.10 ± 0.01	2.06[a]	3.44	2.46[a]	3.63[a]	2.49	0.19	0.03
Lipoic acid	6.41 ± 0.01	2.01	3.45	2.57	3.75	2.60	0.30	0.03
d-Homobiotin	6.29 ± 0.02	2.00	3.38	2.43	3.60	2.48	0.22	0.05
dl-Dethiobiotin	6.35 ± 0.02	1.96	3.37	2.45	3.70	2.48	0.33	0.03
d-Bisnorbiotin	5.86 ± 0.01	1.80	3.11[a]	2.40	3.29[a]	2.26	0.18	−0.14
dl-Bisnordethiobiotin	6.11 ± 0.01	1.89	3.23[a]	2.30	3.43[a]	2.29	0.20	−0.01
d-Tetranorbiotin	4.85 ± 0.01	1.66	2.89	1.92	3.01[a]	1.87[a]	0.12	−0.05
d-Tetranordethiobiotin	5.47 ± 0.01[a]		3.16[a]					
dl-Tetranordethiobiotin	5.48 ± 0.01	1.90	3.14	2.19	3.17	2.01[a]	0.03	−0.18

[a] Average of the calculations of one titration.

$$Me + L \rightleftharpoons MeL \qquad K_{MeL}^{Me} = [MeL]/[Me][L] \quad (2)$$

$$Me(Bipy) + L \rightleftharpoons Me(Bipy)L \qquad K_{Me(Bipy)L}^{Me(Bipy)} = [Me(Bipy)L]/[Me(Bipy)][L] \quad (3)$$

For the case of *d*-biotin and the metal ions, Mn(II), Cu(II), and Zn(II), the results of such titrations are shown in Figure 1, where the pH of the solution is plotted against the neutralization degree. The formation of complexes shifts the buffer region of the ligand to lower pH values. Thus, from Figure 1 the stability of the binary complexes, MeL, follows the series: Mn(II) < Cu(II) > Zn(II), which is in accord with the Irving–Williams (1953) sequence. Since the buffer region is shifted to lower pH values in the presence of Cu(Bipy) as it is with Cu(II) alone, Δ log K_{Cu} according to eq 4 has to be a positive value, which is in agreement with earlier reports (L'Heureux and Martell, 1966; Sigel, 1967; Griesser *et al.*, 1968).[4] The results described so far for biotin are also true for all other ligands investigated during this study.

$$\Delta \log K_{Me} = \log K_{Me(Bipy)L}^{Me(Bipy)} - \log K_{MeL}^{Me} \quad (4)$$

The results obtained for *d*-biotin, lipoic acid, and tetrahydrothiophene ring-modified biotin derivatives are given in Table I. The acidity constants are very similar for all these ligands. The removal of the sulfur atom, however, results in a slight increase in the basicity of the carboxylic acid group, while the oxidation of the sulfur results in a slight decrease.

Also in Table I, the results obtained for *d*-biotin, *dl*-dethiobiotin, and some of their side-chain-modified derivatives are presented. These ligands can be ordered in two different series, within each of which the basicity of the carboxylic acid group decreases: *d*-homobiotin > *d*-biotin > *d*-bisnorbiotin ≫ *d*-tetranorbiotin and *dl*-dethiobiotin > *dl*-bisnordethiobiotin ≫ *dl*-tetranordethiobiotin = *d*-tetranordethiobiotin. The difference within the two given series is the length of the side chain. The longer the side chain is, the more basic the carboxylic acid group becomes. This observation is in agreement with the results for the simple carboxylic acids, where the basicity decreases in the series: valeric acid > propionic acid > acetic acid ≫ formic acid (*cf.* Table II). In addition, a comparison between the biotin and the corresponding dethiobiotin derivatives shows that the carboxylic acid group of the dethio derivatives is always more basic.

If one neglects the ureido group, biotin has two potential binding sites for metal ions (*cf.* Discussion); these are the carboxylic acid group and the sulfur atom of the tetrahydrothiophene ring. It was of interest, therefore, to investigate also the coordination tendency of the two isolated binding sites, *i.e.*, of tetrahydrothiophene and simple carboxylic acids. From nuclear magnetic resonance spectra, it can be seen that there is an interaction between tetrahydrothiophene and Cu(II)[5] and from difference spectra in the ultraviolet region, the stability constant of the Cu(II)–tetrahydrothiophene (1:1) complex could be estimated. This value is given together with those for the simple carboxylic acid complexes in Table II. Another ligand of interest in this connection is tetrahydrothiophene-2-carboxylic acid which has the two binding sites

[4] For a discussion of the possible reasons for the increased stability of ternary Cu(II) complexes containing 2,2′-bipyridyl and another ligand with oxygen atoms as donors, see L'Heureux and Martell (1966) and Sigel (1967).

