Chemical Reviews

Volume 70, Number 1 February 1970

NUCLEAR MAGNETIC RESONANCE STUDIES ON THE BIOCHEMISTRY OF BIOPOLYMERS

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Received November 12, 1968

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I. General Introduction

The application of nmr to the study of polymeric biological macromolecules has reached the state where it was felt that a review of the field would serve not only to collate all the many pertinent studies already published, but would itself possess heuristic value.

Accordingly, the literature has been surveyed through 1967 and presented in three chapters, *viz.*, Proteins, Nucleic Acids, and Polysaccharides. These chapters or sections take the form in which the monomer units are first considered, *i.e.*, the amino acids, purines and pyrimidines, the nucleosides and nucleotides, and the monosaccharides, from the point of view of substituent and solvent effects, configuration and conformation, metal-ion complexing, and molecular interactions generally. Second, nmr studies on peptides, polynucleotides, and disaccharides, oligosaccharides, and cyclic dextrins are considered; and third, nmr reports on the intact biopolymers are reviewed.

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II. Proteins

A. INTRODUCTION

In principle, nuclear magnetic resonance has the ability to distinguish between the various amino acids of proteins on the basis of side-chain proton chemical shifts. However, the 60-MHz proton magnetic resonance spectra of ribonuclease (Figure 1), for example, is almost a continuum as a result of the many near-equivalences of the side-chain protons of the 20 different amino acids.[1] Consequently, to employ nuclear magnetic resonance techniques, one in general resorts to studying the individual amino acids or simple peptide systems. Some protein systems have been studied and will be discussed in the latter part of this chapter. With the advent of the 220-MHz nmr instrument, which produces spectra that are more amenable to the elucidation of structure, future nmr studies of such systems promise to provide a great deal of information concerning not only protein structure but also information about the interaction of proteins.

B. AMINO ACIDS

1. Configuration. Chemical Shifts

The proton magnetic resonance of simple amino acids in aqueous solution was first studied by Takeda and Jardetzky.[2] The resonance absorption of protons in the amino and carboxyl groups in 2–3 *M* solutions of glycine, alanine, β-alanine, cysteine, proline, and hydroxyproline was studied as a function of pH and these protons were found to exchange with water protons, depending upon the acidity of the solution, while protons directly attached to carbon were not affected very much. The $-NH_3^+$ pmr signal was observed only at high acid (H_2SO_4) concentrations. A singlet was found for these

(1) R. C. Ferguson and W. D. Phillips, *Science*, **157**, 257 (1967).

(2) M. Takeda and O. Jardetzky, *J. Chem. Phys.*, **26**, 1346 (1957).

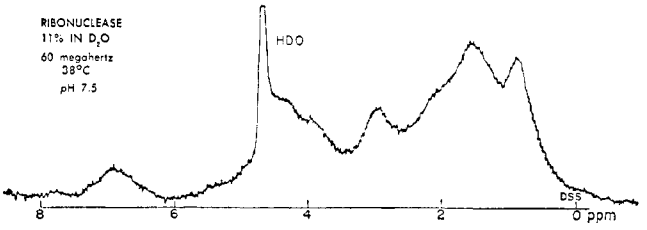

Figure 1. A 60-MHz pmr spectrum of an 11% solution of ribonuclease in D_2O.

protons indicating that the quadrupolar relaxation time of the N nucleus is short compared to the lifetime of a proton in the group as was observed on alkylamines.[3] Ogg and Ray[4] have also shown that nitrogen quadrupole effects are reduced if the nitrogen atom is surrounded by a highly symmetrical arrangement of substituents, as observed in NH_4^+. The chemical shift of the $-NH_3^+$ protons in glycine was found to be close to the center of the triplets observed in acid solution for the NH_4^+ [5] and $CH_3NH_3^+$ [3] ions. The $-NH_3^+$ pmr signal for the amino acids investigated coalesced with the water resonance signal at a pH about 1 owing to rapid proton exchange between water and NH_3^+. This is consistent, in general, with the experiments on the protolysis of the methylammonium ion in aqueous solutions in which the water resonance signal and the NH_4^+ proton resonance signal coalesced at a pH of about 4.[6] The multiplet structure of the CH_2 protons of glycine due to spin–spin coupling with the NH_3^+ protons collapsed to a singlet at pH 0 to −0.3. For the methylammonium ion the collapse of the CH_3 multiplet occurs at pH 3–4.[6] It was suggested that the collapse of the glycine CH_2 multiplet structure at a lower pH indicates that in the presence of an α-carboxyl group the exchange rate of the NH_3^+ protons is faster at a given pH. Multiplet structure due to the NH_3^+ group was not observed with the other amino acids where the splitting resulting from adjacent alkyl groups predominates.

The chemical shift data[2,7] (Table I) obtained for the various groups of other amino acids in different ionic forms suggest that the diamagnetic shielding is reduced by the presence of an adjacent positively charged group and increased by a negatively charged group. These changes in diamagnetic shielding were rationalized in terms of electrical polarization of the electrons by the ionic groups, although it was recognized that changes in bulk diamagnetic shielding associated with changes in solvation might also be a contributing factor. It is of interest to note that a study of the magnetic resonance absorption of protons in polycrystalline glycine, over the temperature range of 25 to −160°, indicates that glycine has a zwitterion structure ($H_3N^+CH_2COO^-$) in the solid state.[8] Kromhout and Moulton[9] have obtained evidence for the existence of glycine in the dipolar form in the solid state. Both α- and β-alanine were also studied in the solid state. Nuclear magnetic resonance studies of ^{14}N in saturated aqueous solutions of glycine and *dl*-alanine also indicate zwitterion structure for these compounds in aqueous solution.[10]

Table I

Chemical Shifts[a] of Proton Absorption Due to Change of Ionic Form

Compound	*Proton group*	*Anion form*	*Neutral molecule*	*Cation form*
Glycine[c,d]	$-CH_2-$	1123 (+11)	1112	1093 (−19)
α-Alanine[c,d]	$-CH_3$[b]	1205 (+7)	1198	1190 (−8)
	$-CH$[b]	1120 (+17)	1103	1180 (−23)
β-Alanine[c,d]	$N-CH_2-$[b]	1137 (+14)	1123	1113 (−10)
	CO_2-CH_2-[b]	1158 (+8)	1150	1135 (−15)
α-Aminobutyric acid[d]	$N-CH_2-$	+4		−8
	$-CO_2-CH_2-$	−2		−17
α-Aminocaproic acid[d]	$N-CH_2-$	11		−3
	$-CO_2-CH_2$	−2		−8

[a] Chemical shifts in cycles per second. Recorded at 40 Mcps. Shifts were measured taking the aromatic proton of toluene at 1000 cycles and methyl protons at 1197 cycles. [b] Center of multiplet. [c] Reference 2. [d] Reference 7.

Jardetzky and Jardetzky[11] have studied a large number of amino acids in aqueous solution using proton magnetic resonance spectroscopy. The proton chemical shifts of 22 of the biologically most important amino acids were investigated with respect to solution pH and the ionic strength of the solution. The chemical shifts observed in water and at the pH extremes are shown in Table II. A perusal of Table II indicates that for all amino acids studied the proton resonance of the NH_2^+ or NH_3^+ groups are observable only in extremely acidic solution which implies a fast rate of exchange of hydrogen in solutions of high pH. The resonance position of protons in the NH_3^+ group in acidic solution occur between −2 and −3.3 ppm compared to the center of the NH_4^+ multiplet which occurs at −2.5 ppm,[5] and, as was observed with the NH_4^+ ion, the resonance position of the NH_3^+ group is strongly dependent upon the pH of the solution. The chemical shift data obtained for the NH_2^+ and NH_3^+ groups in extremely acidic solution show that the electron environment or shielding of the protons attached to nitrogen are affected very little in amino acid side chains.

Table II also indicates that the upfield shift or shielding observed for the α hydrogens of α-amino acids is slightly pH dependent (0 ± 0.15 ppm in acid, 0.85 ± 0.15 ppm in water, and 1.27 ± 0.15 ppm in base); however, those resonance positions of α hydrogens in amino acids that have a sulfur or oxygen attached to the carbon adjacent to the α position showed a downfield shift or a decrease in shielding of the α protons. The greater electronegativity of these substituent atoms probably reduced the electron density associated with the α hydrogens, thereby deshielding them and causing a downfield shift in resonance. It was found that, in general, the greater the distance a group is from the α carbon the less it is affected by changes in pH as would be expected.

(3) J. D. Roberts, *J. Am. Chem. Soc.*, 78, 4495 (1956).
(4) R. A. Ogg and J. D. Ray, *J. Chem. Phys.*, 26, 1339 (1957).
(5) R. A. Ogg, *Discussions Faraday Soc.*, 17, 215 (1954).
(6) E. Grunwald, A. Loewenstein, and S. Melboom, *J. Chem. Phys.*, 25, 382 (1956).
(7) M. Takeda, paper presented at the 131st National Meeting of American Chemical Society, Miami, Fla., 1957, Abstract 50C.
(8) T. M. Shaw, R. H. Elsken, and K. J. Palmer, *Phys. Rev.*, 85, 762 (1952).
(9) R. A. Kromhout and W. G. Moulton, *J. Chem. Phys.*, 23, 1673 (1955).
(10) B. M. Schmidt, L. C. Brown, and D. Williams, *J. Mol. Spectry.*, 3, 30 (1959).
(11) O. Jardetzky and C. D. Jardetzky, *J. Biol. Chem.*, 233, 383 (1958).

Table II[11]

Chemical Shifts in Proton Spectra of Amino Acids and Related Compounds[a]

Compound	Solvent line			$-NH_2^+$ or $-NH_3^+$	CH			CH_2			CH_3			Other			
	H_2SO_4	H_2O	NaOH	H_2SO_4	H_2SO_4	H_2O	NaOH	H_2SO_4	H_2O	NaOH	H_2SO_4	H_2O	NaOH	Nature of group	H_2SO_4	H_2O	NaOH
1. Glycine	−6.98	−0.23	−0.36	−2.27				+0.50	+1.20	+1.55							
2. Alanine	−6.94	−0.21	−0.25	−2.31	+0.07	+0.94	+1.43				+3.05	+3.36	+3.65				
3. β-Alanine	−6.90	−0.22	−0.28	−2.46				α +1.62	+2.00	+2.23							
								β +1.07	+1.33	+1.67							
4. Serine	−6.76	n.o.	−1.10	−2.58	−0.29	n.o.	+0.67	−0.29	n.o.	+1.08							
5. Cysteine	−6.94	−0.14	−0.76	−2.54	−0.11	+0.45	+1.35	+1.06	+1.48	+1.82							
6. Threonine	−6.76	−0.37	−1.06	−2.59	−0.97	0 (?)	+0.40	+0.03	+0.94	+1.31	+2.85	+3.35	+3.56				
7. Aspartic	−7	n.o.	−0.69	−2.47	+0.07	n.o.	+0.93	+1.17	n.o.	+1.94							
8. Methionine	−6.95	−0.24	−0.87	−2.46	0	+0.80	+1.17	+1.26	+1.59	+1.97	+2.00	+2.76	+3.14				
9. Valine	−6.97	−0.09	−0.11	−2.40	α +0.12	+1.14	+1.30				+3.60	+3.79	+3.86				
					+2.23	+2.25	+2.31										
10. Glutamic	−7.01	n.o.	−1.05	−2.41	−0.03	n.o.	+1.29	+1.60	n.o.	+2.36							
								+2.00									
11. Leucine	−7.03	−0.14	−1.02	−2.29	α +0.10	+0.96	+1.36	+2.85	+3.03	+3.33	+3.56	+3.63	+3.73				
					+2.85	+3.03	+3.33										
12. Isoleucine	−6.93	−0.12	n.o.	−2.11	α +0.40	+1.46	n.o.	+3.13	+3.43	n.o.	+3.54	+3.61	n.o.				
					+3.13	+3.43											
13. Lysine	−6.69	−0.69	−0.27	α −2.02	0	+0.78	+1.31	+2.88	+3.01	+3.28							
				−2.74				+1.34	+1.46	+1.81							
14. Ornithine	−5.76	−0.68	−0.53	α −2.19	+0.09	+0.69	+1.02	+2.42	+2.71	+2.76							
				−2.77				+1.19	+1.52	+1.55				NH_2 guanidino	n.o.[b]	−2.28	n.o.
15. Arginine	−5.73	−0.42	−0.30	−2.29	+0.16	+0.71	+1.38	+2.33	+2.74	+3.20							
								+1.29	+1.31	+1.54				NH urea	n.o.	−2.76	n.o.
16. Citrulline	−5.56	−0.98	−0.20	−2.66	+0.19	+1.13	+1.57	+2.56	+2.76	+3.11				NH_2	n.o.	n.o.	n.o.
17. Phenylalanine	−6.99	n.o.	−0.28	−2.14	+0.13	n.o.	+1.32	+1.54	n.o.	+2.07				Aromatic ring	−2.64[c]	n.o.	
18. Tyrosine	−6.74	n.o.	−0.24	−2.42	+0.05	n.o.	+1.17	+1.05	n.o.	+1.64				Aromatic ring	−2.91[c]	n.o.	
19. Dihydroxy-phenylalanine	−6.07	n.o.	−0.34	−2.48	0 (?)	n.o.	?	?	n.o.	?				Aromatic	−2.74[c]	n.o.	−1.96
20. Proline	−7.01	−0.23	−0.64	−2.56	+0.09	+0.49	+1.02	+2.50	+2.85	+2.96							
								+1.11	+1.39	+1.82							
21. Hydroxyproline	−7.14	−0.92	−0.42	−3.34	−0.41	−0.02	+0.90	+0.55	+0.89	+1.43							
					−1.05	−0.23	0	+1.72	+2.21	+2.40							
22. Histidine	−6.93	−0.29	−0.35	−2.47	α −0.10	+1.01	+1.20	+1.08	+1.47	+1.84							
					−3.11	−2.90	−2.38										
					−4.22	−2.90	−3.11										
23. Betaine	−6.89	−0.46	−0.01		+0.49	+0.22	+0.54				+1.46	+1.24	+1.28				
24. Creatine	−5.84	n.o.	−1.22	−2.90	−0.07	n.o.	+0.43	+1.35	n.o.	+1.52							
25. Urea		+0.11												$-NH_2$	n.o.	−1.02	n.o.
26. Guanidine		+0.13	−1.24											NH_2^+	n.o.	−2.26	n.o.

[a] Chemical shifts recorded at 40 Mcps in ppm relative to water (external reference). [b] n.o. = no observation. [c] Centers of very broad lines.

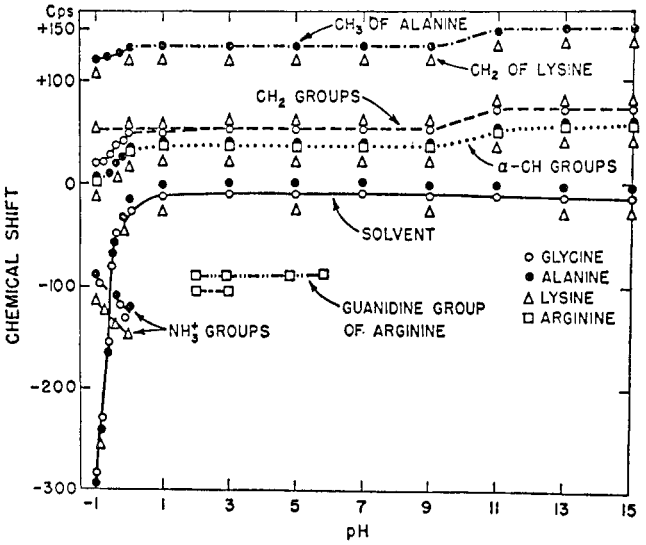

Figure 2. The pH dependence of amino acid proton resonance signals.

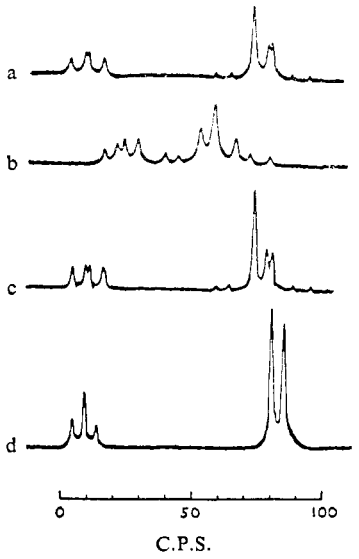

Figure 3. Nmr spectra of amino acids measured at 60.00 Mcps: (a) phenylalanine in 2 *N* HCl, (b) phenylalanine in 2 *N* NaOD, (c) cystine in 2 *N* HCl, (d) cysteine in 2 *N* HCl. In these spectra the magnetic field increases from left to right.

The effect of pH on the proton chemical shifts in amino acids is shown in Figure 2. It is observed that the α-amino protons like those of the ammonium ion[5] are most shielded in nearly anhydrous sulfuric acid, but as the acid concentration decreases, a marked deshielding or downfield shift occurs until at 9–10 *N* acid resonance line broadening begins to take place, and between 3 and 6 *N* acid the resonance peaks coalesce to form a single broad line which becomes narrower upon further dilution. For molecules such as urea, citrulline, guanidine, and arginine, which contain more than one nitrogen group attached to the same carbon, the NH_2 and NH proton resonance signals were not detected in either acid or alkali; however, resonance signals were observed in aqueous solutions over the pH range 2–7 which indicates the existence of at least two exchange mechanisms that are probably acid and base catalyzed, respectively.

Proton chemical shifts of the amino acid groups were found to vary with pH in a manner that reflects the titration curve of the acids. The change in chemical shift from 0 to +0.85 ppm is related to the transition from the cationic form to the dipolar ion, and the change from +0.85 to +1.27 ppm corresponds to the transition from the dipolar ion to the anionic form. Those groups removed from the carboxyl group exhibit the same transitions over a narrow pH range but to a much less extent. The methylene groups of arginine and lysine, however, are mainly affected by the single transition of the adjacent groups from an ionic to a nonionic form.

The dependence of proton chemical shift upon concentration was investigated for solutions of glycine, alanine, arginine, proline, hydroxyproline, guanidine, and urea. The resonance position of the solvent was found to shift to lower fields with increasing concentration; however, the resonance positions of all other amino acid groups was shown to be concentration independent. This would seem to indicate that spontaneous dimerization does not occur in these solutions.

The dependence of proton chemical shift on ionic strength in solutions of alanine, arginine, hydroxyproline, and histidine containing varying amounts of NaCl, $MgCl_2$, or $AlCl_3$ was studied. Consistent decrease in shielding was observed for all groups, particularly the α-CH group, with increasing ionic strength. A very significant shift toward lower fields was observed for the solvent proton resonance in the $AlCl_3$ and $MgCl_2$ solutions but not in the NaCl solutions. This observation is consistent with other nuclear magnetic resonance data obtained for aqueous electrolyte systems concerning the solvation of ions.[12] The chemical shifts in amino acid groups are in the same direction as those for the cationic form of the acids, indicating a decrease in the effective charge on the carboxyl group. Shifts of this type are in general indicative of ion-pair formation.[12] Therefore it is quite reasonable to assume that ion-pair formation between cations and the carboxyl group of the amino acid exists.

The proton magnetic resonance investigations of amino acids discussed thus far have been rather general in nature being restricted to the over-all features of the spectra obtained. However, a more precise nmr analysis of certain proton groups in amino acids has been given by Fujiwara and Arata.[13] The nmr spectra of methylene and methine protons of amino acids of the type

$$\mathrm{R{-}CH_2{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}COOH}$$

where R has no protons spin-coupled with those of interest, have been studied in detail. Cysteine, aspartic acid, and serine, in acid solution, give pmr spectra of three protons, two of which are equivalent. The spectrum of cysteine in acid solution (Figure 3d) indicates that the two protons attached to the β carbon are equivalent since the splitting pattern is the methine triplet (spin–spin coupling of the two equivalent methylene protons with the methine proton) and the methylene doublet (spin–spin interaction with the methine proton). In NaOD solution the cysteine spectra changes to that of a nonequivalent three-spin system. When the R group in the amino acid is bulky such as found in the case of phenylalanine, tryptophane, tyrosine, histidine, and cystine, the spectra obtained for these compounds are that of a nonequivalent three-spin

(12) J. F. Hinton and E. S. Amis, *Chem. Rev.*, 67, 367 (1967).
(13) S. Fujiwara and Y. Arata, *Bull. Chem. Soc. Japan*, 36, 578 (1963).

Table III[13]

Results of Analysis[a] for the Spectra Measured at 60.00 Mcps

			$\nu_A - \nu_C$	$\nu_B - \nu_C$	J_{AC}	J_{BC}	J_{CA}
$H_B\ H_A$	Phenylalanine	2 *N* HCl	67.4	3.7	3.2	14.6	10.0
R—C—C—COOH		2 *N* NaOD	42.1	14.5	4.0	13.3	8.9
$H_C\ NH_2$	Cystine	2 *N* HCl	68.3	4.3	2.0	15.1	9.8
	Cysteine	2 *N* HCl	74.2	0	5.0	...	5.0
		D_2O	55.5	0	4.9	...	4.9

[a] The spectra were analyzed using the method of least squares; values in cps.[15]

system or ABC system[14] in acid and basic media (see Figure 3). The spectral analysis, in terms of coupling constants, for phenylalanine, cystine, and cysteine is shown in Table III.[13,15] The constants shown are considered to be the weighted averages of the chemical shifts and coupling constants over rotational isomers.[16] The relative population of each rotational isomer is a function of the acidity of the solution as can clearly be seen in the spectral data obtained for valine shown in Table IV. The same type of analysis was extended to include aspartic acid and serine.[17]

Table IV[13]

Chemical Shifts and Spin–Spin Coupling Constants (in cps) Obtained from the Spectra of Valine at a Series of pH Values[a]

	pH	J_{23}	$\lvert\nu_4 - \nu_4'\rvert$
(4) CH_3 COOH	14.0	5.1	4.2
	13.0	5.1	4.2
CH—CH	7.0	4.2	3.0
(3) (2)	2.6	4.4	3.0
(4′) CH_3 NH_2	1.0	4.3	1.9
	1.0	4.3	2.1

[a] The spectra were measured at 60.00 Mcps; values in cps.

The pmr spectra of ten simple α-amino acids in acidic and alkaline solution have been analyzed, resulting in the determination of the relative signs of the geminal and vicinal coupling constants.[18] Substituent effects on these coupling constants and on the relative residence times of the rotational isomers were also investigated. The solvent in all cases was either strongly alkaline solutions in D_2O or mixtures of trifluoroacetic acid and D_2O. In general, the spectra observed in alkaline solutions were of the ABC type. First-order spectra were obtained only for α-alanine and α-amino-*n*-butyric acid from which the average CH_3–CH and CH_2–CH coupling constants could be obtained directly. The ABC spectra were analyzed using a computer program for direct analysis of ABC patterns as outlined by Bruegel, Ankel, and Krueckeberg.[19] The computer program gave solutions for all four sign combinations for the coupling constants where this is possible; however, solutions with the two vicinal coupling constants of opposite sign could be rejected after an inspection of the intensities. The two vicinal sets remaining and the geminal coupling constant of the opposite and of the same sign as that of the vicinal couplings along with the intensity differences and mean square deviation of the measured frequencies were presented.

$H^2\ H^1$

R—C—C—COOH

$^3H\ NH_2$

In solution α-amino acids exist as a mixture of three rapidly interconverting rotational isomers with relative residence times *a*, *b*, and *c*, respectively. If the energy of the three rotational isomers is equal (if the relative residence times *a*, *b*, and *c* are $1/3$ each), the two methylene protons will be magnetically equivalent since each of them spends the same time in the same environment giving rise to a AX_2 nmr spectrum as was observed in acidic solutions of cysteine, asparagine, and aspartic acid. For the case in which the rotational isomer energies are unequal, the methylene protons become nonequivalent, producing distinguishable vicinal coupling constants and a chemical shift difference of the two methylene groups. Most of the amino acids investigated in alkaline solution and some in acid solution gave nmr spectra of the type associated with a three-nonequivalent spin system. The vicinal coupling constants are given by assuming a classical staggered conformation with J_t, the coupling constant between two hydrogens in the *trans* position with respect to each other, and

$$J_{12} = bJ_t + (a + c)J_g \quad (1)$$

$$J_{13} = aJ_t + (b + c)J_g \quad (2)$$

J_g, the coupling constant between *gauche* hydrogens being constant for all three isomers. Since $a + b + c = 1$, the vicinal coupling constants may be written as

$$J_{12} = b(J_t + J_g) + J_g \quad (3)$$

$$J_{13} = a(J_t - J_g) + J_g \quad (4)$$

The larger coupling constant may be assigned to J_{13}, assuming *a* to be larger than *b* because of less steric interaction between the two largest groups (R and $-CO_2H$), thereby giving an unambiguous assignment to all three protons. The H^1 proton resonance always occurs at the lowest field owing to the deshielding produced by the amino and carboxyl groups. The intensity differences obtained indicate that the agreement with respect to the two sign combinations favors the assignment with the vicinal and geminal coupling constants of opposite sign. The geminal coupling constants obtained may be divided

(14) H. J. Bernstein, J. A. Pople, and W. G. Schneider, *Can. J. Chem.*, **35**, 65 (1957).
(15) Y. Arata, H. Skimizu, and S. Fujiwara, *J. Chem. Phys.*, **36**, 1951 (1962).
(16) J. A. Pople, *Mol. Phys.*, **1**, 3 (1958).
(17) S. Fujiwara and Y. Arata, *Bull. Chem. Soc. Japan*, **37**, 344 (1964).
(18) K. G. R. Pachler, *Spectrochim. Acta*, **19**, 2085 (1963).
(19) W. Bruegel, Th. Ankel, and F. Krueckeberg, *Z. Elektrochem.*, **64**, 1121 (1960).

into two groups, one having saturated atoms, adjacent to the methylene group, and the other with substituents with double bonds on a carbon adjacent to the methylene groups. The average of the geminal coupling constants of this latter group is approximately 1.5 times greater than that of the first group, in agreement with work done on the effect of hyperconjugation on geminal spin–spin coupling constants.[20] The increase in geminal coupling constants produced by adjacent π electrons is as large as changes due to other substituent effects such as electronegativity. For the vicinal coupling constants where a dependence on the electronegativity of the atoms attached directly to a particular CH–CH part of a molecule has been established,[21–23] no significant effect of adjacent π electrons could be observed. An increase in the geminal coupling constant of about 1.5 cps was observed in going from the alkaline solution ($R–CH_2–CH(NH_2)CO_2^-$) to the acidic solution ($R–CH_2–CH(NH_3^+)CO_2H$).

Information concerning the relative residence times and energies of the rotational isomers can be obtained from the two vicinal coupling constants. In acidic solutions, rotation does not seem to be hindered if the R group is small since AX_2-type spectra are obtained. For histidine, 1-methylhistidine, phenylalanine, and cystine, where the R groups are large, the residence times are no longer the same as seen from the observed spectra pattern. In general the vicinal coupling constants obtained in alkaline solution indicate that the isomer with the larger groups in the *trans* position to each other are more favored. The compounds giving simple AX_2 spectra in acidic solution give ABC-type spectra with nonequivalent methylene protons in alkaline solution, indicating different energies of the rotational isomers.

Taddei and Pratt[24] have investigated a number of amino acids in aqueous solution over a pH range of 1–13. The changes in pmr chemical shifts of the CH protons recorded at 56.45 Mcps, as a function of pH, gave information about the ionization sequences. The coupling constants determined were used to determine the conformations of the ions produced. For an aliphatic group CH–X–H, where X–H is NH_3^+ or COOH, the resonance position of the α-CH proton lies at a lower field compared to its resonance position in the ionized group $CH–X^-$ because X^- is less electronegative than X–H and because the two groups differ in magnetic anisotropy.[25] Since the α-CH proton resonance position is the weighted average of two species, the acid group form and the ionized form, an upfield shift is observed over the pH range in which the acid ionizes. Consequently, a plot of chemical shift *vs.* pH shows a step due to ionization. In the case of γ-aminobutyric acid, the α-CH_2 proton resonance shifts to higher field as the pH is increased because the cation loses a proton from the carboxyl group to give the zwitterion. The γ-CH_2 group proton resonance shifts upfield when the NH_3^+ group ionizes at a higher pH. The chemical shifts of protons that are some distance from the X–H group also exhibit ionization steps. These are produced by changes in inductive effects, although probably small,[25] and long-range shielding which depends on the magnetic anisotropies and spatial arrangement of the X–H and X^- groups.[26] Long-range shielding from groups on other carbon atoms can also contribute to the chemical shift.

The pmr chemical shifts in amino acids such as aspartic acid, cysteine, and histidine, which have three ionizable groups, as a function of pH show three steps corresponding to the three macroscopic dissociation constants. However, these ionization steps usually give only qualitative information concerning which group is ionizing since the ionization of any group affects the chemical shifts of the protons on the other carbon atom. The first ionization for each of these acids is due mainly to the α-carboxyl group; however, the β-CH_2 proton shift accompanying ionization is greatest for aspartic acid in which the loss of the first proton occurs partially at the β-carboxyl. The neutral species of aspartic acid has been shown to be a mixture of the $HO_2CCH_2CH(CO_2^-)NH_3^+$ and $^-O_2CCH_2CH(CO_2H)NH_3^+$ zwitterions in the ratio of 17.5:1.[27] The successive large and small steps observed in the chemical shift of the α-CH proton is in qualitative agreement with this ratio. For cysteine there is evidence[28,29] which indicates that the acid strength of the thiol group in the neutral zwitterion is approximately twice that of the $–NH_3^+$ group, the loss of one proton giving the anions $HSCH_2CH(CO_2^-)NH_2$ and $^-SCH_2CH(CO_2^-)NH_3^+$ in the ratio of 1:2.1. The proton chemical shift *vs.* pH data for cysteine do show a larger step at the third dissociation relative to the second; however, the reliability of this evidence in determining the ionization sequence is in doubt since the over-all shift of the α proton is larger than that in other α-amino acids investigated. This indicates that in cysteine a larger long-range shielding contribution to the shift of the α proton exists.

In histidine solutions the proton chemical shift dependence of the two olefinic CH protons of the imidazole group on pH indicates that for both protons, particularly the one between the nitrogen atoms, shifts change only in the region of the second dissociation step. This clearly shows that the proton is lost mainly from the imidazole cation.

In acid solution the pmr spectra of the two CH_2 protons in aspartic acid, cysteine, and histidine appeared to be AB_2, although the protons are expected to be nonequivalent. However, an increase in pH produces a corresponding increase in shift between the CH_2 protons (H_A and H_B), and the resulting spectra are of the ABC type. Approximate values of the coupling constants were determined assuming the spectra to be of the ABX type (Table V). The values of the vicinal coupling constants J_{AC} and J_{BC} in the case of the completely ionized species indicate that one of the two rotational isomers in which the CH proton is *gauche* to H_B and *trans* to H_A is the preferred conformation in each compound. This conformation is probably the most stable one throughout the third dissociation since the coupling constants do not change very much. The ammonium group, being involved primarily in the dissociation, stays in approximately the same average position relative to the CH_2 protons, probably being closer to H_A, because the shift of this proton experiences greater upfield shift than the H_B proton during the dissociation process. Therefore, the most populated of the *gauche* isomers is the one in which spatial and electrostatic repulsions between the α-carboxyl

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(29) E. L. Elson and J. T. Edsall, *Biochemistry*, **1**, 1 (1962).

Table V[24]

	Aspartic acid			*Cysteine*			*Histidine*		
pH	3.8	9.6	10.2	13.0	10.3	12.7[b]	8.4	10.2	12.7
J_{AB}[a]	17.8	17.0	16.3	15.4	13.1	12.2	15.1	14.6	14.8
J_{AC}	9.3	8.3	8.8	9.9	8.0	8.7	8.8	8.7	8.6
J_{BC}	2.8	3.5	3.7	3.8	3.9	3.6	3.9	3.7	4.3

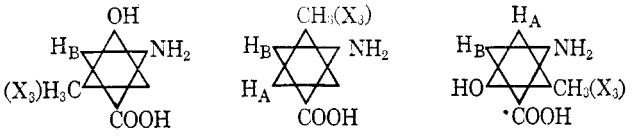
[a] Although the calculations do not give the sign of the geminal coupling constant J_{AB}, it is very likely to be negative relative to those of J_{AC} and J_{BC}, as in many other substituted ethanes. [b] Calculated for ABC type.

group and the group on the β-carbon atom should be at a minimum.

From the chemical shift–pH data obtained for L-lysine, it was determined that the relative proportion of the zwitterions $^{+}H_3N(CH_2)_4CH(CO_2^{-})NH_2$ and $H_2N(CH_2)_4CH(CO_2^{-})NH_3^{+}$ is 5:1, respectively.

It was found that with several amino acids such as valine the first step in the α-CH proton shift was smaller than the second, the first step involving the loss of a proton from the carboxyl group of the cation to give the zwitterion. Table VI[24] shows the coupling constants, which are a weighted average of all the isomers of the ionic species, as a function of pH. A comparison of the coupling constants obtained for valine with a mixture can be determined from the relative intensities of the respective spectral lines.

Aruldhas[33] has examined the 100-Mcps pmr spectra of *dl*-threonine (Figure 4) in D_2O at room temperature, but it has been shown[34] that these data are in error because the sample was contaminated by the allo isomer. The observed spectrum was analyzed erroneously assuming a superposition of spectra arising from different conformations[35–39] and using coupling constant values for the coupling between the single protons[18,36,40–42] and general expression for intensities and transitions in an ABX_3 system.[43,44] Lines 1–13 in Figure 4 were assigned to methyl protons. Aruldhas assumed that conformation I, in which the two hydrogens are *trans*, does not give a separate spectrum but rapidly exchanges with the other two conformations (II and III) of the molecule. The exchange between the latter two conformations (II and III) was reasoned to be slow, thereby producing two superimposed spectra and accounting for the appearance of two doublets.



