Gachelin, G., Goldstein, L., Hofnung, D., and Kalb, A. J. (1972), *Eur. J. Biochem. 30*, 155.
Greer, J., Kaufman, H. W., and Kalb, J. A. (1970), *J. Mol. Biol. 48*, 365.
Hardman, K. D., and Ainsworth, C. F. (1972), *Biochemistry 11*, 4910.
Kalb, A. J., and Levitzki, A. (1968), *Biochem. J. 109*, 669.
Kalb, A. J., and Lustig, A. (1968), *Biochim. Biophys. Acta 168*, 366.
Levitzki, A., and Reuben, J. (1973), *Biochemistry 12*, 41.
Lindskog, S., and Nyman, P. O. (1964), *Biochim. Biophys. Acta 85*, 462.
Pauling, L. (1960), The Nature of the Chemical Bond, Ithaca, N. Y., Cornell University Press, p 518.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.
Schubert, J. (1954), *J. Amer. Chem. Soc. 76*, 3442.
Shoham, M. (1972), M.S. Thesis, The Weizmann Institute of Science, Rehovot, Israel, p 36.
Sillén, L. G., and Martell, A. E. (1964), *Chem. Soc. Spec. Publ. No. 17*, pp 150, 290–293.
Sumner, J. B., and Howell, S. F. (1936a), *J. Biol. Chem. 115*, 583.
Sumner, J. B., and Howell, S. F. (1936b), *J. Bacteriol. 32*, 227.
Vallee, B. L., and Wacker, W. E. C. (1970), *Proteins 5*, 11.
West, T. S. (1969), Complexometry with EDTA and Related Reagents, Poole, Dorset, BDH Chemicals, Ltd.
Williams, M. N. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1292.
Yariv, J., Kalb, A. J., and Levitzki, A. (1968), *Biochim. Biophys. Acta 165*, 303.

Interactions of Metal Ions with Biotin and Biotin Derivatives. Complexing and Hydrogen-Bond Formation of the Ureido Group†

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ABSTRACT: Earlier studies have shown that two of the three potential binding sites of *d*-biotin can coordinate with metal ions in solution; namely, the carboxylate and the thioether groups. It is shown in this paper that the third potential site, the ureido group, can also interact with metal ions. This is evidenced by line-broadening studies of the nuclear magnetic resonance spectra of biotin and some derivatives in the absence and presence of increasing amounts of the paramagnetic divalent manganese ion in deuterated dimethyl sulfoxide as solvent. That the presence of the sulfur in the biotinyl moiety enhances the interaction between Mn^{2+} and the ureido group follows from a comparison with dethiobiotin and ethyleneurea (2-imidazolidone) as ligands. The nucleophilicity of the ureido group becomes evident by the intermolecular formation of hydrogen bonds between phenol and the biotin model, ethyleneurea. Studies of the infrared spectra in chloroform revealed a significant shift of the carbonyl-stretching frequency to longer wavelengths.

The early work of Lynen and his coworkers (Lynen *et al.*, 1961; Lynen, 1967; *cf.* also Knappe, 1970) concerning carboxylation reactions in biological systems led to the conclusion that, during the enzymatic process, a nucleophilic attack occurs by the nitrogen of the ureido group of *d*-biotin at the carbon of carbon dioxide or bicarbonate. Later, Bruice and Hegarty (1970) concluded, based on experiments with model compounds, that, although the ureido group shows no nucleophilicity in *inter*molecular reactions, it is an effective nucleophile in *intra*molecular reactions with nearby electrophiles. In addition, these authors gave evidence that the oxygen of the ureido group is more nucleophilic than the nitrogen in such intramolecular reactions. They proposed a mechanism for the enzymatic carboxylation reaction with an O-carboxylated biotin as an intermediate. However, in a recent review, Moss and Lane (1971) attacked this theory. Indeed, the model used by Bruice and Hegarty in their study is so different from the biotin molecule as it occurs in the enzymatic surroundings that no unequivocal decision appears possible at this time, even though the hypothesis of Bruice and Hegarty has some appealing features.