[5] These nuclear magnetic resonance experiments were carried out in D_2O solutions containing 50% D_6-acetone. Tetrahydrothiophene was 0.2 M and $Cu(ClO_4)_2$ when added, 5×10^{-4}, 10^{-3}, and 2×10^{-3} M.

TABLE II: Negative Log Acidity Constants, pK_{HL}^{H}, and Log Stability Constants of Binary, Log K_{MeL}^{Me}, and Ternary, Log $K_{Me(Bipy)L}^{Me(Bipy)}$, Mn(II), Cu(II), and Zn(II) Complexes with Biotin, Tetrahydrothiophene-2-carboxylic Acid, Tetrahydrothiophene, and Some Aliphatic Carboxylic Acids, Determined in Water Containing 50% Dioxane (I = 0.1; T = 25°).

		Binary Complexes			Ternary Complexes			
Ligand	pK_{HL}^{H}	Log K_{MnL}^{Mn}	Log K_{CuL}^{Cu}	Log K_{ZnL}^{Zn}	Log $K_{Cu(Bipy)L}^{Cu(Bipy)}$	Log $K_{Zn(Bipy)L}^{Zn(Bipy)}$	ΔLog K_{Cu}	ΔLog K_{Zn}
d-Biotin	6.22 ± 0.01	2.07	3.39	2.45	3.64	2.49	0.25	0.04
Tetrahydrothiophene-2-carboxylic acid	5.58 ± 0.01[b]	1.80[b]	4.31[b]	2.35[b]	4.32	2.30	0.01	−0.05
Tetrahydrothiophene			~0.4[a]					
Valeric acid	6.44 ± 0.01	1.91	3.49	2.47	3.68	2.43	0.19	−0.04
Propionic acid	6.29 ± 0.01[b,c]	1.92[b]	3.45[b,c]	2.41[b,c]	3.60[c]	2.38[c]	0.15	−0.03
Acetic acid	6.01 ± 0.01[b,c]	1.97[b]	3.36[b,c]	2.32[b,c]	3.51[c]	2.21[c]	0.15	−0.11
Formic acid	4.75 ± 0.01[b,c]	1.82[b]	2.80[b,c]	1.97[b,c]	2.84[c]	1.83[c]	0.04	−0.14

[a] Value in water containing 75% dioxane: log $K_{CuL}^{Cu} \cong 0.1$ (I = 0.1; T = 25°). [b] Values taken from Sigel *et al.* (1969). [c] Values taken from Griesser *et al.* (1968).

in a favorable stereochemical way, *i.e.*, there is the possibility of forming a five-membered ring chelate, the values of which are also given in Table II.

A comparison of the acidity constants of *d*-tetranorbiotin, *d*-tetranordethiobiotin (Table I), tetrahydrothiophene-2-carboxylic acid, and valeric acid (Table II) results in the series: valeric acid > tetrahydrothiophene 2-carboxylic acid ~ *d*-tetranordethiobiotin > *d*-tetranorbiotin, within which the basicity decreases. So it can be seen that the presence of the ureido ring as well as the tetrahydrothiophene ring leads to a further acidification of the carboxylic acid group by approximately 1 log unit.

The acidity constant of *d*-biotin and the stability constants of the corresponding binary complexes with Cu(II) and Zn(II) were also determined in water (*cf.* Table III). Both acidity and stability constants are affected very strongly by the change of the solvent. Both sorts of constants become smaller by about the same order. However, the constants of the simple carboxylic acids, like valeric and acetic acid, are influenced in the same way (Tables II and III), so again the values of *d*-biotin are of the same order as those of the simple carboxylic acids. Thus, it is possible to make at least a first estimation for the values which would be found for all investigated ligands in water, by comparing the constants given in Tables I and II with the constants presented in Table III.