I II III

Table VI

Proton Resonance Spectra of Valine, Threonine, and Allothreonine

	pH 1			*pH 6*			*pH 12*		
	Val	*Thr*	*Allothr*	*Val*	*Thr*	*Allothr*	*Val*	*Thr*	*Allothr*
Shift of α-CH proton[a]	−2.74	−2.81	−2.90	−2.36	−2.34	−2.60	−1.83	−1.85	−2.10
Shift of β-CH proton[a]	−1.14	−3.21	−3.16	−1.03	−3.02	−3.12	−0.73	−2.69	−2.82
J (α-CH to β-CH)[b]	4.3	3.8	3.6	4.4	4.8	4.2	5.1	4.9	4.6
Shift of CH_3 protons[a]	+0.16[c]	−0.12	−0.09	+0.21	−0.08	+0.04	+0.32	+0.04	+0.13
	+0.19			+0.26			+0.39		
J (β-CH to CH_3)[b]	6.7[d]	6.6	6.6	7.0	6.7	6.6	6.8	6.7	6.6

[a] Shifts are given in ppm, negative values indicating a line on the low-field side of the reference, Me_3COH. Shifts were recorded at 56.95 Mcps using *t*-butyl alcohol as an internal reference. [b] Coupling constants are given in cps and are assumed to be positive. [c] The upper set of values refers to one of the methyl groups in valine and the lower set refers to the other. [d] The coupling to the β-CH proton is the same for the two methyl groups.

those of other aliphatic compounds in which the vicinal coupling constants are in the ranges 1–3 and 9–12 cps between CH protons in the *gauche* and *trans* arrangements,[22,30–32] respectively, indicates that in valine one or both of the *gauche* rotational isomers is more predominant than the *trans* form. This is surprising since the *trans* isomer should be the more stable form if steric repulsions determine the relative stabilities. The methyl proton spectrum in valine consists of two doublets due to the nonequivalence of the two methyl groups, and the pH dependences of the chemical shifts of the methyl groups are slightly different owing to long-range shielding. The dependences of the α-CH, β-CH, and methyl proton shifts and coupling constants of L-threonine are similar to those found for valine. It should be noted that at any given pH the pmr spectrum of threonine is different from that of allothreonine; consequently, the relative amount of each compound in

However, Bak and Nicolaisen[34] suggest that this is an unsatisfactory explanation of the occurrence of the two spectra since the interconversion II → III could still occur rapidly enough through rotamer I to produce only one averaged spectrum for each amino acid. It was also indicated that the experimental data of Aruldhas[33] is in disagreement with that

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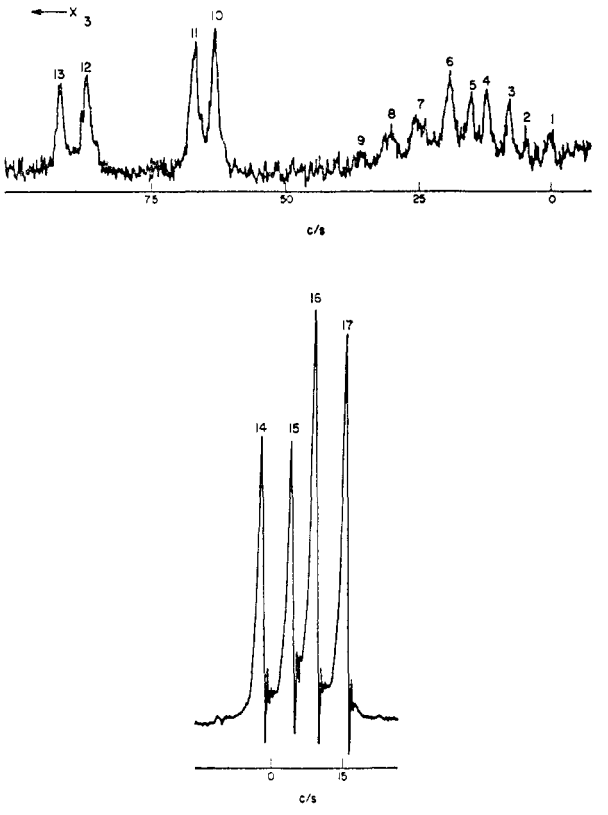


Figure 4. (Top) Observed spectra of A (lines 1–9) and B (lines 10–13) protons of DL-threonine. (Bottom) Methyl group of DL-threonine.

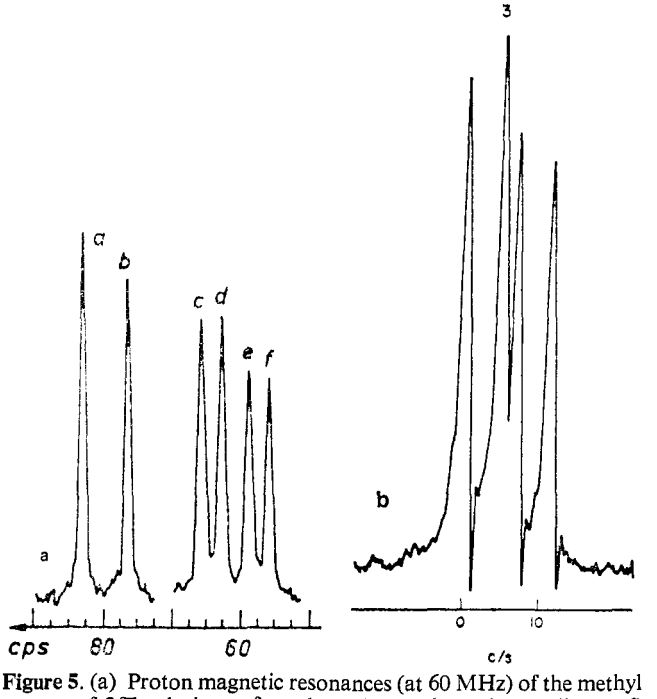
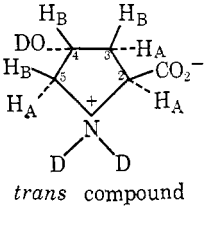


Figure 5. (a) Proton magnetic resonances (at 60 MHz) of the methyl groups of 2% solutions of DL-threonine (a, b) and DL-valine (c–f) in D_2O. Increasing field from left to right. Chemical shifts in cycles sec^{-1} (cps) relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). (b) Methyl group of DL-valine. Field increasing from left to right.

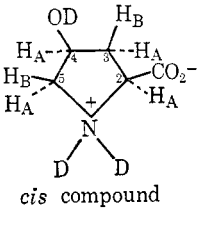
of Taddei and Pratt.[24] These authors,[24] under experimental conditions similar to that of Aruldhas,[33] observed one methyl group spin doublet for *dl*-threonine and one for allothreonine (the chemical shift difference being 0.12 ppm) at 60 Mcps. At 100 Mcps this corresponds to a proton chemical shift difference of 12 cps. Figure 4 (top), according to Bak and Nicolaisen,[34] indicates that there is a chemical shift difference between the two methyl doublets recorded by Aruldhas[33] of 11.5 cps. It was concluded, therefore, that the *dl*-threonine sample used by Aruldhas was contaminated by the allo isomer to the extent of 30–40%. Bak and Nicolaisen obtained the pmr spectrum of *dl*-threonine in D_2O at 60 Mcps (Figure 5a) and found the methyl group appeared as a very distinct doublet in contrast, as they indicate, to the triplet to be expected according to Aruldhas.

In the case of *dl*-valine in which there is no allo isomer, two methyl group doublets were observed by Aruldhas and interpreted by analogy with *dl*-threonine. This feature of the spectrum was interpreted by Taddei and Pratt[24] by assigning different chemical shifts to the two methyl groups of valine which are, in general, magnetically nonequivalent owing to the unequal lifetimes of the rotamers. A spin coupling constant, $J[H(\beta), CH_3(\gamma)] = 7.0$ cps, was obtained by Taddei and Pratt[24] for each of the two methyl groups, whereas Aruldhas obtained values of 5.0 and 5.1 cps. Bak and Nicolaisen[34] recorded the methyl group resonances of *dl*-valine in D_2O at 60 Mcps (Figure 5a) and presented three possible interpretations: (1) two spin doublets separated by 3 cps; (2) two spin doublets separated by 6.8 cps; (3) one spin doublet separated by 3.8 cps and a second separated by 9.8 cps. According to Bak and Nicolaisen, Aruldhas' spectrum (Figure 5b) may be interpreted as (1) two spin doublets separated by 5 cps; (2) two spin doublets separated by 7 cps; or (3) one spin doublet separated by 2 cps and the other spin doublet separated by 12 cps. Therefore, since spin couplings are independent of the field intensity, the solution common to both interpretations is $J[H(\beta), CH_3(\gamma)] = 6.8$ cps. It was concluded that no evidence has been presented to disprove that the pmr spectra of α-amino rotamers at room temperature are effectively averaged to one spectrum.

The pmr spectra of *trans*-hydroxyl-L-proline and *cis*-(allo)-hydroxyl-L-proline in D_2O have been studied in detail.[21,45] The pmr spectrum (60 Mcps) of both compounds was obtained and analyzed giving the coupling constants between all of the nonexchangeable protons in the molecules as well as the chemical shifts of these protons. Both compounds exist as zwitterions in D_2O. The N–H and O–H protons rapidly exchange with the solvent producing one coalesced peak; consequently, the complex spectra of the compounds are due to the six remaining protons. The spectrum obtained for the *trans* compound is shown in Figure 6, the assignments being in agreement with those reported previously.[11] There are two pairs of strongly coupled nuclei, the proton pairs on C_3 and C_5 which



trans compound



cis compound

(45) R. J. Abraham and K. A. McLauchlan, *Mol. Phys.*, 5, 195 (1962).

are denoted AB and A'B', respectively. The C_5 protons are coupled to the C_4 proton (X) giving an A'B'X' system, and the C_3 protons are coupled to the C_4 proton (X) and the C_2 proton (R) giving an ABXR system (the nomenclature is that of Pople and Schaeffer[46]). In the AB regions these systems should give rise to 8 and 16 lines, respectively. The spectrum obtained, however, shows more than this number of lines in these regions; in particular, the A and B' parts are further split. This additional splitting can be accounted for on the basis of a long-range coupling between one of the C_3 protons and one of the C_5 protons with no observable coupling between the other pair. Such long-range couplings have been observed[47,48] and are known to be dependent on the orientation of the two protons.[49] The chemical shifts and coupling constants (cps) obtained for the two systems are

(a) For the A'B'X' system

$J_{A'B'} = 12.69$	$\delta_{A'B'} = 8.4$	
$J_{A'X'} = 4.09$	$\tau_{A'} = 6.69$	$\tau_X = 5.52$
$J_{B'X'} = 1.22$	$\tau_{B'} = 6.83$	

(b) For the ABXR system

$J_{AB} = 14.06$	$J_{AR} = 7.66$	$\tau_A = 7.77$
$J_{AX} = 1.41$	$J_{BR} = 10.44$	$\tau_B = 8.05$
$J_{BX} = 4.31$	$\delta_{AB} = 16.8$	$\tau_R = 5.83$

with the long-range splitting between A and B' being equal to 1.28 cps.

The pmr spectrum of allo-hydroxyproline was found to be very similar to that obtained for the *trans* compound and was analyzed in an analogous manner. The allo compound spectrum was divided into an A'B'X and ABXR splitting pattern with the additional long-range coupling between A' and B. The chemical shifts and coupling constants (cps) obtained are

(a) For the A'B'X system

$J_{A'B'} = 12.50$	$\delta_{A'B'} = 3.63$	$\tau_X = 5.60$
$J_{A'X} = 0.94$	$\tau_{A'} = 6.75$	
$J_{B'X} = 4.57$	$\tau_{B'} = 6.81$	

(b) For the ABXR system

$J_{AB} = 14.23$	$J_{AR} = 10.48$	$\tau_A = 7.69$
$J_{AX} = 4.71$	$J_{BR} = 3.84$	$\tau_B = 7.95$
$J_{BX} = 2.09$	$\delta_{AB} = 15.58$	$\tau_R = 5.96$

with the long-range splitting between A' and B being equal to 1.37 cps. Although a complete analysis of the two spectra was given, it was not possible to make complete assignments for all the groups in the spectrum since there is no way of determining from the spectrum which of the A and B parts of the 3H and 5H groups belong to the protons $C_{3a,b}$ and $C_{5a,b}$.

A perusal of the coupling constants determined shows a wide range of values suggesting that the compounds in solution exist predominantly in one configuration. This conclusion is also supported by the existence of the long-range coupling between only one pair of C_3 and C_5 protons. If the molecules were changing between two or more conformations, the observed coupling constants would be the weighted average of the constants for each conformer and the values determined would not exhibit such a large variation. The variation observed for the couplings is also thought to be too large to be attributed only to substituent group effects in the ring.[28] One possible mechanism that might contribute to the values obtained is that of a variation in the dihedral angle; however, such a mechanism could only produce the values obtained if the ring were buckled. Although the preceding conclusions are valid for both the *trans* and allo compounds, the fact that the coupling values are different for the two compounds suggests different conformations for them.

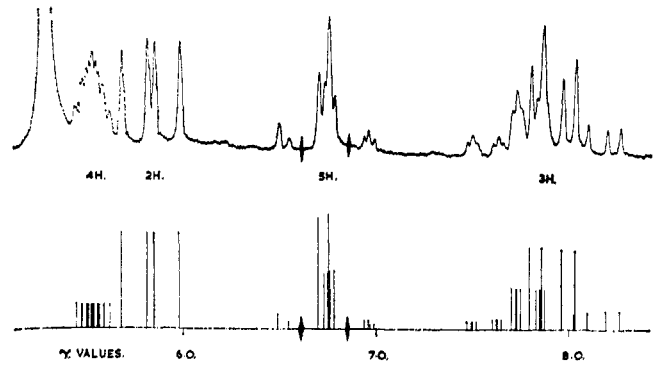


Figure 6. The observed and calculated nmr spectra at 60 Mcps of *trans*-hydroxyproline in D_2O.

From a consideration of the data for *trans*-hydroxyproline there is assumed to be an equivalent symmetry in the ring conformation about C_4, since the 3H–4H and 4H–5H couplings are very similar. This suggests that the ring exists in an "envelope" conformation analogous to the C_s form of cyclopentane[50] in which C_4 is out of the plane containing the other four ring nuclei. This conclusion is supported by the proton coupling constants obtained in camphane-2,3-diols,[47] compounds in which the cyclohexane ring exists in the strained boat form and in which the conformation of the 3, 4, and 5 carbon atoms of the ring is quite similar to that of the same numbered atoms in the "envelope" proposed for *trans*-hydroxyproline. The values of the coupling constants obtained for the camphane-2,3-diols were: $J_{3\text{-}exo\text{-}4} = 4.4$ cps; $J_{3\text{-}exo\text{-}5\text{-}exo} = 1.3$ cps; $J_{3\text{-}endo\text{-}4} = 0$; and $J_{3\text{-}endo\text{-}5\text{-}endo} = 0$. These values are very similar to the corresponding 3H–5H and 3H–4H coupling constants in the *trans* compound. Long-range coupling in the camphane-2,3-diols occurs only between the C_3 and C_5 equatorial protons which suggests, by analogy, that the C_3 and C_5 protons in *trans*-hydroxyproline possessing long-range coupling are the pseudo-equatorial protons. This suggests the following assignment for *trans*-hydroxyproline, where the A and B' of the spectrum are assigned to H_{3a} and H_{5a}, respectively (values in cps).

trans Compound

	$\tau_{2a} = 5.83$	$\tau_{4b} = 5.52$
	$\tau_{3a} = 7.77$	$\tau_{5a} = 6.83$
	$\tau_{3b} = 8.05$	$\tau_{5b} = 6.69$
$J_{2a\text{-}3a} = 7.66$	$J_{3a\text{-}4b} = 1.41$	$J_{4b\text{-}5a} = 1.22$
$J_{2a\text{-}3b} = 10.44$	$J_{3b\text{-}4b} = 4.31$	$J_{4b\text{-}5b} = 4.09$
$J_{3a\text{-}3b} = 14.06$	$J_{3a\text{-}5a} = 1.6$	$J_{5a\text{-}5b} = 12.69$

(46) J. A. Pople and T. Schaeffer, *Mol. Phys.*, **3**, 547 (1960).
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(50) J. E. Kilpatrick, K. S. Pitzer, and R. Spitzer, *ibid.*, **69**, 2483 (1947).

Allo Compound

$\tau_{2a} = 5.96$	$\tau_{4a} = 5.60$	
$\tau_{3a} = 7.69$	$\tau_{5a} = 6.81$	
$\tau_{3b} = 7.95$	$\tau_{5b} = 6.75$	
$J_{2a\text{-}3a} = 10.48$	$J_{3a\text{-}4a} = 4.71$	$J_{4a\text{-}5a} = 4.57$
$J_{2a\text{-}3b} = 3.84$	$J_{3b\text{-}4a} = 2.09$	$J_{4a\text{-}5b} = 0.94$
$J_{3a\text{-}3b} = 14.23$	$J_{3b\text{-}5b} = 2.0$	$J_{5a\text{-}5b} = 12.50$

The same general arguments are valid for the allo compound. However, the fact that the 3H–4H couplings are not equal to the 4H–5H couplings suggests that in this molecule the conformation is not the simple "envelope" conformation of the *trans* compound. Assuming that the long-range coupling occurs only between the 3H and 5H protons that are in pseudo-equatorial environments, assignments can be made for the compound as shown above.

Abraham and McLauchlan[51] calculated the dihedral angles in a regular pentagon as functions of the amount of buckle for the two symmetric deformations of the ring and applied these results together with an equation of the Karplus[52] type to the observed proton coupling constants in *trans*-hydroxy-L-proline and *cis*-(allo)-hydroxy-L-proline to obtain the conformations of these molecules in solution. The equation derived theoretically by Karplus[52] relates the vicinal coupling constants to the dihedral angles between the relevant C–H bonds. The Karplus equation has been used by others in studying the conformations of five-[53,54] and six-membered rings.[55–57] It was found that the *trans* and allo molecules exist in the envelope conformation with the C_4 atom projecting out of the plane of the other ring atoms in the *trans* compound and the C_5 atom in the allo compound. The buckle angle of the ring was found to be 53° for the *trans* compound and approximately 70° for the allo compound.

The degree of ring buckle found in the *trans* molecule is surprising since there are no interactions between substituent groups except the normal steric interactions and the electrostatic attraction in the zwitterion, which is not dependent on the conformation. Five-membered rings are generally assumed to be planar or slightly buckled, and the two dominant interactions determining the molecular conformation in such systems, the tendency to form staggered bonds, and the tendency to obtain tetrahedral carbon angles oppose one another,[60] resulting in a conformation that is a compromise between these two factors. The results obtained suggest that the tendency to form staggered bonds constitutes the stronger interaction and that the carbon atoms can be more readily distorted than has been assumed. Support for the postulate can be obtained from a comparison of the energy, 1.9 kcal/mole,[58] required to decrease a C–C–C angle to 100° (the values obtained in the proposed conformation of the *trans* molecule) with the much greater energy difference, 5–10 kcal/mole,[59] between the eclipsed and staggered configurations of a substituted ethane.

The proposed conformation of the allo molecule is considerably different from that proposed for the *trans* molecule. One reason for this difference could be the formation of an intramolecular hydrogen bond between the carboxyl and hydroxyl groups. This intramolecular hydrogen bonding might also be responsible for the increased buckling of the allo molecule compared with the *trans* molecule.

Pachler[60] has shown that the average coupling constant [$J_{av} = {}^1/_3(J_t + 2J_g)$] for the $C\text{–}CH_2\text{–}CH(NH_2)CO_2^-$ system does not change appreciably even though the molecules containing this fragment are chemically quite different. The parameters derived were used to calculate relative residence times and free-energy differences for the rotational isomers of some α-amino acids in alkaline solution.

The normalized residence times (*a*, *b*, *c*) for the three staggered configurations of α-amino acids are related to the two vicinal coupling constants by the equations

$$J_{12} = aJ_g + bJ_t + cJ_g \quad (5)$$

$$J_{13} = aJ_t + bJ_g + cJ_g \quad (6)$$

$$a = (J_{13} - J_g)/(J_t - J_g) \quad (7)$$

$$b = (J_{12} - J_g)/(J_t - J_g) \quad (8)$$

$$c = 1 - (a + b) \quad (9)$$

assuming classical staggered configurations with dihedral angles of 60° and identical coupling constants for all coupling hydrogen atoms in *trans* or *gauche* position to each other for all three rotational isomers. Therefore the relative residence times may be determined for all three rotational isomers from the two measured coupling constants, J_{12} and J_{13}, if one has reliable values for J_g and J_t.

Since the magnitude of the average vicinal coupling constant [$J_{av} = {}^1/_3(J_t + 2J_g)$] is primarily a function of the electronegativity of the substituents on a particular CH–CH fragment,[21–23] the coupling constants in molecules containing exactly the same fragment would be expected to be in close agreement. The dependence of vicinal proton–proton coupling constants on the electronegativity of substituents on a particular CH–CH fragment has been described by the equation[21]

$$J_{av} = 18.0 = 0.80\sum_{i=1}^{6} E_i \quad (10)$$

A value of $J_{av} = 6.35$ cps is obtained using the average electronegativity values[61–63] for atoms directly attached only to the C–C fragment. A value of $J_{av} = 6.32$ cps was also obtained using the parameters derived for the Karplus[52] equation for *trans*- and allo-proline by Abraham and McLauchlan.[51] Pachler[60] also showed that the average coupling constant, J_{av}, can also be derived from the coupling constants measured for the different amino acids which are subject to rotational isomerization. A plot of vicinal coupling constants, J_{12} and J_{13}, *vs.* the chemical shift difference, $\nu_2 - \nu_3$, obtained by Pachler[18] consists of two straight lines which intersect at a value of $\nu_2 - \nu_3 = 0$ and gives a corresponding value of $J = 6.30$ cps. This value was shown to be equivalent to the average coupling constant J_{av}. A mean value of $J_{av} = 6.25 \pm 0.15$ cps was obtained from these values and from values obtained from

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other sources. Using the value of $J_{av} = 6.25$ cps and the ratio $J_t/J_g = 5.22$,[51] one obtains the necessary parameters $J_t = 13.56$ cps and $J_g = 2.60$ cps to calculate the relative residence times for the three rotational isomers of some α-amino acids in alkaline solution. The relative residence times obtained are shown in Table VII.

Table VII[59]

No.	Compound	a	b	c	ΔF_{ab}[a]	ΔF_{ac}[a]
1	α-Amino-*n*-butyric acid	0.33	0.33	0.33	0.0	0.0
2	1-Methylhistidine	0.43	0.31	0.26	−0.200	−0.300
3	Phenylalanine	0.47	0.26	0.27	−0.360	−0.340
4	Histidine	0.48	0.23	0.29	−0.440	−0.300
5	Asparagine	0.57	0.21	0.22	−0.600	−0.570
6	Aspartic acid	0.64	0.14	0.22	−0.910	−0.640

[a] ΔF in kcal/mole.

The residence time data obtained also permit one to calculate the relative free energy differences (ΔF) of the rotational isomers from the relationships; $a/b = \exp(-\Delta F_{ab}/RT)$ and $a/c = \exp(-\Delta F_{ac}/RT)$. These free energy values are shown in Table VII.

The close agreement of the average coupling constants obtained for the $C\text{–}CH_2\text{–}CH(NH_2)CO_2^-$ fragment by different methods indicates that the vicinal coupling constants do not vary much with changes in a molecule at positions more than one bond apart from the fragment of interest. The data also indicate that the deviation of the dihedral angle from 60° in a compound such as α-amino-*n*-butyric acid is not large since no significant effect on the average coupling constant was observed.

Although the assumption of classical staggered configurations without angle distortions is probably incorrect for the compounds studied and there may be different *gauche* and *trans* coupling constants in the three rotation isomers of a particular compound,[21,64] the values obtained provide a semiquantitative relationship between the factors influencing the relative residence times and free energy differences of rotational isomers and steric repulsion and electrostatic interactions discussed previously.[18] It is observed from Table VII that isomer *a*, which has the α-carboxyl group *trans* to the R group, is the most stable form in all of the compounds. The relative residence times of the other two isomers are approximately equal, suggesting that the energies of the rotational isomers are determined primarily by interactions of the R and α-carboxyl groups while the amino group does not make an essential contribution to the relative energies.

The pmr spectra measured at 100 Mcps of the three CH protons of L-serine in aqueous solution at a series of hydrogen ion concentrations have been analyzed by the method of least squares.[65] All spectra obtained were of the ABC type, and the chemical shifts were found to be strongly dependent on the hydrogen ion concentration. The nmr parameters of α-amino acids are assumed to be independent of the ionic strength of the solution.[66] The nmr parameters obtained are summarized in Table VIII.

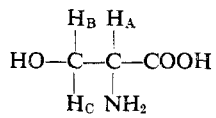
Table VIII

Nmr Parameters for the Three CH Protons of Serine in Aqueous Solution with Various Hydrogen Ion Concentrations[a]

	$\nu_A - \nu_C$	$\nu_B - \nu_C$	J_{AB}	J_{AC}	J_{BC}
5 *M* NaOH	−42.6	−17.5	6.7	4.1	−11.0
4 *M* NaOH	−42.1	−15.9	6.6	4.0	−11.0
3 *M* NaOH	−41.3	−13.0	6.6	3.9	−11.2
pH 13.9	−40.3	−9.4	6.1	4.2	−11.1
12.5	−39.2	−5.6	5.8	4.3	−11.2
10.4	−36.9	−5.4	5.8	4.2	−11.3
9.6	−31.0	−5.3	6.0	4.1	−11.6
9.2	−26.6	−4.5	6.2	3.7	−11.8
8.9	−22.9	−4.1	6.2	3.7	−12.1
8.0	−14.0	−4.1	5.5	3.7	−11.9
7.7	−13.8	−3.8	5.6	3.4	−12.0
6.8	−13.4	−3.9	5.8	3.2	−11.8
6.2	−13.8	−4.6	5.9	3.7	−12.6
5.0	−14.1	−3.6	5.4	3.8	−12.2
4.7	−13.6	−3.9	5.8	3.5	−12.3
3.9	−10.8	0	4.7	4.7	...
3.5	−9.8	0	4.6	4.6	...
2.8	−6.0	0	4.5	4.5	...
2.3	~0	~0	...	...	...
1.2	14.8	6.4	4.6	3.6	−12.7
0.9	19.5	7.1	4.4	3.4	−12.5
0.7	18.8	7.4	4.3	3.5	−12.2
0.4	20.1	7.8	4.3	3.5	−12.5
0.1	21.7	7.1	4.4	3.4	−12.6
3 *M* DCl	23.6	7.1	4.3	3.4	−12.5
6 *M* DCl	24.5	6.0	4.3	3.4	−12.6

[a] All values are in cycles per second.[65] Spectra recorded at 100 Mcps with *t*-butyl alcohol as reference.

A plot of the chemical shifts of the A and B protons of serine



taking the C proton resonance as a reference, as a function of pH also showed the A proton chemical shift to be much more affected by solution pH than that of the B proton. Inflection points were observed in the A proton curve at pH 9.2 ± 0.2 and 2.0 ± 0.5 which correspond to the values of $pK(NH_3^+) = 9.15$ and $pK(COOH) = 2.21$ which were obtained thermodynamically for serine.[67] The data obtained indicate that the cation NH_3^+RCOOH, the zwitterion $NH_3^+RCCO^-$, and the anion NH_2RCOO^- are the dominant ionic forms of serine in the range of pH <1, 4–8, and >10, respectively. Proton A exhibits a low-field shift of 0.25 and 0.35 ppm when the NH_2 and COO^{2-} groups are protonated, respectively, which is comparable in magnitude to those obtained for protons of aliphatic amines and carboxylic acids.[68,69] The coupling constants were found to be pH dependent. The change in the electronegativity of the amino and carboxyl groups is thought to be responsible for the change in the geminal coupling constant.

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Table IX

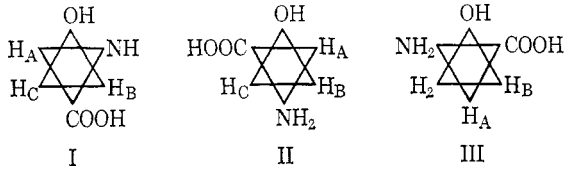
Nmr Parameters of the CH Protons of Serine at Several Temperatures[65] [a]

	$\nu_A - \nu_C$[b]	$\nu_B - \nu_C$[b]	J_{AB}[b]	J_{AC}[b]	J_{BC}[b]	p_I	p_{II}	p_{III}	E_V[c]	E_{VI}[c]
pH 13.9										
28°	−40.3	−9.4	6.1	4.2	−11.1	0.32	0.15	0.53	0.46	−0.30
37°	−40.4	−9.5	6.1	4.3	−11.0	0.32	0.15	0.53	0.46	−0.30
55°	−40.8	−9.7	6.3	4.4	−11.2	0.34	0.17	0.49	0.47	−0.25
74°	−40.4	−9.9	6.2	4.4	−11.1	0.33	0.17	0.50	0.47	−0.29
pH 5.0										
28°	−14.1	−3.6	5.4	3.8	−12.2	0.28	0.11	0.61	0.56	−0.48
55°	−14.6	−4.4	5.9	3.6	−12.2	0.30	0.09	0.61	0.76	−0.41
65°	−14.4	−4.6	5.9	3.9	−12.3	0.30	0.12	0.58	0.61	−0.44
84°	−14.5	−4.8	6.0	4.1	−12.4	0.31	0.14	0.55	0.58	−0.42
3 *M* HCl										
28°	21.8	6.8	4.4	3.4	−12.6	0.17	0.07	0.76	0.49	−0.90
37°	21.7	6.2	4.7	3.4	−12.8	0.19	0.08	0.73	0.55	−0.82
46°	21.1	5.3	4.8	3.2	−12.9	0.20	0.06	0.74	0.82	−0.82
55°	20.8	4.1	5.1	3.3	−13.2	0.22	0.06	0.70	0.83	−0.76
65°	20.5	3.4	5.8	3.0	−13.2	0.29	0.04	0.67	1.41	−0.56

[a] Spectra recorded at 100 Mcps with *t*-butyl alcohol as reference. [b] In cps. [c] *E* in kcal/mole.

The dependence of the nmr spectra on temperature was investigated at three different hydrogen ion concentrations, in which the anion, zwitterion, and cation exist as the dominant species. The spectra of the anion and zwitterion were found to be only slightly temperature dependent; however, the spectra of the cation showed a marked temperature dependence.

The experimental data obtained were treated in terms of the statistical average of the contribution from the three rotational isomers of staggered form in a manner similar to that of Pachler.[60] Equations 5–9 were used for the three rotational isomers, the only difference being that the term fractional populations, p, was used instead of relative residence time. Values used for J_t = 13.6 cps and J_g = 2.6 cps were those determined by Pachler.[60] It was also assumed that J_t and J_g do not change with the hydrogen ion concentration. This assumption is supported by the fact that in alanine ($p_I = p_{II} = p_{III}$) the spin–



spin coupling constant between protons is independent of pH. This suggests that J_g and J_t are also independent of pH. The results obtained from the data obtained using a least-squares method of Arata, Shimizu, and Fujiwara[15,17] are shown in Table IX.

The rotational isomers I and II are not distinguishable from each other since it was assumed that J_g and J_t are only dependent of the dihedral angle. Therefore, p_I may be taken for the fractional population of rotational isomer II and p_{II} that of isomer I. The data indicate that the rotational isomer (III) which has the hydroxyl group *gauche* to both the amino and carboxyl groups is the dominant species in solution. X-Ray diffraction studies of DL-serine have shown only isomer III to be present in the solid state.[70] Using data previously obtained for cysteine,[17] which contains an SH group instead of an OH group, fractional populations at pH ~14 and 28° of p_I = 0.45, p_{II} = 0.17, and p_{III} = 0.38 were obtained. A comparison of the results obtained for both amino acids indicates that through hydrogen bonding the OH group is an important factor in determining the configuration of serine. Hydrogen bonding of the SH group is generally much weaker than that of the OH group.[71]

Since it is generally assumed that the entropy difference for rotational isomers is zero,[72] the free energy differences, ΔF, are equivalent to the potential energy differences, ΔE.[60] The relative energies [$\exp(-E_{II}/RT) = p_{II}/p_I$ and $\exp(-E_{III}/RT) = p_{III}/p_{II}$] obtained for serine are shown in Table IX. The relative energies E_{II} and E_{III} are seen to be temperature independent at pH 5 and 13.9, and the spectra changes were discussed in terms of increasing population of the rotational isomers I and II with an increase in temperature according to the Boltzman distribution law. In 3 *M* HCl the relative energies are temperature dependent. It is thought that under these conditions the rotational isomer III becomes less stable at higher temperature than expected from the Boltzman law; consequently, the assumptions made in describing the conformation of rotational isomers are not as accurate in the 3 *M* HCl solution.

The pH dependence of spin–spin coupling constants and chemical shifts of malic, O-methylmalic, and aspartic acids have been investigated.[73] The vicinal coupling constants were discussed in terms of the relative residence times and the relative free energies of the rotational isomers. The geminal coupling constants were discussed with respect to the orientation of the β-carboxyl groups.