Though it is obvious that the reactions occurring at biotin during the carboxylation process are still disputed, the following is definite. In biotin-containing enzymes, the carboxylate group of biotin is amide linked with an ϵ-amino group of lysine, which is part of the peptide chain of the enzymes and probably has no other function than attaching the coenzyme to the protein by a covalent bond. That both remaining functional groups, *i.e.*, the thioether and the ureido groups, are of importance during the enzymatic reaction has been shown independently with two different carboxylases by the carboxylation rates of free biotin as compared to different free biotin derivatives (Lynen *et al.*, 1961; Stoll *et al.*, 1968); it turned out that dethiobiotin and a biotin without the car-

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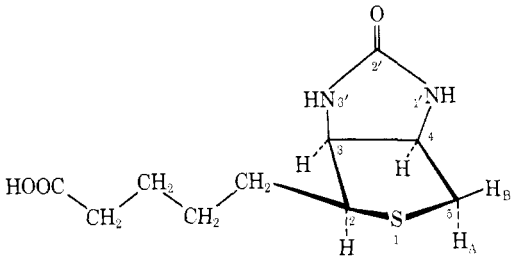
bonyl group in the second five-membered ring are inactive. As a consequence of these results, a number of papers dealt with the possible role of the several functional groups of *d*-biotin (*cf.*, *e.g.*, Caplow, 1965; Mildvan and Scrutton, 1967; Bowen *et al.*, 1968).

The structure of *d*-biotin is given in structure A. The

Structure A



relative configuration at the asymmetric carbon atoms of the biotin molecule was shown to be an all-cis structure by X-ray crystallographic analysis (Traub, 1956, 1959; Bonnemere *et al.*, 1965). By measuring the anomalous dispersion of X-rays, Trotter and Hamilton (1966) were able to elucidate the absolute configuration of this molecule.[1]

As known so far, all carboxylases require divalent metal ions, namely, Mg^{2+} or Mn^{2+}, for the enzymatic reaction. Some of the carboxylases and transcarboxylases additionally contain tightly bound metal ions, *viz.*, Mn^{2+} in the case of carboxylases (Scrutton *et al.*, 1966) or Co^{2+} and Zn^{2+} in the case of transcarboxylases (Northrop and Wood, 1969). Therefore, we started to study the interaction of metal ions with *d*-biotin and biotin derivatives (Sigel *et al.*, 1969b; Griesser *et al.*, 1970). Such studies are of interest for two reasons: (i) the role of the metal ions during the biological process so far is not really understood; (ii) a metal ion can be considered as an electrophile; hence, it may be used as a probe to identify nucleophilic sites by its coordination.

So far, it has been shown that metal ions do interact with two of the three possible binding sites of biotin, *i.e.*, with the carboxylate and the thioether groups (Sigel *et al.*, 1969b). Whether or not the third potential binding site, the ureido group, is also able to coordinate to metal ions was still open to question. Since molecules like urea are known to show some coordination tendency toward transition metal ions (Penland *et al.*, 1957), there was some possibility of finding such a quality for the ureido group of *d*-biotin. Such interactions are expected to be rather weak; therefore, nuclear magnetic resonance (nmr) line-broadening measurements are especially suitable for such an investigation (*cf.*, *e.g.*, Cohn and Hughes, 1962; Sigel *et al.*, 1969b). Indeed, as shown herein, an interaction between metal ions and the ureido group of *d*-biotin and of other biotin-type molecules can be demonstrated by using deuterated dimethyl sulfoxide as a solvent.

In addition, a search for the possible formation of hydrogen bonds between the oxygen of the urea group of biotin and an H donor was included in the present study for the following reasons. According to the reaction scheme devised by Lynen *et al.* (1961) to describe the enzymatic carboxylation, the first step entails the carboxylation of biotin by nucleophilic attack of a nitrogen of the ureido group at bicarbonate. The nucleophilicity of this nitrogen is very low, so that somehow an activation would have to take place during the enzymatic process. A direct deprotonation of the NH group with formation of a strongly nucleophilic anion is not very probable because amide groups are deprotonated only in strongly alkaline media. A more probable way of activation would be the polarization of the carbonyl double bond of the urea part by formation of a hydrogen bond or, at the extreme, protonation of the oxygen. This would lead to an increase in the double bond character of the carbonyl carbon–nitrogen bond and to a concomitant higher acidity of the NH group. Under such conditions, the NH group could be deprotonated more easily by suitable groups of the enzyme in its vicinity. The resulting nitrogen anion could then react with the bicarbonate ion to form the carboxylated biotin. Indeed, some evidence will be given herein for the possible formation of such a hydrogen bond.