Nuclear Magnetic Resonance Spectra. From a comparison of the stability constants of the structurally related complexes, it is often possible based on such indirect evidence to make a statement about the structure of the complexes. A direct method for such investigations is offered by nuclear magnetic resonance spectroscopy. The line widths of the signals due to protons near the binding sites are broadened in the presence of the paramagnetic ions, Cu(II) and Mn(II), as is well known (Swift and Connick, 1962; Cohn and Hughes, 1962). Thus, it is possible to learn from such measurements which binding sites of a ligand are involved by the complex formation with the metal ion. The nuclear magnetic resonance spectra of *d*-biotin with increasing amounts of Mn(II) are shown in Figure 2, the measured line widths of the proton signals are given in Table IV.

As expected, the signal due to the protons of the methylene group neighboring to the carboxylic group (C_{13}) is broadened very strongly. That the carboxylic acid group is involved with the complex formation was already obvious from the stability constants. However, the hydrogens on C_2, next to the sulfur atom, are influenced, too. 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 that 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

TABLE III: Negative Log Acidity Constant, pK_{HL}^{H}, and Log Stability Constants, Log K_{MeL}^{Me}, of Mn(II), Cu(II), and Zn(II) 1:1 Complexes with Biotin, Determined in Water (I = 0.1; T = 25°). For Comparison, the Values from the Literature Are Also Given for Some Monocarboxylic Acids.

Ligand	pK_{HL}^{H}	Log K_{MnL}^{Mn}	Log K_{CuL}^{Cu}	Log K_{ZnL}^{Zn}
d-Biotin	4.51 ± 0.02	*a*	1.63	0.82
Valeric acid	4.86[b]		1.92[c]	
Propionic acid	4.66[d]		1.86[c]	1.01[e]
Acetic acid	4.55[d]	0.61[f]	1.79[c]	1.03[e]
Formic acid	3.49[d]		1.53[c]	0.60[g]
Chloroacetic acid	2.60[d]		1.03[c]	

[a] The shift of the buffer region to lower pH was not significant enough for the calculation of a stability constant. [b] Dippy (1938); (25°; → 0).[h] [c] Rossotti *et al.* (1964); (25°; 3; $NaClO_4$).[h] [d] Perrin (1959); (20°; 1; $NaClO_4$).[h] [e] Cannan and Kibrick (1938); (20°; 0.2; KCl).[h] [f] Li *et al.* (1957); (25°; 0.16?).[h] [g] Hershenson *et al.* (1957) (25°; 2; $NaClO_4$). [h] The values in parentheses are temperature, ionic strength, and medium.

TABLE IV: Line Widths[a] of Nuclear Magnetic Resonance Spectra of *d*-Biotin with Increasing Amounts of Mn(II) (*cf.* Figure 2).

$[Mn^{2+}]$	>CH(R) S	H(A)	H(B)	CH_2COO	$(CH_2)_3$
	~15	4.7	4.4	6.1	14.5
2.5×10^{-4}	~18	6.1	4.3	13	16.5
5×10^{-4}	~21	11	5	18	18
10^{-3}	~25	~13	~6.5	~25	~21

[a] Given in cycles per second; measured at the half-height of the peaks. The sum of the line width of all peaks within a group is given.

the metal ion (Sternlicht *et al.*, 1965) this experimental result means that Mn(II) is closer to H(A) than to H(B). This indicates that Mn(II) is bonded to sulfur from below the plane (*cf.* structure A), *i.e.*, a *d*-sulfoxide-like complex is formed between *d*-biotin and Mn(II).[6]

With Cu(II) the same experiments were carried out under the same conditions as given in Figure 2. In this case, however, a precipitation, probably of copper hydroxide,[7] occurred. A repetition of the experiments at pD ~ 7, where it is difficult to keep *d*-biotin in solution, gave clear evidence that the influence of Cu(II) on the proton nuclear magnetic resonance spectrum of *d*-biotin is the same as with Mn(II). So Cu(II) forms also a *d*-sulfoxide-like complex with *d*-biotin. An attempt to observe a chemical shift in the nuclear magnetic resonance spectrum of *d*-biotin in the presence of Zn(II) (*cf.* Cohn and Hughes, 1962) was without result. *d*-Biotin–Zn-$(ClO_4)_2$ (1:1) mixtures at pH 7.5 precipitate.