The conformational equilibria of cysteine, histidine, and their derivatives have been studied by an analysis of the pmr spectra of these compounds.[74] Chemical shifts and spin–spin coupling constants were obtained for the three-spin systems in cysteine, histidine, and their derivatives as a function of the charge on the molecule. The effect of size, as well as charge,

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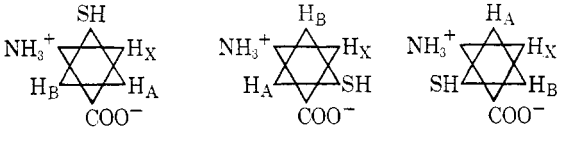
Table X[74]

Proton Magnetic Resonance Parameters [a]

Compound	*Charge*	ν_X	ν_A	ν_B	$-J_{AB}$	J_{AX}	J_{BX}
Cysteine	+	121.5	197.5			5.2	
	+−	151.5	208.0			5.1	
	−1	169.5	209.4	218.4	13.9	3.9	8.2
	−1.5	182.9	211.0	227.2	13.2	3.6	8.7
	−2	195.4	213.2	235.5	12.8	3.3	9.5
Cysteine methyl ester	+1	118.5	197.4			5.2	
	0	152.5	209.7			5.3	
	−1	170.0	220.6			5.2	
S-Methylcysteine	+	124.5	197.4			6.4	
	+−	152.7	205.0	209.5	15.0	2.3	9.7
	−	181.7	218.1	224.1	14.0	3.7	8.9
Thiazolidine-4-carboxylic acid	+[b]	99.5	176.3			6.4	
	−[c]	161.3	189.0	216.3	10.2	6.8	8.1
Histidine	++	116.5	176.9			6.9	
	++−	140.0	185.7			6.7	
	+−	153.0	202.9			6.5	
	−	182.9	216.4	226.4	14.9	4.5	8.7
Histidine methyl ester	++	113.0	176.0			6.8	
	0	162.0	212.4			6.2	
Acetylhistidine	+	102.7	191.7			7.4	
	−	125.5	206.1	214.7	15.0	3.4	9.9
Glycylhistidine	++−	115.8	194.7	200.4	16.0	3.1	10.5
	+−				15.2	2.9	10.5
	−	122.5	205.2	211.6	15.2	3.0	10.6

[a] With respect to benzene as external reference in cps at a frequency of 60 Mcps. [b] For the pair of protons on C-2, the average value of two peaks separated by 1.2 cps is 116.2 cps. [c] For the pair of protons on C-2, ν = 124.1 and 143.5 and J = 9.4 cps.

in determining conformational preferences was sought in this investigation. The nmr parameters obtained are shown in Table X in which A and B refer to the two hydrogens bound to the same carbon and the separate hydrogen is denoted as X. The three staggered forms used to describe the rotamers of cysteine are



where *t*, *g*, and *h* are used to designate the mole fractions (*i.e.*, relative residence times or fractional populations as described previously) of a *trans* and two *gauche* rotamers, respectively. In the *trans* rotamer the sulfhydryl and carboxylate groups are placed *trans*, and in the *gauche* rotamer, *h*, the three largest groups are nearest to each other. The labeling of the H_A and H_B protons in the rotamers shown above involves a commitment to the designation of the highest field hydrogen, H_B, to the position *trans* to H_X in the *t* isomer. This assignment was made by analogy with data obtained on malic acid.

For a given amino acid and its derivatives, the main factor which determines the chemical shifts appears to be the charge on the molecule. A comparison of the data in Table X for cysteine, S-methylcysteine, and cysteine methyl ester shows that the chemical shifts for each of the three protons for each compound are similar when compared at the same over-all net charge per cysteine molecule. The frequency of the lowest field or α hydrogen shows a marked dependence on charge since it is adjacent to two ionizing groups. With respect to the two β hydrogens, the one at the highest field, H_B, is the more dependent upon charge. It was also observed that the difference, $\nu_B - \nu_A$, decreases as the pH decreases from alkaline solutions and finally disappears near zero net charge on the cysteine molecule. Similar conclusions can be drawn concerning the dominant role of charge in determining chemical shifts for histidine and its derivatives.

In determining the mole fraction (*t*, *g*, and *h*) Martin and Mathur[74] followed a different procedure from that used by Pachler[60] and Ogura, Arata, and Fujiwara.[65] By considering three limiting cases in the rotamer distribution, it is possible to simplify the equations relating the coupling constants J_{AX} and J_{BX} to the mole fractions and the *trans* and *gauche* coupling constants. The three cases considered were: (I) $g = h$; (II) $h = t$; and (III) $g = t$. Substitution of these limitations into eq 5 and 6 (where *a*, *b*, and *c* correspond to *t*, *g*, and *h*, respectively, and $J_{12} = J_{AX}$ and $J_{13} = J_{BX}$) and recognizing that $t + g + h = 1$, one may obtain the following relationships

$$\text{I} \quad 2J_{AX} + J_{BX} = 2J_G + J_T \tag{11}$$

$$\text{II} \quad J_{AX} + 2J_{BX} = 2J_G + J_T \tag{12}$$

$$\text{III} \quad J_{AX} = J_{BX} \tag{13}$$

The expression $(2J_G + J_T)$ is of importance since it is directly related to the average coupling constant. From the data contained in Table X it can be shown that for cysteine $2J_{AX} + J_{BX} = 16.0 \pm 0.1$ over a complete unit of charge; consequently, case I ($g = h$) applies. This value which is also equal to $(2J_G + J_T)$ is considerably less than 18.76;[60] therefore the values previously recommended for J_G and J_T do not seem to be applicable to cysteine. This difference might be due to deviations from the 60 and 180° dihedral angles. In order to

obtain mole fractions, values of J_G and J_T must be assumed (only one value must be assumed since one value determines the other). If $J_T = 12$ and $J_G = 2$, for cysteine with a double negative charge $g = h = 0.12$ and $t = 0.75$. The conclusion that rotamer t is the predominant rotameric form for cysteine is a direct consequence of the assignments of the H_A and H_B hydrogens and the fact that $J_T > J_G$ for such compounds.[51] The greater thermodynamic stability of rotamer t in basic solutions is the result expected for the favored *trans* positions of the bulkiest and identically charged groups. Greater populations of the *trans* rotamers are also indicated by the data obtained for histidine and its derivatives by arguments analogous to those presented for cysteine.

If one assumes that $2J_{AX} + J_{BX} \simeq 16.0$ in acidic solutions of cysteine where only an average coupling constant was obtained (see Table X), then $J_{AX} = J_{BX}$ within experimental error and all rotamers are equally populated in acid solutions where the carboxylic acid group is in its acid form. A similar average coupling constant of approximately 5 cps has been obtained for cysteine in trifluoroacetic solutions.[75] For cysteine methyl ester it appears that all three rotamers are equally stable throughout the entire pH range since approximately the same average coupling constants were obtained for all charged species. Therefore, the bulky ester group seems not to be as important in determining the preferred rotamer conformation as is a negatively charged carboxylate group. This conclusion is also supported by the appearance of an AB_2 spectrum in the most basic solutions of histidine methyl ester, although histidine exhibits an ABX spectrum even in neutral solutions. Therefore steric effects appear less important in determining conformation than charge. Intramolecular hydrogen bonding may also be an important factor in determining rotamer stabilities.

Thiazolidine-4-carboxylic acid and the cysteine chelates of zinc and nickel ions have coupling constants that are approximately the same as that of the parent compound; structures analogous to rotamer t in the parent compound are indicated for the conformation about the α and β carbons. This similarity in coupling constants suggests that rotamer g is not favored in cysteine.

Rotamer populations are also related to the chemical shift between the two β hydrogens, and this property was discussed in detail in order to gain further insight into the rotamer distributions. It appears the major factor in effecting a large chemical shift difference between the two β hydrogens is due to an asymmetric amino group.

The pmr spectra of L-cystine, *meso*-cystine, and their dimethyl esters in basic and acidic solutions have been obtained and analyzed.[76] Chemical shifts and coupling constants were obtained for each compound in both acidic and alkaline media. Previous analysis of the nmr spectra of simple amino acids in solution were based on the classical rotamer structures which neglect intramolecular interactions other than steric hindrance in the *trans* and *gauche* configurations. The data obtained for L-cystine indicate that the stabilization of the actual configuration of L-cystine in acid solution is due to intramolecular interaction between the two moieties. This stabilization appears not to occur in *meso*-cystine as evidenced by the equivalence of the spectrum of *meso*-cystine to that of the esters of both *meso*-cystine and L-cystine. Additional evidence is given by the large decrease in the shift, δ_{AB}, from L-cystine to *meso*-cystine in acid solution, indicating that the β protons have become almost equivalent magnetically in *meso*-cystine. This observation suggests that the moieties in *meso*-cystine rotate about the disulfide bond more freely than in L-cystine. It was also observed that the coupling constants J_{AX} and J_{BX} for L-cystine and *meso*-cystine are relatively independent of pH and are comparable to those of L-cysteine. Therefore, it would seem that the classical rotamer analysis does not apply to the cystines.

The pmr signal of the NH proton of an amino acid in general cannot be observed in aqueous solution because of rapid exchange. However, when N-(2,4-dinitrophenyl), DNP, derivatives of amino acids are dissolved in dehydrated dioxane, the exchange of the NH proton is slow enough to permit the observation of the pmr signal.[77] The spectra obtained for the protons on the NH and DNP groups were found to be useful for the identification of DNP amino acids. By this method DNP amino acids such as DNP-threonine and DNP-allothreonine, DNP-leucine, and DNP-isoleucine can easily be discriminated.

Nuclear magnetic resonance spectra of derivatives of cycloserine have been obtained and analyzed.[78] The data indicate that acetylation of the 3-isoxazoidone ring of N-acetylcycloserine occurs on nitrogen while sulfonylation occurs on the carbonyl oxygen. The change in conformation due to the introduction of a double bond into the heterocyclic ring is shown in a consistent shift of the parameters of the nmr spectra (ABX system).

Bovey and Tiers[75] have made an extensive nmr study of amino acids and peptides in trifluoroacetic acid. Trifluoroacetic acid was chosen because it is an excellent solvent for these compounds and because in using trifluoroacetic acid instead of water tetramethylsilane could be employed as the internal reference.[79] Proton magnetic resonance signals were obtained for peptide and amide protons as well as the sulfhydryl group of cysteine; however, hydroxyl and carboxyl protons were unobservable due to rapid exchange with the solvent. Chemical shifts and coupling constants were obtained from which information concerning charge distributions, inductive effects of polar groups and positive charges, rates of proton exchange with solvent, and molecular conformation were obtained.

The nmr spectra of two diastereoisomers of γ-hydroxy-γ-methylglutamic acid have been obtained and their configurations determined from the spectra.[80]

An nmr study of the protolysis kinetics of sarcosine hydrochloride and sarcosine methyl ester hydrochloride has been conducted by Sheinblatt.[81,82] Exchange mechanisms and the rate constants obtained from the nmr data are discussed. Sheinblatt[83] has also studied the proton exchange between the amino group of glycine and water by measuring the effect of exchange upon the nmr spectrum of the methylene group in glycine. In the most acidic ($\sim[H^+] = 4.5\ M$) solutions the proton exchange is slow and the spectrum of the methylene group consists of a quartet. In less acidic solutions ($\sim[H^+] =$

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3.0 *M*), the exchange rate increases and the methylene quartet broadens and collapses to a single resonance line which becomes narrower as the rate of exchange increases. The mean lifetime between exchanges of protons in the NH_3^+ group was measured as a function of glycine concentration and of hydrogen ion concentration. A similar study was also extended to the glycine methyl ester. Exchange mechanisms were found to be separable by the study of the change in the mean lifetime with pH and glycine or ester concentration and the rate constants determined for the reactions. A comparison of the results obtained for glycine, glycine methyl ester, the sarcosine systems studied,[81,82] and simple amines[84] suggests that the proton-exchange mechanism for positively charged amino groups in strongly acidic solutions is the same in the different types of systems studied.

Del Re, Pullman, and Yonezawa[85] have applied molecular orbital theory to the study of the distribution of the electronic charges in the α-amino acids of proteins in their neutral, dipolar, cationic, and anionic forms. The results were correlated with the proton chemical shifts observed in the nuclear magnetic resonance of these compounds. A parallelism was found to exist between the proton shifts (δ_H) and the σ-electronic charges of the hydrogens (Q_H) as well as between the shifts and the charges of the neighboring carbons (Q_C). The results obtained were represented by the equation

$$\delta_H = -9.92Q_C - 133.93Q_H + 9.67 \qquad (14)$$

for the data relating to acid and alkaline solutions. This relationship indicates that the contributions of Q_C to the proton chemical shift is much smaller than that of Q_H.

2. *Metal-Ion Complexes*

The interaction of metal ions with proteins and polypeptides is of very important biological significance. However, in order to study these systems it is necessary to first study the interaction of metal ions with simple model compounds which are active components of the complicated polypeptide chains.

Cobalt ions are of biological significance since they act as activators for cholinesterase, carboxylase, and phosphoglucomutase and afford protection of brain respiration against high oxygen pressure.[86] The cobalt(II)–histidine complex is of particular interest due to the ability of the complex to absorb oxygen reversibly; a number of effects of cobalt in biological systems may be due to this property of the complex. McDonald and Phillips[86] have studied the structures of cobalt(II)–histidine complexes using proton contact interaction shifts obtained from nmr spectra.

The presence of a paramagnetic metal ion such as Co(II) in a coordination compound can produce a number of effects that are observable in the nmr spectrum of the coordinating group. In general one observes a marked decrease in the transverse nuclear relaxation time or line-width parameter[87] and a change in the electronic relaxation time.[87,88] As the electronic relaxation time of a paramagnetic system decreases, large shifts in the nmr spectra are observed that result from nucleus–electron isotropic hyperfine contact interaction[89] and pseudocontact interactions.[90] Contact interaction shifts have been observed mainly in the ligands of transition metal chelates in which the contact shifts are caused by effects of the unpaired electrons of the metal ion on the local magnetic environments of the ligand's nuclei. The unpaired electrons of the paramagnetic metal ions can be transferred to π orbitals of conjugated ligands owing to $p\pi$–$d\pi$ bonding or spin polarization effects. Spin density can be introduced into the σ orbitals of the ligands by similar mechanisms. Alternatively, the nmr frequencies of the atoms of ligands or paramagnetic chelates can be affected by field effects orginating at the paramagnetic ion. Additional information concerning metal–ligand interactions and ligand spin density distributions can be found in the literature.[91–95]

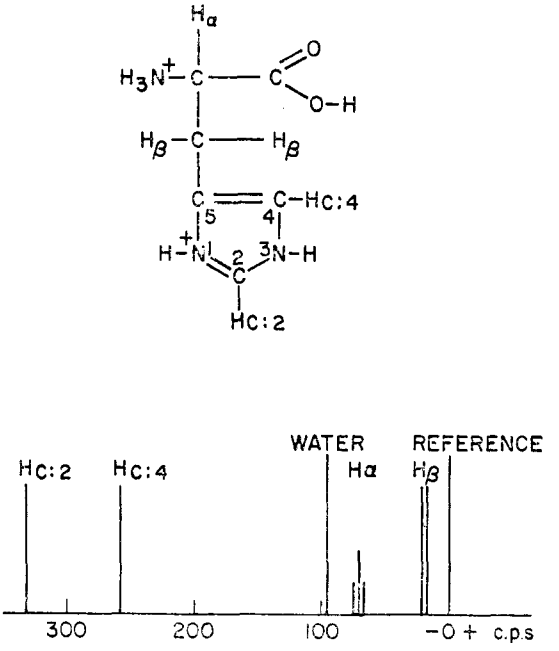

Figure 7. Nmr spectrum at 60 Mcps of aqueous histidine at pH 0.9.

The nmr spectrum of histidine in aqueous solution (D_2O) at pH 0.9 is shown in Figure 7. The nmr spectra of complexes of Co(II) with histidine were obtained over a pH range of 0.5–12 in D_2O solutions which were 0.4 *M* in histidine and contained varying concentrations of Co(II). Large contact shifts were observed and used to elucidate the characteristics of the different types of Co(II)–histidine complexes formed.

The addition of Co(II) ions to pure water produces a shift to a lower applied magnetic field in the water proton resonance. This shift is produced by a contact shift of approximately 5100 cps at 60 Mcps for the protons of water in the first coordination sphere of the Co(II) ions. The whole contact shift is not observed because of the fast proton exchange between bound and bulk water; consequently, the proton resonance signal of this system is a single composite line (for

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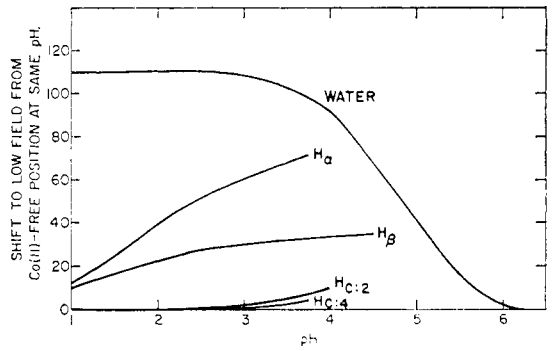

Figure 8. Effect of pH on contact shifts of nmr spectrum of complex I: D-histidine 0.4 *M*, Co(II) 0.2 *M*.

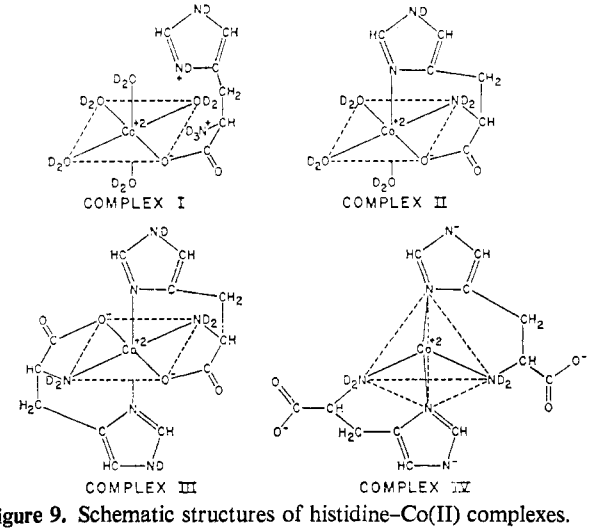

Figure 9. Schematic structures of histidine–Co(II) complexes.

additional literature references on this subject see ref 12). The replacement of water molecules in the coordination sphere of Co(II) ions by other ligands produces a high-field shift in the water proton resonance toward the cobalt-free position.

At a pH <1, where histidine is completely protonated, the addition of Co(II) to the solution causes no change in the nmr spectrum of histidine, while the water proton resonance undergoes a low-field shift proportional to the Co(II) added. This indicates that under these conditions there is no interaction of Co(II) with histidine.

Over the pH range 1.0–3.5 small contact shifts for the H_α and H_β protons to lower fields were observed (Figure 8). This carboxyl group on the histidine molecule completely ionizes in this pH range. Apparently, the carboxyl anion competes with water for a coordination site on the Co(II) ion, forming a histidine–Co(II) complex I as shown in Figure 9. The whole contact shift is not observed because of the fast exchange of free and bound histidine molecules as indicated by the observance of only one histidine nmr spectrum and not that of free and bound histidine. That the histidine ion competes unfavorably with water for a site on Co(II) in this pH range is supported by the very small high-field shift observed in the water proton resonance. The examination of the interactions of Co(II) with histamine and histidine methyl ester at pH 1–3.5 showed no proton contact shifts. This observation supports the contention that only the carboxyl group of histidine is involved in the binding of this ligand to Co(II) in complex I. Complex I was also found to be octahedrally coordinated.

As the pH of the 0.4 *M* histidine–0.2 *M* Co(II) solution is increased from 3 to 7, the HDO proton resonance (HDO being formed by exchange between D_2O and exchangeable protons in histidine) shifts to high field to that of water free of Co(II) indicating complete displacement of water from the first coordination sphere of Co(II) by histidine. At a pH of about 4.5 the histidine spectrum shows large contact shifts due to a new complex (II). At a pH of about 5, an additional and separate spectrum appears and is attributed to complex III. The spectrum of complex II disappears at approximately pH 7. The intensity of the spectrum of complex III increases with pH until complex II disappears and then remains constant at pH 10.5. If the histidine–Co(II) ratio is greater than 2, the ratio of complex III:II is increased, and a broadened spectrum of uncomplexed histidine remains for pH values greater than 7. However, if the histidine–Co(II) ratio is 1, complex II is favored and not entirely converted to complex III until a pH of about 8.

It was concluded that Co(II) replaces the proton at the 1 nitrogen and that the amine and carboxyl groups also bind to Co(II), displacing a proton from the protonated amine group. Complex II is considered to be a 1:1 Co(II)–histidine complex containing three water molecules. Complex III is considered to be a 1:2 Co(II)–histidine complex in which all of the water molecules are replaced by tridentate histidine.

At pH >11.5 the nmr spectrum of a new histidine–Co(II) complex (IV) appears. The color of the solution of this system changes from pink to blue and the magnetic susceptibility decreases, properties characteristic of tetrahedral Co(II) complexes.[96,97] Complex IV is thought to be a 2:1 histidine–Co(II) tetrahedral complex in which the 1 nitrogen position and either the carboxyl or amine nitrogen group binds to Co(II). The amine nitrogen group is the most probable second binding group since it is more basic than the carboxyl group and since an X-ray study[98] of a solid zinc–histidine system has shown that no coordinate binding between zinc and the carboxyl group exists in the tetrahedrally coordinated zinc.

McDonald and Phillips also found that $\Delta F°$ for the 2:1 octahedral histidine:Co(II) complex composed of one D-histidine and one L-histidine is approximately 0.7 kcal/mole less than the complex containing two histidine molecules of the same configuration.

In the presence of oxygen both the paramagnetic susceptibility of the solution and the intensity of the nmr spectrum of the original histidine–Co(II) complex decrease. The nmr of the histidine–Co(II) complexes in the presence of O_2 behave in a manner that is consistent with studies of the magnetic characteristics of these complexes.[99,100]

Carlson and Brown[101] have studied the divalent metal ion complexes of α-alanine and L-histidine in D_2O using nmr. The data obtained indicate a bidentate chelate structure of the 1:1 Zn(II)- and Cd(II)–histidine complexes at pD 5.0 and 5.9, respectively. The imidazole resonances of the complexes appear between the resonances observed for free histidine at

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pD 5.5 and 8.6 where the imidazole ring is protonated and nonprotonated, respectively, which is consistent with metal ion coordination to the imidazole moiety. The imidazole resonances for the Zn(II) complex occur at lower fields than those of the Cd(II) complex suggesting that Cd(II) does not interact with the imidazole moiety as strongly as does Zn(II). The nmr data also suggest the formation of a 1:1 Zn(II)–histidine complex at pH >11. The imidazole resonances in the Zn(II) complex at pH 11.8 occur upfield to those observed in the Zn(II) complex at pD 5.0 and in the spectra of free histidine at pD 8.6 and 12.4. The high-field shift is related to an increase in electron density about the ring protons at pD >11 due to the ionization of the "pyrrole" hydrogen. The data obtained also support amino coordination in the Zn(II)–histidine complex at pD 11.8.

Milner and Pratt[95] have studied the nickel complexes of histidine, L-proline, β-alanine, norvaline, and sarcosine using nuclear magnetic resonance techniques. A qualitative explanation for the large proton chemical shifts observed is given in terms of an isotropic contact interaction between the protons and a small unpaired electron spin density produced by the paramagnetic metal ion.

The effect of metal ion complexation on the pmr spectra of a number of amino acids has been used to elucidate the nature of binding sites in the acids and to provide information on the assignment of resonance frequencies.[102] The Cd(II) and Cu(II) complexes of alaninate, serinate, and prolinate were studied. In each case the largest downfield shift observed upon the addition of Cd(II) was that of the CH proton which lies between the binding sites. The addition of Cu(II) to an alaninate solution caused the collapse of the quartet due to the CH proton while the CH_3 resonance signal was only slightly broadened. The effect of Cd(II) on the pmr spectra of glycinate was also investigated. The Zn(II) and Cd(II) complexes of histidine and the Zn(II), Ni(II), and Cu(II) complexes of cysteine have been studied by Martin and Mathur.[74] The diamagnetic cysteine–nickel 2:1 complex gave a pmr spectra which showed very large upfield shifts. The large upfield shifts observed with the diamagnetic nickel complexes were found to be in marked contrast to the smaller upfield shifts observed with the zinc and cadmium complexes when compared with ligands of the same net charge as the complex. The data obtained for the cadmium and zinc complexes of histidine indicate that the favored structure is the *gauche* rotamer in which the three bulkiest groups are nearest to each other. The zinc and nickel complexes of cysteine gave coupling constants that differed very little from the parent compound. The data for these complexes indicate structure like the *trans* rotamer in the parent compound. In all cases of diamagnetic nickel complexes, the HDO (D_2O used as solvent) resonance line did not show broadening.

Jardetzky and Wertz[103] studied weak complexes of the sodium ion in aqueous solution by observing line-width broadening and changes in signal amplitude of the Na^{23} nmr absorption. Of the amino acids systems studied, only histidine exhibited any pronounced effect.

The aqueous pyruvate–glycinate–Zn(II) system has been investigated by Leussing and Stanfield using nmr.[104] This technique was employed to study the complexes of Zn(II) with pyruvate and glycinate ions separately and in mixed systems to determine which forms are predominant in the complexes. The related alanate–glyoxalate system was also studied. In the presence of a base such as CN^- the pyruvate ions were found to be dimerized, and the dimer formation constant of about 6 was obtained from the nmr data. The data obtained also indicate that the formation of higher polymerization products is negligible in these particular experiments. It was observed that Zn(II) ions behave as protons toward pyruvate ions by increasing the degree of hydration. This seems to be the result of the cooperative operation of a field effect (promoting the stability of Zn $Pyruv^+_{diol}$) and an inductive effect (destabilizing Zn $Pyruv^+_{keto}$).

In 1 *M* solutions glycinate and pyruvate ions were found to react incompletely to produce a solution of pyruvate dimer, *cis* and *trans* forms of the ketimine, and probably carbinolamine. The addition of Ca(II) or Zn(II) was found to inhibit dimerization as a result of complexing the reactants. The ligands in the mixed metal ion–pyruvate–glycinate complexes seem to be condensed as either the carbinolamine or the Schiff base. Transamination to produce alanine was found to be negligible over a 24-hr period. Alanate and glyoxalate ions were found to react giving a Schiff base that is slow to tautomerize. The reaction was found to be dramatically catalyzed by Zn(II).

C. PEPTIDES AND POLYPEPTIDES

1. Configuration. Chemical Shifts

The elucidation of the nmr spectra of proteins in terms of structure requires that one have specific information correlating the changes in nmr spectra which accompany the polymerization of amino acids and their subsequent arrangement in specific secondary and tertiary structures. Consequently, nmr studies of polypeptide systems are essential to the final utilization of the technique for the study of the more complex protein systems.

The nmr studies of glycylglycine,[2] glycylglycinate, and triglycinate[1] in aqueous solution have shown that the CH_2 groups in these molecules are nonequivalent; two CH_2 resonance signals were observed in the first two compounds and three CH_2 resonance signals were observed for the triglycinate molecule. In trifluoroacetic acid only one resonance signal was observed for the CH_2 groups in glycylglycine.[75] Peptide hydrogen resonance signals were discernible in trifluoroacetic acid (TFA) appearing near τ 2, while the methylene τ value remained nearly constant for the polyglycines although it was 0.1 ppm less than that for glycine. The τ values for the α protons for the glycyl peptides of alanine, leucine, and isoleucine in TFA were found to be 0.3–0.4 unit lower than in amino acids, while the side-chain proton τ values of the glycyl peptides were greater. This suggests that the peptide linkage exerts a very strong deshielding or electron-withdrawing effect felt most strongly by the α protons. The coupling constants for the peptide hydrogen and the β hydrogen with the α hydrogen are approximately the same (5–6 cps) in these glycyl peptides. One interesting feature of the spectra of the glycyl peptides is the narrow multiplet resonance observed for the peptide protons which has been discussed by Bovey and Tiers.[105] Studies of the isomeric pairs

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(105) G. V. D. Tiers and F. A. Bovey, *J. Phys. Chem.*, **63**, 302 (1959).

of dipeptides, glycylvaline–valylglycine, glycylleucine–leucylglycine, and glycylproline–prolylglycine, showed that the nmr spectra were appreciably different for each pair. In TFA protons of and near the benzene ring in the aromatic peptides were found to have relatively low τ values as a result of the ring current in the benzene ring;[106–109] however, protons in positions near the face of the ring, perhaps as the result of a folded structure, have large τ values since these protons are in the region of the ring where a field exists that is opposite to the direction of the applied field. A comparison of the nmr spectra of D-leucyl-L-tyrosine and L-leucyl-L-tyrosine with leucine showed that the methylene peak of the DL isomer is shifted upfield appreciably with respect to the methylene peak of leucine while that of the LL isomer is shifted only a small amount indicating that these protons are markedly closer to the face of the benzene ring than those of the LL isomer. These data suggest a more compact structure for the DL isomer. The nmr spectra of several other peptides in TFA were obtained.

An nmr study of the *cis*-peptide bonds in diastereomeric piperazinediones in TFA has been conducted.[110] It was suggested that the differences in chemical shifts between the members of diastereoisomeric pairs of piperazinediones might be used to determine the absolute configuration of amino acids or of the extent of racemization resulting from the formation of peptide bonds. The use of nmr in the measurement of racemization in peptide synthesis has also been proposed by Halpern, Chew, and Weinstein,[111] the technique being based upon a method for the determination for steric purity of model peptides.[112] It had been reported previously that magnetic nonequivalence had been observed in the diastereoisomeric peptide pair alanyl-tyrosine,[113] although van Gorkom[114] has reported that diastereoisomeric alanyl peptides have identical nmr spectra. A more complete study of alanyl peptides has, however, shown that a small change in chemical shift of the methyl resonances exists between L–L and L–D peptides[112] (see Table XI[113,115]). It is seen (Figure 10a) that the observed chemical shift is enough to allow the detection of the contamination of one diastereoisomer with the other in an artificial mixture. The presence of an aromatic amino acid in the peptide was found to enhance the methyl shift and, as can be seen in Figure 10b–d, the optical purity, the configuration, and the location of the alanyl unit in either the C-terminal or N-terminal position can be simultaneously determined by inspection of the nmr spectrum. Similar information can be obtained from the presence of an alanyl residue in a tripeptide (Figure 10e,f). Ring current deshielding was felt to be the cause of the increased chemical shift of the alanyl methyl group in phenylalanine- or tyrosine-containing diastereoisomers. It is obvious that chemical shifts and resonance peak intensities can be used in the study of racemization in peptide synthesis. It was shown that the methyl resonances in several N-acylated peptide derivatives could be used for the quantitative analysis of N-protected diastereoisomeric peptide reaction mixtures.

Table XI[113]

Methyl Resonances of Alanyl Peptides

Peptide[a]	*N-Terminal*[b]	*Central*	*C-Terminal*
L-Alanyl-L-alanine	92.8	...	80.9
D-Alanyl-D-alanine	93.0	...	80.8
L-Alanyl-D-alanine	91.1	...	81.1
L-Alanyl-L-alanyl-L-alanine	93.1	84.1	79.6
L-Alanyl-D-alanyl-L-alanine	91.2	83.0	78.9
D-Alanyl-L-alanyl-L-alanine	91.5	83.9	79.6
D-Alanyl-D-alanyl-L-alanine	92.9	83.4	79.0

[a] All spectra were determined on a Varian A-60 spectrometer with the center of gravity of the chemical shift given in cps downfield from sodium dimethylsilapentylsulfonic acid (SDSS). The compounds were dissolved in deuterium oxide, and the pH values were adjusted to 5–6 with addition of deuterioacetic acid or sodium deuteroxide. The error of measurement was ±0.5 cps. [b] N-Terminal and C-terminal methyl resonances were assigned by observing shift in spectral lines with varying pH.[115]

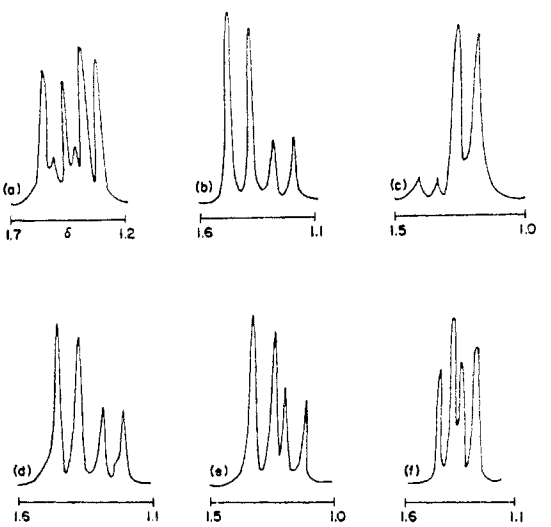

Figure 10. Nmr spectra in the region of the methyl resonance for alanyl peptides: (a) L-alanyl-D-alanine and D-alanyl-D-alanine (1:9, D_2O, pH 5, SDSS); (b) L-alanyl-L-phenylalanine and L-alanyl-D-phenylalanine (3:1, D_2O, pH 3, SDSS); (c) L-phenylalanyl-L-alanine and L-phenylalanine-D-alanine (1:9, D_2O, pH <1, SDSS); (d) DL-alanyl-DL-phenylalanine (commercial sample, D_2O, pH 5, SDSS); (e) glycyl-DL-phenylalanyl-D-alanine (D_2O, pH 5, SDSS); (f) glycyl-DL-alanyl-L-phenylalanine (D_2O, pH >10, SDSS).