Experimental Procedures

Materials. *d*-Biotin was purchased from Hoffmann-La Roche, Inc., Nutley, N. J., ethyleneurea (2-imidazolidone) was purchased from Aldrich Chemical Co., Milwaukee, Wis., and was recrystallized from methanol before use. Dethiobiotin (Tepper *et al.*, 1966) and the biotin amides (McCormick, 1973) were synthesized as described.

The manganese perchlorate used for the nmr measurements was purchased from Alfa Inorganics, Beverly, Mass. The water of crystallization was removed by repeated refluxing of the salts with 99.5% D_2O and evaporation of the solvent. The dry salt was kept in a vacuum desiccator over P_2O_5 whereupon the pink solid turned white, which indicates that the D_2O was removed from the manganous salt. The amorphous solid was dissolved in 99.5% Me_2SO-d_6 (Diaprep, Inc., Atlanta, Ga.) or 99.9% Me_2SO-d_6 (J. T. Baker Chemical Co., New York, N. Y.) for use.

Measurements. The nmr measurements were done at 60 or 30 MHz. For the 60-MHz spectra, a Jeol JNM-MH-60 spectrometer, equipped with a temperature control unit, was used in the field-locked mode with sweep widths of 540 cps and a sweep time of 300 sec. The 30-MHz spectra were recorded with a Varian EM-300 spectrometer, sweep widths of 150–300 cps and sweep times of 124–240 sec. Tetramethylsilane was used as an internal standard.

The infrared measurements were performed with a Perkin-Elmer Model 137B infracord spectrophotometer, equipped with a scale expander and an external recorder.

Results

Interaction of Metal Ions with the Ureido Group of d-Biotin. Paramagnetic metal ions, like Cu^{2+} or Mn^{2+}, bound to an organic ligand affect the line widths of the ligand protons; *i.e.*, line broadening occurs. The measurement of such broadening of characteristic nmr lines in the presence of Mn^{2+} or Cu^{2+} is a convenient method for studying possible interactions of these metal ions with potential ligand atoms (Cohn and Hughes, 1962; Swift and Connick, 1962). Owing to the fast exchange between free and coordinated ligands, only trace amounts of the metal ions are necessary for the observa-

[1] After the first X-ray papers, the structure was drawn erroneously, with the side chain on the other side, *i.e.*, where H_B is actually located. In other words, H_B and the side chain were interchanged. This incorrect structure was also shown in our earlier papers (Sigel *et al.*, 1969b; Griesser *et al.*, 1970; Sigel and McCormick, 1970). However, this is without any influence on the results of these papers. Additionally, none of the conclusions are affected, including the statement that the coordination of the metal ion to the sulfur of the tetrahydrothiophene ring occurs predominantly from the open side of the molecule, *i.e.*, trans to the side chain.

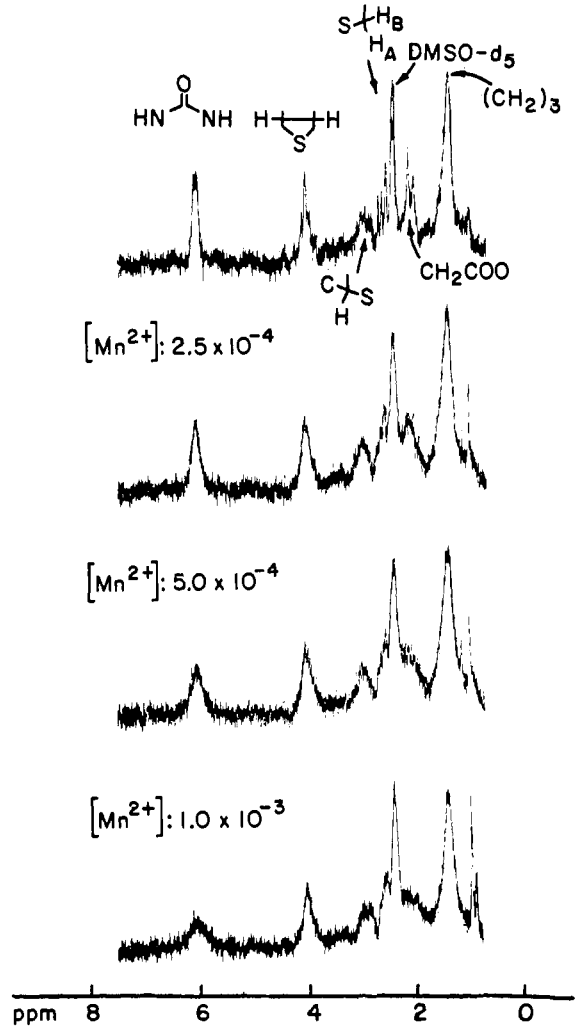


FIGURE 1: Nuclear magnetic resonance spectra of 0.18 M *d*-biotin alone (top) and with increasing concentrations of $Mn(ClO_4)_2$, measured at 60 MHz in Me_2SO-d_6; $T = 37°$.

