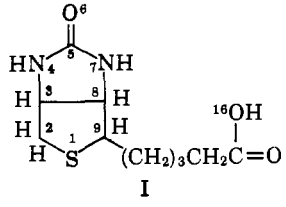
A Nuclear Magnetic Resonance Investigation of Biotin. The Biotin Sulfonium Ion*

Jay A. Glasel

ABSTRACT: The high resolution proton magnetic resonance spectra of D-biotin and D-biotin methyl ester in a variety of solvents have been analyzed. In acid solutions the results show that the biotin sulfonium ion is readily formed. Analysis of the spectra of the methyl ester shows that the hydrogen-bonding solvent, $CHCl_3$, interacts with the sulfur atom. It has also been observed that the two imide protons have differing exchange rates with the hydroxyl group of ethanol which was introduced in small amounts in the chloroform solutions. These observations are discussed in terms of the biological activity of biotin.

Several papers have appeared dealing with the possible role of the molecular configuration of D-biotin (I) in the physiological action of the molecule (Traub, 1956, 1959; Mistry and Dakshinamurti, 1964).



I

The skeletal arrangement of the atoms as shown above has been known since the early chemical work which led to the synthesis of biotin (Hofman *et al.*, 1941). However, recent X-ray crystallographic work has shown that in the solid state there is a possibility that a hydrogen bond exists between O-16 and O-6 (Traub, 1959). It was postulated that the presence of such a bond would presumably affect the keto–enol equilibrium in the ureido ring. It has been postulated that one of the mechanisms of action of biotin in carboxyl transfer may take place by a proton transfer mechanism which would be affected by this shift in keto–enol equilibrium (Lichstein, 1950). Moreover, crystallographic work has shown conclusively that the carboxyl group, during the formation of *N*-carboxy biotin, adds preferentially to the nitrogen atom farthest away from the valeric acid chain (Bonnemere *et al.*, 1965). This work confirms earlier chemical studies (Knappe *et al.*, 1963).

In view of these solid-state investigations it is of some interest to gather information on the configuration of biotin in solution. It is evident that the configuration of the molecule plays an important role in its physiological activity. Thus, whereas oxybiotin and biotin sulfoxide retain some physiological activity, L-biotin, where the valeric acid chain is *trans* to the junction between the two rings, has no activity (György, 1954). The presence of an intact ureido ring is necessary for the binding of biotin and its analogs to avidin. This is emphasized by the binding of ethyleneurea to avidin. This effect has been discussed in terms of an interaction between the ureido ring and a tryptophan ring in the protein (Green, 1963). The extra stabilization of the binding of D-biotin has been suggested to be due to interactions between properly placed, nonpolar, side chains on the protein and the valeric acid side chain on the biotin molecule (Green, 1963).

As a first step towards an investigation of these possible interactions by proton magnetic resonance it seemed worthwhile to find out how much information could be derived from an investigation of biotin and biotin analogs by themselves. A stumbling block to such an investigation is the insolubility of biotin in aqueous media. This may explain why there have been no published reports of nmr[1] spectroscopic analyses of the molecule or its analogs. However, the solubility of biotin in water <pH 2 and >pH 10 is quite high. In addition it has been found that biotin is very soluble in trifluoroacetic acid and dimethyl sulfoxide. The nmr spectra obtained for biotin and biotin analogs in these various solvent systems are quite interesting, and have revealed some new facets of the chemistry of this potent molecule.

Experimental Section

The nmr spectra were taken with a Varian Associates, Inc., DA-60 spectrometer operating at 60 MHz. The signal:noise ratio on the standard 1% ethyl benzene in carbon tetrachloride was 25:1. The temperature of

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[1] Abbreviations used in this paper are: nmr, nuclear magnetic resonance; TFA, trifluoroacetic acid; DMSO-d_6, deuterated dimethyl sulfoxide; TSS, 3-(trimethylsilyl-1-propanesulfonic acid) sodium salt; TMS, tetramethylsilane; megahertz, 1,000,000 cycles/sec; hertz, 1 cycle/sec.