Long-range shielding of protons in a series of peptides in their different ionization states has been studied by van Gorkom.[114] It was observed that dissociation of a –COOH or $-NH_3^+$ group, produced by raising the pH of the solution, caused an increase in shielding of the protons in the ion or molecule. The change in shielding was related to charge density and magnetic anisotropy differences between the conjugated acid and base groups. Long-range shielding effects over 11 bonds were observed in dialanylalanine and in alanylglycylglycine. From a study of nine di- and tripeptides a correlation between the average magnitude of the dissociation shifts of protons and their separation by the number of

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bonds from the dissociating proton was obtained. The same correlation was found to hold for a single peptide suggesting that head-to-tail association of the charged terminal groups of the zwitterions is negligible.

The nmr spectra of a polyamino acid in both the random and helical form was first studied by Bovey, Tiers, and Filipovich.[116] A fine-line spectrum was obtained for poly-γ-benzyl-L-glutamate in trifluoroacetic (TFA) acid in which the molecule exists in the random-coil conformation. A broadened spectrum was obtained in trichloroethylene containing a small amount of TFA in which the molecule is in the helical conformation. Another nmr study of poly-γ-benzyl-L-glutamate in $CDCl_3$–TFA solution showed that the α-CH and NH peptide resonance peaks appear at a TFA concentration between 20 and 30% in good agreement with the charge in Moffitt parameter[117] and specific rotation over this solvent range.[118] The appearance of these two resonance peaks is the result of the helix–random-coil transition. Side-chain interactions were also observed. The helix–coil transition of poly-γ-benzyl-L-glutamate in mixtures of deuteriobenzene and TFA has been studied by comparing proton magnetic relaxation behavior with optical activity studies.[119] The transition from coil to helix was found to occur at lower temperature in the nuclear magnetic relaxation measurements compared to the optical activity measurements. This apparent difference is thought to be due to the fact that relaxation measurements detect the slowing down of motional modes, whereas changes in optical activity are associated with the stabilization in position of several monomer units in succession. Therefore, at a temperature slightly less than that necessary for maintaining a stable helical form, successive segments probably occupy helical conformation long enough to effectively slow down molecular motion. The decrease observed in the spin–spin relaxation time for the phenyl and α-hydrogen atoms occurs because the formation of a helical rod inhibits the free motion of the chemical groups. As would be expected the effect was much more pronounced in the α-hydrogen resonance than in the phenyl resonance. The transition point was found to be concentration dependent. This dependence was rationalized in terms of the binding of the more polar solvent to the polypeptide in the coil conformation. The *effective* concentration of the unbound polar solvent is reduced as the polypeptide concentration increases; consequently, the helix-breaking tendency is reduced with increasing polypeptide concentration, and the helix is also formed more easily at a lower temperature when the polypeptide is present in higher concentration. Similar studies have been made with poly-γ-ethyl-L-glutamate in TFA–trifluoroethanol mixtures.[120] At 25° the critical helix–coil transition point was found to be in the range of 62–64% TFA content. It was further concluded that the ratio of the area of the NH peak to the methyl peak was proportional to the random-coil content of the polymer and that this technique could be used for studying helix–coil transitions in polypeptides.

Helix–random-coil interconversion in model polypeptide systems has been investigated by Stewart, Mandelkern, and Glick.[121–123] Pmr spectra were obtained of poly-L-alanine (PLA) and poly-DL-alanine (PDLA) in trifluoroacetic acid (TFA)–chloroform-*d* solution.[24] This solvent system has been postulated to support the helix–random-coil or interconverting configuration of poly-L-alanine but only the random-coil configuration of the DL polymer. The pmr spectrum of a dilute solution of PLA in TFA contains three main resonance groups resulting from the side-chain methyl protons, the methylene proton, and the amide proton. The Moffitt parameter, b_0, has been used extensively in investigating the helical–random coil or interconverting structure occurring in dilute solutions of polypeptides. Plots of b_0 *vs.* % TFA for solutions of poly-L-alanine (PLA) and poly-L-leucine (PLL) were presented and interpreted in terms of predominance by a particular configuration: low acid, helix configuration; highest acid, random coil; and interconversion of helix to random coil occurring in the region of sharply varying b_0. The correlation between the b_0 parameter and pmr chemical shift when also plotted as a function of % TFA was shown. The chemical shift variations were found to parallel that of the b_0 parameter. The difference in the pmr features of poly-DL-alanine and those of PLA lend support to the structure conclusion since PDLA has the same chemical structure as PLA but possesses the random-coil form at all acid concentrations.[124–127] The difference in chemical shifts observed between corresponding groups in helical PLA and random-coil PDLA at comparable TFA concentrations was interpreted as evidence for the predominance of the indicated configurations. The pmr chemical shift differences observed between protons in the helical compared with those in the random-coil configuration are attributed to anisotropic shielding effects. In the helix configuration the positions of the different groups are fixed with respect to each other; consequently, anisotropic effects associated with the carboxyl bond, and to a lesser extent with that of other bonds, could make a large contribution to the shielding constant of the proton held near the bond axis. In the random-coil form of the polypeptide, the motion of the different groups with respect to each other can occur more freely, resulting in a significantly decreased anisotropic contribution. In the α-helical structure of PLA, the methylene proton is *cis* to the carbonyl bond, the methyl group is *trans*, and the amide proton is *trans* to the carbonyl bond.[128] It has been shown[129–131] that the resonance of the methyl proton of an N-ethyl group in diethylamides or of an N-methyl group in dimethylamides occurs at a higher field when the group is *cis* to the carbonyl oxygen than when it is *trans*. A comparison of the observed chemical shifts for the amide, methyl, and methylene protons in PDLA and PLA

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at a comparable acid concentration shows that the differences are consistent with the proposed structures. In the low TFA concentration range the pmr shifts for PLA and PDLA are different but converge in 100% TFA which indicates that PLA and PDLA have the same configuration in 100% TFA. The data obtained also suggest that both PLL and PLA are in the random-coil configuration in 100% TFA. It was found that the 100% TFA value for each b_0 is not zero; a conventional interpretation would indicate a residual helical content for each peptide. It was concluded that since the pmr behavior confirms the fact that PLA is random coil in 100% TFA the empirical assignment of helical content through b_0 values cannot be made for PLA and PLL. Nonzero b_0 values have also been found for other substances.[132-136] An examination of the chemical shifts observed for the various peptide protons suggests that there is some interaction between solvent and polypeptide; however, the data indicate that the interaction is not protonation of the peptide units. This conclusion was drawn from the fact that the chemical shift for the TFA protons experiences no downfield shift indicative of protonation. The TFA acid proton exhibits a total shift of about 0.267 ppm over the range of 25–100% TFA, whereas simple amides produce more than an order of magnitude displacement in this proton.[123] It was proposed that the principal acid–polymer interaction is hydrogen bonding without proton transfer. Intramolecular hydrogen bonding is suppressed in favor of intermolecular hydrogen bonding between polypeptide and TFA as the acid concentration is increased. It is thought that this is the driving force converting the polypeptide from the helical to the random-coil form. Another important aspect of this work is that the proton resonance peaks of PLA were observable over the entire range of TFA concentrations studied, this being the first reported case in which pmr features due to protons attached to the backbone have been observed in the helical conformation of the polypeptide. Earlier studies of several polynucleic acids suggest that these species exhibit an nmr absorption only when the polymer possesses a disordered structure.[137,138]

Proton chemical shifts and line-width data have been used to study poly-L-alanine, poly-β-benzyl-L-aspartate, poly-γ-benzyl-L-glutamate, poly-L-glutamic acid, and poly-L-methionine in TFA–$CDCl_3$ solution throughout their helix–coil transition region.[139] A rapid interconversion between helical and nonhelical regions of the polymers was indicated by the sharpness of the spectra obtained with the lifetime of a residue in either state having an upper limit of approximately 6×10^{-3} sec. The proton resonance peak corresponding to the α-CH on the peptide backbone was found to undergo the greatest shift on helix formation; consequently, this chemical shift should be an accurate measure of the per cent helicity. For the case where single resonance peaks are observed the chemical shift is given by

$$\delta_{obsd} = \alpha\delta_H + (1 - \alpha)\delta_R \quad (15)$$

where α is the fraction helix, $(1 - \alpha)$ is the fraction random coil, and δ_H and δ_R are the chemical shifts of the totally helical and random polymers. The time-averaged per cent helicity is, therefore

$$\% \text{ helicity} = \frac{\delta_{obsd} - \delta_R}{\delta_H - \delta_R} \times 100 \quad (16)$$

Upon helix formation the α-CH resonance peak shifts upfield. This shift is attributed to the anisotropy of the peptide bond. In a right-handed helix the proton on the α carbon is almost perpendicular to the plane of the peptide linkage preceding it and almost in the plane of the peptide linkage following it. The conjugated π-electron system following the α-CH would produce a downfield shift while that preceding the α-CH would tend to produce an upfield shift. Since the observed shift is upfield, the main influence seems to be that from the peptide linkage on the amino terminal side. In a left-handed helix the position of the α-CH is reversed with respect to its neighboring peptide linkages; however, the α-CH peak of poly-β-benzylaspartate, a polymer throught to form a left-handed helix,[140] shifts upfield. Assuming that the helix is left-handed, it is difficult to determine if the upfield shift is produced by diamagnetic anisotropy arising from side-chain groups or whether the effect of the peptide bond on the carboxyl terminal side dominates in this particular instance.

The chemical shift of the proton on the peptide nitrogen upon helix formation is determined by two opposing factors: (1) difference in hydrogen bonding (participation of a proton in hydrogen bonding causes a downfield shift in the NH peak); (2) magnetic anisotropy of the adjacent peptide bond (the proton will be directly above the plane of the peptide bond preceding it causing an upfield shift in the NH peak). A downfield shift in the NH peak was observed in all polymers except poly-β-benzyl-L-aspartate. Hydrogen bonding in this polyamino acid may not play a significant role in determining the direction of chemical shift since it is known that the polymer forms an unstable helix.[135]

The resonance peaks of side-chain protons were found not to be significantly shifted on helix formation.

It was thought that any increase in the α-CH line width upon helix formation would be due primarily to relaxation of residues while they were in the helical state by means of intramolecular magnetic dipole fields; therefore, the line width might be expected to be a measure of the rigidity and per cent helicity of the polymer. Experimentally, the line width was observed to go through a maximum. A second relaxation mechanism must be involved in the transition region. One such mechanism might involve the contribution to the line broadening from the rate of helix–coil exchange as the helices begin to form. The NH resonance peak was found to broaden first and even disappear in some polymers in the transition region. This broadening is thought to be the result of intermolecular dipole interactions occurring in hydrogen-bond formation.

The chemical shifts observed for poly-L-alanine were consistent with those reported previously;[122] however, a different interpretation was given. The line-width data obtained suggest

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that the rate of interchange between helix–coil is very fast except through the initial transition range and has a maximum value around 60% $CDCl_3$; then the rate decreases perhaps because of aggregation of helices. It had been suggested previously[122] that the gradual change in proton chemical shift over the range of 30–90% $CDCl_3$ is the result of a tightening of the helix with an accompanying increase in magnetic anisotropy produced by the decrease in motion. Assuming this to be correct, the line width would be expected to increase over this range rather than decrease. Markley, Meadows, and Jardetzky[139] interpret their data as indicating that the cooperative transition to the helix configuration occurs at about 20% $CDCl_3$ but that the helix is kinetically very unstable until sufficient $CDCl_3$ is present to cause aggregation of helices. The increased concentration of $CDCl_3$ causes the helical form to become thermodynamically more stable.

The 100-Mcps pmr spectra of poly-L-leucine and poly-β-methyl-L-aspartate have been investigated over the whole TFA–$CDCl_3$ concentration range.[141] Separate resonance peaks were observed for the helical and coil conformations for both the peptide NH and the α-CH protons. Because separate resonance peaks were obtained, it was possible to obtain lower limit of the order of 10^{-2} for the lifetimes of the protons in the two conformations. Also since separate peaks for the helical and coil conformations are observable, it is possible to estimate directly the "conformational" purity of the various solutions.

The 100-Mcps data were found to confirm the previous conclusion[122] that TFA is not hydrogen-bonded to the amide groups of the helix. The coil α-CH proton resonance peak occurs downfield relative to that of the helical α-CH proton resonance peak. The shift difference between the left-handed helix poly-β-methylaspartate and the coil is greater than the shift difference between the right-handed helix poly-L-leucine and the coil which is in agreement with the theoretical predictions of Sternlicht and Wilson.[142] The coil NH proton resonance was found to occur upfield relative to the helical resonance peak. This might be indicative of the breaking of the intramolecular peptide hydrogen bond without the subsequent formation of an intermolecular solvent NH hydrogen bond.

A number of synthetic poly(L-α-amino acids) have been studied in the solid state over a temperature range of 77–470°K using nmr techniques.[143]

Sheinblatt[115] has proposed a method for the determination of the sequence of amino acids in di- and tripeptides using an nmr technique. The method depends upon the fact that the resonance absorption position of a group adjacent to a potentially ionizable group is a function of the state of ionization of that group. Removal of a proton of an acidic group produces high-field shifts in the resonance absorption positions of its neighboring groups while protonation of the basic group causes low-field shifts in the resonance positions of the neighboring groups. A neutral peptide molecule in aqueous solution exists as a zwitterion. The addition of a base to an aqueous peptide solution neutralizes the NH_3^+ group which should cause the spectrum of the adjacent group to shift toward higher field. Addition of an acid to an aqueous peptide solution results in the protonation of the ionized carboxylic group which should produce a low-field shift in the resonance of the adjacent group.

These observations suggest that the sequence of amino acid residues in di- and tripeptides could be determined by observing the shifts of the resonance positions as a function of pH. For a dipeptide XY where X and Y are different amino acid residues, if the sequence is XY (X = NH_3^+RCO; Y = $NHR'COO^-$), the addition of base will cause the resonance positions of the R group in X to shift toward high field relative to their positions when the molecule is a zwitterion. The addition of an acid would be expected to produce a low-field shift in the resonance position of the R′ group. For a reversal of the dipeptide sequence the resonance position of the Y(R′) group should shift toward higher field with the addition of base, and the X(R) group resonance should shift toward lower field with the addition of acid. This case was tested by observing the nmr spectra of glycylalanine and alanylglycine in acidic, neutral, and basic solutions. For glycylalanine the resonance position of the CH_2 group of glycine undergoes an upfield shift in basic solution while the resonance positions of the α-CH and to a lesser extent the resonance positions of the CH_3 group of alanine shift toward lower field in acidic solution. In the case of alanylglycine, the resonance positions of α-CH and CH_3 of alanine shift toward higher field with the addition of base whereas the resonance position of the CH_2 group of glycine shifted toward lower field with the addition of base.

The amino acid sequence in a tripeptide can be determined in a similar manner. For a tripeptide composed of three different amino acid residues (XYZ), if the peptide sequence is XYZ (Z = NH_3^+RCO; Y = $NHR'CO$; Z = $NHR''COO^-$), addition of base produces a high-field shift in the resonance position of R while the addition of acid produces a low-field shift in the resonance position of R″. The resonance position of R′ should only be slightly affected by pH changes since Y is relatively distant from the ionizable centers. Thirteen pairs of dipeptides as well as a number of tripeptides were analyzed in the manner indicated above.

Nakamura and Jardetzky[144,145] have shown that the chemical shifts produced by the incorporation of an amino acid into a polypeptide chain can be arranged in terms of a few relatively simple rules. The predominant utility of this method is for the study of secondary and tertiary structure of peptides and not for the analysis of the primary sequence of large peptides. The chemical shifts of the glycine–CH_2 proton resonance positions in peptides containing a glycine residue are shown in Table XII. The data obtained indicate a number of interesting properties. (1) All of the chemical shifts of the glycine–CH_2 are grouped about six mean values: (a) glycyl amino acid cations, 231.8 cps; (b) glycyl amino acid zwitterions; (c) glycyl amino acid anions, 199 cps; (d) aminoacylglycine cations, 242.5 cps; (e) aminoacylglycine zwitterions, 227.2 cps; and (f) aminoacylglycine anions, 225.0 cps. These data indicate that the shifts due to differences in the chemical nature of the nearest neighbor are smaller than the changes in the chemical shift produced by titration or peptide bond formation. (2) The chemical shifts of glycine in glycyl amino acid cations and glycyl amino acid zwitterions are approximately the same and so are the chemical shifts observed in aminoacylglycine zwitterions and acylglycine anions suggesting that

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Table XII[144]

Chemical Shifts of Glycine CH_2 Protons in Peptides Containing a Glycine Residue[a]

Peptide	*Cation*	*Zwitterion*	*Anion*
Glycine	231.2	212.8	185.6
Glycyl-L-alanine	231.4	228.8	199.3
Glycyl-L-valine	233.7	232.6	201.3
Glycyl-L-leucine	232.3	229.9	199.4
Glycyl-L-isoleucine	233.5	231.7	200.8
Glycyl-L-methionine	232.9	230.7	201.2
Glycyl-L-serine	234.8	233.8	202.1
Glycyl-L-proline	239.8	236.8	206.2
Glycyl-L-aspartic acid	232.5	230.9,[c] 228.9[d]	199.4
Glycyl-L-glutamic acid	233.2	230.7,[e] 230.2[f]	200.1
Glycyl-L-lysine	233.0	233.5[g]	206.9,[h] 200.3
Glycyl-L-histidine	231.9	230.1,[i] 220.4[j]	196.3
Glycyl-L-tryptophan	227.4 221.2	...[b]	189.0
Glycyl-L-phenylalanine	226.5	227.1 220.3	192.9
Glycyl-β-alanine	226.9	226.4	196.6
L-Alanylglycine	243.8	231.3 224.8	226.1
L-Valylglycine	244.8	235.2 222.4	226.7
L-Leucylglycine	244.0	233.5 221.5	225.5
L-Serylglycine	245.5	235.2 226.4	227.4
L-Prolylglycine	244.8	230.5 225.5	225.8
L-Hydroxyprolylglycine	244.8	228.3	225.8
L-Lysylglycine	243.8	228.2,[k] 226.3[l]	225.9
L-Histidylglycine	245.8 240.2	235.1 221.7,[m] 226.3[n]	223.1
L-Tryptophylglycine	239.2 232.6	239.7 215.4	216.3
L-Phenylalanylglycine	239.0	231.6 213.3	223.4 217.3
L-Tyrosylglycine	239.9	232.0 214.2	225.6 219.4
β-Alanylglycine	241.0	226.0	225.2

[a] Cps from DSS as an internal reference (60 Mc, 0.05 *M*). [b] Solubility is not adequate for measurement. [c] pD 3.61. [d] pD 6.94. [e] pD 3.79. [f] pD 6.85. [g] pD 3.59. [h] pD 8.47. [i] pD 4.80. [j] pD 7.60. [k] pD 5.88. [l] pD 9.58. [m] pD 4.11. [n] pD 6.61.

the glycine amino group dominates the shift while the charge on the neighboring amino acid has only a small effect. (3) The CH_2 resonance position shift in aminoacylglycine anions is about 40 cps to lower field compared to the glycine anion. The large shift can be related to the formation of the peptide bond at the N-terminal site since the state of protonation of glycine is the same in each case. (4) However, the CH_2 resonance position shifts in glycyl amino acid cations were found to be the same as observed in free glycine, indicating that peptide bond formation at the C terminal does not contribute very much to the over-all chemical shift. (5) The spectra of some of the aminoacylglycines and glycyl amino acids showed a chemical shift nonequivalence for the two methylene protons. This feature of these spectra was discussed in detail. Mandel[146] has observed this for the middle residue of leucylglycylglycine, and it has been discussed by Morlino and Martin.[147]

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From an analysis of the data in Table XII, Nakamura and Jardetzky concluded that the chemical shifts produced by incorporating a given amino acid residue into a polypeptide with a definite primary and secondary structure form two separate classes when grouped according to their magnitude. It was further concluded that shifts are additive to a very high degree of approximation. The terms first order and second order were used to designate all shifts falling into the range of 0.3–1 and 0.01–0.2 ppm, respectively. First-order shifts due to changes in primary or secondary structure were observed only for α-C protons, while second-order shifts were observed for both side-chain protons and the α-C protons.

Three types of first-order shifts are evident from Table XII, the shift due to the titration of the N terminal, the shift due to the C terminal, and the N-terminal peptide shift. The N-terminal titration shift, δ_{tN}, is given by

$$\delta_{tN} \equiv \delta(A)^{\pm} - \delta(A)^{-} \simeq \delta(AX)^{\pm} - \delta(AX)^{-} = 0.45 \pm 0.01 \text{ ppm} \quad (17)$$

where $\delta(A)^{\pm}$ and $\delta(A)^{-}$ are the chemical-shift values of the α-CH protons of the free amino acid A in its zwitterionic and cationic forms. The terms $\delta(AX)^{\pm}$ and $\delta(AX)$ are the chemical shifts of the α-CH protons of amino acid A forming the N-terminal residue of a dipeptide. δ_{tN} is a positive quantity indicative of a downfield shift. The C-terminal titration shift, δ_{tc}, is given by

$$\delta_{tc} \equiv \delta(A)^{+} - \delta(A)^{\pm} \simeq \delta(AX)^{+} - \delta(AX)^{\pm} = 0.3 \pm 0.01 \text{ ppm} \quad (18)$$

where $\delta(AX)^{+}$ and $\delta(AX)^{\pm}$ represent the chemical shifts of the α-CH protons of amino A which forms the C-terminal residue of a dipeptide. The value of δ_{tc} is also positive denoting downfield shifts. The N-terminal peptide shift, δ_{pN}, is given by

$$\delta_{pN} \equiv \delta(XA)^{-} - \delta(A)^{-} \simeq 0.66 \pm 0.01 \text{ ppm} \quad (19)$$

The value of δ_{pN} was obtained by disregarding the chemical shifts observed for L-tryptophylglycine, L-phenylalanylglycine, and L-tyrosylglycine. An additional first-order shift must be considered, that being the helix formation shift, δ_h, given by

$$\delta_h = \delta(A)_{helix} - \delta(A)_{coil} \simeq 0.4 \text{ ppm} \quad (20)$$

Second-order shifts must also be taken into account. These are the C-terminal peptide shift

$$\delta_{pc} \equiv \delta(AX)^{+} - \delta(A)^{+} = 0.00 - 0.02 \text{ ppm} \quad (21)$$

the N-terminal neighbor shift

$$\delta_{nN} \equiv \delta(XA)^{-} - \delta(YA)^{-} \simeq 0.05 - 0.1 \text{ ppm} \quad (22)$$

and the C-terminal neighbor shift

$$\delta_{nc} \equiv \delta(AX)^{+} - \delta(AY)^{+} \simeq 0.0 - 0.2 \text{ ppm} \quad (23)$$

where X and Y represent different amino acids. Therefore, the chemical shift of a glycine residue in the interior of a peptide chain may be approximated by

$$\delta_{obsd} \simeq \delta(A)^{+} + \delta_{tc} + \delta_{tN} + \delta_{pN} + \delta_h + (\delta_{pc} + \delta_{nN} + \delta_{nc}) \quad (24)$$

This approximation was verified for several glycine-containing dipeptides as shown in Table XIII.

An investigation of a series of oligoglycines also confirmed the conclusion concerning the additivity of chemical shifts.

Table XIII[144]

Comparison of Observed and Calculated Line Positions for Glycine-Containing Dipeptides

Residue	*Calculated*[a]	*Observed*
Gly (2) in triglycine (±)	4.11	4.07
Gly (2) in tetraglycine (±)	4.11	4.10
Gly (3) in tetraglycine (±)	4.06	3.98
Gly (2) in pentaglycine (±)	4.11	4.12
Gly (3) in pentaglycine (±)	4.06	4.00
Gly (4) in pentaglycine (±)	4.06	3.98

[a] δ_{calcd} from eq 24, using values (ppm) $\delta(Gly)^+ = 3.55$, $\delta_{tN} = 0.45$, $\delta_{tc} = 0.3$, $\delta_{pN} = 0.66$, $\delta_{pc} = 0$.

resulting for different effects.[147] It was also shown that the resonance shift of a given residue in a peptide chain is dependent on the state of ionization. In some cases the resonance shift depends upon the structure of its nearest neighbor. For the case of an extended peptide chain, the resonance shift of a given amino acid was found to be independent of its removed and nearest neighbors.

Nmr techniques have also been applied to the study of a number of cyclic peptides.[148,149]

The rate of exchange of the peptide hydrogen of glycylglycine has been measured by the nmr technique.[150] It was observed that the predominant exchange of the peptide hydrogen occurs with OH^- ions. At 23° the rate constant for this reaction was determined to be $k = 7.8 \times 10^8$ $(M \text{ sec})^{-1}$. A comparison of the results obtained for glycylglycine with that obtained for N-methylacetamide[151,152] indicates that the rate of exchange of the peptide hydrogen with OH^- is 1.5×10^2 times greater for glycylgycine than for N-methylacetamide. It was also observed that for glycylglycine no exchange occurs in acidic solution.

Proton exchange of poly-L-alanine in chloroform–trifluoroacetic acid solutions has been investigated using the nmr technique and mechanisms postulated for the exchange reaction.[121]

2. Metal-Ion Complexes

A number of metal complexes of peptides have been studied using nmr method.[102,153–155] Zinc and magnesium complexes of glycylglycinate and glycinamide in aqueous solution were investigated to determine the effect of chelating metal ions on the chemical shifts of the methylene proton.[153]

Two methylene resonance peaks were observed for glycylglycinate indicating that the two CH_2 groups are nonequivalent. The addition of zinc ions to the peptide solution produces downfield shifts in both resonance positions. However, the shifts of the two CH_2 groups were found to be unequal, probably because in glycylglycinate one of the CH_2 groups lies between the two coordination sites. It has been shown that the coordination sites in glycylglycinate toward zinc are the peptide oxygen in the immediately adjacent amide group and the terminal amino group.[156] Downfield shifts were also observed in the Mg^{2+} solution although the shifts produced were smaller than those observed in the Zn^{2+} solution, indicating weaker binding by the Mg^{2+} ions. The data obtained also indicate that zinc chelates strongly to glycinamide. An nmr study of the metal ion complexes of glycyl-L-alaninate, glycylserine, and glycylprolinate indicated that in glycyl peptides the binding sites for a metal ion are the terminal amino group and the adjacent amide group.[102] The Co^{2+} and Mn^{2+} complexes of glycylglycinate in aqueous solution, before and after oxygenation, have been investigated by nmr.[155] In the case of the cobalt–glycylglycinate complex the attachment of oxygen is reversible, whereas it is irreversible for the manganese complex. The oxygenated cobalt–dipeptide complex was found not to form with glycylprolinate. The effects of charge and metal ion on the nmr shifts of glycyl peptides was investigated by Mathur and Martin.[154] The addition of zinc ions to a glycinate anion solution produced a downfield shift in the glycinate resonance, relative to the glycinate anion, in agreement with other results[102,153,157] on the effect of a diamagnetic metal ion on the free base form of a ligand. Chemical shifts of the diamagnetic nickel tetraglycine complexes were obtained. The pronounced upfield shifts caused by the diamagnetic nickel ion were concluded to be a feature of the metal ion and not of the ligands involved.

The simplest molecule which contains a peptide bond and which may be considered as a fragment of a polypeptide chain is N-methylacetamide. The complete spectrum of this compound in the pure state has been published[158,159] as well as in aqueous solution[160] and aqueous solutions of $AlCl_3$.[161] The N–H coupling constant has also been obtained.[3] The *cis* and *trans* configurations of the peptide bond in N-methylacetamide have been studied using nmr.[162–164] Proton-exchange studies of N-methylacetamide have been conducted using nmr techniques.[151,152,165,166]

D. PROTEINS

1. Configuration. Chemical Shifts

The first protein for which a nmr spectrum (40 Mcps) was obtained was ribonuclease.[167] Of the four resonance peaks observed, peak assignments were made for aromatic hydrogens (the lowest field peak) and for hydrogens on aliphatic carbon atoms attached only to other aliphatic carbons (the highest field peak). This spectrum was further interpreted by Jardetzky and Jardetzky[168] on the basis of measurements of the spectra of amino acids and peptides in H_2O, D_2O, and

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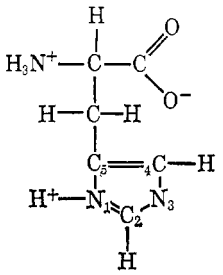
concentrated sulfuric acid.[2,11] It was concluded that the lowest field peak was due to the phenylalanine hydrogens, tyrosine hydrogens, and probably the CH protons on the imidazole group of histidine, in agreement with the initial assignment.[167] Proceeding upfield, the second peak was assigned to the α-CH protons of all amino acids, the β protons of serine, β protons of threonine and glycine protons, and protons of the CH_2 group in arginine. The third peak comprises the CH_2 groups of cysteine, cystine, methionine, aspartic acid, lysine, proline, histidine, phenylalanine, and tyrosine. The highest field peak was thought to be a fusion of two broad peaks. One of the peaks comprises the aliphatic CH groups of leucine, isoleucine, and valine, the CH_2 groups of leucine, isoleucine, proline, lysine, and arginine, and the CH_3 group of methionine. The second peak is thought to be due to the CH_3 groups of alanine, valine, leucine, isoleucine, and threonine.

Wishnia and Saunders have studied the nature of the slowly exchanging protons attached to nitrogen atoms in ribonuclease.[169,170] It was observed from the nmr spectra of ribonuclease in D_2O that 24 $\pm$ 5 protons exchanged much more slowly than the others, whereas in guanidinium ribonuclease 54 $\pm$ 7 protons exchange at a comparable rate. It was suggested that in both cases all of the slowly exchanging protons are guanidinium protons.

The nmr spectra of a number of proteins in D_2O have been obtained by Kowalsky.[171] The proteins investigated were ribonuclease, bovine serum albumin, β-lactoglobulin, pepsin, pepsinogen, chymotrypsin, chymotrypsinogen, ovalbumin, aldolase, insulin, cytochrome *c*, myoglobin, and lysozyme. The shapes of the resonance absorption peaks were shown to be characteristic of the internal mobility or flexibility of the peptide chain and the composition of the protein. The spectra of some proteins in trifluoroacetic acid have been obtained.[116] Temperature effects on the nmr spectra of ribonuclease, oxidized ribonuclease, and lysozyme have been studied.[172] The spectrum of the aromatic ring protons of ribonuclease was found to change over the temperature range 50–70°, and above 70° narrowing of some of the resonance absorption peaks was observed. Some line narrowing with increasing temperature was observed with oxidized ribonuclease.

The nmr technique has been successfully employed to distinguish between the histidine residues of ribonuclease.[173]



The chemical shifts of the C-2 and C-4 protons of L-histidine and four histidine derivatives (glycyl-L-histidine, L-histidylglycine, poly-L-histidine, and imidazole) were studied as a function of pD. A downfield shift of approximately 0.9 and 0.4 ppm was observed for the C-2 and C-4 protons, respectively, upon deuteration of the imidazole group over the pD range 1–11, in agreement with previous work.[86] The observed shifts are due to their proximity to the charged group. The nmr spectrum of DL-histidyl-DL-histidine was examined as a function of pD, and it was observed that the C-2 protons of the two imidazole groups gave a single resonance peak at low and high pD values, but in the intermediate range two and in some instances three peaks appear. The two peaks observed in the intermediate range are due to the two different magnetic environments of the C-2 protons. The third peak was felt to be due to the fact that the sample consists of a mixture of two different racemates which have slightly different spectra. The spectrum of oxidized ribonuclease in D_2O was measured as a function of pD. The spectrum at pD 10.15 showed the same characteristic peaks as had been reported previously.[146,171,174] The C-2 histidine protons were identified in the spectrum and found to undergo a downfield shift as the pD of the solution was reduced below a value of 9, and at pD 6.5 a second resonance peak appeared. The assignment of the C-2 histidine protons was also based upon the intensity of the resonance peak relative to the aromatic resonance peaks of phenylalanine, tyrosine, and C-4 histidine protons which are included in the phenylalanine and tyrosine peaks. The relative peak area measurements indicate that the four imidazole groups in oxidized ribonuclease are equivalent.

A comparison of the ribonuclease and oxidized ribonuclease spectra indicates, as observed previously,[146,171,174] that an opening of the protein structure by oxidation produces a more distinct spectrum. The C-2 protons of the four imidazole groups in ribonuclease were assigned on the basis of the agreement between the chemical shifts observed as a function of pD and those for the various model compounds studied. Below pD 4.85 a single resonance absorption was observed; however, at higher pD values a triplet was observed which collapsed again to a singlet at pD >8.2. Area measurements of the triplet indicate that two peaks of the triplet represent one histidine residue, and the third peak represents two histidine residues.