tion of an effect. As solvent, Me_2SO-d_6 was chosen because compounds structurally related to biotin are rather insoluble in most of the other organic solvents. Additionally, an aprotonic solvent was needed, since the protons of the ureido group exchange too fast in protonic solvents, like D_2O, so that the peaks of interest cannot be observed (Glasel, 1966; Sigel *et al.*, 1969b). In Me_2SO-d_6, the ureido protons do not exchange (Glasel, 1966).

At the top of Figure 1, the spectrum of 0.18 M *d*-biotin in Me_2SO-d_6 is shown. The general features of the spectrum are the same as in D_2O (Glasel, 1966; Sigel *et al.*, 1969b), but, in addition, one sees the peaks for the ureido protons at 6.4 ppm downfield from internal tetramethylsilane. The spectra in the lower part of Figure 1 were recorded in the presence of increasing amounts of $Mn(ClO_4)_2$. It is obvious from looking at the spectra that the line widths of the ureido protons and of the methylene group next to the carboxylate increase more than the line widths of the other signals in the spectra. The line widths taken from these spectra are summarized in Table I, in addition to the widths for the biotin spectrum in the presence of 2×10^{-3} M Mn^{2+}.

The results assembled in Figure 1 and Table I demonstrate, unequivocally, a broadening of the signals due to the ureido protons in the presence of Mn^{2+} and, hence, an interaction

TABLE I: Line Widths[a] of Nuclear Magnetic Resonance Spectra of *d*-Biotin[b] with Increasing Amounts of Mn^{2+}.

$[Mn^{2+}]$	H(1′),H(3′)	H(3),H(4)	CH_2COO	$(CH_2)_3$
	8.5	10.0	~12	11.5
2.5×10^{-4}	12.0	12.0	~24	16.5
5.0×10^{-4}	18.5	15.0	vb[c]	18.0
1.0×10^{-3}	24.0	12.0	vb	15.0
2.0×10^{-3}	vb	16.0	vb	31.0

[a] Spectra were measured at 60 MHz. The line widths are given in cycles per second, measured at the half-height of the peaks. [b] Biotin was 0.18 M in Me_2SO-d_6; $T = 37°$. [c] vb means very broad.

between these metal ions and the ureido group in Me_2SO-d_6. The observation of line broadening of the methylene protons next to the carboxylate group shows that the metal ions coordinate to this carboxylate as well. This is already known for D_2O solutions (Sigel *et al.*, 1969b). Unfortunately, the line widths of the methylene group next to the sulfur cannot be studied in detail in MeSO-d_6 because of the rather strong signal of the only partially deuterated Me_2SO-d_6 at ~2.6 ppm, which overlaps with the multiplets for H_A and H_B. In D_2O, the interaction between the thioether group and Mn^{2+} or Cu^{2+} had previously been proved (Sigel *et al.*, 1969b; Griesser *et al.*, 1970), and it may be seen from Figure 1 that the corresponding signals are broadened, too, in Me_2SO-d_6.

Since it was of interest to see whether or not the carboxylate and the thioether groups in biotin have an observable effect on the interaction of the metal ion with the ureido group, the measurements have been extended to some related molecules, *i.e.*, ethyleneurea (2-imidazolidone) and dethiobiotin. In these latter ligands, at least a part of the additional coordination sites present in biotin are missing. The line widths measured in the spectra of the two model compounds and of *d*-biotin, in the absence and presence of increasing concentrations of Mn^{2+}, are listed in Table II. These data have been determined at 30 MHz from spectra in MeSO-d_6 solutions containing the ligands in 0.32 M concentrations. As these experimental conditions are somewhat different from

TABLE II: Line Widths[a] of Nuclear Magnetic Resonance Spectra of Ethyleneurea, Dethiobiotin, and *d*-Biotin with Increasing Amounts of Mn^{2+}.