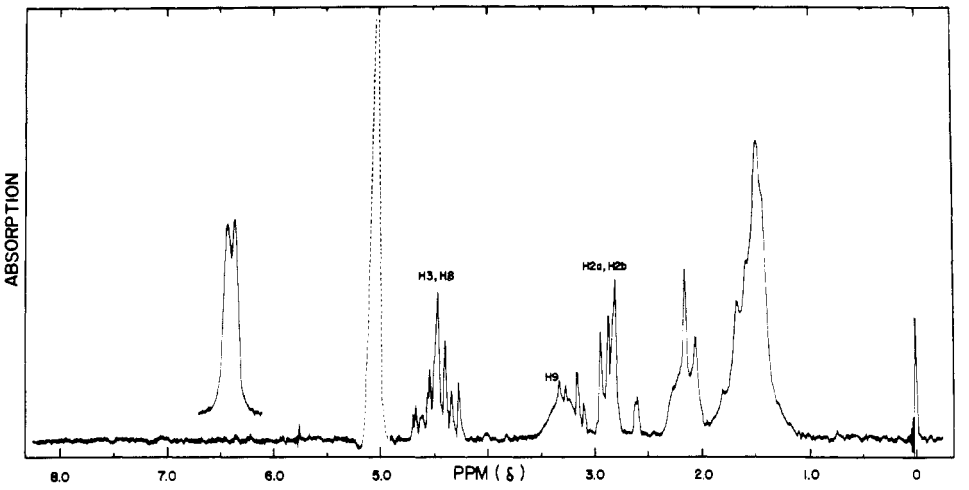


FIGURE 1: Spectrum of D-biotin in D_2O, pH 11.3. Reference is TSS. See results section for labeling and assignments for protons. Inset: the imide protons for solution of D-biotin in DMSO-d_6, referenced to TSS. Gain different from main spectrum. Carboxyl proton at δ 11.8 is not shown.

the sample in the nmr probe was 26 ± 1°. An internal standard of TSS was used interchangeably with one of TMS. All spectra were taken immediately following solution, on precalibrated charts. The samples were contained in 5-mm diameter precision tubes. The samples were not degassed.

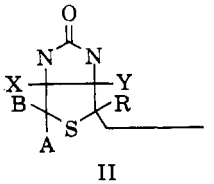
Deuterated solvents were commercial high purity quality purchased from Merck Sharp and Dohme of Canada, Ltd. Solutions of NaOD were prepared by adding D_2O to NaO. D-Biotin was obtained from Calbiochem and was used directly.

Biotin methyl ester was synthesized from D-biotin by a standard method (Du Vigneaud *et al.*, 1941), mp 150° after sublimation (lit. 166.5°).[2]

Ethyleneurea (2-imidazolidone) and thiolane were obtained from Aldrich Chemical Corp. and Eastman Chemical Corp. and were used without further purification.

Spectral Analysis

The spectra of basic interest in this paper are those of D-biotin and D-biotin methyl ester in acidic or basic media. It has been possible to analyze these spectra on the basis ABXYR II. [The standard nomenclature



II

(Pople *et al.*, 1959), is followed in this paper.] The analysis is a simple form of a subspectral analysis (Diehl, 1965) containing two separated strongly coupled spin pairs. The spectrum for D-biotin in basic solution as shown in Figure 1 is typical. The spectra taken in DMSO-d_6 were less well defined due to their higher viscosity. Experimental parameters were measured from the 250- and 100-hertz scans on the instrument. The identification of the various protons is discussed more fully in the results section.

Analysis proceeds from the identification of the AB system due to the methylene protons on the thiolane ring. This presents a sharp eight-line system on which to base further analysis (see Figure 2A). The sharpness of the lines indicates negligible coupling of either A or B to Y or R or to the rapidly exchanging imide protons. Continuing around the ring in a clockwise direction it is clear that X and Y are the second tightly coupled group in the molecule. This is not unexpected since their chemical shifts on the basis of the molecular geometry should be almost identical. Hence, the four-line system arising from the coupling of X to A and B (forming an ABX system) is split into eight lines by the formation of a second-order two-spin system with Y. Again, coupling with the imide protons is negligible to either X or Y.