It is obvious that the use of nmr techniques in obtaining structure information concerning proteins seems to be very limited by the overlap of the chemical shifts of the large number of structurally and environmentally nonequivalent protons contained in these molecules in their native, folded conformations in neutral aqueous solutions. The study of the most pronounced resonance peaks of proteins is one alternative to the problem; however, since chemical shifts are linear functions of the applied polarizing magnetic field, the better alternative would be to investigate proteins at the highest field possible. The use of very high fields has the effect of pulling the spectrum apart, thereby exposing more of the fine structure of the spectrum. The majority of nmr studies on proteins have thus far been conducted at 40 or 60 Mcps. A typical 60-Mcps spectrum of a protein was shown in Figure 1. The nmr spectra of ribonuclease, oxidized ribonuclease, lysozyme, cytochrome *c*, and 20 of the common L-amino acids as well as representative di- and tripeptides have been investigated at 100 Mcps.[146] A correlation was made between the ribonuclease spectrum and amino acid protons. The striking difference between spectra observed with low applied fields and very high fields can be seen from a com-

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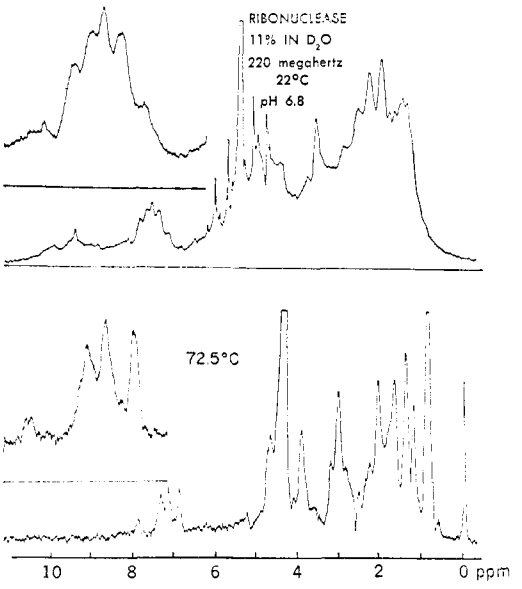

Figure 11. Pmr spectra (220 MHz) of ribonuclease in its native conformation (top) and of thermally denatured ribonuclease (bottom). Inserts are expansions of the 6.8–7.8-ppm region of the spectra.

parison between a 60-Mcps spectrum of ribonuclease (Figure 1) and that of a 220-Mcps spectrum of the same compound seen in Figure 1.[1,175] The manifestations in high-frequency (220 Mcps) pmr spectroscopy of the conformational differences that exist between the denatured and native forms of ribonuclease, lysozyme, and cytochrome *c* have been investigated.[175] As seen in Figure 11 the spectra of ribonuclease at 220 Mcps is fairly well resolved; however, improved resolution can be obtained by thermal denaturation. The spectrum of denatured ribonuclease has been fairly well analyzed.[146,171,173–177] In the denatured state it seems that the side chains of component amino acids of proteins are largely unconstrained and exhibit chemical shifts for the various structurally nonequivalent protons that do not strongly reflect the amino acid sequence of the protein. Conformational proximity effects and nearest neighbor interactions do not seem to contribute greatly in determining the chemical shifts of the side-chain protons; therefore, all residues of a given amino acid produce a spectrum of resonance quite similar to that of the free amino acid so that the nmr absorption of the denatured form of the protein can be approximately represented by a superposition of the resonances of the component amino acids, weighted by the amino acid composition of the protein. Since the nmr spectrum of the protein changes as a function of temperature because of denaturation, it would seem possible to observe protein conformational changes associated with denaturation or renaturation. It was also predicted that this approach might be sensitive enough to reflect conformational changes that may accompany the interaction of proteins with substrates, effectors, inhibitors, lipids, nucleic acids, and metal ions.

Thermal denaturation of lysozyme also gave rise to a relatively simple, sharp spectrum that was characteristic of ribonuclease. The most interesting feature in the nmr spectrum of lysozyme due to the conformational changes accompanying denaturation is observed in the extreme high-field region. The sharp high-field resonance that occurs in the denatured form is assigned to the highly shielded methyl groups of leucine, isoleucine, and valine. In the renaturation process this high-field resonance decreases in intensity accompanied by the appearance of new resonances which are not found in the spectra of component amino acids or in spectra of lysozyme or other proteins in the denatured form. These resonances seem to reflect aspects of the folded conformation of the protein. The same type high-field resonance was also observed in cytochrome *c* but only in the folded, native conformation. The unusual positions of these resonances were attributed to high-field ring current shifts that are induced in the greatly shielded C–H protons of amino acid residues whose side chains are proximal to the faces of the aromatic groups of histidine, phenylalanine, tyrosine, and tryptophan residues in the proteins' folded conformation. In the spectra of the reduced and oxidized forms of folded cytochrome *c*, an additional high-field shift was observed and is felt to be due to side chains of component amino acids that experience in large ring current associated with the highly conjugated porphyrin ring. The differences in nmr spectra between ferricytochrome *c* and ferrocytochrome *c* suggest that important conformational dissimilarities exist between the oxidized and reduced forms of the protein which are the result of differences in side-chain coordination at the heme iron. Nuclear magnetic resonance studies of cytochrome *c* have also been conducted by Kowalsky.[176]

The nmr spectra of myoglobin and hemoglobin were also investigated.[175] A very broad high-field resonance characteristic of the folded conformation was observed for native myoglobin. The broadening of this resonance peak is attributed to paramagnetic relaxation by the high-spin heme iron, although the effect could originate in differences in electronic relaxation times of iron in the reduced and oxidized forms of the protein. Conversion of myoglobin to the cyano-coordinated derivative (the low-spin form) leads to the appearance of two sharp resonances in the high-field region of the nmr spectrum. Paramagnetic substances are very effective in producing spin–lattice relaxation or accelerating transitions between nuclear spin states. This effect can be measured by nmr techniques, and in principle the method might be applied to heme proteins to obtain information about the position of the paramagnetic iron ions in these compounds, the extent of protein hydration, and exchange rates of protein-bound water molecules. A number of such studies have been conducted on ferrihemoglobin and other heme proteins.[178–183]

Transitions between the energy levels of atomic nuclei can be induced by a number of processes, some of which depend upon the relative movement of neighboring nuclei. The measurable parameters of a system obtainable for nmr spectra which relate to transition probabilities are the spin–lattice

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and spin–spin relaxation times. The effect of temperature on the spin–lattice relaxation times of aqueous solutions of ribonuclease and human serum albumin has been investigated.[184] Aqueous solutions of the proteins were subjected to periods of controlled heating and the spin–lattice relaxation times of water protons measured. The relaxation time variations observed suggest that a configurational change, related to a disordering of the protein structure, occurs which involves an increase in various modes of internal mobility. The relaxation time measurements also suggest a close, ordered protein. A study of the temperature effect on the relaxation time of the protons of ribonuclease indicated thermally induced unfolding of the compact native structure of the protein.[185] Spin–spin relaxation times of water sorbed on solid bovine serum albumin have been determined as a function of temperature and water content of the albumin samples.[186] The data obtained indicate that the sorbed water, depending upon its amount, may exist in three different states: (1) direct binding of water molecules to available polar groups on the protein; (2) the formation of a hydration shell, the structure of which is very different from that of liquid water and is determined by the protein substrate; (3) water that exists in a structure approaching that of liquid water. Proton magnetic relaxation time studies of protein hydration have been made in the ovalbumin system.[187] Several nmr studies of the water protons in keratin fibers have shown that the mobility of the water is considerably less than in the bulk liquid,[188–191] and relaxation time measurements of the protons of water in hair have shown that the water molecules exist in a continuous range of environments with a wide spread of rates of molecular rotation.[192] It has also been shown from relaxation methods that in a system, such as water absorbed on collagen, where water molecules are preferentially ordered with respect to the helical chains of a fibrous protein, the shape and width of the water proton absorption peak may give details of the structure.[193]

Relaxation time measurements may be used to study the binding sites of biologically active substances.[194] The restriction of the thermal motion of a chemical group causes the relaxation time of the nuclear spins which it contains to be shortened, and the nmr peak attributed to this particular group will be broadened. For small molecules (molecular weight ≤ 600) which have an nmr spectrum consisting of more than one peak, the ratio of line widths at half-height for any two peaks from the molecule remains unchanged by changes in the composition of the solution; therefore, nonspecific effects such as viscosity changes can be automatically corrected. Selective broadening of one or more peaks can, therefore, be interpreted in terms of specific interaction involving the chemical groups to which the peaks correspond. The stoichiometry of the binding can be determined by plotting the line width (or its inverse, the relaxation time) against the relative concentration of the interacting molecular species, the plot indicating an inflection point at the value of the relaxation time corresponding to the stoichiometric ratio of the complex formed. The mechanism of penicillin binding to serum albumin has been investigated using nuclear magnetic relaxation techniques to identify the parts of the penicillin molecule involved in the interaction.[195] The addition of serum albumin to solutions of penicillin G produced marked broadening of the resonance peaks of penicillin. As a result of the relatively large increment in the relaxation rate of the phenyl peak, it was suggested that this portion of the molecule is the binding site. Temperature, ionic strength, and inhibition effects were also studied and a model for the penicillin–albumin interaction presented. The mechanism of the binding of sulfonamides to bovine serum albumin has been investigated using relaxation rate techniques.[196,197] This technique has been used to study the nature of binding sites in other molecules.[198–200]

The structure and binding sites of enzymes have been investigated using a 100-Mcps nmr spectrometer.[201] The aromatic resonance absorption regions of the enzyme bovine pancreatic ribonuclease, staphylococcal nuclease, and hen egg-white and human lysozymes were studied and the imidazole C-2 proton resonances of the four histidine residues of ribonuclease and staphylococcal nuclease and the single histidine residues of the two lysozymes observed in the nmr spectra. The effects of pH changes and the presence of metal ions and inhibitors of enzymatic activity on each of these resonance peaks were obtained. From plots of the shifts of these peaks as a function of pH, pK values were obtained. The addition of nucleotide inhibitors to ribonuclease produced selective broadening of two of the four histidine C-2 peaks, whereas the presence of such inhibitors had no effect on the histidine regions of the staphylococcal nuclease or hen egg-white lysozyme spectra. The formation of the hen egg-white lysozyme-inhibitor did produce a sharpening of the aromatic region of the spectrum, suggesting the dissociation of enzyme aggregates. The addition of Ca^{2+} ions to staphylococcal nuclease produced a downfield shift (16 cps) in one of the histidine C-2 peaks, while the other three peaks were only slightly affected.

The enzyme–coenzyme complex formed as a result of the binding of diphosphopyridine nucleotide (DPN) to the enzyme yeast alcohol dehydrogenase (YADN) has been investigated using the selective broadening technique.[199] The data obtained provided evidence for the involvement of the pyridine ring in the interaction between DPN and the enzyme. The effect of the enzymes yeast alcohol dehydrogenase (YADH) and equine liver alcohol dehydrogenase (LADH) on the nmr spectra of oxidized nicotinamide–adenine (NAD^+), reduced nicotinamideadenine dinucleotide (NADH), ethanol, and acetaldehyde has been examined to obtain information

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about substrate binding.[202] It was observed that the resonances of the adenine C-2 and C-8 protons of NAD^+ and NADH are broadened in the presence of YADH. The nicotinamide proton resonances for NAD^+ were not noticeably broadened. The C-2 proton resonance peak of NADH was not broadened in the presence of YADH; however, the C-4 proton peak was strongly affected by either broadening or by an intensity decrease or both. LADH was found to produce an intensity decrease in the spectra of both the nicotinamide and adenine moieties of NAD^+ and NADH. The data obtained suggest a preferential interaction of YADH with the adenine moiety of NAD^+ and NADH in the absence of substrate. These results are consistent with a relatively rapid turnover of the coenzymes. The effect of LADH suggests binding of both the nicotinamide and adenine moieties to the enzyme with relatively slow turnover. Other data obtained appear to eliminate the possibility of rigid binding of substrates with rapid turnover and suggest that the presence of coenzyme is required for binding of the substrate in the rigid mode required to account for the stereospecificity of the enzymes.

Enzyme–substrate interactions have been studied using changes in proton and fluorine chemical shifts to indicate the interaction between lysozyme and acetamido sugars[203] and for interactions between chymotrypsin and N-acetyl-DL-*p*-fluorophenylalanine, N-acetyl-DL-*m*-fluorophenylalanine, and N-acetyl-D- and -L-phenylalanine.[204]

The nmr spectra of porcine and bovine insulin and of the A and B chain of bovine insulin have been obtained and examined.[205] Nuclear magnetic resonance techniques have also been employed in the study of the N terminus of the lens protein α-crystallin.[206]

2. *Metal-Ion Complexes*

A number of nmr studies have been conducted on metal ion interactions with proteins.[183,207–212] The interaction of manganous ion with bovine serum albumin gave information related to the association constants and to the structure of metal–protein complexes at the binding site.[209] The investigations of the interaction of divalent cations[210] and substrates[213] with rabbit muscle pyruvate kinase indicates an enzyme–metal–phosphoenol–pyruvate bridge structure as an intermediate in the pyruvate kinase reaction. Such an enzyme–metal linkage has been illustrated by Mg^{2+} and Mn^{2+} binding studies using epr measurements and proton relaxation rates.[210] The interaction of F^- and FPO_3^- with manganese and with the manganese–pyruvate kinase complex has been investigated by fluorine nuclear magnetic resonance and by proton relaxation rate studies to obtain evidence for the existence of a short-lived enzyme–metal–fluorophosphate bridge structure.[211] A continuation of the study of enzyme–metal–substrate bridge complexes provided direct evidence for the existence of pyruvate carboxylase–Mn^{2+}–pyruvate and pyruvate carboxylase–Mn^{2+}–α-ketobutyrate bridge complexes in solution.[212]

A "halogen ion probe" method has been developed for studying sites of metal binding in proteins.[214] The method has also been used to study the reactivity of sulfhydryl groups of hemoglobin,[215] antibody–hapten interactions,[216] and helix–coil transitions.[217]

III. Nucleic Acids

A. INTRODUCTION

While a number of different techniques have been widely used in investigations of the structure and interactions of the nucleic acids, *e.g.*, ultracentrifugation, optical rotary dispersion, and optical density measurements, none of these is capable of yielding detailed information at the molecular level. Recent applications of nmr to the study of nucleic acids described in the following chapter of this review have already not only suggested many new approaches to a variety of problems, but also yielded much important information that as yet no other single technique can provide so directly.

Although a majority of published reports have presented nmr data on the monomer units (and their derivatives) of the naturally occurring nucleic acids, considerable progress has been made in the elucidation of nmr results obtained directly from studies on synthetic polynucleotides and on intact nucleic acids, particularly since the advent of high-frequency instruments. Structural studies apart, the application of nmr spectroscopy to the investigation of monomer–monomer, and monomer–polymer interactions affords a particularly powerful method of approach to this field of molecular biology. Accordingly, in this chapter, nmr studies on monomer units and their mutual interactions will first be considered, and then applications to the investigation of intact nucleic acids and polynucleotides.

B. MONOMERIC UNITS

1. *Purines and Pyrimidines*

In aqueous solution, the nmr spectrum of purine displays three resonant frequencies, exchange preventing the observation of the N-9 proton. The high-field peak is assigned to H-8, the intermediate peak to H-2, and the low-field peak to H-6.[218–220] Earlier reports made the following assignments: H-8, H-6, H-2[221] and H-6, H-8, and H-2.[222] The latter assignment was made on the basis of charge density considerations

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at particular carbon atoms and of substituent effects. Thus the most shielded proton belongs to the C-6 atom because this atom is bonded to a carbon and a nitrogen atom, whereas both the C-2 and C-8 atoms are bonded to two nitrogen atoms. In order to distinguish between the H-2 and H-8 resonant frequencies, a comparison was made between the spectra of the free purines and their ribose derivatives in solutions of sodium peroxide and D_2O. All measurements were made at 40 Mcps, against a toluene (aromatic peak) external standard. The H-8, H-6, H-2 assignment was made on the basis of a study of N-acetylpurines, and took into consideration ring-current anisotropy differences between five- and six-membered rings. The low- and midfield peaks were assigned to C-2 and C-6 protons, respectively, and H-2 was placed below H-6 on consideration of inductive effects.

Desulfurization of 8-mercaptopurine[220] led to the assignment of the high-field peak to H-8. 8-Mercaptopurine in dioxane containing 1% D_2O was partially desulfurized with Raney nickel, and the high-field peak was shown to diminish by 47%. An exchange reaction, 50% complete at the end of 45 min when purine was heated at 105° in D_2O, took place. At the end of 4 hr, the high-field peak completely disappeared, and the other two were unaffected.

In an analogous experiment with 6-mercaptopurine, the relative area of the low-field peak decreased by 47% on desulfurization. Under desulfurization conditions some exchange of purine hydrogen took place: the H-8 signal decreased by about 20% and that of the H-6 by about 10%. Little exchange was observed in pure dioxane, even though the catalyst contained an excess of 80% deuterium.

It should be emphasized that these results were obtained for spectra measured in neutral solution, whereas the earlier reports concerned measurements in chloroform (H-8, H-6, H-2) and in basic aqueous media (H-6, H-8, H-2). However, the results reported for acetylated purine[221] are readily interpretable in the light of later peak assignments. The chemical shifts observed when purine is acetylated at N-9 are, working downfield, −18.0, −7.1, and −5.3 cps. The corresponding shifts for the N-7 acetylated purine are, therefore, −16.3, 12.3, and −19.8, cps, in accord with the H-8, H-2, and H-6 protons, respectively.

In a comparison of the nmr spectra of purine and 2- and 9-deuteriopurines,[219] convincing corroboration of the latter results was obtained.

Results of an nmr study comparing the spectral assignments of purine, 6-deuteriopurine, and 8-deuteriopurine in basic, neutral, and acidic media are given in Table XIV.

Chemical shifts are measured relative to hexamethyldisiloxane external standard. Methanol is added to the deuterium solution of 6-chloropurine for solubility reasons.

The C-8 proton of purine appears at high field under all conditions of acidity, in contrast to the behavior of the same proton in 6-substituted purines. Here a "cross-over" effect is noted, and the C-8 proton appears at low field in acidic solutions. In nucleosides, ribose exhibits a deshielding effect, and the C-8 proton appears at low field even in neutral solutions.

The pyrimidine moieties appear to be protonated at N-1. However, observed shifts of the C-8 protons suggest some delocalization of charge into the imidazole ring in acid solution, accounted for by considering the cation as a linear combination of the resonance structures in which the N-1 and N-7 become positively charged. To account for the experimentally observed differences in behavior of the 6-substituted purines compared to purine in acidic media, changes in the π-electron density at the carbon to which the observed proton is bound are invoked. Linear correlations between charge density and chemical shifts have been made.[223–226] In purines the inductive effect of the positively charged nitrogen atom in the ring structure causes such changes, and a contribution from the third resonance structure, *e.g.*, for adenine, in which the amino nitrogen atom becomes positively charged, could cause further delocalization of the charge from C-2, which would lead then to a smaller downfield shift at this position compared to that of the C-8 position.

Table XIV

pH Effect on the Chemical Shifts of Some Purines[218]

Compound	*Medium*	*C-8*	*C-2*	*C-6*
Purine	3 *M* NaOD	522	542	554
	D_2O	527	545	555
	0.6 *M* D_2SO_4	563	579	589
6-Chloropurine	D_2O (40% MeOH)	525	531	
	0.6 *M* D_2SO_4	568	549	
Adenine	0.3 *M* NaOD	503	512	
	0.6 *M* D_2SO_4	532	527	
Adenosine	D_2O	504	498	
	0.6 *M* D_2O	533	527	

Nmr techniques have been used to study the behavior of purine in an acid medium.[227] Results of such studies, at three different concentrations of purine as a function of pH in the range pH 0–8, are given in Table XV.

An essentially parallel dependence of the chemical shift of each proton with pH is apparent.[228] In addition, shifts become progressively less dependent on concentration as the pH decreases, and below about pH 2.5, the C-2 and the C-6 proton resonances exhibit a splitting due to the coupling constant $J_{2,6}$. Within the margin of experimental error, a plot of $J_{2,6}$ *vs.* the chemical shift of C-6 is linear.[227,229]

The relationship between the chemical shift and pH can be expressed in terms of a simple equilibrium: $BH^+ \rightleftarrows B + H^+$. If the dissociation constant is K_a, and if f is the fraction of the base protonated, then the relationship between pK_a and f is given by

$$f = [1 + \text{antilog}\,(\text{pH} - \text{p}K_a)]^{-1} \tag{25}$$

and the experimental value of the chemical shift v_i can then be expressed by

$$v_i = fv_i^+ + (1 - f)v_i^0 \tag{26}$$

given v_i^0 and v_i^+ are respectively the corresponding shifts for B^+ and BH^+.

A plot of v_i *vs.* f should be linear, and by extrapolation to zero concentration, a value of pK_a is obtained. A plot of v_i values at three concentrations yields a pK_a of 2.75,[227] which

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Table XV[a]

Chemical Shifts of Aqueous Purine as a Function of Concentration and pH

pH	*C-6*	*C-2*	*C-8*	*Concn, M*
0.04	−169	−156	−141	2.63
1.09	−162	−150	−134	
1.53	−155	−143	−128	
2.03	−139	−128	−115	
2.30	−128	−119	−107	
2.82	−111	−104	−93	
4.03	−100	−95	−85	
5.60	−100	−95	−85	
8.01	−97	−93	−83	
				0.531
0.12	−170	−159	−144	
1.20	−166	−155	−139	
1.56	−164	−153	−137	
2.03	−154	−143	−127	
2.47	−143	−133	−111	
2.98	−127	−199	−104	
4.00	−120	−112	−97	
5.71	−119	−112	−97	
8.03	−117	−109	−94	
				0.205
0.13	−170	−159	−144	
1.13	−168	−157	−141	
1.50	−166	−155	−139	
2.03	−160	−149	−133	
2.52	−151	−140	−123	
2.92	−140	−130	−112	
3.98	−131	−122	−105	
5.80	−129	−120	−103	
8.00	−129	−120	−102	

[a] Frequencies are given in cps relative to external benzene.

compares reasonably well with other reported values of 2.39 and 2.52.[230,231]

In trifluoroacetic acid (TFA) solution, spin–spin coupling has been observed between the 2 and 6 protons of purine.[232] In dimethyl sulfoxide (DMSO) solution, the proton resonances assigned to C-2 and C-8 protons of purine, observed at a sweep rate of 0.1 cps/sec, were broad compared to the H-6 signal, suggesting a quadrupole broadening effect due to the adjacent nitrogen atoms at positions 2 and 8. Since in TFA, where H-2 and H-6 appear as an AB pair, with ν_0 ($\delta_6 - \delta_2$) = 22.63 ± 0.05 cps and $J_{2,6}$ = 1.05 ± 0.05 cps, aqueous solutions were examined for evidence of spin–spin coupling; in TFA the purine is most probably protonated at N-1.[233] Nmr spectra of purine in water were measured between pH 6 and 0, and at pH 2.4, when *ca.* half the solute molecules would be protonated, given $pK_{a'}$ = 2.39 and pK_{a2} = 8.59 in water at 20°, splitting is indicated by a slight broadening of the H-2 and H-6 proton resonance signals, while below pH 0.5, splitting is clearly apparent. This result confirms and extends the nmr data referred to above.[227]

In addition, at pH 0.28, the H-6 signal was further split, caused by coupling with H-8, by about 0.3 cps. These assignments were made by measuring the spectra of purine-6-*d* and purine-8-*d*. Nitrogen quadrupole broadening of the resonances of H-2 and H-8 makes the detection of possible long-range coupling between these centers difficult.

A linear correlation between the chemical shift of the proton in the 8 position of 2,6-disubstituted purines and the sum of the σ constants[234] for the 2- and 6-position substituents has been reported.[232] This is given by

$$\delta_{H\text{-}8} = (8.658 \pm 0.017) + (0.342 \pm 0.016)\sum_{2,6}\sigma_p^+ \quad (27)$$

A correlation coefficient of r = 0.984 and a standard deviation in $\delta_{H\text{-}8}$ of ±0.066 ppm was observed for the 17 compounds studied, using DMSO as solvent. However, it was found that the chemical shifts of purines containing amino and diethylamino groups fitted the equation less well. A similar effect was noticed in comparative results obtained from TFA solutions of 6-disubstituted purines, including the two amino-containing compounds.

Again using Brown's electrophilic substituent constants, a linear correlation with the H-8 proton was reported in an examination of the spectra obtained from eight 6-substituted purines.[232] Equation 28 shows a correlation coefficient,

$$\delta_{H\text{-}8} = (8.653 \pm 0.013) + (0.359 \pm 0.019)\sigma_6^+ \quad (28)$$

r = 0.991, and a standard deviation of ±0.035 ppm. The largest deviation was given by 6-iodopurine, and a similar shielding effect was observed for 2,6-dibromopurine. However, 2-chloro-, 6-chloro-, and 2,6-dichloropurines were slightly less shielded than predicted. It is concluded that the resonance effect outweighs the inductive effect of the larger more polarizable halogens substituted in a strongly electrophilic ring system, *e.g.*, that of purine, since 2,6-dibromo substitution appears to be electron donating. Table XVI summarizes the chemical shifts observed in DMSO. It is apparent that on this basis the $\delta_{H\text{-}8}$ for 2-, 6-, and 2,6-substituted purines can be predicted to within 5% of the 1.25-ppm variation observed in the 27 compounds studied.

The effect of protonation on chemical shifts seems to suggest that all the basic sites in purine are involved to some extent. Since the observed shifts on protonation for H-2, H-6, and H-8 are −39, −41, and −41 cps, respectively, exclusive protonation at N-1 is difficult to justify. Nevertheless, precise interpretation of such results is difficult.[227] A consideration of the ^{13}C–H couplings probably provides a less ambiguous method of approach to this problem. The additivity relation of Malinowski (eq 29), where α and β

$$J_{CH_i} = \sum \zeta_{\alpha\beta} \quad (29)$$

designate the atoms α and β to carbon, is widely applicable, and values of ζ are known for many aromatic heterocycles.[172,235,236] Assuming values of ζ for five- and for six-membered rings, calculated couplings are found to be in close agreement with those experimentally observed, *viz.*, J_{CH_6} = 187.5 (187.4) cps, J_{CH_2} = 206.0 (207.0) cps, and J_{CH_8} = 211.0 (213.5) cps, where the experimental value is in parentheses. To include the species BH^+ in these calculations,

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Table XVI

Chemical Shifts of the 8 Proton of 2-, 6-, and 2,6-Disubstituted Purines in DMSO

Substituent 2	Substituent 6	σ_{p2}^+	σ_{p6}^+	$\sum_{2,6}\sigma_p^+$	$\sigma_{H\text{-}8}$, [a] ppm	d [b]
NMe_2	NH_2	−1.7	−1.3	−3.0	7.72	0.11
NH_2	NH_2	−1.3	−1.3	−2.6	7.76	0.01
NH_2	SMe	−1.3	−0.604	−1.9	7.93	−0.06
SMe	NMe_2	−0.604	−1.7	−2.3	7.97	0.11
NH_2	Me	−1.3	−0.311	−1.6	7.98	−0.12
SMe	NH_2	−0.604	−1.3	−1.9	8.02	0.03
F	NMe_2	−0.073	−1.7	−1.8	8.03	−0.01
H	NMe_2	0.000	−1.7	−1.7	8.07	0.00
NH_2	H	−1.3	0.000	−1.3	8.08	−0.12
Cl	NH_2	0.114	−1.3	−1.2	8.13	−0.11
H	NH_2	0.000	−1.3	−1.3	8.14	−0.06
F	NH_2	−0.073	−1.3	−1.4	8.16	−0.02
SMe	SMe	−0.604	−0.604	−1.208	8.32	0.08
H	OMe	0.000	−0.778	−0.778	8.38	−0.01
Cl	OMe	0.114	−0.778	−0.664	8.45	0.03
H	SMe	0.000	−0.604	−0.604	8.47	0.02
H	Me	0.000	−0.311	−0.311	8.53	−0.02
Et	Cl	−0.295	0.114	−0.181	8.58	−0.01
H	I	0.000	0.135	0.135	8.63	−0.07
Cl	Me	0.114	−0.311	−0.197	8.64	0.05
H	H	0.000	0.000	0.000	8.68	0.03
Br	Br	0.150	0.150	0.300	8.70	−0.06
H	Cl	0.000	0.114	0.114	8.71	0.02
Cl	H	0.114	0.000	0.114	8.76	0.07
Cl	Cl	0.114	0.114	0.228	8.77	0.04
H	CN	0.000	0.659	0.659	8.91	0.03
CF_3	Cl	0.612	0.114	0.726	8.97	0.06

[a] In ppm. [b] $d = \delta_{H\text{-}8}$(obsd) − $\delta_{H\text{-}8}$(calcd).

if the fraction of time a proton spends at N-1, N-2, and on the imidazole ring (N-7, N-9) are denoted by a, b, and c, respectively, and θ is the change produced in ζ_{NC} by complete protonation, changes in J_{CH} on protonation can be estimated by solving the following equations for a, b, and c.

$$a + b + c = 1 \tag{30}$$

$$a\theta = \Delta J_{CH_6} = 9.0 \text{ cps} \tag{31}$$

$$(a + b)\theta = \Delta J_{CH_2} = 12.1 \text{ cps} \tag{32}$$

$$c\theta = \Delta J_{CH_5} = 5.0 \text{ cps} \tag{33}$$

The per cent protonation at each site considered is N-1, 54 ± 12%; N-3, 17 ± 12%; imidazole, 29 ± 7%. Experimental data for ^{13}C–H coupling constants are given in Table XVII.

A number of nmr studies on naturally occurring pyrimidines and their corresponding nucleosides have been reported[237–241] and pyrimidine chemical shifts, substituent effects, and tautomeric structure discussed in detail.[242] The proton resonance assignments of unsubstituted pyrimidines

Table XVII

^{13}C–H Coupling Constants for d_3-Purine (2.77 M) in D_2O [a]

pD	$J(^{13}C)$ H-6	$J(^{13}C)$ H-2	$J(^{13}C)$ H-8
0.15	196.6	219.1	218.5
1.09	196.1	218.8	217.3
2.72	191.4	211.5	215.2
3.22	188.8	208.6	213.8
5.63	187.4	207.0	213.5

[a] In cps at 60 Mcps from external cyclohexane.

are straightforward, in that the magnetic equivalence of H-4 and H-6 are predictable from the symmetry of the molecule.[241] It is evident that $w_2 < w_4 = w_6 < w_5$, so (in chloroform at 40 Mcps) H-2 occurs at −370.4 cps, H-4 and H-6 occur at −351.0 cps, and H-5 occurs at −294.4 cps relative to TMS. The coupling constant $J_{4,6}$ can be obtained by inspection of the ^{13}C lines associated with H-4, and the $J_{4,5}$ (5 cps) and $J_{2,5}$ (1.5 cps) values are in accord with analogous observations made on aromatic systems, although the value is somewhat larger than the *para* coupling in substituted benzenes.[243] Assuming the linear dependence of the ^{13}C–H coupling constants on the s-electron density of the C atom involved,[244] it is proposed that the use of ^{13}C–H couplings is a more reliable index of s charge density and orbital character (C–H) in the pyrimidine ring than is apparent from the corresponding chemical shifts.[241] While for pyrimidine itself the relationship between the ^{13}C–H coupling constants and chemical shifts is not exactly linear, it has been found that a plot of the methyl shifts of the 2-methyl-, 4-methyl-, and 5-methylpyrimidines *vs.* the ^{13}C–H coupling constants yields a satisfactory straight line. The inductive withdrawal of charge from the methyl group is in the order C_5, C_4, C_2.

It has been suggested that cytosine has a zwitterionic structure,[238] but subsequent studies of uv and nmr spectra have shown this to be incorrect.[240,245] An early report[246] attributed the imino structure to deoxycytidine, the relevant nmr spectra later being shown to be those of the hydrochloride.[247,248]

In an nmr investigation of the proton mobility in substituted uracils,[248] an attempt was made to relate chemical shift with other molecular properties. A plot of H-6 chemical shifts in uracils *vs.* group moments for seven substituents (NO_2, COOH, Br, F, I, H, CH_3) at the C-5 position showed that all the experimental points except that of COOH fell close to a straight line. The group dipole moments were taken from ref 249 and 250.

A similar observation, of a plot of the N-3 and N-1 protons *vs.* group moments, was made.[238] The COOH group point, probably because of hydrogen-bonding properties and acidity of the group, again displayed no linear correlation. The points corresponding to Br, F, and I appeared as a cluster, but the F point became increasingly nonlinear, the best fit being obtained when plotted against the H-6 chemical

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shift, the least satisfactory against the N-1. It is suggested that this is because F is a more effective electron donor than the other halogens examined in the conjugated system formed by the lone pair of electrons at F and N and the C_5–C_6 double bond. Thus the N-1 proton would be expected to be considerably displaced to high field in the 5-fluorouracil derivative relative to 5-bromouracil and 5-iodouracil.

A plot of the two N–H proton shifts *vs.* the Hammett σ constants for substituents in the 5 position (CH_3, H, F, I, Br, COOH, NO_2) gave a linear plot, the experimental point for the COOH group again excepted.[238]

An nmr study of 14 2-substituted, six 5-substituted, five 4-substituted, and five disubstituted pyrimidines has been reported.[242] 2-Substituted pyrimidines exhibit an essentially simple spectrum: a doublet (H-4 and H-6) and 1:2:1 triplet corresponding to H-5. The doublet lines are generally broader than that of the C-5 proton, attributable to a moderately rapid quadrupole relaxation of the nitrogen nuclei, and to coupling between the hydrogen atoms at positions 6 and 4. Long-range coupling between the 2-methyl group (J = 0.55 cps) and H-5 of 2-methylpyrimidine is shown by the fine structure apparent in the H-5 signal.[241,242] The line widths in the spectrum of 2-hydroxypyrimidine (a potentially tautomeric compound) are sensitive to the presence of small amounts of acid or base, and are generally broader than those of other 2-substituted pyrimidines, in pure solution. However, in aqueous solution, the C-4 and C-6 proton signal is a well-resolved doublet in the presence of traces of hydrogen chloride.

The spectra of 5-substituted pyrimidines are less complex than those of their 2-substituted analogs, consisting of two bands of unequal intensity. The coupling $J_{2,4}$ of less than 0.6 cps gives rise to three unresolved lines in the H-2 band and two lines in the H-4, H-6 band. In addition, the H-2 signal is broadened because of coupling with both ring nitrogen atoms, and the H-4 and H-6 signals are less strongly coupled to their respective *para* nitrogens than their *ortho* nitrogens.