	Ethyleneurea[b]		Dethiobiotin[b]	Biotin[b]	
$[Mn^{2+}]$	NH	CH_2-CH_2	NH	NH	CH(N)-CH(N)
	11.1	3.0	11.8	5.7	7.2
2.0×10^{-4}	13.5	2.7	12.3	7.8	7.8
4.0×10^{-4}	14.4	3.0	15.0	10.8	7.2
8.0×10^{-4}	19.5	4.8	17.7	16.5	7.8
1.2×10^{-3}			19.5	21.0	8.4

[a] Spectra were measured at 30 MHz. The line widths are given in cycles per second, measured at the half-height of the peaks. [b] Ligands were 0.32 M in Me_2SO-d_6.

TABLE III: Line Widths[a] of Nuclear Magnetic Resonance Spectra of the Amide[b] of Biotin and Histamine with Increasing Amounts of Mn^{2+}.

[Mn^{2+}]	Imidazole Group H(2'')[c]	Imidazole Group H(4'')[c]	CH_2-*NH*-CO[d]	H(1')-, H(3')	H(3),-H(4)
	3.5	4.0	13	9.0	10.0
2.0×10^{-4}	vb[e]	vb	14	13.0	13.0
4.0×10^{-4}	vb	vb		vb	13.0
8.0×10^{-4}	vb	vb		vb	14.5
1.2×10^{-3}	vb	vb		vb	20.5

[a] Spectra were measured at 60 MHz. The line widths are given in cycles per second, measured at the half-height of the peaks. [b] Amide was 0.13 M in Me_2SO-d_6; $T = 37°$. [c] The line width of the most intensive peak of a multiplet is given. [d] Measured on rather broad and weak peaks with low signal-to-noise ratio; therefore, not very accurate. [e] vb means very broad.

those of Figure 1 and Table I, the spectra of biotin were recorded again. Obviously, the signals due to the protons of the urea group are broadened with all three ligands (*cf.* Table II).

To obtain a somewhat broader basis concerning the interaction between metal ions and biotin, three model peptides, *viz.*, the amides of biotin with histamine, tryptamine, and tyramine, were also included in this study. Line width measurements (*cf.* Tables III–V) indicate, again, an interaction with the ureido group. In the case of the biotin amide of histamine (Table III), the extensive broadening of the imidazole protons shows that Mn^{2+} coordinates to the imidazole moiety also. Such an interaction is already known from studies with histidine and histamine (Sigel *et al.*, 1969a; Sigel and McCormick, 1971). In contrast, the data for the biotin amides of tryptamine (Table IV) and of tyramine (Table V) suggest that, under the conditions of our measurements,

TABLE IV: Line Widths[a] of Nuclear Magnetic Resonance Spectra of the Amide[b] of Biotin and Tryptamine with Increasing Amounts of Mn^{2+}.

[Mn^{2+}]	Indole NH	CH_2-*NH*-CO[c]	H(1'),-H(3')	H(3),-H(4)	$(CH_2)_3$
	4.5	9.5	9.0	15.0	17.0
2.5×10^{-4}	6.0	13.0	13.0	15.0	18.0
5.0×10^{-4}	6.0	13.0	26.0	16.5	20.0
1.0×10^{-3}	10.0[c]	16.0	vb[d]	18.0	22.0
1.5×10^{-3}	12.0[c]	18.0	vb	25.0	26.0

[a] Spectra were measured at 60 MHz. The line widths are given in cycles per second, measured at the half-height of the peaks. [b] Amide was 0.16 M in Me_2SO-d_6; $T = 37°$. [c] Measured on rather broad and weak peaks with low signal-to-noise ratio; therefore, not very accurate. These line widths can only be considered as approximations. [d] vb means very broad.

TABLE V: Line Widths[a] of Nuclear Magnetic Resonance Spectra of the Amide[b] of Biotin and Tyramine with Increasing Amounts of Mn^{2+}.