The numerical values of the constants for the ABX system were derived manually (Pople *et al.*, 1959). The values for same constants were derived using an ABC computer analysis (Stanley *et al.*, 1964) adapted for use on an IBM 7094 computer operating under the IBSYS Version 13 system. The combination of possible constants was screened using a more convenient computer program for this purpose (Cavanaugh, 1963). The values of the constants derived from the ABX and ABC analyses were the same and hence confidence may be placed in the more tractable ABX computations. In the computer analysis the chemical shift, AX, was incremented over ±30 hertz from the approximate

[2] The synthesis was performed by Dr. Mella Adlersberg of this department. The sublimed material contained 1 mole of methanol of crystallization. This accounts for the difference in the melting points.

experimental value. There was negligible change in the predicted pattern of the AB portion which was the same as the directly observable experimental pattern within the limits of accuracy of the experiment. This bears out the ABX nature of the system.

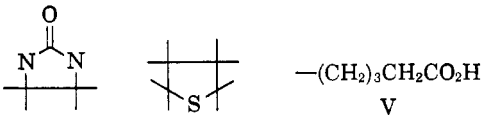
The tightly coupled XY analysis was performed manually according to the usual scheme (Pople *et al.*, 1959). The two Y lines resulting from this are each split up into a doublet by a first-order YR coupling. Finally, the R proton is coupled in a complex fashion to the methylene protons on the valeric side chain resulting in a broad system. Since this proton is, however, coupled most tightly to the methylene group next to it, one may pick out approximate coupling constants for this interaction on a first-order basis. The assumptions underlying this analysis are summarized in Table I.

TABLE I: Assumptions Used in the Subspectral Analysis of the Spectrum of D-Biotin.

$J_{AB} \approx \delta_{AB}$
$J_{AX} \ll \delta_{AX}$
$J_{BX} \ll \delta_{BX}$
$J_{AY} \approx 0\ \delta_{AY} \gg J_{AY}$
$J_{BY} \approx 0\ \delta_{BY} \gg J_{BY}$
$J_{XY} \approx \delta_{XY}$
$J_{YR} \ll \delta_{YR}$

Results

It is convenient for the analysis of the spectra to consider the molecule I to be made up of three building blocks. These are: an ethyleneurea ring III, joined to a thiolane ring IV, to which is attached a valeric acid side chain V.



III IV V

A common solvent system for these compounds is dimethyl sulfoxide, and their spectra in this solvent is the basis for the assignments.

The spectrum of neat *n*-valeric acid displays a sharp peak representing one proton at δ 12. This is the carboxyl proton. In DMSO solutions this line broadens considerably and changes position in a concentration- and temperature-dependent way due to hydrogen bonding to the proton-accepting solvent. Thus, the very broad peak at δ 11.8 in D-biotin in DMSO, which disappears in biotin lyophilized from D_2O and in biotin methyl ester, is identified with this carboxyl proton. This feature is not shown in Figure 1.

The spectrum of ethyleneurea in DMSO displays a broad peak at δ 5.99 representing two protons and a sharp peak at δ 3.22 representing four protons. The former is, therefore, identified with the magnetically equivalent imide protons. Upon close examination of the methylene peak at δ 3.22 it can be seen that the apparently single peak is really a triplet, which on a first-order basis yields $J_{az} = 0.5$ hertz. This presumably is the ^{14}N–C–H coupling. The imide protons are clearly not coupled to the methylene protons. When these results are applied to the biotin spectrum it is apparent that the two peaks at δ 6.38 (Figure 1, inset) in biotin separated by 4 hertz are the two imide peaks which are not equivalent due to the asymmetry of the biotin molecule. This band also disappears in biotin lyophilized from D_2O. It has the same position and ap-