Because of unresolved long-range couplings to methyl group protons, the ring hydrogen signals in the spectrum of 5-methylpyrimidine are unusually broad, and long-range couplings between H-6, H-2, and H-4 and the methyl group cause the latter to become further split, and to have the appearance of a 1:3:3:1 quartet.

Since the spectra of the potentially tautomeric 5-hydroxypyrimidines have the same appearance as other 5-substituted pyrimidines, the existence of a stable zwitterionic form is ruled out. Some of these results are summarized in Table XVIII.[251]

An nmr study of a number of 5-unsubstituted and 5-halogenopyrimidines in solvents of varying acidity has been reported.[251] In 5-bromopyrimidine, the presence of bromine, causing an upfield shift of H-2 and downfield shifts of the H-4 and H-6 signals, is also manifested by a downfield shift of the methyl resonance in the analogous compound with a methyl group substituted in the 6 position. The C-5 chloro compound exhibits similar shift changes. However, no correlation between the deshielding effect on the C-6 methyl proton resonances and the particular halogen was possible. While the inductive effect of the halogen is a probable cause of the observed downfield shifts, it is also necessary to invoke magnetic moment anisotropy and steric effect factors.[251,252]

Table XVIII

Chemical Shifts (ppm) of Substituted Pyrimidines Relative to the Hydrogens of Pyrimidine

	Solvent: acetone		*Solvent: cyclohexane*	
Substituent	δ_5	$\delta_{4,6}$	δ_5	$\delta_{4,6}$
2-CN	−0.38	−0.24		
2-CO_2CH_3	−0.21	−0.19		
2-SCN	−0.07	−0.04	0.03	0.07
2-I	−0.09	0.22	0.03	0.32
2-Br	−0.09	0.08	0.02	0.22
2-Cl	−0.07	0.02	0.02	0.13
2-CH_3	0.25	0.12	0.24	0.11
2-SCH_3	0.34	0.19	0.36	0.27
2-OCH_3	0.41	0.20	0.38	0.24
2-NH_2	0.92	0.53		
2-$NHCH_3$	0.96	0.52	0.82	0.45
2-$N(CH_3)_2$	1.00	0.49	0.86	0.46
1-H, 2-O (2-OH)	1.08	0.47		
	δ_2	$\delta_{4,6}$	δ_2	$\delta_{4,6}$
5-CN	−0.27	−0.46	0.17	−0.05
5-Br	0.02	−0.16	0.21	0.15
5-CH_3	0.18	0.15	0.21	0.15
5-OCH_3	0.39	0.30	0.44	0.35
5-OH	0.48	0.41		

The spectra of 5-chloro- and 5-bromo-2,4-dimethoxy-6-methylpyrimidines in carbon tetrachloride exhibit two nonequivalent methoxy proton resonances, separated by 0.1 ppm, an effect not observed in the spectra of 2,4-dimethoxy-6-methylpyrimidine. The chemical shifts of the nonequivalent methoxy resonances are apparently independent of halogen change.

Increasing acidity narrowed the C-2 proton resonance in 4-hydroxy-6-methylpyrimidine: in neutral solvents the nitrogen quadrupole effect broadens this signal considerably. A similar effect in the spectra of 2-methylthio-6-methylpyrimidine for the C-4 and C-5 proton resonances was observed. The $J_{4,5}$ coupling constant (4.8 cps) in carbon tetrachloride is increased on protonation in acetic acid (5.7 cps), and in TFA (6.1 cps). This contrasts with a report[22] that in olefinic compounds of the type CH =CHX the vicinal proton couplings are reduced by electronegative substituents according to the relationships

$$J_{trans} = 19 - 3.3\Delta E \quad (34)$$

$$J_{cis} = 11.7 - 4.0\Delta E \quad (35)$$

where ΔE is the difference in electronegativity between X and the hydrogen atom replaced. In accord with some delocalization of charge through a protonated nitrogen atom, the resonance peaks of the nuclear protons exhibit a marked downfield shift with increasing acidity. This effect is particularly evident at C-2 and has been previously noted for α and β hydrogens in pyridine[253] and in 4-substituted pyridine[254] and for the H-4 resonance of 2-substituted thiazole.[255] The acid dependence of the shift of the signal from the C-6 methyl substituted in the pyrimidine ring has been noted in

(251) T. Nishiwaki, *Tetrahedron*, 22, 2657 (1967).

(252) C. Haigh, M. Palmer, and B. Semple, *J. Chem. Soc.*, 6004 (1965).
(253) V. Gil and J. Murrell, *Trans. Faraday Soc.*, 60, 248 (1964).
(254) A. R. Katritzky and J. Lagowsky *J. Chem. Soc.*, 43 (1961).
(255) G. Clarke and D. Williams, *ibid.*, 4597 (1965).

observations of the spectra of picoline, lutidine, and quinaldine.[256]

The nonequivalent methyl signals shown by 2,6-dimethyl-4-hydroxypyrimidine in deuterated chloroform are shifted downfield in acetic acid or TFA. The larger separation of the two bands (0.17 to 0.34 ppm) in acid solution may be due to the increased influence of the protonated nitrogen atom felt by the C-2 methyl group, which accordingly is assigned to the low-field resonance in view of the spectral assignments made for H-2 and H-4 of 5-bromopyrimidine.[242]

In TFA, 2,6-dimethyl-4-hydroxypyrimidine exhibits a coupling constant ($J_{5,6}$ = 0.5 cps) considerably higher than that reported for 6-methylpyrimidine[241] ($J_{6Me,5}$ = 0.5 cps) determined from the ^{13}C–H coupling. In an acidic solvent, the spectrum of 2-methylthio-6-methylpyrimidine indicates a similar effect.

The methoxy resonance of 2,4-dimethoxy-6-methylpyrimidine appearing as a singlet in carbon tetrachloride (3.83 ppm) or acetic acid (4.00 ppm) in TFA gives rise to two distinct bands (4.33 and 4.27 ppm). The methyl group appears as a doublet when the spectrum is recorded in TFA at a reduced sweep rate, each signal exhibiting an additional splitting suggestive of coupling to the –NH arising from protonation. In acetic acid, this splitting is not so marked.

The downfield shift with increasing acidity is shown by N-methyl groups substituted in pyrimidines.

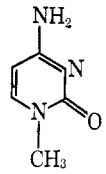
A concentration dependence of the C–H signals of 4-hydroxypyrimidines over the range 3–20% solution in deuterated chloroform, accompanied by a downfield shift, suggests a hydrogen-bonding interaction.[251] This is summarized in Table XIX.

Table XIX

Effect of Concentration on the Nmr Spectra of 4-Hydroxypyrimidines in $CDCl_3$ at 25°

Compound	Concn, %	Chemical shifts 2H	5H	Me	SEt
4-Hydroxy-6-methylpyrimidine	1	8.12	6.31	2.35	
	3	8.13	6.33	2.33	
	5	8.13	6.33	2.33	
2,6-Dimethyl-4-hydroxypyrimidine	3		6.15	2.30, 2.47	
	5		6.15	2.29, 2.46	
	7		6.15	2.29, 2.46	
	10		6.15	2.29, 2.46	
	15		6.14	2.29, 2.45	
	20		6.15	2.30, 2.48	
4-Hydroxy-2-ethylthiopyrimidine	3	4H 7.88	6.23		1.39, 3.22
	5	4H 7.88	6.23		1.36, 3.20
	10	4H 7.90	6.27		1.41, 3.22
	15	4H 7.90	6.26		1.41, 3.22
	20	4H 7.88	6.24		1.38, 3.20

Several nmr studies of cytosine and its nucleosides have been reported.[168,245,248,257,258] In a preliminary report, 1-methylcytosine has been shown to be protonated at N-3 and to have the following tautomeric structure in DMSO.[259]



In Tables XX and XXI, some chemical shifts of cytosine derivatives in DMSO and sulfur dioxide are compared.

The spectra of 1-methylcytosine and 1-methyl-5-^{15}N-cytosine in DMSO show doublets at 5.67 and 7.62 ppm, assigned to the H-5 and H-6 protons, respectively, and coupled by *ca.* 7 cps. Methyl substitution in the related compound uracil was the basis of the literature assignment of the higher field doublet to H-5 and the lower field doublet to H-6,[222] but coupling between N-1 and H-6 provides firmer evidence.[260] The 1-methyl protons exhibit a sharp line at 3.23 ppm. At 7.00 ppm, a broad line with an area equivalent to two protons is split into a 90-cps doublet by ^{15}N substitution into the amino group not only establishing the assignment of the amino protons, but also proving the tautomeric structure has the amino form.

1-Methylcytosine hydrochloride and 1-methyl-7-^{15}N hydrochloride SO_2 spectra show three broad peaks due to the NH protons in the ^{14}N compound, each with an area equivalent to one proton. The nonequivalence of the two amino protons[240,258] arises from the partial double-bond character of the C_4N_7 bond. The ^{15}N compound exhibits two resonances at 7.42 and 7.82 ppm, split into doublets by 92.6 and 91.4 cps, respectively, readily assignable to the amino protons. In DMSO solution only two lines for the latter are seen. 1-Methylcytosine in SO_2 exhibits a 7-cps splitting of the H-5 line due to coupling with H-6, which is further split by 2.5 cps by the proton giving rise to the 11.43-ppm peak, confirmed by decoupling. That this peak is due to H-3 is confirmed by the ^{15}N results. The 2.5-cps coupling also eliminates the possibility of protonation occurring at the oxygen.[259]

In DMSO solution, in the spectrum of the ^{15}N analog, the H-5 resonance is further split by 0.7 cps by coupling with the ^{15}N confirming the higher field doublet assignment to H-5.

1-Methylcytosine hydriodide proton resonances are analogous to those of the hydrochloride, but the NH signal is markedly shifted. This is in accord with the suggestion that cytidine hydrochloride exists in ion pairs in solvents like DMSO.[258]

To make an unambiguous assignment of the amino resonances of the hydriodide, it is insufficient to assume that the hydrochloride signals occur in the same relative order, and so by analogy conclude a correspondence. Accordingly, an equimolecular mixture of the hydriodide and hydrochloride was examined in SO_2 solution. Peaks occurring exactly midway between those of either species alone were observed, confirming that the two low-field and the two high-field assignments corresponded (Table XX).

The spectra of substituted cytosine salts vary considerably with temperature change. The H-3 resonance[239] broadens with increasing temperature, and the resonances due to H-7 broaden and merge with concomitant frequency shifts in SO_2. In DMSO the resonances arising from H-5 and H-6 *cis* and *trans* isomers also merge. In principle such data can be

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(257) J. P. Kokko J. H. Goldstein, and L. Mandell *J. Am. Chem. Soc.*, **83**, 2909 (1961).
(258) O. Jardetzky· P. Pappas, and N. G. Wade, **85**, 1657 (1963).
(259) H. T. Miles R. B. Bradley, and E. D. Becker, *Science*, **142**, 1569 (1963).
(260) J. P. Kokko, Ph.D. Dissertation, Emory University, 1964

Table XX

Chemical Shifts for Cytosine Derivatives in SO_2 at −60°

Compound	Chemical shifts, ppm						
	1-CH_3	3-CH_3	7-CH_3	5-H	6-H	3-H	7-H
1-Methylcytosine hydrochloride (**1**)	3.55			6.28	7.82	11.44	7.45, 7.83
1-Methylcytosine-7-^{15}N hydrochloride (**2**)	3.55			6.27	7.82	11.42	7.42, 7.82
1-Methylcytosine hydroiodide (**2**)	3.58			6.33	7.87	11.02	7.28, 7.45
1,3-Dimethylcytosine hydrochloride	3.58	3.65		6.42	7.80		7.25, 8.00
1,3-Dimethylcytosine hydriodide	3.58	3.67		6.37	7.82		7.00, 7.82
1,7-Dimethylcytosine hydrochloride	3.54		3.23	6.38	7.70	11.09	8.65
	3.52		3.18	6.52	7.90	11.54	8.03
1,7-Dimethylcytosine-7-^{15}N hydrochloride	3.54		3.25	6.35	7.68	11.00	8.60
	3.52		3.18	6.25	7.88	11.50	8.03
1,7-Dimethylcytosine hydriodide	3.58		3.33	6.35	7.77	11.50	7.50
	3.58		3.25	6.35	7.97	10.92	7.23
1:1 mixture of **1** and **2**	3.58			6.32	7.85	11.22	7.35, 7.65

Table XXI

Chemical Shifts for Cytosine Derivatives in DMSO-d_6 at 23°

Compound	Chemical shifts, ppm						
	1-CH_3	3-CH_3	7-CH_3	5-H	6-H	3-H	7-H
1-Methylcytosine	3.23			5.67	7.62		7.00
1-Methylcytosine-7-^{15}N	3.23			5.67	7.63		7.02
1-Methylcytosine hydrochloride	3.35			6.18	8.12		8.85, 10.00
1-Methylcytosine-7-^{15}N hydrochloride	3.35			6.18	8.10		8.84, 9.45
1,3-Dimethylcytosine	3.20	3.18		5.70	7.02		7.35
1,3-Dimethylcytosine hydriodide	3.40	3.43		6.13	8.05		9.34
1,7-Dimethylcytosine-7-^{15}N	3.27		2.80	5.75	7.62		7.50
1,7-Dimethylcytosine hydrochloride	3.40		3.05	6.40	8.32		9.54
	3.37		3.02	6.35	8.02		11.02
1,7-Dimethylcytosine-7-^{15}N hydrochloride	3.42			6.40	8.34		
	3.38		3.02	6.33	8.02		11.00
1,7-Dimethylcytosine hydriodide	3.37		2.97	6.03	7.97	10.89	9.87
	3.37		2.97	6.33	8.25		8.39
1,7-Dimethylcytosine-7-^{15}N hydriodide	3.40		3.01	6.10	8.02		9.94
	3.40		3.01	6.38	8.30		
Deoxycytidine				5.80	7.85		7.32
Deoxycytidine hydrochloride				6.25	8.30		8.82, 9.94
5-Methyldeoxycytidine					7.68		6.67, 7.80
5-Iododeoxycytidine					8.31		6.91, 7.37

interpreted in terms of exchange rates and the activation energy for internal rotation and can lead to estimations of the ratios of geometrical isomers present at different temperatures.

In general, protonation causes a downfield shift of the amino hydrogen resonances, which is in turn affected by the nature of the solvent.

In a pmr and ^{15}N magnetic resonance study of several pyrimidine derivatives, 1-methylcytosine-$^{15}N_3$ has been shown[261] to protonate at N-3, in agreement with the work described above. 2,4-Dimethoxypyrimidine-$^{15}N_2$, 1-methyl-4-methoxy-2-pyrimidine-$^{15}N_2$, 1-methylcytosine-$^{15}N_3$, uracil-$^{15}N_2$, and 2,4-dichloropyrimidine-$^{15}N_2$ exhibited long-range ^{15}N–H couplings, a particularly large coupling (12.5 cps) being noted for 2,4-dichloropyrimidine-$^{15}N_3$. An unambiguous synthesis is given in Figure 12.

2. *Nucleosides and Nucleotides*

That the association of bases and nucleosides in neutral aqueous solution is an energetically favorable process has been established by osmotic studies.[262,263] It was apparent that the interactions between nucleosides and purine bases were more favorable than the corresponding cross-interactions

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(263) P. O. P. Ts'O and S. I. Chan, *ibid.*, 86, 4176 (1964)

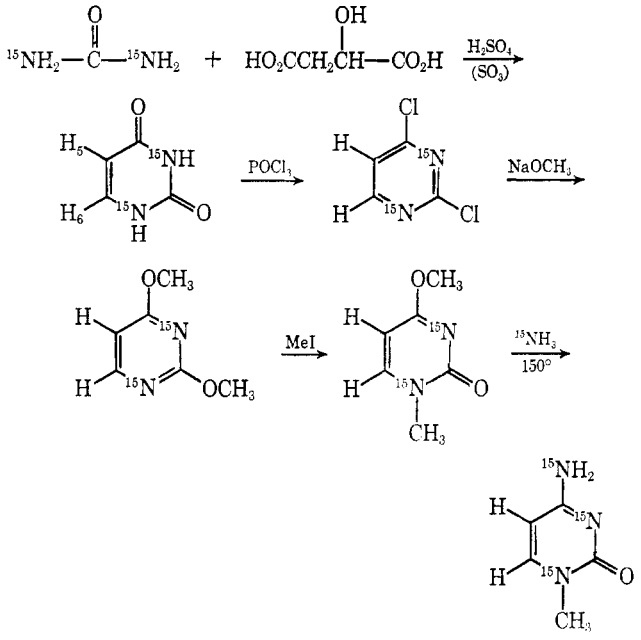

Figure 12. Synthesis of 1-methylcytosine-$^{15}N_3$.

between pyrimidine bases and nucleosides and purine. Least favored interactions observed were between pyrimidine bases and nucleosides themselves. These results have been confirmed and extended by nmr studies.[237,264,265]

To elucidate the nature of the complex formed, the interaction between purine and 6-methylpurine in aqueous solution was examined by nmr as a function of concentration over the range 0.05–1.0 *M*.[228]

While downfield dilution shifts for aromatic systems are well known and are attributed to the magnetic anisotropy of neighboring molecule ring currents,[38] similar proximity effects for purine ring systems are of an order of magnitude greater and are interpreted in terms of a molecular association. For example, the dilution shift observed for dioxane in azulene is 1 ppm for the five-membered ring protons and 0.25 ppm for the seven-membered ring protons for a difference of 20 to 3 mole % solutions.[106] The corresponding dilution effect for purine from *ca.* 2 mole % solution to infinite dilution is 0.60–0.70 ppm for protons of the pyrimidine ring, and rather less for the proton on the imidazole ring.

Both purine and 6-methylpurine exhibit parallel concentration dependencies with respect to the protons on the pyrimidine ring, while the imidazole proton shift deviates at low concentration.

It was shown that the concentration shift at 50° was *ca.* 50–60% that at 25° which is entirely consistent with the idea of molecular association. In nonaqueous solvents (DMSO and DMF (dimethylformamide)), the proton resonances were essentially independent of concentration over the range 0.20–1.00 *M*. However, there is some evidence that hydrogen bonding between nucleosides can occur[266,267] in DMSO. However, DMSO and DMF are hydrogen-bond acceptors and interact with purine with the result that solute molecules cannot associate as readily as in neutral aqueous solutions. Additions of 50% water to DMSO solutions results in concentration shifts of the purine ring protons similar to those observed in water alone. Hydrogen bonding is unlikely to play a part in purine–purine interactions in aqueous solution since the chemical shifts would be in the opposite direction.[222,246] However, it has been pointed out that concentration-dependent chemical shift data alone cannot rule out a combination of both effects.[237] Similar concentration-dependent upfield shifts have been observed for the ring protons of *para*-substituted toluenes in benzene[268] and are interpreted in terms of π complexing. However, since the concentration shifts of protons undergoing π complexing would be expected to be dependent on their relative acidities, the observed parallel dependence of the pyrimidine ring protons of purine on concentrations eliminates this possibility. Accordingly, the type of molecular association proposed is one of vertical stacking, with the ring axis somewhat displaced.[228]

An nmr study of the association in aqueous solution of purine nucleosides has shown that hydrogen bonding is not involved.[265] In accord with previous work on purine, 6-methylpurine, and pyrimidine nucleosides,[228,262–264] association through a mechanism of vertical stacking is shown to operate even more favorably than in the case of purine alone. Vapor-pressure osmometry studies substantiate this conclusion. Association occurs even when the potential sites for hydrogen bonding have been methylated, *e.g.*, 1-methylinosine and N-6-dimethyladenosine. Upfield shifts of the base protons, in particular H-2, those of the methyl group on the bases, and the anomeric proton H-1′, increase progressively as the concentration of the solute increases for each of the eleven compounds examined. The protons furthest away from the base, *e.g.*, H-5′ and H-5″, do not show a concentration-dependent shift. The H-2 proton chemical shift of purine nucleosides was found to decrease in the following order: ribosylpurine, ribosyl-6-chloropurine, 1-methylinosine, 6-thioinosine, adenosine, N-6-methyladenosine, inosine, N-6-2′-methyldeoxyadenosine 2′-O-methyladenosine, 2′-deoxyadenosine, 3′-deoxyadenosine, N-6-dimethyladenosine, ribosyl-2,6-diaminopurine, and 1-methylguanosine.

From Table XXII it is clear that the concentration-dependent chemical shift for H-2′ is greater than that observed for H-8. Also the anomeric shift, H-1′, is larger in six instances, but similar in magnitude for the remaining nucleosides. In view of the results obtained for purine and 6-methylpurine, a preferred orientation (average) of the nucleoside bases in vertical stacks appears best to account for the experimental data, because the H-2 proton will be shielded strongly by the six-membered ring of the neighboring bases most of the time, and also the H-8 proton and H-1′ proton will be shielded mainly by the five-membered ring. Additionally, the latter protons will spend less time shielded by the five-membered ring relative to the time spent by the H-2 proton in the proximity of the six-membered ring. Given that the thermal energy kT is of the same order as the standard free-energy of association, the breaking up and the re-forming of molecule stacks must occur rapidly, since no significant line broadening or splitting was observed. In addition the base type in the nucleoside influences to some extent the preferred orientation of the

(264) M. P. Schweitzer, S. I. Chan, and P. O. P. Ts'O, *J. Am. Chem. Soc.*, 87, 5241 (1965).

(265) A. D. Broom, M. P. Schweitzer, and P. O. P. Ts'O, *ibid.*, 89, 3612 (1967).

(266) L. Katz and S. Penman, *J. Mol. Biol.*, 15, 220 (1966).

(267) R. R. Shoup, H. T. Miles, and E. D. Becker, *Biochem. Biophys. Res. Commun.*, 23, 194 (1966).

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stacked molecules, as an inspection of Table XXII shows. For example, the concentration-dependent chemical shift of the H-2 proton in the dimethylnucleoside is smaller than that of the monomethylnucleoside.

Table XXII

Concentration Dependence of Chemical Shifts for 11 Purine Nucleosides (0.0–0.2 *M*) in D_2O

Compound	Temp, °C	Δδ, cps H-2	H-8	H-1′
Inosine	32	6.4	5.3	7.1
1-Methylinosine	33	8.9	6.4	6.8
Ribosylpurine	30	10.7	6.4	8.8
Purine	25–27	12.6	9.6	
2′-O-Methyladenosine	31	13.7	7.5	8.8
6-Methylpurine	25–27	19.4	13.3	
2′-Deoxyadenosine	30	19.8	13.0	13.6
N-6-Methyl-2′-deoxyadenosine	32	26.0	15.8	14.0
N-6-Dimethyladenosine	28	27.2	14.5	14.4
N-6-Methyladenosine	26	32.6	17.5	12.6
2′-Deoxyadenosine	30	14.8	10.0	9.8
Adenosine	32	14.8	8.3	6.9
3′-Deoxyadenosine	25	15.8	9.0	9.6

The existence of hydrogen bonding between the 2′-OH group of the pentose and the N-3 atom of the purine ring is suggested by a downfield shift of several cycles per second of the ring proton resonances of adenine ribonucleosides compared to the corresponding adenine-2′-deoxy- or 2-substituted nucleosides.

Uv absorption spectroscopy[269] and ir spectroscopy[270,271] data support the concept of intramolecular hydrogen bonding and may account for the differences in conformation between polydeoxyribonucleotides and polyribonucleotides as observed by ORD.[272] By the same criteria, 3′-deoxyadenosine does not appear to undergo intramolecular hydrogen bonding involving the 2′-OH group, which appears to have different properties in these two compounds. Adenosine, for example, has a lower pK_a of hydroxyl ionization than does 3′-deoxyadenosine.[273]

Of the 6-substituted nucleosides, only 1-methylinosine exhibits a chemical shift of H-2 lower than that of H-8 and is also lower by 8.5 cps in comparison to the same proton resonance of inosine. However, the H-8 proton resonance of 7-methylguanosine is shifted downfield by more than 1 ppm when compared to the corresponding shift exhibited by the H-8 proton of 1-methylguanosine, due to quaternization of the imidazole ring upon methylation of the 7 position. Therefore, compared to inosine itself, the substantial and selective deshielding of H-2 in 1-methylinosine is in accord with an extensive degree of quaternization occurring at the N-1 position.

The coupling constants of the furanose protons H-1′ and H-2′ are shown to be slightly concentration dependent: the value of $J_{1',2'}$(adenosine) is 6.1–6.4 cps and that of 3′-deoxyadenosine 2.1–2.3 cps. It has previously been concluded[274] that the ribose residue in adenosine probably exists in the $C_{2'}$-*endo* configuration, and earlier reports of the nmr spectrum of 3′-deoxyadenosine[275,276] made no mention of the small value of $J_{1,2'}$ compared to that of adenosine. Broom and coworkers propose that some of the difficulties in applying the Karplus relationship,[52] including substituent perturbations and considerations of C-1′–C-2′ bond lengths, are minimized[277,278] in making comparisons between adenosine and 3′-deoxyadenosine. In consequence, the dihedral angle $\theta_{1',2'}$ of 3′-deoxyadenosine will be smaller than that of adenosine, such that C-2′ is only slightly puckered out of the plane in the furanose ring.

When the 6′ carbon is in the *endo* position, from stereochemical considerations, hydrogen-bond formation between the 2′-OH group and the N-3 atom is favored, in agreement with the observation that intramolecular hydrogen bonding was observed for adenosine and not for 3′-deoxyadenosine.

The concentration dependence of chemical shifts for cytidine, thymidine, and uridine protons has been studied.[264] The magnitude of the observed shifts is of roughly the same order as might be expected from the diamagnetic susceptibility of the bulk solutions. Similarly, monitoring the proton shifts of thymidine as a function of cytidine concentration revealed only slight shielding effects, again probably due to magnetic bulk susceptibility increases. Thus in comparison to similar studies[228,237] on purine bases, the concentration shifts for the pyrimidine nucleosides studied are negligible. It is suggested that the pyrimidine nucleosides do not support ring currents as do aromatic purine bases.[264] However, cytidine and uridine have both been shown to associate in aqueous solution by osmotic pressure lowering.[263] The effect of purine on the proton resonances of thymidine, uridine, and cytidine has been studied.[264] Upfield concentration shifts are observed particularly for the thymine base protons and the anomeric proton signals. There is, however, a progressive decrease in the concentration effect proceeding around the deoxyribose ring from C-1′ to C-5′. This suggests that the purine–pyrimidine nucleoside interaction localizes at the pyrimidine base of the nucleoside in accord with a vertical stacking of the purine and pyrimidine bases. In general, the concentration shifts are 50–60% those observed for the protons of purine and 6-methylpurine under similar conditions.

The complementary base-pairing of nucleosides and nucleoside derivatives has been observed in nonaqueous solution.[266] A large downfield shift at the resonant frequency of the donor protons implicates hydrogen bonding[88] between specific bases and provides nmr support for the Watson and Crick base-pairing model.[279] Similar evidence has been adduced for mixtures of simply substituted purines and pyrimidines.[267] This is in contrast to the studies in aqueous systems described above which have shown that the principle phenomenon observed is a stacking interaction between bases, shown by a small temperature- and concentration-dependent upfield shift

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in the nonexchangeable ring proton resonances.[228,237,264,265] For example, the C-8 ring proton and the ribose proton C-1′ of guanosine in aqueous solution show this shift. In DMSO[266] the same protons show no change with concentration, and accordingly stacking interactions can be considered negligible in this solvent, in concentrations up to 0.5 *M*. A slight downfield shift of the N-1 and of the amino proton resonance is considered indicative of a small degree of self-association. Hydrogen bonding between nucleoside nitrogen protons and DMSO would be expected, and in a temperature-dependence study[266] a large upfield shift of the N-1 and the amino proton resonances of 9-ethyladenine and 9-ethylguanine (0.25 *M* in DMSO) is shown as the temperature increases. Hydrogen-bond formation is disrupted with increasing temperature.[71] The protons not expected to be involved in hydrogen bonding (C-2 and the ethyl methylene) showed no change with temperature.

A dramatic downfield shift of the hydrogen-bonding protons is observed in the system guanosine–cytidine–DMSO, which increases as the ratio of cytidine to guanosine increases, shown in Figure 13. The apparently linear dependence of the downfield shift suggests a 1:1 complex. No such effect is observed with adenosine– or uridine–guanosine mixtures, and cytidine interacts only with guanosine.

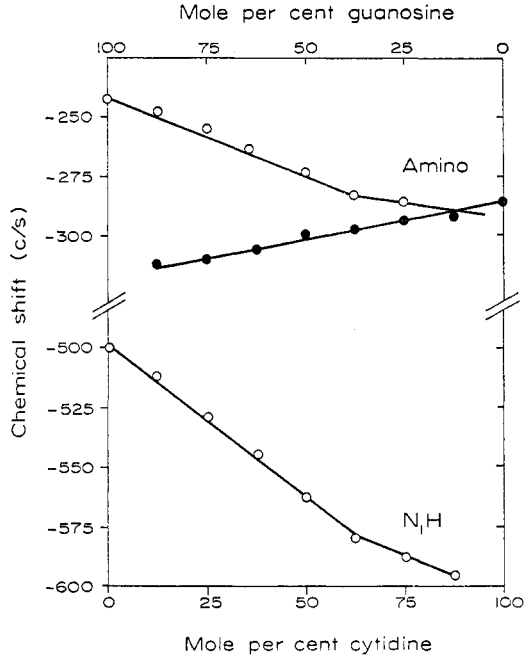

Figure 13. Chemical shift of the hydrogen-bonding protons of guanosine (○) and cytidine (●) in mixtures of the two species in DMSO. The measurements were made at 16° on solutions containing a total nucleoside concentration of 0.5 *M*.

No interaction between adenosine and uridine is observed in DMSO[266] which is in accord with the comparatively weaker hydrogen-bonding interaction involved[280] and with solvent competition.

In view of an ir study[281] in which characteristic hydrogen-bonding absorption bands were reported for mixture of 9-ethyladenine and 1-cyclouracil in chloroform, the same system has been examined by an nmr technique.[266] A large downfield shift of the N-3 uracil proton signal was observed in the presence of adenine, whereas the adenine amino proton showed a smaller downfield shift. In the absence of the complementary base, it is concluded from dilution studies that considerable self-association of the bases occurs in chloroform, but that the effects of interspecies association predominate.

A deviation from linearity of the adenine proton resonance at high uracil to adenine ratios suggests that the association is not a 1:1 complex, since the data are consistent with a quadratic dependence upon concentration. An A + 2U structure has been postulated.[282,283]

To effect a comparison between the guanine–cytosine and the adenine–uracil association under conditions where these were measurable and all the bases soluble, an equal volume mixture of benzene and DMSO has been used.[266] These results are summarized in Table XXIII.

Table XXIII

Shifts in the Resonant Frequency of the N_1 Proton of Guanosine and the N_3 Proton of Uridine upon the Addition of Other Nucleosides[a]

Nucleoside added	*Shift of N_1 proton, cps*
Guanosine	
Adenosine	−0.1
Guanosine	−7.1
Cytidine	−134.7
Uridine	−1.2
Uridine	
Nucleoside added	*Shift of N_3 proton, cps*
Adenosine	−8.2
Guanosine	0
Cytidine	−0.6
Uridine	0

[a] The solvent was an equal volume mixture of dimethyl sulfoxide and benzene. The chemical shifts were measured at 60 Mcps and −4°. The dimethyl sulfoxide resonance was used as an internal standard. The N_1 proton of guanosine and the N_3 proton of uridine were measured at a base concentration of 0.05 *M*. Then additional nucleosides were added to bring the total molarity to 0.2 *M* and the proton frequency measured again. The table gives the added compound and the net shift between the two measurements. Because of broadening due to the ^{14}N quadrupole moment, the accuracy of the relative shift measurement was limited to about ±1.0 cps.[266]

The cytosine–guanine complex has been studied at temperatures down to −10° using a 1:1 mixture of DMSO-d_6 and DMF-d_7.[267] Methylcytosine, labeled at N-7 with ^{15}N, permitted the clear observation of the C–NH_2 signal which, while broad at room temperature, was split into two peaks at lower temperatures owing to hindered rotation. The interaction shift of the cytosine protons is halved upon changing the guanosine:cytosine molar ratio from 1:1 to 1:2, demonstrating a sufficiently rapid interchange between complexes and uncomplexed bases to permit averaging of the chemical shifts. A mixture of 1-methylcytosine and 9-ethylguanine

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(Figure 14) showed downfield shifts of the thymine nitrogen proton, but virtually no shift was observed for the adenine amino protons. Comparatively larger shifts observed for the 1-methylthymine and 2-aminoadenosine mixtures are in accord with the formation of three interbase hydrogen bonds. If the noninteracting bases are mixed in pairs, the observed chemical shifts are the same as those measured under the same conditions on the individual components; hence no interaction occurs. The ^{15}N spin–spin coupling effect, shown by both the free and the complexed base, rules out the possibility of a change in tautomeric form on base pairing.

Nmr studies of the concentration dependence of relaxation times in aqueous solutions of nucleic acid derivatives confirms the tendency toward self-association.[237]

When Cu(II) binds to a ligand, the protons near the binding sites are rapidly relaxed by the paramagnetic ions. In the case of amino acids and peptides, this is shown by a broadening of the corresponding nmr lines.[101] Direct evidence of copper binding to nucleotide bases has been obtained by nmr studies.[284] In addition, the method indicates which sites on the bases are involved. The spectrum of dAMP in D_2O reveals that on addition of copper (2×10^{-5} or 1×10^{-4} *M*) the lines corresponding to H-2 and H-8 are broadened, the latter to a considerably greater extent. For dAMP and other nucleotides, the sugar proton lines are little influenced by the addition of Cu(II). These results confirm earlier suggestions that the site of attachment is N-7.[285–287]

In the D_2O spectrum of dGMP there is only one base proton (H-8) giving rise to a resonance peak broadened in the presence of copper ions, which therefore bind to the N-7 site.[284] While chelation would cause loss of a proton through participation of the C-6 oxygen atom, with concomitant displacement of the negative charge to the oxygen atom, the nmr data show that copper complexing does remove the N-1 proton in neutral solutions. The effect of Cu(II) on the H-2 of dAMP could be explained by a weaker binding to N-1 or N-3.