[Mn^{2+}]	HO	CH_2-*NH*-CO	H(1'),-H(3')	H(3),-H(4)	$(CH_2)_3$
	11.0	14.0	9.0	12.0	17.0
2.5×10^{-4}	11.0	13.0	20.0	14.0	18.0
5.0×10^{-4}	12.0	12.0	vb[c]	14.0	20.0
1.0×10^{-3}	16.0	18.0	vb	22.0	22.0

[a] Spectra were measured at 60 MHz. The line widths are given in cycles per second, measured at the half-height of the peaks. [b] Amide was 0.16 M in Me_2SO-d_6; $T = 37°$. [c] vb means very broad.

neither the phenolic group of the tyramyl nor the indole nitrogen in the tryptamyl portions show a considerable coordination tendency toward Mn^{2+}. In higher concentrations of this metal ion, some effects were observed, but, due to the general line broadening under these conditions, no conclusive statement is possible as to whether or not a very weak interaction may occur.

Formation of Hydrogen Bonds with the Ureido Group of d-Biotin. As already mentioned, a possible acidification (and, hence, activation) of the NH group of the urea part by polarization of the carbonyl double bond through formation of hydrogen bonds between the carbonyl oxygen and an H donor is also of interest. In principle, the mentioned amides of biotin appear to be suitable for such a study, since *intra*molecular hydrogen bonds could be formed between the carbonyl group of the ureido part of the biotin portion and the functional groups of the side chain, *i.e.*, the ring NH group from the histamine or tryptamine portion, and the phenolic group from the tyramine. Unfortunately, all these compounds are very insoluble in common organic solvents, with the exception of dimethyl sulfoxide, so that the presence of hydrogen bonds in suitable nonpolar solution could not be checked. It is not surprising that all chemical shifts of the biotinyl group hydrogens in the nmr spectra of the amides in Me_2SO are the same as for biotin because any hydrogen bonds will be broken in this polar solvent.

To demonstrate the existence of *inter*molecular hydrogen bonds with the aid of infrared measurements, again *d*-biotin is not soluble enough in suitable solvents. However, ethyleneurea and phenol are easily soluble in chloroform in concentrations that allow the measurement of infrared spectra. The shift of the CO-stretching frequency at constant amounts of ethyleneurea with and without increasing concentrations of phenol up to a molar ratio of 1:18 can be observed. Phenol showed no absorption in the 1700 cm^{-1} area, so that an observed shift of the CO band has to be due to hydrogen bonding between phenol and the ureido group. In chloroform solutions of 0.02 M ethyleneurea, the peak for the CO-stretching frequency is at 1711 ± 2 cm^{-1}. As the results listed in Table VI unequivocally demonstrate, in the presence of phenol, this peak is shifted toward smaller wave numbers, *i.e.*, longer wavelengths, thus indicating a decreasing double-bond character. We conclude from this finding that a hydrogen bond is formed between the phenolic hydroxyl as proton donor and the carboxyl oxygen of ethyleneurea.

Discussion

Interaction between Metal Ions and the Biotinyl Moiety. As mentioned, carboxylases require divalent metal ions. In fact, Mildvan and Scrutton (1967) showed that during the enzymatic process an enzyme–metal ion–substrate complex is formed. Additionally, it was suggested that the carboxylated biotin intermediate is coordinated to the metal ion (Mildvan *et al.*, 1966; Mildvan and Scrutton, 1967), thus facilitating the transfer of CO_2 to the substrate. However, it has not as yet been elucidated whether or not the metal ions also interact directly with the biotinyl moiety before carboxylation.

Based on potentiometric measurements, the interaction of metal ions with the carboxylate group of the side chain of biotin and with the α-amino acetate group of biocytin (ϵ-*N*-*d*-biotinyl-L-lysine) was shown. The coordination tendency of these groups determines completely the stability of the complexes, which is of the same magnitude as observed for simple carboxylate complexes in the case of biotin (Sigel *et al.*, 1969b) or amino acid complexes in the case of biocytin (Griesser *et al.*, 1970). However, these results do not exclude the possibility of weaker interactions between other groups and the metal ions. It can only be concluded that such additional interactions do not significantly contribute to the global stability constant of these coordination compounds.

In fact, our earlier measurements with biotin (Sigel *et al.*, 1969b) and biocytin (ϵ-*N*-*d*-biotinyl-L-lysine) (Griesser *et al.*, 1970) have unambiguously shown that metal ions coordinate to the sulfur, also. This interaction occurs preferably from the more accessible and sterically less-hindered side. In contrast herewith, protonation of the sulfur occurs from the sterically less-accessible side of the bicyclic system (Griesser and McCormick, 1973). That protonation and coordination of metal ions to the sulfur occur from different sides of the heterocyclic system is not too surprising. The coordination of a metal ion, especially in the solvated form, will be more influenced by steric hindrance than the protonation. The proton may just fit into the "pocket" formed by the bicyclic ring system of biotin, whereas the more bulky metal ion will not.