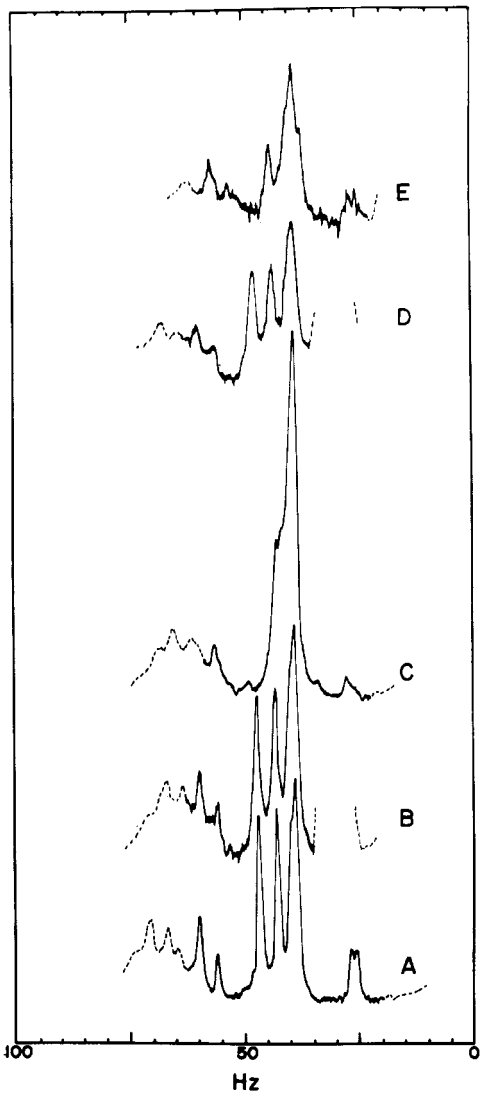


FIGURE 2: Comparison of the H2aH2b spectrum in various solvents for D-biotin and D-biotin methyl ester. (A) D-biotin in D_2O, pH 11.3. (B) D-biotin in DMSO-d_6. (C) D-biotin in 38% DCl in D_2O. (D) D-biotin methyl ester in DMSO-d_6. (E) D-biotin methyl ester in $CHCl_3$. Note expanded frequency scan when comparing with Figure 1.

TABLE II: Chemical Shifts for D-Biotin in Various Solvent Systems (Parts per Million from TMS).

Solvent	Chain CH_2	Terminal CH_2	A, B (C-2)	R (C-9)	X, Y (C-3, -8)	NH	OH
DMSO	1.39	2.17	~2.67	~3.04	~4.16	6.38	11.81
D_2O (pH >11)	1.47	2.15	~2.87	~3.27	~4.45	Exchanged	
TFA (100%)	1.73	2.62	~3.10	~3.47	~4.81	Exchanging	
D_2O (pH <0)	1.58	2.48	~3.08	~3.44	~4.82	Exchanged	

pearance in the spectrum of biotin methyl ester in DMSO. The deshielding is expected since the ring in biotin is a disubstituted ethyleneurea ring.

The spectrum of a 1:1 molar mixture of ethyleneurea and valeric acid in DMSO (total 20% w/v solution) was no different from the spectra of the components taken separately and added together. Furthermore, in the spectrum of ethyleneurea there was no evidence of an enol component within an estimated upper limit of 1%.

The spectra of *n*-valeric acid and the other aliphatic carboxylic acids up to *n*-caproic acid reveal separate systems of peaks at δ 1.4 and 2.3 which become less well defined as the number of carbon atoms goes up. These can be identified with the methylene groups on the chain. Integration of the areas shows that the system at δ 2.3 represents the α-methylene group while the remainder are grouped around δ 1.4. These two broad bands are present in the spectrum of biotin and, therefore, can be identified.

Further assignment of the components of the spectrum follows since the AB part of an ABX spectrum can be seen at δ 2.67. This must represent AB coupled to X. The fact that it is clearly a single AB portion rules out discernible long-range coupling to Y or R. Furthermore, in DMSO solutions of biotin lyophilized from D_2O the complex system at δ 4.2 sharpens without other changes in the spectrum except for the exchangeable protons. This shows that there is negligible coupling of the AB system to the imide protons. This is consistent with the results for pure ethyleneurea. The system of protons containing the X part of the ABX system is contained in the complex pattern at δ 4.2. This assignment is made on the basis of integration, the pattern fit, and the sharpening of lines in solutions of deuterated biotin. The system of lines forming the X part of the ABX pattern is itself split due to coupling with Y.

The broad peak at δ 3.1 representing a single proton is the only feature remaining to be identified. This must be due to R, more highly shielded than X or Y due to proximity to the side chain, and spin coupled to both the side chain and Y.