Discussion

Stability of Complexes. For a closer consideration of the stability of the investigated complexes from which we may be able to learn something about the structure of the complexes, it is necessary to take the basicity of the carboxylic acid group of the several ligands into account. 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). That this is true for the binary complexes, MeL, formed with simple carboxylic acids, had already been shown (Sigel *et al.*, 1969). The straight "reference lines" from this report for the metal ions, Mn(II), Cu(II), and Zn(II), are also used in Figure 3. All the values of the binary complexes, MeL, presented in Tables I and II, are shown in Figure 3. It can be seen that all values are on the straight lines, within the accuracy of measurements. The only definite exception is the Cu(II)–tetrahydrothiophene-2-carboxylic acid (1:1) complex. Thus, for all the Me(II) complexes, except the last one mentioned, it is obvious that the stability-determining factor is the basicity of the carboxylic acid group. The stability increase of the Cu(II)–tetrahydrothiophene-2 carboxylic acid (1:1) complex by about 1.1 log units, compared with the basicity of the carboxylic acid group, definitely shows that in this case a chelate is formed (Sigel *et al.*, 1969), *i.e.*, eq 5 is on the right side. Surprising in this connection is that no stability increase with *d*-tetranorbiotin is found, since this ligand seems to have a structure similar to tetrahydrothiophene-2-carboxylic acid.

$$\text{simple carboxylic acid complex} \rightleftharpoons \text{chelate} \qquad (5)$$

From the fact that all these values lie on the lines given by the simple carboxylic acid complexes, it cannot, however, be concluded that there is no interaction between the metal ions and the other possible binding sites. This means that eq 5 has not to be on the left side in each case. It can only be definitely concluded that such interactions, if they occur, do not significantly increase the stability of complexes, *i.e.*, these groups can coordinate only as strongly as did the dislodged water molecule; therefore, no visible gain of free energy, ΔG, results. These problems will be discussed further with respect to the structure of the complexes in the next section.

The reference line in Figure 4 that indicates the formation of simple carboxylic acid complexes with the Cu(II)–2,2′-bipyridyl 1:1 complex was taken from an earlier report (Griesser *et al.*, 1968). For comparison, the (interrupted) line due to the formation of the binary Cu(II)–carboxylic acid (1:1) complexes is also given. Again the values for all the investigated complexes lie on the straight line with the only exception of the ternary 2,2′-bipyridyl–Cu(II)–tetrahydrothiophene-2-carboxylic acid complex, which is more stable than one would expect on the basis of the basicity of the carboxylic acid group alone. Thus, in the ternary complexes, too, the carboxylic acid group is the stability-determining one. The increase in stability of the ternary complexes is in accordance with these results (*cf.* also Tables I and II), since positive $\Delta \log K_{Cu}$ values (*cf.* eq 4) were always found for the formation of ternary complexes between Cu(II)–2,2′-bipyridyl and oxygen-coordinating ligands (L'Heureux and Martell, 1966; Sigel, 1967; Griesser *et al.*, 1968).[4] For the ternary Zn(II) complexes, the values are also shown in Figure 4. In this case, however, the stability constants of the ternary complexes (Tables I and II) are hardly significantly different from those obtained for the binary complexes (*cf.* also Griesser *et al.*, 1968).

Structure of Complexes. *d*-Biotin has, besides the carboxylic

[6] In this case the metal ion is bound to the sulfur as is oxygen in *d*-biotin *d*-sulfoxide.

[7] It may also be that a Cu(II)–*d*-biotin complex precipitates. From water solutions (pH ~ 8) containing $Cu(ClO_4)_2$–*d*-biotin in the ratio 1:2, a Cu(II)–*d*-biotin (1:2) complex that contains five water molecules precipitates. *Anal.* Found: C, 37.70; H, 5.48; N, 8.62; S, 10.01. Calcd.: C, 37.52; H, 6.30; N, 8.75; S, 10.02.