Hence, by also taking into account the present results, one has to conclude that all three potential binding sites of biotin, *i.e.*, the carboxylate, the thioether, and the ureido groups, do, indeed, interact with metal ions. Whether these interactions are independent of each other or macrochelates are formed with a simultaneous coordination of one metal ion to two or even three sites has not been decided unequivocally (Sigel *et al.*, 1969b). As the carboxylate group of biotin in the enzyme is amide linked to the protein and, therefore, no longer available for metal ion coordination, this group shall not be considered further. However, it seems worthwhile to discuss the possibility of a simultaneous coordination of a metal ion to the ureido and the thioether groups.

A superficial comparison of the line widths data given in Table II for the ureido groups of ethyleneurea, dethiobiotin, and biotin shows only that the line widths increase in all three systems. However, a more careful analysis of these results allows further conclusions. In Figure 2, a plot of the normalized (*cf.* Sigel and McCormick, 1971; see also Ihnat and Bersohn, 1970) line widths, $(\nu_M - \nu_0)/\nu_0$, of the three systems *vs.* the concentration of $Mn(ClO_4)_2$ is shown.[2] Obviously, in the case of biotin the line widths for the ureido protons increase much faster. Thus, it becomes evident that the sulfur in biotin has an influence on the interaction of metal ions with the ureido group. However, the nature of this influence is not immediately obvious.

[2] ν_0, line widths in the absence of Mn^{2+}, and ν_M, in the presence of Mn^{2+}.

TABLE VI: Shift of the Carbonyl Infrared-Stretching Frequency of Ethyleneurea in the Presence of Increasing Concentrations of Phenol.[a]

Phenol][b]:[Ethyleneurea][c]	Carbonyl-Stretching Frequency in cm^{-1}
0:1	1711
1:1	1708
2:1	1706
3:1	1704
6:1	1698
9:1	1694
12:1	1692
15:1	1691
18:1	1690

[a] Measurements were in NaCl cells with 0.5-mm path length. [b] Phenol varied from 0 to 0.36 M. [c] Ethyleneurea = 0.02 M.

Whether the metal ions are coordinated to the ureido group through the carbonyl oxygen or one of the nitrogens does not follow from our experiments, but, since metal ions coordinate to the amide group, CONH, quite generally through the oxygen atom (Freeman, 1966), it seems reasonable to assume that O coordination is also preferred in systems containing the ureido group. Coordination through oxygen would also be in agreement with the observation that, in solid metal ion complexes of ethyleneurea, most metal ions coordinate throuh the oxygen (Berni *et al.*, 1963).

Formation of Hydrogen Bonds with the Ureido Group. As mentioned, Bruice and Hegarty (1970) favor a mechanism

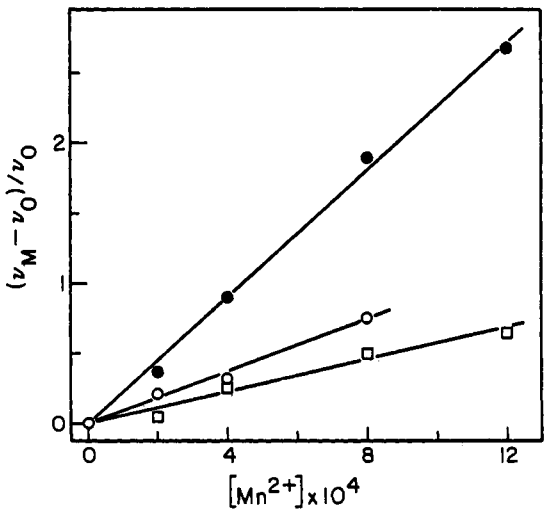


FIGURE 2: Relation between the normalized line widths, $(\nu_M - \nu_0)/\nu_0$, for the NH protons of the ureido part in *d*-biotin (B, ●), dethiobiotin (DB, □), and ethyleneurea (EU, ○), and the concentration of $Mn(ClO_4)_2$ in 0.32 M solutions of the ligands Me_2SO-d_6. (The data used for the plots are those of Table II.)

for the carboxylation half-reaction where the oxygen of the ureido part is carboxylated during the enzymatic process. In addition, these authors showed that the carbonyl oxygen possesses a considerable nucleophilicity. This is in agreement with our results from the infrared studies (*cf.* Table VI); that is, that hydrogen bond formation occurs between the CO group of ethyleneurea and the OH group of phenol. Moreover, this result concerning the formation of an H bond is also of interest from another point of view of the enzymatic process. Mildvan and Scrutton (1967) suggested a mechanism for the carboxylation half-reaction where an activation of the ureido group occurs by polarization of the CO group through a phenolic or similar group present at a suitable position in the enzyme chain. As previously outlined, this would facilitate the deprotonation of the ureido NH group.