Binding to N-1 or the amino group of dCMP could explain the broadening of both H-4 and to a greater extent H-5 resonance signals on the addition of copper. The nmr spectra of dTMP are unaffected by Cu(II), contraindicating complex formation.

Complementary studies in DMSO-d_6[284] were carried out using deoxyribonucleosides in place of deoxyribonucleotides, since the latter are not very soluble in DMSO-d_6. The NH and NH_2 peaks are visible under these conditions. On copper complexing, generally similar nmr data were obtained, indicating that binding behavior in DMSO-d_6 was like that previously observed in water. The NH and NH_2 lines were relatively unaffected by copper complexing in each of the four nucleosides examined (dAMP, dCTP, dTMP, and dGMP). This confirms earlier proposals that copper does not bind to the deoxyadenosine group.[286,287] In deoxycytidine the N-1 site is implicated in copper binding, and in deoxyguanosine the broadening of the H-8 resonance confirms that the binding site is N-7. The deoxythymidine proton resonances are

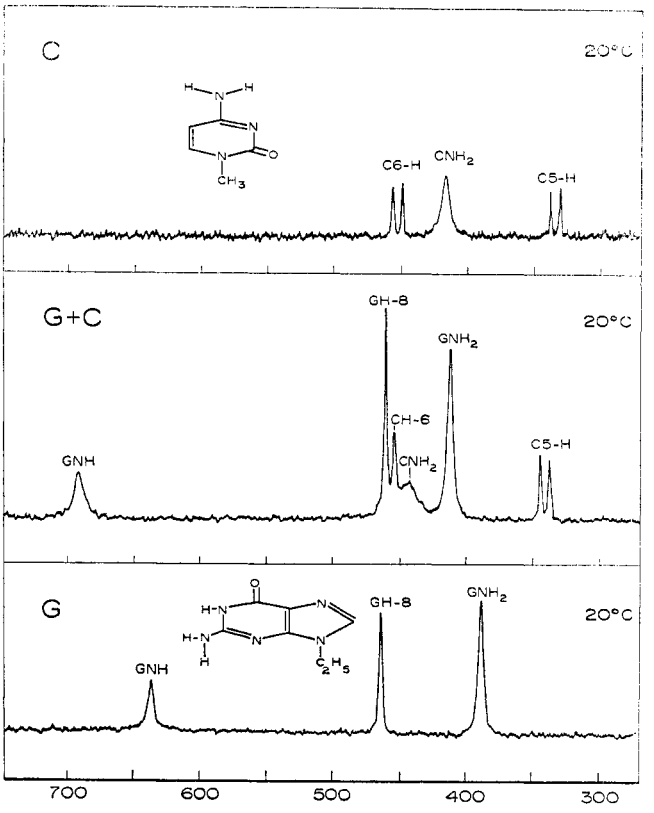

Figure 14. Nmr spectra of 9-ethylguanine (G) and 1-methylcytosine (C) in DMSO-d_6. The spectra were measured with a Varian A-60 spectrometer. Each spectrum was calibrated by means of audiofrequency side bands. The abscissa is given in cps as downfield shifts from an internal TMS standard.

again relatively unaffected. ^{31}P spectra of the deoxyribonucleotides of adenine and thymine show considerable broadening of the phosphate resonances in the presence of Cu(II), in accord with earlier reports of copper binding to the phosphate moieties.[285,288]

The three resonance peaks of purine (0.1 *M*) are shifted downfield in the presence of added $ZnCl_2$ (0.01 *M*) by 2.0, 2.7, and 3.7 cps for the H-2, H-6, and H-8 proton resonances, respectively, indicating that the preferred binding site is N-7.[289] On addition of $CuCl_2$ (0.1 *M*) to purine (0.1 *M*) in DMSO, the line broadening observed was greatest for the H-6 resonance, somewhat less for the H-8 resonance. The H-2 resonance is relatively unaffected. The preferred binding site in purine for both Cu(II) and Zn(II) is therefore N-7.

It is of interest to calculate the formation constants of the metal complexes. For a 1:1 complex, the following equation can be shown to apply.[289]

$$\frac{1}{\nu - \nu_f} = \frac{1}{\nu_c - \nu_f} + \frac{1 + B_0K}{K(\nu_c - \nu_f)} \frac{1}{M_0} \qquad (36)$$

ν_f and ν_c are the characteristic frequencies of the free and complexed molecule, respectively, B_0 is the initial concentration of the base, (MB) is the equilibrium concentration of the metal complex, and K is the equilibrium constant of the reaction M + B = MB. M_0 is the initial concentration of the metal ion. The values of K and $(\nu_c - \nu_f)$ can be calculated

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from a linear plot of $1/(\nu - \nu_t)$ *vs.* $1/M_0$ where ν is the observed frequency.

Taking ν_t as 560 cps, the frequency of H-8, for a 0.1 *M* solution of purine in DMSO at 36°, $ZnCl_2$ addition between 0.1 and 0.5 *M* gave a linear plot from which *K* was calculated to be 4.41 $mole^{-1}$, and $\nu_0 - \nu_t$, 15.8 cps. Computations of (MB) and *K* were performed using an IBM 1620 computer assuming different values of ν_0. The *K* value of the $Zn(purine)^{2+}$ complex became constant at 5.2 ± 0.5 cps only when ν_0 = 529.5 ± 0.5 cps, in very fair agreement with the graphically obtained results.

The addition of $ZnCl_2$ to a 0.1 *M* solution of cytosine in DMSO causes the H-5 and the H-6 signals to be shifted downfield to an equal extent. $CuCl_2$ (10^{-3} *M*) addition to 0.1 *M* purine in DMSO causes a slightly greater line broadening for the H-5 than for the H-6 signal. Thus the N-3 site is preferred for both Zn and Cu(II) ions binding in cytosine. The NH_2 line is relatively unaffected by Cu(II) ions.[284] A computation similar to that described for purine was performed assuming ν_t = 439.7 cps and by varying the value of ν_0. *K* for the $Zn(cytosine)^{2+}$ complex became relatively constant at 34.3 ± 3.0 cps in the concentration range 0.100–0.84 *M* $ZnCl_2$, when ν_0 is assumed to be 457.5–458.0 cps.

A long-range coupling effect of the 5-fluoro group on the C-1′ proton has been shown for 1-β-D-arabinofuranosyl-5-fluorocytosine, a cancer chemotherapeutic agent.[290] This effect in the 5-fluoropyrimidine series is also shown by 1-β-D-arabinofuranosyl-5-fluorouracil by the appearance of a pair of doublets for the anomeric hydrogen in place of the expected doublet. This coupling is visible in the spectrum of the 2,3,5-tri-*O*-acetate of 5-fluorouracil. Another long-range coupling in α-5-fluoro-2-deoxyuridine, where the anomeric proton appears as a split triplet, and as a pair of doublets in the β anomer, has been noted.[291]

Acetylated nucleosides do not provide reliable nmr data for the determination of the configuration of the sugar moiety.[292]

Magnetic shielding of the phosphorus nucleus by electrons gives rise to the chemical shifts observed in ^{31}P nmr spectroscopy, and changes in the importance of ionic and double-bonded structures may be responsible for the shifts between homologous compounds.[293] In a recent study of cyclic phosphate and phosphite triesters[294] it was observed that nucleoside cyclic phosphates have chemical shifts 3–4 ppm lower than the mean value of a group of five-membered phosphate esters. This was attributed to inductive effects of the furanose ring or to the pyrimidine ring. That five-membered cyclic esters show less electron shielding of the phosphorus nucleus is consistent with a diminution in the $d\pi$–$p\pi$ double-bond character of the cyclic P–O bonds of the five-membered ring.

When an alkoxy group is replaced by an aryloxy group, a change to more positive values of the phosphate chemical shift is observed. This is consistent with an enhancement of $d\pi$–$p\pi$ overlap by conjugation with the aromatic system. In agreement with the deduction that bonding is diminished in the five-membered cyclic phosphates is the observation that the increment in the chemical shifts of the six-membered phosphates is twice that of the five-membered analogs. It is concluded that the nmr approach can provide direct experimental verification of the hypothesis[295] that the $d_{x^2-y^2}$ orbitals are responsible for strong π bonding in phosphates, and that by matching available p orbitals of O–(C) ligands with these d orbitals the bond orders of the P–O(–C) bonds in phosphate diesters can be determined.

The 100-Mcps spectra of three dinucleotides, ApGp, CpGp, and UpGp, in D_2O solution have been described.[296] The H-8 guanine signal of ApGp at −8.05 ppm overlaps with the absorption due either to the H-2 or the H-8 proton of the adenine base. The corresponding signals of CpGp and UpGp appear at −7.93 and *ca.* 8.2 ppm and partially obscure one another. Unlike the H-8 signal of the guanine moiety which exhibits a marked broadening in the order ApGp > CpGp > UpGp, the signals assigned to the 5′ terminal nucleotide narrow in the same order. The narrowing of absorption signals could be due to a cleavage of the stacked conformation, leading to a breakdown of the dipole–dipole interaction between neighboring bases, suggesting increased rotational motion of the 5′ terminal base. The broadening of the H-8 proton resonance in the order given can be plausibly explained in terms of hindered rotation about the bond between the N-9 of the 3′ terminal guanine and the anomeric carbon in the dinucleotide, an effect which decreases as the stacked conformation becomes more favored. Whereas the H-6 proton signal of uracil in UpGp is quite sharp, indicating free rotation about the glycosidic linkage, rotation about the same linkage of the guanine base is strongly restricted, indicated by $\Delta\nu_{1/2}$ = 54 cps.

In contrast, ApGp, where a strong base–base interaction operates, rotation is completely restricted. CpGp appears to be an intermediate case.

Since thymidine dinucleotide phosphate[297] and polyU[138] both show sharp proton signals, the hindered rotation of the 3′ base residue in the unstacked dinucleotide UpGp underlines the importance of nucleotide sequence and suggests caution in applying line broadening of nmr signals observed under different conditions for naturally occurring and synthetic polymers to simple physical explanations.

In studies on the mechanism of action of chloramphenicol,[298] nmr data are presented that strongly suggest that the most probable conformation of this antibiotic closely resembles that of a pyrimidine ribonucleotide not only in size, but also in the orientation of individual moieties and in the distribution of electronegative groups. In accord with extensive data[299–303] published on chloramphenicol analogs, the

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Table XXIV[237]

Chemical Shifts in the Spectra of Polynucleotides and Their Monomers[a]

	Chemical shift, cps[b]							
	H-8	*H-2*	*H-6*	*H-5*	*H-1'*	H_2O	*H-5'*	Acetone
PolyU			−76.4	+36.9	+41.0	+111.4	+142.5	+262.0
UMP			−91.0	+23.5	+28.5	+109.4	+154.5	+261.2
Polymerization shift			+14.6	+13.5	+12.5	...	−12.0	...
PolyC			−71.7	+47.1	+56.3	+110.7	+132.7	+251.3
CMP			−89.8	+23.7	+34.2	+111.1	+164.7	+261.0
Polymerization shift			+18.1	+23.4	+22.1	...	−32.0	...
PolyA	−74.1	−67.8			+61.8	+112.2	+127.7	+261.4
AMP	−117.6	−91.6			+40.1	+111.4	+159.5	+261.1
Polymerization shift	+43.5	+23.8			+21.7	...	31.8	...

[a] Concentration 0.2 *M*, pH 7.0. [b] Referred to benzene as an external standard.

loss of biological activity following alteration of the propanol residue can most probably be understood in terms of loss of similarity to the ribose ring. Since the L-(+)-*threo* and -*erythro* isomer series cannot form nucleotide-like conformations, the lack of activity of these compounds follows, given the initial assumption, as does the inactivity of *para*-substituted chloramphenicol compounds which cannot serve as hydrogen-bond acceptors.

The conformation of puromycin has been discussed,[304] and the ribose shifts compared to those of other adenine derivatives. Applications of nmr to pharmacological research generally have been described.[305]

Catecholamine–adenine nucleotide interactions have been examined by nmr spectroscopy.[200] It was found, for example, that at pH 5.6 three molecules of epinephrine interact with one molecule of ATP, and one molecule of epinephrine interacts with one molecule of AMP, although an additional catecholamine molecule may interact at high pH values. The proposed structure of the epinephrine–AMP complex involves an ionic bond between the catecholamine nitrogen and the phosphate moiety of the nucleotide, and a hydrogen bond between the β-hydroxyl group of the catecholamine and a phosphate oxygen.

C. POLYNUCLEOTIDES

Comparison of the nmr spectra of polyU and polyA with the spectra of the corresponding monomers shows that almost equal resolution obtains in both instances. Furthermore, the areas of corresponding peaks in spectra obtained from equal monomer and polymer concentrations are very nearly equal, indicating that such spectra represent residues from the entire polymer and not merely isolated regions.[237] This means that the correlation times of all the bases in the polymer are sufficiently short to average the dipole–dipole interactions.[198,306,307] From a consideration of the observed relaxation times, it is concluded that the bases in the polymer can undergo internal rotation with a time constant of the order of 10^{-9} sec.

In Table XXIV, polymerization shifts are shown, clearly indicating a mutual shielding of the bases; *i.e.*, each base spends an appreciable time in the diamagnetic region of another base.

Temperature-dependent downfield shifts of 15–25 cps in the proton resonances of polyA have been observed in D_2O solution as the temperature increased from 25 to 60°.[308] This suggests a partial ordering of the polyA bases at room temperature with a concomitant high-field shift being reversed by heating as a more random-coil configuration is assumed. The spectral line widths decrease at the same time. Analysis of similarly obtained polyI and polyG spectra showed line sharpening with temperature increase, but no corresponding chemical shifts.

Only the HDO resonance was observed when a solution containing equal concentrations of polyA and polyU in D_2O at pH 7.0 was examined.[283] However, low-field resonances are observed corresponding to those of single-stranded polyA and polyU when the temperature is raised to 35°. The formation of poly(A + U) and poly(A + 2U)[40,309] can be quantitatively followed from the intensity of the nmr spectral lines.[283] "Melting" curves of a solution of polyA and polyU were plotted expressing the averaged observed spectral intensities as a fraction of the expected intensities of polyA and polyU assuming random-coil configuration. The complexing and "melting" were shown to be completely reversible, the melting temperature being raised by the addition of an electrolyte (NaCl). An initial "melting" at 36.5° corresponding to *ca.* 20% of the ultimate spectral intensities suggested the presence of a lower molecular weight fraction.

Solutions containing various ratios of polyA and polyU were examined as a function of temperature. Results plotted as fractions of the expected intensities assuming no complexing showed (a) no appreciable complexing occurs above 65°, and (b) at 25° poly(A + U) forms when the mole fraction of polyU is less than 0.5 and poly(A + 2U) forms when this is greater than 0.66. Calf thymus DNA similarly examined showed that individual resonances underwent considerable sharpening with temperature increase throughout the melting range. It is suggested that discrete structural regions in individual DNA double strands could account for this observation.[283]

Spectra of polyU in D_2O obtained at 31 and 72° exhibited resonance signals only slightly broader than those of the

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(309) A. R. Rich and D. R. Davies, *J. Am. Chem. Soc.*, **78**, 3548 (1956).

monomer.[188] In these measurements 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt was used as an internal reference, since it is water soluble and has the same chemical shift as TMS. The H-6 peak (δ 8.1 ppm) and the combined peak due to the ribose H-1′ and the H-5′ proton resonance areas do not change with temperature, indicating that there is no helical or other ordered structure. An examination of the spectra of polyA (pH 6.1) at 32 and 78° showed that the peak areas at the higher temperature are significantly larger and indicates considerable ordered structure. By this criterion, lowering the pH to 4.5 decreases the ordered structure.[188] However, the disordered fraction is dependent on the polymer concentration and the conditions used in lowering the pH.[810] The system polyA and polyU in D_2O undergoes a sharp temperature-dependent transition. At 45° only the reference and the solvent peaks are visible, whereas at 54° the ribose and aromatic peaks are clearly visible (δ 8 and 6). If the temperature is raised further, the peak areas continue to increase since the polyA chain possesses some order even after the complete dissociation of the polyA–polyU complex.

Analogous experiments indicate, within the precision of the method, that polyI is completely ordered at 31° whereas polyC possesses only a slight degree of order under the same conditions.

The line width of the water signal in solution of transfer RNA is considerably broadened, an effect noted[141,811] for DNA in comparison with spectra obtained from synthetic polynucleotides. Similar broadening of the ribose and base resonances is also noted.[188,812]

D. RIBONUCLEIC AND DEOXYRIBONUCLEIC ACIDS

In some early work, nmr data were presented[813] which tended to confirm the hypothesis that when DNA is dissolved in water, an increased ordering of the water structure results, with concomitant heightened viscosity and dielectric constant of the DNA solutions.[814] In repeating this work later, no broadening of the water peak which could not be accounted for by ordinary viscosity effects was noted.[815]

In a study of collagen hydration, the value of using molecularly orientated samples for nmr studies on biological macromolecules was demonstrated.[193] It has been shown that the diple–dipole interactions responsible for the very broad lines in the nmr spectra of solids should in theory be averaged out if the sample is rotated around an axis inclined $\cos^2\theta = {}^1/_3$ to the direction of the static magnetic field, *i.e.*, giving $\theta = 54.7°$.[816] In a preliminary study, NaDNA, films were wet spun[817] and a signal band occurring at 14.8 Mcps showed a strong angular dependence. A plot of the inverse peak-to-peak amplitude of the recorded signal derivative as a function of the angle between the direction of the molecular orientation and the field exhibited a minimum at *ca.* 55°. In another study, of the hydration of silk fibroin, keratin, and salmon sperm, some water signals were found to show an angular dependence.[818]

The spectra of RNA in deuterated aqueous pyrophosphate buffer have been studied as a function of pH[819] and measurements made between pH 4 and 11 shown to provide a means of estimating fractional helical content. Proton signal assignments were made on the basis of a comparison with known assignments for nucleic acid components. An "apparent area" (S_n) increase, in the region of absorption of the non-exchangeable protons at C-2 and C-8 of the purine bases, and C-4 of the pyrimidines, of the RNA absorption peaks was noted in going from a neutral to an environment of higher pH. S_n should be proportional to the concentration in g/1000 ml (n) of the polymer, but inversely proportional to the mean molecular weight (M_n). The number of protons per M_n is N_n. If the corresponding quantities for an appropriate reference compound such as a mononucleotide are S_r, r, M_r, and N_r, then the following equation is obtained.

$$S_n/S_r = (n/r)(N_n/N_r)(M_r/M_n) \tag{37}$$

or

$$N_n/M_n = (r/n)(N_r/M_r)(S_n/S_r) \tag{38}$$

A plot of N_n/M_n *vs.* pH at 35° shows that for pH 4–8, N_n/M_n has a constant value of 0.0020, and thereafter increases sharply to pH 11. A value of 0.0040 for N_n/M_n can be calculated if it is assumed that all the protons in RNA had given rise to detectable signals. The low values of N_n/M_n observed for yeast RNA are not due to exchange of the aromatic protons under the conditions employed, and are therefore ascribed to complete nonresolution of certain protons, in line with observations on solids, viscous liquids, and polymers.[138] The secondary structure implied by intramolecular hydrogen bonding, *i.e.*, G-C, A-U,[820] should give rise to a restriction of motion of some segments of the nucleic acid chain. As a result, averaging out of local fields by protons located within these ordered segments is not possible. The variations with pH can therefore be interpreted as a result of cleavage of the intramolecular hydrogen bridging due to ionization of amide protons in the nucleic acid backbone, with concomitant increase in the rotational and diffusional motion of the molecules concerned.

If x is the value of N_n/M_n for RNA molecules with completely random secondary structure, and y is the corresponding value for molecules with maximum secondary structure due to base pairing, then an estimate of the fractional helical content can be made. *E.g.*, if x and y are assumed to be 0.0040 and 0.0000, respectively, then substituting in $100(x - 0.0200)/(x - y)\%$, where 0.0020 is the observed value at pH 7.0, and in $100(x - 0.0200)/(x - y)\%$, where 0.0028 is the observed value at pH 11, the yeast RNA in question can be estimated to contain *ca.* 50% helical structure at pH 7.0 and only 30% helical structure at pH 11.

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A method for the determination of nearest neighbor base frequency ratios in DNA by nmr at 220 Mcps has been reported.[321] Two well-resolved thymine methyl resonances exhibited by single-stranded DNA in neutral aqueous solution are attributed to the presence of two nonequivalent thymine methyl environments. Examination of the spectra of thymine containing 3′–5′ linked deoxyribonucleoside monophosphate dimers (TpT, d-TpC, d-TpA, d-TpG, d-CpT, d-ApT, and d-GpT) in neutral D_2O solution at 20, 50, and 90° showed that all thymine-containing dimers including TpT gave only one T–CH_3 resonance, occurring from 1.81 to 1.84 at 20° and from 1.86 to 1.89 ppm at 90° relative to DSS where thymine is in the 5′-neighbor position. Similar results were noted where thymine has a 5′-neighbor pyrimidine. When, however, the thymine has a purine neighbor, the T–CH_3 resonances are shifted to higher field values at 20°, an effect which is decreased with higher temperatures. *E.g.*, at 20, 50, and 90° d-ApT exhibits T–CH_3 shifts of 1.61, 1.71, and 1.78 and d-GpT 1.68, 1.75, and 1.81 ppm, respectively. The temperature shift is in accord with the observation that purines and pyrimidines exhibit a stacking interaction at low temperatures in neutral aqueous solutions.

Thus the high-field shifts of the T–CH_3 resonances of d-APT and d-GpT can be ascribed to nonrandom stacking of the purine and pyrimidine bases in which the methyl group lying within the ring field current of the purine is the favored configuration. Since the resonance signal observed is narrow, fast exchange between the stacked and randomized configurations seems to be likely.

The spectrum of single-stranded calf thymus DNA at 90° exhibits five distinct spectral regions (see Figures 15 and 16). The resonance signals from 7.3 to 8.3 ppm comprise the H-2 and H-8 protons of adenine, the H-8 protons of guanine, and the H-6 protons of cytosine and thymine; those from 5.8 to 6.4 ppm include signals from the H-5 protons of cytosine and the H-1′ deoxyribose residue. The H-3′ signals occur in the region from 2.1 to 2.8 ppm, and the T–CH_3 proton resonances cover the range 1.6–1.9 ppm. The HDO resonance largely obscures the individual contributions of the H-4′ and H-5′ protons. As can be seen from the spectrum, the two T–CH_3 signals at 1.71 and 1.83 ppm are completely resolved, with a frequency separation of *ca.* 28 cps. This is in contrast to the 7-cps separation observed at 60 Mcps.[288] While at lower temperatures the reversion of DNA to a helical configuration tends to broaden the 1.73 and 1.84 resonances, addition of DMSO (10%) enabled well-resolved spectra to be recorded down to 50°. Neither the temperature change nor pD changes between 6 and 11 affected the separation or the relative intensities of the spectral lines.

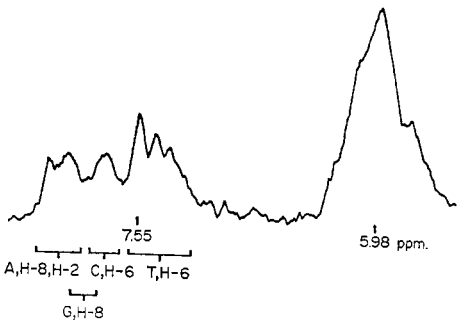

Figure 15. High-field regions of pmr spectrum of calf thymus DNA, 220 Mcps: concentration 20 mg/ml in D_2O, pD 7.0, 93°, 20 spectra averaged in computer of average transients.

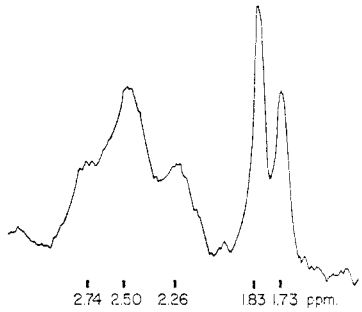

Figure 16. Low-field regions of pmr spectrum of calf thymus DNA at 220 Mcps: concentration 30 mg/ml in D_2O, pD 7, 93°, 50 spectra averaged.

Accordingly, the intensity ratios of the two T–CH_3 resonances of calf thymus DNA, and DNA from calf marrow, calf spleen, salmon sperm, sperm, and several bacteriophage species were measured at 90° in neutral D_2O. A computer of average transients gave $(I_{1.71}/I_{1.84}) = 0.67 \pm 0.02$ for (Sigma) calf thymus DNA. Results are recorded in Table XXV.

Table XXV

Relative Intensities of DNA T–CH_3 Resonances

DNA source	$I_{1.71}/I_{1.83}$[a]	$\frac{ApT + GpT}{TpT + CpT}$[b]
Calf thymus (Sigma)	0.67	0.77
Calf thymus (CalBiochem)	0.66	
Calf marrow	0.69	
Calf spleen	0.69	
ϕX-174	0.61	0.82
C_{16}	0.75	
Salmon sperm	0.79	0.85
T_6	0.85	0.92
$T4_0$	0.89	0.95

[a] From nmr analysis, equal to (ApT + GpT)/(TpT + CpT).
[b] Calculated from Kornberg, *et al.*[321]

While there is qualitative agreement between the nearest neighbor frequency ratios calculated by two very different methods, the nmr approach appears to indicate a greater departure from randomness in favor of pyrimidines over purines at the 5′-neighbor position.

The application of nuclear magnetic relaxation methods to the study of specific molecular interactions in biological systems has been ably reviewed.[179] However, the application of proton relaxation enhancement methods to the study of transition metal binding in DNA solutions will be described briefly here.[322]

The longitudinal relaxation time T_1 (the time constant for decay of nonequilibrium distributions or spin–lattice relaxation) depends primarily on the dipolar field of the paramagnetic ion. The transverse relaxation time T_2 (or spin–spin relaxation time) is dependent on T_1 and also the isotropic

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hyperfine interaction between the magnetic ion and the proton.

For ions such as Mn^{2+}, Cu^{2+}, and Cr^{3+} it can be shown[323,324] that

$$\frac{1}{NT_1} = \frac{6}{15}\frac{S(S+1)g^2\beta^2\gamma I^2}{r^6}p\tau_c \quad (39)$$

and

$$\frac{1}{NT_2} = \frac{7}{15}\frac{S(S+1)g^2\beta^2\gamma I^2}{r^6}p\tau_c + \frac{1}{3}\frac{S(S+1)A^2}{h^2}p\tau_e \quad (40)$$

where N is the molar concentration of the paramagnetic ions, γI the proton gyromagnetic ratio, p the probability a proton in a 1 M solution of ions is in the hydration sphere of the paramagnetic ion, τ_c the correlation time for the dipolar interaction, A the isotropic indirect hyperfine interaction, r the ion–proton internuclear distance, and τ_e the correlation time for the isotropic hyperfine interaction.

$1/\tau_c$ is defined by $1/\tau_s + 1/\tau_h$ where τ_s is the electron spin relaxation time and τ_h the correlation time for the proton chemical exchange in the hydration sphere. $1/\tau_c$ is defined by $1/\tau_s + 1/\tau_r + 1/\tau_h$ where τ_r is the rotation correlation time, and in general $1/\nu_h$ does not contribute to $1/\tau_c$.

If p^* and $p^*T_c^*$ denote corresponding values of the bound ion, then the enhancement factor ϵ_1 is [322]

$$\epsilon_1 = \frac{1/T_1^* - 1/T_1^*(0)}{1/T_1 - 1/T_1(0)} \simeq \frac{T_1}{T_1^*} \simeq \frac{p^*\tau_c^*}{p\tau_c} \quad (41)$$

Thus the experimental value of ϵ_1 can be interpreted in terms of changes in τ_c or p. In general when[322] $\epsilon_1 = 1$, (a) the ion is in solution and not bound, or (b) the ion is bound to an outside site, but $\tau_s^* = \tau_s < \tau_r$. If $\epsilon_1 > 1$, the ion is bound to an outside site, but if $\epsilon_1 < 1$, the ion is bound (a) to a site inaccessible to water protons ($p^* \ll p$), or (b) to an accessible site, but $\tau_c^* < \tau_c$.

Experimentally, T_1 and T_2 were determined by the Carr–Purcell method of pulsed nmr.[325] The T_1 of water protons was observed to decrease slightly on addition of DNA to distilled, deionized water, and at a concentration of 2 mg/cc the following values (sec) were obtained: calf thymus Sigma Type II, 2.38; calf thymus Worthington, 595, 2.53; herring sperm, 2.47; and deionized water, 2.83. Graphical methods were used to evaluate T_i[322] on the addition of Mn^{2+}, Cu^{2+}, and Cr^{3+} to DNA solutions. An outside site for Mn^{2+} is indicated by $e_1 \sim 8$, and N_0 (the capacity of DNA for Mn^{2+}) is 7×10^{-3} per mg of DNA, *i.e.*, the number of available absorption sites. Cu^{2+} and Cr^{3+} ions also showed relaxation enhancements $e_1 \sim 1$, but unlike Mn^{2+} these ions could cause precipitation of DNA. Evaluation of T_e gave similar values for the three ions (Mn^{2+}, 2.5×10^{-10}; Cr^{3+}, 1.7×10^{-10}; and Cu^{2+}, 2.4×10^{-10}) suggestive of the same residual motion in the bound state.

In a comparison of the relaxation enhancement caused by Mn^{2+} bound to DNA, RNA, polyA, polyI, polyU, polyC, and *E. coli* ribosomes, the equilibrium constants for binding and the concentration of binding sites have been calculated from the dependence of proton relaxation enhancement on the concentration of the manganese ions.[326] Assuming a value of unity for water, the enhancement factors were shown to range from 3.7 for polyU to 16.7 for polyA.

IV. Polysaccharides

A. INTRODUCTION

The original nuclear magnetic resonance studies of fully acetylated pentopyranoses and hexopyranoses conducted by Lemieux, Kullnig, Bernstein, and Schneider a decade ago initiated the development of this unique method for the conformational analysis of carbohydrates. Thus, extensive application of nmr spectroscopy to the elucidation of the configuration and conformation of monosaccharides and their derivatives has been used to supplement established methods of structural analysis.

Nmr spectroscopic analysis has been further extended to the identification of oligo- and polysaccharide structures. The selection of solvents, which enable both carbon–proton and hydroxyl–proton signals to be observed and which ensure the dissolution of high molecular weight carbohydrates, coupled with the establishment of 100- and 220-MHz spectral procedures has enhanced structural investigations of polysaccharides.

Some pertinent nmr studies of monosaccharides and their derivatives since 1964[327] are described firstly in the section, followed by a consideration of recent investigations of oligo- and polysaccharide spectra.