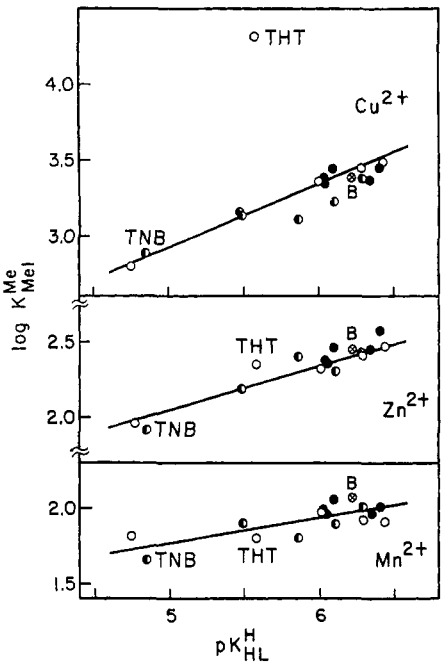


FIGURE 3: Relation between log K_{MeL}^{Me} and pK_{HL}^{H} for the binary Me(II) 1:1 complexes (eq 2) of *d*-biotin (B, ⊗), *d*-tetranorbiotin (TNB), tetrahydrothiophene-2-carboylic acid (THT), and other ligands of Table I (●), (◐), and Table II (○). The reference line due to 1:1 complexes of monodentate carboxylic acids is taken from an earlier report (Sigel *et al.*, 1969).

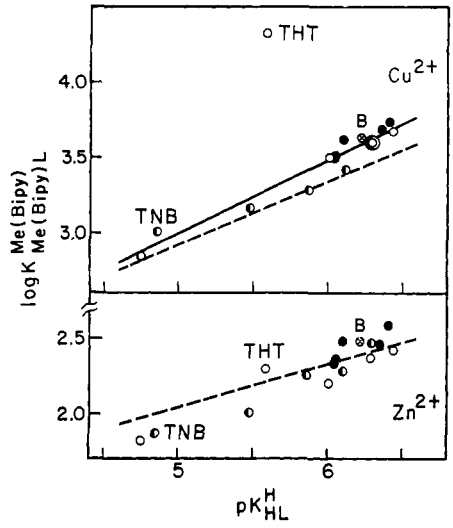


FIGURE 4: Relation between log $K_{Me(BipyL)}^{Me(Bipy)}$ and pK_{HL}^{H} for the ternary 2,2′-bipyridyl-Me(II) ligand complexes (eq 3) of *d*-biotin (B, ⊗), *d*-tetranorbiotin (TNB), tetrahydrothiophene-2-carboxylic acid (THT), and the other ligands of Table I (●), (◐), and Table II (○). The reference line due to the ternary Cu(II) complexes of monodentate carboxylic acids is taken from an earlier report (Griesser *et al.*, 1968); the interrupted lines are the reference lines of the binary complexes (*cf.* Figure 3).

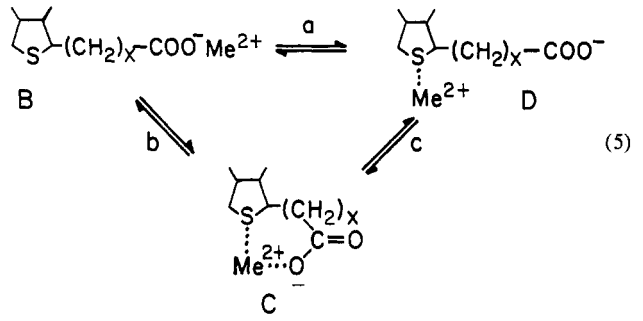
group which is definitively involved with the coordination of the metal ions, two other possible binding sites; namely, the ureido group and the sulfur atom of the tetrahydrothiophene ring. From the stability constants given, we saw that in each case the interaction with one of these two possible binding sites has to be very weak, since there is no significant increase of the stability compared with that expected on the basis of the basicity of the carboxylic acid group alone.