Hence, an unambiguous statement as to whether the mechanism proposed by Mildvan and Scrutton (1967) (*cf.* also Lynen *et al.*, 1961) or the one discussed by Bruice and Hegarty (1970) is more probable is not possible. It can only be emphasized that the nucleophilic qualities of the CO group of the ureido part are now evident; whether or not they are used for a direct nucleophilic attack at the bicarbonate or for the formation of an H bond and, thus, for an activation of the NH group remains open to question.

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References

Berni, R. J., Benerito, R. R., Ayres, W. M., and Jonassen, H. B. (1963), *J. Inorg. Nucl. Chem. 25*, 807.

Bonnemere, C., Hamilton, J. A., Steinrauf, L. K., and Knappe, J. (1965), *Biochemistry 4*, 240.

Bowen, C. E., Rauscher, E., and Ingraham, L. L. (1968), *Arch. Biochem. Biophys. 125*, 865.

Bruice, T. C., and Hegarty, A. F. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 705.

Caplow, M. (1965), *J. Amer. Chem. Soc. 87*, 5774.

Cohn, M., and Hughes, T. R., Jr. (1962), *J. Biol. Chem. 237*, 176.

Freeman, H. C. (1966), *in* The Biochemistry of Copper, Peisach, J., Aisen, P., and Blumberg, W. E., Ed., New York, N. Y., Academic Press.

Glasel, J. A. (1966), *Biochemistry 5*, 1851.

Griesser, R., and McCormick, D. B. (1973), submitted for publication.

Griesser, R., Prijs, B., Sigel, H., Föry, W., Wright, L. D., and McCormick, D. B. (1970), *Biochemistry 9*, 3285.

Ihnat, M., and Bersohn, R. (1970), *Biochemistry 9*, 4555.

Knappe, J. (1970), *Annu. Rev. Biochem. 39*, 757.

Lynen, F. (1967), *Biochem. J. 102*, 381.

Lynen, F., Knappe, J., Lorch, E., Jütting, G., Ringelmann, E., and Lachance, J. P. (1961), *Biochem. Z. 335*, 123.

McCormick, D. B. (1973), *J. Heterocycl. Chem.* (in press).

Mildvan, A. S., and Scrutton, M. C. (1967), *Biochemistry 6*, 2978.

Mildvan, A. S., Scrutton, M. C., and Utter, M. F. (1966), *J. Biol. Chem. 241*, 3488.

Moss, J., and Lane, M. D. (1971), *Advan. Enzymol. 35*, 321.

Northrop, D. B., and Wood, H. G. (1969), *J. Biol. Chem. 244*, 5801.

Penland, R. B., Mizushima, S., Curran, C., and Quagliano, J. V. (1957), *J. Amer. Chem. Soc. 79*, 1575.

Scrutton, M. C., Utter, M. F., and Mildvan, A. S. (1966), *J. Biol. Chem. 241*, 3480.

Sigel, H., Griesser, R., and McCormick, D. B. (1969a), *Arch. Biochem. Biophys. 134*, 217.

Sigel, H., and McCormick, D. B. (1970), *Accounts Chem. Res. 3*, 201.

Sigel, H., and McCormick, D. B. (1971), *J. Amer. Chem. Soc. 93*, 2041.

Sigel, H., McCormick, D. B., Griesser, R., Prijs, B., and Wright, L. D. (1969b), *Biochemistry 8*, 2687.

Stoll, E., Ryder, E., Edwards, J. B., and Lane, M. D. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 986.

Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37*, 307.

Tepper, J. P., McCormick, D. B., and Wright, L. D. (1966), *J. Biol. Chem. 241*, 5734.

Traub, W. (1956), *Nature (London) 178*, 649.

Traub, W. (1959), *Science 129*, 210.

Trotter, J., and Hamilton, J. A. (1966), *Biochemistry 5*, 713.