The details of the derivation of the constants from this spectrum are discussed in the previous section. The chemical shifts for various groups are summarized in Table II for some of the solvent systems used. Table III gives the constants derived for D-biotin in acidic and basic solutions.

TABLE III: Coupling Constants and Chemical Shifts of Protons on Biotin in Acidic and Basic Solutions.

Constant	Acid (pH <0)		Basic (pH >11)
J_{XY} (hertz)	7.6		8.0
δ_{XY}	11.0		10.8
J_{YR}	4.4		4.0
δ_{YR}	64.0		74.5
J_{XA}	0.5		0.6
J_{XB}	3.6		4.5
δ_{XB}		95.0	
J_{AB}	−13.3[a]		−13.0[a]
δ_{AB}	5.5		11.5

[a] Arbitrarily given negative sign.

The spectra of thiolane taken neat and in DMSO are identical and extremely complex as is expected. However, the spectrum of thiolane in acidic DMSO, with which it is miscible in certain proportions, is composed of two species with completely different spectra. In the spectra of biotin taken in acidic media similar changes take place for AB as may be seen from Figure 2C and Table III. The results are simpler to analyze in biotin due to the fortuitious simplicity of the spectrum. The high viscosity of the DMSO solutions makes the spectra only poorly resolved. The spectrum of biotin in basic solution is identical with that in DMSO with the exception of the exchangeable protons and small chemical shifts which are associated with the removal of the imide protons in basic solution. Using very high gain and good filtering the spectrum of biotin in water at pH 5 was obtained. The pattern was identical with that in DMSO. In acidic media, however, the chemical shift δ_{AB} is half the value of that in neutral or basic media.

The spectra of biotin methyl ester in DMSO is identical with that of D-biotin with the exception of the carboxyl proton and the methyl group. However, biotin methyl ester is soluble in chloroform, and the spectrum in this medium is extremely interesting. The chemical shift δ_{AB} decreases almost to the value it has in acidic solutions of biotin (Figure 2E), whereas in DMSO it had the same value as in biotin. Moreover, the two

imide protons become nonequivalent by 20 as compared to 5 hertz in pure biotin in DMSO solutions.

Discussion

The results indicate that there is no significant hydrogen-bond formation between the valeric acid chain and the ureido ring in DMSO solutions of biotin. There are no features of the spectra which are compatible with an interpretation based on the assumption of enol formation. Since neither the imide nor the carboxyl protons can be observed in other solutions a direct comparison cannot be made with the DMSO results.

The change in the chemical shift δ_{AB} between acidic and basic solutions of biotin, and which takes place without similar changes in the XY system is evidently due to the protonation of the adjoining sulfur atom in acidic solution. This results in the formation of a biotin sulfonium ion. The spectra of highly acidic solutions of biotin are, therefore, those of this ion. The situation is similar in the experiments involving acidic solutions of thiolane, the result being the formation of thiolane sulfonium ion.

The ease with which the sulfur atom accepts protons from donating groups is illustrated by the similar shift which takes place when biotin methyl ester is placed in the hydrogen-bonding solvent, $CHCl_3$. It is probable, therefore, that relatively strong interactions between the sulfur atom and proteins to which biotin is bound can take place. This may in part explain differences in activity between D-biotin and analogs such as D-oxybiotin and D-biotin sulfoxides and sulfones.

In chloroform solutions the imide protons are separated much more than they are in DMSO solutions. It was found that the hydroxyl group of ethanol exchanges more rapidly with one of these protons than with the other. This was discovered by observing the relative positions of the hydroxyl signal and the two imide signals as small amounts of ethanol were added to chloroform solutions of biotin methyl ester. As the concentration of ethanol increases the hydroxyl signal and that of the high-field proton coalesce before the whole system does. This result is consistent with unequal exchange rates for proton-donating molecules due to steric hindrance by the rapidly moving valeric acid side chain. Thus, at least part of the difference in reactivity between the ureido imide protons can be explained, for unbound biotin, on the basis of steric factors.

The results of this study indicate that both O-6 and S-1 should be considered as potential hydrogen-bond formers with polar groups on a protein to which biotin binds. The asymmetry of the reactivity of the imide protons may be a reflection of steric factors due to the valeric acid chain.

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