On the basis of an X-ray crystallographic analysis (Traub, 1956), it has been suggested that *d*-biotin may be capable of forming an intramolecular hydrogen bond in solution between O_{16} and O_6 (Traub, 1959). The formation of such a hydrogen bond should result in a higher basicity of *d*-biotin. A comparison of the acidity constants of *d*-biotin, lipoic acid (Table I), and valeric acid (Table III) shows, however, that the two latter molecules are more basic (less acidic) than *d*-biotin, even though they are not capable of forming such a hydrogen bond. Thus, it is unlikely that under the conditions of this investigation a hydrogen bond between O_{16} and O_6 is formed. Glasel (1966), on the basis of his nuclear magnetic resonance study where he used dimethyl sulfoxide as a solvent, also comes to the conclusion that there is no significant hydrogen bond. This is meaningful with regard to the interaction of metal ions with the urea group, since it is well known from the investigations of metal ion–peptide complexes that the oxygen of the carbonyl group is a better ligand atom than the amide nitrogen as long as the latter is not deprotonated (*cf.*, *e.g.*, Freeman, 1966). As no significant hydrogen bond between O_{16} and O_6 is formed in *d*-biotin, it is improbable that a metal ion is much more effective in forming a macrochelate between the carbonyl and the carboxylic acid groups. In agreement with this assumption is the fact that the stability constants for *d*-biotin and *dl*-dethiobiotin[8] are both comparable on the basis of the basicity of the carboxylic acid group with that for lipoic acid wherein such a macrochelate cannot be formed (*cf.* Table I and Figures 3 and 4). As expected, there was no indication that the amide nitrogens of the ureido ring are deprotonated under the conditions involved (*cf.* Figure 1). An interaction with the neutral amide nitrogen is unlikely as already mentioned (Freeman, 1966). In agreement with this assumption are the stability constants found for the Me(II) complexes of *d*- and *dl*-tetranordethiobiotin[8] (Table I and Figures 3 and 4). These ligands are most favored for such an interaction, since six-membered chelates can be formed; there was, however, no significant increase in stability of these complexes. The results can be explained on the basis of the basicity of the carboxylic acid group alone. In summary, from the results presented in this paper, no conclusion can be drawn as to whether there is or is not a slight interaction between the metal ions and one of the atoms of the ureido group.

The second possible binding site of *d*-biotin, besides the carboxylic acid group, is the sulfur atom of the tetrahydrothiophene ring. That a sulfur atom thus bound is able to coordinate with metal ions is obvious from the results already presented. Tetrahydrothiophene-2-carboxylic acid forms a chelate with Cu(II) (Figures 3 and 4; Sigel *et al.*, 1969). Also, evidence is given for the existence of a simple Cu(II)–tetrahydrothiophene complex (Table II), and especially the nuclear magnetic resonance results obtained with *d*-biotin and Mn(II) or Cu(II) (Figure 2) show definitely the interaction

[8] In the dethio compounds, the *d* and *l* forms are equivalent with regard to the possibility that a metal ion already bound to the carboxylic acid group might interact with the urea group.

with the sulfur atom. Thus, complexes B, C, and D and equilibria, given in eq 6, may exist in solution.



(5)

From the stability constants given, we know that the stability-determining factor of these complexes is the carboxylic acid group. As already discussed, this fact does not exclude the structure of a chelate (C). Some few percentages of a simple sulfur complex (D) in equilibrium with B would not significantly influence the calculation of the stability constants.[9] Also the nuclear magnetic resonance results can be due to a mixture of a simple sulfur complex (D) and a chelate (C), since space-filling molecular models show that a metal ion can be coordinated at the same time to the carboxylic acid group and the sulfur of *d*-biotin.[10] Thus, from these results it cannot be deduced which one of the possible complexes, B, C, or D, predominates in solution. However, it is very likely that to a certain degree all three equilibria, a, b, and c, exist.

The nuclear magnetic resonance results (*cf.* Figure 2) indicate that the interaction between the sulfur of *d*-biotin and Mn(II) or Cu(II) occurs in a stereospecific way, *i.e.*, *d*-biotin *d*-sulfoxide like complexes are formed. With this interpretation, both complex structures, either C or D, are in agreement. That the side below the plane of the tetrahydrothiophene ring is more open for an interaction with sulfur can also be seen from the kinetic preference for the formation of the *d*-sulfoxide of *d*-biotin, while the *l*-sulfoxide is the thermodynamically more stable one (Ruis *et al.*, 1967). In this connection it is also of interest that the protonation of the sulfur atom of *d*-biotin in 38% DCl in D_2O produces mainly a shifting of the signals of H(A) at C_2 (Glasel, 1966); the same behavior can be observed in the nuclear magnetic resonance spectrum of *d*-biotin *d*-sulfoxide (D. B. McCormick and H. Ruis, 1966, unpublished data).

Along the same lines, *i.e.*, the formation of structure-specific metal ion complexes with sulfur, lie the results obtained for *d*-tetranorbiotin. This ligand should also be able to form five-membered chelates as does tetrahydrothiophene-2-carboxylic acid, at least with Cu(II). However, the stability constants indicate no significant chelate formation (*cf.* Table I and Figures 3 and 4). The only reasonable explanation for this experimental result is the fact that a chelate formation can occur in this case only if the metal ion is bound in an *l*-sulfoxide-like way. Since C_{10} and N_7 are only separated by 2.8 Å (Traub, 1959), it would seem difficult for a still, at least partly, hydrated metal ion to fit "between" the two five-membered rings (*cf.* A), with the result that either no chelate formation occurs, or if it does, there is no significant increase in complex stability. A definite conclusion about the situation in eq 5 on the basis of these results alone cannot be made so far. A preliminary nuclear magnetic resonance study of *d*-tetranorbiotin in the presence of Mn(II), however, gives evidence that mainly a simple carboxylic acid complex is formed, since the broadening of the signal due to the proton of C_9 is greater by about a factor of 2.5 than the broadening of the signals due to the protons at C_2.

Conclusions with Regard to Possible Enzyme–Metal Ion–Substrate Complexes. In the discussion of the structures of the Me(II)–*d*-biotin complexes, in which the sulfur participates, no definite decision between structures C and D in eq 6 could be made. However, such a decision with regard to enzyme–metal ion–substrate complexes is irrelevant in each case, since the coenzyme form of *d*-biotin is amide linked through the ε-amino of L-lysine to the enzymes (Lane and Lynen, 1963), *i.e.*, the carboxylic acid group is no longer available as a binding site. From the investigation of the Cu(II)–tetrahydrothiophene complex (Table II), we know that it is possible to form a simple metal ion–thioether complex. Furthermore, the stability constants given in Table II for water solutions containing 50 and 75% dioxane suggest that possibly such a complex is even somewhat more stable in water as a solvent.

That such small complex stabilities are enough to originate a special structure can be seen, for example, from the log stability constant, log $K_{Cu(Ade)}^{Cu}$ = 0.84, of the Cu(II)–adenosine (1:1) complex (Schneider *et al.*, 1964). The interaction between N_7 of the adenine portion and Cu(II) is strong enough to produce a macrochelate in the Cu(II)–ATP complex, while the stability of the last-mentioned complex is not significantly different from that of the Cu(II)–methyl triphosphate complex (Schneider *et al.*, 1964). Thus, it is a reasonable assumption, based on the results presented herein, that the interaction between certain metal ions and the sulfur of a *d*-biotin-containing enzyme is strong enough to create an enzyme–metal ion–substrate complex with the right arrangement relating to space and reaction. Probably in these cases, too, *d*-sulfoxide-like complexes between the metal ion and the sulfur atom will be formed, *i.e.*, the "open" side of the sulfur atom is used for coordination. Moreover, these metal ion–thioether complexes seem to be labile enough to allow the rapid rearrangement of the complexes necessary for the enzyme to act as a catalyst. This does not necessarily mean that the whole complex of higher order has to be taken to pieces. For example, the adenine portion can be pushed out of the coordination sphere of the metal ion in a Cu(II)–ATP complex, simply by adding 2,2′-bipyridyl in the ratio of 1:1, while the phosphate groups are still bonded to the metal ion (Sigel *et al.*, 1967); the same is true for other ternary 2,2′-bipyridyl–Cu(II)–nucleoside triphosphate complexes (Sigel, 1968).

On the basis of the over-all results presented, it seems reasonable to consider that a possible function of the sulfur atom of *d*-biotin is to complex with metal ions.

[9] This is true, since the differences between K_{HL}^{H} and K_A' (*cf.* eq 1 and Figure 1) are not very great, and in a complex with structure D, the carboxylic acid group would be acidified, too, although of course somewhat less than in B or C. No significant "movement" of the values of K_A', within the calculations from a titration curve, could be observed for any case.

[10] A further possibility under the conditions of the nuclear magnetic resonance measurements, where the concentration of the ligand is high, is the formation of complexes wherein the metal ion is bonded to a carboxylic acid group of one ligand and also to a sulfur atom of another ligand molecule.

Acknowledgments

The measurements of complex stabilities were performed with the skillful technical assistance of Miss R. Baumbusch, Miss G. Baum, and Mr. A. Tàlos. We also wish to thank Professor S. Iwahara and Dr. H.-C. Li for making the chain-shortened biotin derivatives available.

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