B. MONOSACCHARIDES

In dimethyl sulfoxide solvent, hydroxyl proton signals are generally displayed as well-resolved peaks at fields below τ 6.[328] Hydroxyl proton resonances sometimes superimpose one another in DMSO, producing a spectrum which shows loss of multiplicity.[329] Glucose and oligomers of glucose give characteristic resonances whose chemical shifts are independent of concentration under given conditions (5–20% w/w).[330] Nonanomeric C–H proton signals are observed at fields greater than τ 6 using deuterated DMSO (DMSO-d_6) as solvent (Figure 17).[331] The anomeric C–H signal occurs at fields below τ 6 in DMSO, and the O–H signals are distinguished from this signal by deuteration or temperature variation.[330,332]

The chemical shifts of the O–H signals in DMSO depend upon the equatorial or axial orientation of the O–H group and are influenced by the accessibility of the O–H groups for hydrogen bonding with the solvent.[325,331,333] Strong hydrogen bonding with the DMSO solvent results in a shift in the position of the O–H proton signal downfield, and a reduction in the rate of proton exchange. Equatorial O–H groups absorb at lower field than axial O–H protons, and the addition of heavy water to the solvent results in deuteration and disappearance of the O–H signal.[331]

The 60- or 100-Mcps spectra of glucose, galactose, and xylose derivatives in DMSO reveal O–H and C-1–H signals

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(327) L. D. Hall, *Advan. Carbohydrate Chem.*, **19**, 51 (1964).
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(332) A. S. Perlin, *Can. J. Chem.*, **44**, 539 (1966).
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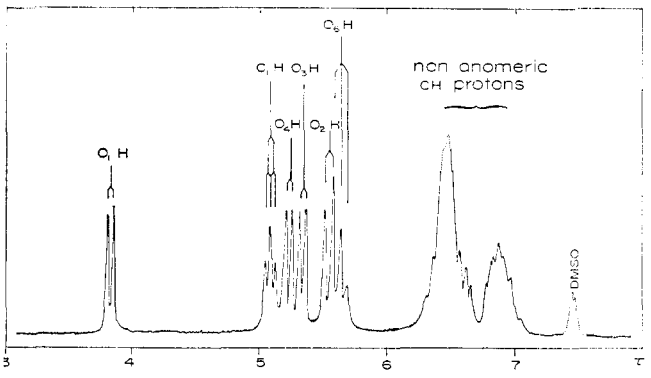

Figure 17. Pmr spectrum of α-D-glucose in DMSO-d_6 at 28°.

below τ 6, the O-1-H proton giving the most downfield signal.[331] The large downfield shift (3.70–4.05 ppm, for α anomers; 3.40–3.58 ppm for β anomers (relative to internal TMS)) is attributed to the inductive effect of the ring oxygen, which makes the anomeric O–H proton more acidic and more strongly bonded to the DMSO solvent than the other O–H functions. The C-1–H signals appear as doublets when the O–H group at the anomeric center is substituted, while this signal is a pair of doublets due to coupling of the C-1–H proton with the C-2–H and O-1–H protons when the O–H at the anomeric center is unsubstituted.[334] The signal for the anomeric proton is displaced upfield with respect to the shift of the signal in D_2O solution (α-D-glucose, τ 5.06 in DMSO and 4.78 in D_2O), while the splitting of the C-1–H signal is of the order of 3 and 7.5 cps for α and β anomers in DMSO, D_2O, and $CDCl_3$.[331,334]

Upon addition of D_2O to α-D-glucose in DMSO, the O–H proton signals disappear, leaving as the remaining signal a doublet assigned to the most strongly deshielded proton, C-1–H, which is coupled with the anomeric O–H proton.[332] Other doublets in the DMSO spectrum are due to the secondary O–H protons, O-2–H, O-3–H, and O-4–H. The pyranose (O-4–H) or furanose (O-5–H) forms of monosaccharides are distinguished by comparing the spectra of α-D-glucose and α-D-glucose deuterated in the 5 position. The signals of the secondary O–H protons in both α-D-glucose and α-D-glucose-5,6,6′-d_3 spectra are similar, indicating that the 5 position is engaged in pyranose ring formation. In a furanose ring, the O-5–H signal is replaced in the spectrum of the deuterated sample by a strongly modified signal.[332]

Assignments for the O-2–H, O-3–H, and O-4–H proton signals of glucose in DMSO-d_6 are based upon double resonance experiments using frequency-swept spin-decoupling, since these proton signals are not well shifted from one another.[335,336] Each proton is irradiated at the appropriate resonance frequency, which causes one or another of the hydroxyl proton doublets to become spin decoupled. α-D-Xylose in DMSO gives a spectrum with four well-resolved C–H proton doublets that correspond in chemical shift and spacing to the O–H proton signals of O-1–H, O-2–H, O-3–H, and O-4–H of α-D-glucose. The presence of these four secondary O–H groups indicates that α-D-xylose is a pyranose sugar in this solvent. The nonanomeric O–H groups give individual peaks (doublets at τ 5.34–5.68 for α anomers, closed doublets at τ 5.25 for β anomers) with characteristic splitting (3.5–6.5 cps) that suggests differences in the geometry of the O–H groups and strength of the hydrogen bonds.[331]

The preferred ring size of fused-ring derivatives has been studied in the DMSO solvent.[332] D-Mannose-2,3-carbonate and related compounds were shown to behave chemically as furanoses, and the hydroxyl signals in the DMSO spectrum indicate a furanose structure.[332]

The spectrum of D-mannose-2,3-carbonate in DMSO is similar to that of the compound in D_2O, except for the O–H proton signals (two doublets and a triplet). The absence of coupling between the C-4–H proton and an O–H group of the carbonate is noted from the production of the same quartet signal for C-4–H with either solvent. This signal is not affected by the addition of D_2O, which suggests that position 4 of the carbonate does not have a free O–H group. Coupling of the C-5 proton with an O–H group is not apparent since its signal is obscured by those of the C-6–H protons. Such coupling is demonstrated with the 6-O-trityl derivative of the mannose carbonate using the double-resonance technique. The resulting spectrum of this compound showed the C-4–H and other signals as in the spectrum of the mannose carbonate, excepting the C-5–H signal which was isolated. Irradiation at the resonance frequency of the latter signal caused the doublet at 5.9 ppm to be spin-decoupled and appear as a broad singlet rather than a doublet at 5.9 ppm. These results indicated that the ring structure of D-mannose-2,3-carbonate is furanose rather than pyranose. Similar procedures also suggested that D-xylose-2,3-carbonate has a similar ring configuration in this solvent.

The apparent coupling constants (obtained by measuring directly the peak splitting) of the monosaccharides given in Table XXVI suggest that these sugars exist in the $^4C_1$[337] conformation in the DMSO solvent as in D_2O.[338–340] In the C1 conformation, the C-1–H bond is equatorial while the B1 and 3B conformations have this bond in the axial orientation. The splitting of the anomeric proton resonance has been used in the past to determine the dihedral angle between the dihedral angle between the H–C_1–C_2 and C_1–C_2–H planes using the modified Karplus[52] equation,[341] but these values for the coupling constants depend upon factors other than the dihedral angle.[327,342] However, the angles of the C1, B1, and 3B forms (60, 60, and 0°, respectively) differ sufficiently to allow measured splitting to indicate either an axial–equatorial (C1 or B1) or diaxial (3B) orientation of the C-1–H and C-2–H bonds.[331]

The absorption of the O-1–H proton of β anomers at 0.3–0.4 ppm lower field than α anomers and the assumption of the C1 ring conformation of the pyranose ring in these monosaccharides suggest that the equatorially oriented O–H protons absorb at lower field than the protons of the axially oriented O–H functions.[328,331]

Similar results (Table XXVII) have been obtained from the pmr spectra of epimeric cyclohexanols in DMSO, in

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Table XXVI[331]

Chemical Shifts[a] and Coupling Constants[b] of Some Monosaccharides in DMSO

Monosaccharide	*O-1-H*		*C-1-H*		*O-2-H, O-6-H O-4-H doublets*	*O-6-H triplet*
	Eq	*Axial*	*Eq*	*Axial*		
α-D-Glucose		3.85 (4.5)	5.08 (4.5, 3.0[c])		5.28 (5.0) 5.40 (4.5) 5.60 (6.5)	5.70 (5.0)
β-D-Glucose	3.50 (6.5)			5.70 (6.5, 6.5[c])	5.25 (3.5)[d]	5.58 (5.5)
α-D-Galactose		3.95 (4.5)	5.05 (4.5, ~2.0[c])		5.57 (5.0) 5.74 (7.0) 5.78 (4.0)	5.54 (5.0)
α-D-Xylose		3.90 (4.5)	5.15 (4.5, 3.5[c])		5.24 (4.0) 5.36 (3.5) 5.60 (6.5)	

[a] Chemical shifts given as τ values (ppm) relative to internal TMS. [b] Coupling constants (cps) given in parentheses. [c] Value obtained from the deuterated product. Deuteration of these sugars before dissolution in DMSO was accomplished by dissolving the sample in D_2O, and pumping off the liquid phase. [d] Three hydroxyls.

Table XXVII[333]

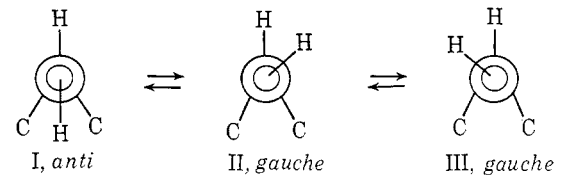
Hydroxyl Proton Chemical Shifts[a] and Coupling Constants[b] of Epimeric Cyclohexanols in DMSO[c]

Equatorial alcohol		*Axial alcohol*	
Cyclohexanol[d]	5.70 (4.2)		
trans-2-*t*-Butylcyclohexanol	5.98 (5.7)	*cis*-2-*t*-Butylcyclohexanol	6.20 (4.4)
cis-3-Methylcyclohexanol	5.67 (4.5)	*trans*-3-Methylcyclohexanol	5.89 (3.5)
trans-4-*t*-Butylcyclohexanol	5.65 (4.5)	*cis*-4-*t*-Butylcyclohexanol	5.92 (3.0)
Epiandrosterone	5.67 (4.5)	Androsterone	5.92 (3.0)

[a] Chemical shifts given as τ values relative in internal TMS. [b] Coupling constants (cps) given in parentheses. [c] At <0.12 mole fraction, 40°. [d] Prevailing conformation.

which the axial O–H proton occurs between 0.21 and 0.27 ppm upfield from the equatorial function.[328,333] With both axial and equatorial alcohols, vicinal substitution causes the O–H signal to shift to higher field, the shift increasing with the bulk of the substituent. The equatorial alcohol coupling constants are 1.0–1.5 cps greater than those of the axial epimers, and vicinal substitution increases the coupling in both epimers.[338,343]

Rader has related the differences in the coupling constants for the epimeric cyclohexanols to the effect of the dihedral angle and the rotational isomers about the C–O bond in which the O–H groups may be arranged either between the two C–C bonds (I), the C–H bond (II), or the C–C bond (III).



I, *anti* II, *gauche* III, *gauche*

The rate of rotation about the C–O bond and the formation of solute–solvent hydrogen bonds is greater than the change in the chemical shifts between the hydroxyl protons of the three isomers, and the coupling constant (I) varies with the mole fractions (N) of conformers I–III according to the equation

$$J = N_I J_I + N_{II} J_{II} + N_{III} J_{III} \quad (42)$$

With an equatorial O–H group, both rotamer types should be present, while an axial O–H system would have rotamer I restricted owing to steric interactions of the axial 3,5 hydrogens with the hydroxyl hydrogen. In axial and equatorial systems the *gauche* forms would be essentially equivalent in the absence of vicinal substitution, and a symmetrical O–H stretching band and an unsymmetrical band have been noted from ir data for the homogeneous axial O–H group and the conformationally heterogeneous equatorial epimer.[338,344]

The hydroxyl–carbinol proton coupling constants can be applied qualitatively to the Karplus expression

$$J_{vic} = A \cos^2 \theta + B \quad (43)$$

where A and B are constants for a given system and are functions of HCC bond angles, C–C bond angle length, and the electronegativity of the substituents attached to the two carbon atoms,[342,345] so that

$$J_I > J_{II} = J_{III} \quad (44)$$

An equatorial alcohol has a larger coupling constant than its axial epimer because of the contribution of the *anti* conformer (I) to the equatorial epimer.

The epimers of 4-*t*-butylcyclohexanol and androsterone–

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(344) H. S. Aaron, G. E. Wicks, Jr., and C. P. Rader, *ibid.*, **29**, 2248 (1964); H. S. Aaron, C. P. Ferguson, and C. P. Rader, *J. Am. Chem. Soc.*, **89**, 1431 (1967).

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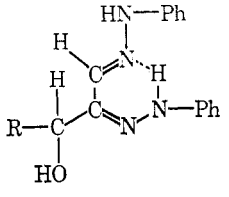
epiandrosterone systems have identical coupling constants (Table XXVII), and the J value for the two axial epimers is equivalent to J_{gauche} for these alcohols if the mole fraction of *anti* conformer is neglected.[333] Upon vicinal substitution, conformers II and III are not similar and an increase in J may result from a change in conformer mole fractions, the minimum energy dihedral angles of the conformers, or a change in one of the factors affecting constants A and B of the Karplus equation.[333]

The difference in the chemical shifts between the epimeric alcohols has been related to the solute–solvent hydrogen bonding. Solute–solute hydrogen bonding is neglected since no variation of the chemical shifts with concentration occurs under the controlled conditions used. Hydrogen bonding affects the chemical shift of the participating proton and causes the signal to be shifted qualitatively downfield as the strength of the bond increases.[331,338]

The relative strength of the axial and equatorial O–H solvent hydrogen bonds will be determined by steric effects, since polar contributions to the bond strengths is similar. It is predicted that a given electron donor forms a hydrogen bond with an equatorial epimer which is stronger than that formed by the epimer owing to greater steric accessibility.[338]

The differences in the chemical shifts of the axial and equatorial O–H functions in monosaccharides have been explained by the difference in the strength of the hydrogen bonding of the O–H groups with the DMSO solvent, magnetic anisotropy, and the different accessibility of the O–H groups to the solvent molecules. Pmr and ir data suggest that all of the O–H groups of both anomers of glucose are strongly hydrogen bonded to the solvent, and that the intrahydrogen bonds in glucose are broken by the DMSO solvent.[331,346] The nonanomeric O–H signals of α-glucose give individual peaks with characteristic splitting, while the β anomer shows apparently equivalent O-2–H, O-3–H, and O-4–H proton signals in this respect.

The solubility of sugar osazones in DMSO, and the reduced rate of proton exchange enabling the observation of spin–spin splitting of O–H protons and sharp N–H resonance lines, allows sugar osazone structure to be studied in this solvent.[347] The chelate structure



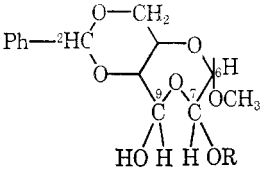
has been assigned[347–350] to the initial structure of sugar osazones on the basis of the pmr spectra of osazones and related compounds in DMSO and other solvents. At 60 Mcps, the spectra of sugar osazones reveal the chelated N–H proton signal between 12 and 13 ppm relative to TMS. The nonchelated imino proton signals occur between 9 and 11 ppm. In deuteriochloroform, this free imino proton signal appears between 7 and 8 ppm and disappears upon addition of D_2O.[351]

Replacement of the α-imino hydrogen in the phenylhydrazone residue on C-1 by a methyl group, as in tetra-*O*-acetyl-D-glucose 1-methylphenyl-2-phenylosazone, does not alter the position of the signals of the chelated N–H group, but the signal of the nonchelated N–H group disappears. Thus, the position of the methyl group in the 1-methylphenyl-2-phenylosazones is identical with the position of the nonchelated proton in the sugar osazones.[349] A long-range spin–spin coupling between the C-1 proton and the nonchelated N–H proton has been noted, and weak splitting of this proton disappears during exchange of the nonchelated N–H proton with D_2O to give a more intense peak. Coupling of the C-1 proton with this nonchelated N–H proton has also been confirmed with the double resonance method at 100 Mcps.

The cyclic or acylic forms of sugar hydrazones can be distinguished by pmr in DMSO.[332,347]

D-Erythrose 2,5-dichlorophenylhydrazone in DMSO displays two doublets (O-2–H and O-3–H) and a triplet (O-4–H), and D-fucose *p*-toluenesulfonylhydrazone produces four doublets (O-2–H to O-5–H). Hydroxyl signals produced by D-mannose phenylhydrazone are too closely grouped to allow the determination of individual O–H functions, but the intensity of these signals relative to the phenyl protons corresponds to the presence of five free O–H groups. In the spectra of unsubstituted hydrazones, the signals of the N–H groups are found in the region 8.5–9.5 ppm, and disappear after the addition of D_2O. The N–H group of D-erythrose 2,5-dichlorophenylhydrazone exchanges with the deuterium more slowly than does the N–H group of other hydrazones.[332]

The dialdehyde produced by cleaving the 2,3-diol group of 4,6-*O*-benzylidene methyl-α-D-glucopyranoside and its stereoisomers forms a crystalline dihydrate whose spectrum in DMSO favors the hemialdal monohydrate structure. The spectrum contains two O–H doublets (6.74 and 6.80 ppm). The alcoholates produced by treating this hemialdal



with various alcohols have been examined by pmr in DMSO,[332] and the spectra indicate only one O–H doublet at 6.85 ppm with either the methyl or ethyl derivative. The proton α to this O–H group (H-9) was identified by deuteration, and a doublet at 4.54 ppm was assigned to the proton α to the O–R group at C-7. This latter signal showed coupling with a proton to produce a doublet at 4.25 ppm, H-6, and the dialdehyde methanolate and ethanolate were assigned a structure in which the alkoxyl group is at position 7 and the free O–H group at C-9.

Products derived from the glycol-cleavage oxidation of methyl aldopentopyranosides are regarded as dialdehyde enantiomorphs and hemialdals, with those derived from a pair of anomers constituting mirror image forms. Examination of the periodate oxidation products of methyl-α- and

(346) A. J. Mitchell and H. G. Higgins, *Tetrahedron*, **21**, 1109 (1965).
(347) O. L. Chapman, R. W. King, W. J. Welstead, Jr., and T. J Murphy, *J. Am. Chem. Soc.*, **86**, 4968 (1964).
(348) H. El Khadem, M. L. Wolfrom, and D. Horton, *J. Org. Chem.*, **30**, 858 (1965).
(349) L. Mester, E. Moczar, and J. Parello, *J. Am. Chem. Soc.*, **87**, 596 (1965).
(350) L. Mester, E. Moczar, G. Bass, and A. Schimple, *Carbohydrate Res.*, **5**, 406 (1967).
(351) L. Mester, *Angew. Chem. Intern. Ed. Engl.*, **4**, 574 (1965).

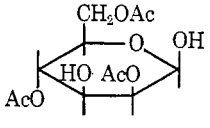
β-xylopyranosides in D_2O by pmr (100 Mcps) revealed an indistinguishable spectrum in which the multiplicity of signals and the varied intensities indicated a mixture of products. Investigation of the products in DMSO and DMSO-d_6 allowed the determination of the presence of the hemialdal (two O–H groups) and the hydrated form of the acyclic dialdehyde (four O–H groups) in the mixture.[332,335]

A freshly prepared solution of the sample in DMSO did not produce a spectrum containing well-resolved peaks, and a change in the composition occurred producing at equilibrium a spectrum in which only two O–H signals (6.2–6.5 ppm) were displayed. After deuterium exchange, a spectrum was obtained in DMSO-d_6 which contained a triplet (5.4 ppm), a methoxyl signal (3.5 ppm), and doublets at 4.9, 4.35, and 3.6 ppm. The relative intensities of the triplet and doublets were 1:1:1:2, respectively. By spin-decoupling, the triplet and doublets were assigned to C-4–H, C-2–H, C-1–H, and the methylenic 5 protons, respectively. The spectrum indicated one component of the aqueous oxidation mixture as predominant in the DMSO solvent, and this component had a hemialdal structure.

Similarly, the spectra obtained for the products prepared from 5-deuterated methyl-α- and β-D-xylopyranosides indicated that the 1-methoxyl group of each hemialdal is axial and the O-4–H group equatorial. The structure derived from the β anomer suggested an inverted chair conformation relative to that of the other hemialdal and the parent glycosides. An axial orientation was suggested for the O-2–H group of each hemialdal.[335]

Derivatives containing strongly deshielding substituent groups produce O–H and C–H signals in the same region of the DMSO spectrum and are differentiated by the addition of D_2O.[328,332]

The spectra of β-2,4,6- and α-3,4,6-tri-*O*-acetyl-D-mannoses (I and II) has been studied by Perlin.[332] The spectrum of I reveals two O–H proton doublets (6.8 and 5.25 ppm relative



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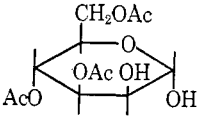


II

to the methyl resonances of the solvent). The lower field signal has the same spacing (7.4 cps) as the anomeric C–H signal at 4.78 ppm and was assigned to the O-1–H proton. Since the anomeric position is free and the primary carbinol group substituted, the two C–H signals unchanged upon deuteration were assigned to those protons α to the secondary acetoxyl groups. The C-2–H proton was assigned to the signal at 5.05 ppm which showed weak coupling with the anomeric proton, but was not coupled with the doublet at 4.65 ppm, which was assigned to the nonadjacent carbon, C-4–H, thus confirming the 2,4,6-triacetate structure of I.

The spectrum of II in DMSO revealed that the anomeric O–H position is unsubstituted, the signals occurring at 6.7–6.8 ppm. The other free O–H was secondary at 5.2–5.3 ppm. A doublet at 4.99 ppm (2.2 cps) was overlapped by a broad multiplet caused by protons α to the acetoxyl groups. Neither signal was coupled with C-1–H and were assigned to C-3–H and C-4–H. The doublet was assigned to C-3–H since its spacing was similar to that expected for this proton at C-3 in the C-*manno* series, thus confirming the 2,4,6-triacetate structure II.

Methylated monosaccharides display OMe signals in deuterated DMSO as sharp singlets between τ 6.50 and 6.78 relative to internal TMS.[334] These signals superimpose the nonanomeric C–H signals and are assigned on the basis of correlation with the position and orientation of substituent groups. In the 60-Mcps spectra of *O*-methylated glucoses (Table XXVIII), the C-1–H and O–H signals are affected by the adjacent OMe groups. Upon methylation at O-1, the anomeric C–H signal shifts upfield 0.37 and 0.25 ppm for α and β anomers, respectively, in DMSO-d_6, while methylation at O-2 results in a downfield shift of *ca.* 0.21 ppm for α anomers. Such effects are additive, and the shift occurring for 1,2-O-methylated α anomers is +0.18 ppm.

In deuterated chloroform, the anomeric C–H signals of monosaccharides unsubstituted at O-1 are at lower field (0.17–0.29 ppm) than in DMSO-d_6, and the O–H signals are concentration dependent. Methylation at O-1–H produces a smaller solvent effect. The splitting of the anomeric proton signal by coupling with C-2–H is 3 cps for α and 7.5 cps for β anomers in both solvents.

The electron-releasing effect of the methyl group, and the magnetic anisotropy of the O-1–Me bond can contribute to the shielding of the anomeric proton. The downfield shift of the anomeric C–H signal upon methylation at O-2 could occur because the O-2–Me bond in its more probable conformations is oriented with respect to the C-1–H signal to produce a long-range deshielding of the anomeric proton.[334]

Methylation of adjacent O–H groups affects the shift of the unsubstituted O–H functions in monosaccharides (Table XXIX).[334] O-Methylated glucoses in which the O-1–H group is unsubstituted, with one OMe substituent, have a −0.13 to −0.16 ppm downfield shift of the O–H groups adjacent to the OMe group (*e.g.*, 3-*O*-methyl-α-glucose). Two OMe groups α and α′ to the O–H group produce approximately twice this downfield shift in the signal of the unsubstituted O–H group (2,4,6-tri-*O*-methyl-α-glucose, 2,3,6-tri-*O*-methyl-α-D-glucose). Two OMe groups in the α and β position produce a −0.15 to −0.19 ppm shift in the O–H signal (2,3-di-*O*-methyl-α-D-glucose). Three OMe groups (α, β, γ) produce a larger downfield shift of the O-1–H signal. With β anomers, the downfield shift of the anomeric O–H signal is about 0.04 ppm greater than that of the α anomers, and the downfield shift is larger for O-methylated glucosides than for O-methylated glucoses.

From these results, a sequence of OMe groups causes a larger downfield shift of the O–H group signal adjacent to the sequence than a single isolated OMe group.

The O-1–Me signal of methyl α-D-glucoside in DMSO (τ 6.78) occurs at 0.17 ppm upfield with respect to that of the β anomer (τ 6.61). These results are similar to those obtained in D_2O solution (τ 6.64 and 6.48).[346] The O-1–Me signal of α anomers shifts about 0.06 ppm downfield upon methylation at O-2. O-6–Me signals occur at the highest field within the OMe resonance range in DMSO. In $CDCl_3$, provided C-1–H bears an O–H or OMe substituent, α anomers display the O-2–Me signal at τ 6.46–6.52 and 6.64–6.55 in DMSO-d_6. β anomers produce signals of this group at τ 6.51–6.55 in DMSO-d_6. O-3–Me and O-4–Me signals occur at lowest field within the O–Me signal range and are slightly affected by the configuration at the anomeric center.

The observed effect of the O–Me group at C-1 and C-2 on

Table XXVIII[334]

Chemical Shifts[a] and Coupling Constants[b] of Some O-Methylated Glucoses in DMSO-d_6 at 38°

Compound	*O-1-H doublet*	*C-1-H pair of doublets[c]*	*Nonanomeric O-H's[i]*	*OMe singlets*
3-*O*-Methyl-α-D-glucose	3.78 (4.5)	5.08[d] (4.5, ~2[e])	5.12 (~3) O-4-H 5.44 (~5) O-2-H 5.70 (5.5) O-6-H	6.51 O-3-Me
3-*O*-Methyl-β-D-glucose	3.45 (6.5)	5.68[d] (6.5, 7.0[e])	*g*	6.51 O-3-Me
2,3,6-Tri-*O*-methyl-α-D-glucose	3.66 (4.5)	4.90 (4.5, 3.0[e])	4.94 (5.0[d]) O-4-H	6.55 O-3-Me 6.67 O-2-Me 6.78 O-6-Me
2,3,6-Tri-*O*-methyl-β-D-glucose[f]	*h*	5.64[e] (h, 7.0[e])	*h*	6.52 O-2-Me 6.55 O-3-Me 6.76 O-6-Me
2,3,4,5-Tetra-*O*-methyl-α-D-glucose[f]	3.62 (4.5)	4.87 (4.5, 3.0)		6.54 O-3-Me 6.58 O-4-Me 6.66 O-2-Me 6.73 O-6-Me
2,3,4,6-Tetra-*O*-methyl-β-D-glucose[f]	3.24 (6.5)	5.62 (6.5, 7.0)		6.51 O-2-Me 6.54 O-3-Me 6.58 O-4-Me 6.73 O-6-Me
Methyl α-D-glucoside		5.45 (3.0)	5.22 (5.0) O-4-H 5.32 (4.0) O-3-H 5.39 (6.0) O-2-H 5.62 (5.5) O-6-H	6.78 O-1-Me
Methyl β-D-glucoside		5.95 (7.0)	5.04 (4.0) O-2-H 5.16 O-3-H 5.16 O-4-H 5.56 (6.0) O-6-H	6.61 O-1-Me

[a] Chemical shifts given as τ values (ppm) relative to internal TMS. [b] Coupling constants (cps) given in parentheses. [c] Doublet when coupling with O-1-H has been removed. [d] Assignment from spectrum at higher temperature. [e] Value observed after addition of 5% CF_3COOH. [f] Value observed from anomeric mixture. [g] Value not observed from anomeric mixture. [h] Value not observed due to presence of CF_3COOH. [i] Secondary = doublets; primary = triplet.

Table XXIX[334]

Effects[a] of OMe Substituents on Unsubstituted O–H Signals in O-Methylated Glucoses and Glucosides in DMSO-d_6 (60 Mcps)

Compound	*O-4-H*	*O-3-H*	*O-2-H*	*O-1-H*
3-*O*-Methyl-α-D-glucose	−0.16		−0.16	
2,4,6-Tri-*O*-methyl-α-D-glucose		−0.34		
2,3,6-Tri-*O*-methyl-α-D-glucose	−0.34			−0.19
2,3-Di-*O*-methyl-α-D-glucose				−0.15
2,3,4,6-Tetra-*O*-methyl-α-D-glucose				−0.23
Methyl α-D-glucoside			−0.21	
Methyl β-D-glucoside			−0.21	
Methyl 2,3-di-*O*-methyl-α-D-glucoside	−0.28			

[a] Expressed as τ difference, ΔO-H, TMS internal standard.

the anomeric proton of α-glucose derivatives suggests the range characteristic of equatorial anomeric proton bonds for unsubstituted sugars in DMSO (τ 4.83–5.35) be extended to τ 4.61–5.45.[334] The anomeric proton signals of O-methylated β anomers between τ 5.58 and 5.95 are within the characteristic field of axial C-1–H bonds of unsubstituted compounds. Splitting of the C-1–H signal (3 cps) for all derivatives of α-glucose and β-glucose suggests the C-1–H/C-2–H dihedral angle of about 60 and 180°, respectively. The orientation of the anomeric C–H bond in each anomeric form suggested the C1 conformation for the pyranose units in these O-methylated monosaccharides.[334]

The downfield shift of the signals of the O–H protons adjacent to the O–Me groups indicate a stronger hydrogen bonding with DMSO. From ir data ν_{O-H} absorption (unsubstituted sugars in the range of 3400–3320 cm^{-1}) is shifted to lower frequencies in most of the methylated monosaccharides. The largest shift is given by the O-1–H of O-2 methylated compounds (ν_{O-H} = 3275–3230 cm^{-1}). The O-1–H seems the most strongly hydrogen-bonded hydroxyl group, and ir data indicate it is able to give intermolecular association in very dilute CCl_4 solution.[334,346]

The stronger hydrogen bonding in the O-methylated sugars is explained by the fact that bulky solvent molecules are prevented by mutual repulsion from closely approaching the O–H groups in unsubstituted sugars, while a O–H group surrounded by O–Me groups is less bulky and is more effective in attracting a DMSO molecule.[334] With the anomeric O–H group, substantially the same splitting is observed for unsubstituted and O-2 methylated glucose (4.5 cps for α

and 6.5 cps for β anomers), and it is implied that the O-1–H bond is oriented substantially the same in the two types of products.

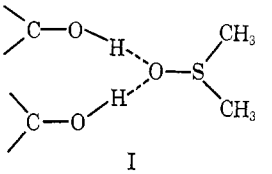
The spectrum of a reducing sugar in DMSO is largely that of the anomeric form of the solid sugar, and enables the purity and anomeric ratio of the sugar to be determined.[330,352] The anomeric composition and configuration of glucose and related sugars has been determined from the signal of the anomeric O–H group in DMSO.[353] The splitting and chemical shift of this proton, characteristic of the anomeric configuration, is easily detected in the τ 3–5 region of the spectrum owing to slow mutarotation in the dry DMSO solvent. The spectra of sugar solutions (10%) after immediate dissolution in DMSO allows the ratio of the areas under the peaks of the two anomers to be determined, which corresponds to the weight ratio of the anomers in the solid sugar sample.

β-D-Arabinose freshly dissolved in DMSO produces a spectrum (100 Mcps) in which each O–H group displays a doublet, indicative of the pyranose ring structure.[332] The anomeric O–H proton signal occurs at 5.88 ppm relative to the methyl protons of the solvent. After 24 hr, an additional overlapping signal appears at 5.94 ppm together with a less intense hydroxyl signal at 6.23 ppm (ascribed to the pyranose ring form). The dissolution in DMSO of a concentrate of the mutarotated sugar produces a spectrum in which the O-1–H signal at 6.23 ppm predominates, while the overlapping signal at 5.94 ppm (ascribed to the furanose form) is a minor signal. Storage of the mutarotated sugar for 2 days in DMSO results in this signal becoming more intense.

These assignments are based upon the signal of O–H protons displayed by solutions of D-arabinose and D-arabinose-5,5′-d_2. The undeuterated sample produces a signal at 4.52 ppm which becomes more complex and intense as the proportion of the assumed furanose form increases. The spectrum of the deuterated sugar gives a sharp singlet in this region, suggesting the primary carbinol group is liberated as the anomeric O–H signal (5.94 ppm) becomes more intense. This anomeric O–H signal is caused by D-arabinofuranose.[332]

Similar results were obtained with D-galactose. Different samples of D-ribose gave spectra in which a number of O–H proton signals are overlapped. No evidence for the presence of a primary carbinol multiplet was obtained, and both anomers of D-ribose were presumed to be pyranoses. A third O–H signal that became apparent was considered to be that of D-ribofuranose, together with a triplet at 4.36 ppm.[332]

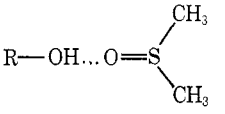
A more positive specific rotation of α-D-glucose and α-D-xylose samples in DMSO compared with values obtained in aqueous solution was originally considered as a preference of the DMSO solvent for the α anomeric forms of these sugars *via* complex formation.[354] In contrast, the rotation values of D-mannose were less positive in DMSO, suggesting that the solvent stabilized the β anomer in this case. α-D-Glucose and α-D-xylose were considered as doubly hydrogen bonded to the DMSO molecule across the two *cis*-hydroxyl groups at C-1 and C-2, with distortion of the pyranose ring and a decrease in the C-1–H/C-2–H dihedral angle.



I

The ring conformation of glucose and xylose derivatives, however, is substantially the same in DMSO as in D_2O, namely C1, which apparently allows accommodation of the bulky solvent molecules by the O–H groups.[331]

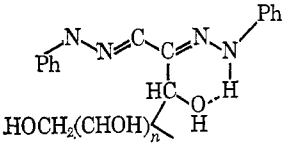
The chemical shifts of the anomeric O–H signals (τ 3.85 for α-glucose, 3.96 for 2-deoxy-α-glucose, 3.50 for β-glucose, and 3.58 for 2-deoxy-β-glucose) suggest the anomeric O–H functions of these compounds are similarly hydrogen bonded with one DMSO molecule, and since 2-deoxyglucoses do not associate with DMSO as in I, it is suggested the anomeric hydroxyl groups of glucoses should associate with one DMSO molecule as in II.



II

Perlin has also suggested that the chain-like structure of DMSO is not likely to favor one anomeric form of a sugar to another in spite of the strong hydrogen-bonding ability of the solvent. Furanose forms are prominent in DMSO for those sugars showing complex mutarotation in water. The more inherently stable ring form of a sugar appears to be more apparent in DMSO than in water, and this solvent seems more satisfactory for the comparison of conformational stability of interconvertible forms of sugars than water.[332]

Chapman[347] has examined the spectra of equilibrated mixtures of sugar osazones and their mutarotated isomers in DMSO and $CDCl_3$ and has observed the appearance of two new resonance signals in the region characteristic of the solvent N–H resonances. Also, a new aldimine C–H signal at slightly higher field was noted together with a downfield shift (0.4–1.0 ppm) of the hydroxyl signal of C-3. It was suggested that mutarotation involves the opening of the chelate ring with concomitant geometric isomerization about the C-2–N bond to give as the final product the O-chelated



Mester[350,355] has suggested that the position of the new N–H signals reported by Chapman support a nonchelated rather than an O-chelated structure, and that many structural changes could displace the C-3 hydroxyl signal. Mester examined the 60-MHz spectra of osazones during mutarotation in deuteriopyridine and *p*-dioxane (Table XXX) and found that the initial form revealed a strongly chelated and solvent-bonded N–H proton, while the final form displayed two N–H signals in the solvent-bonded region.

(352) B. Casu, M. Reggiani, G. G. Gallo, and A. Vigevani, *Tetrahedron Letters*, 2839 (1964).

(353) M. Reggiani, "Investigation on the Conformation of Glucopyranose Rings in Amylose Corn Starches and in Linear and Cyclic Dextrins Prepared from These Starches," Final Report, Project No. UR-E-15-(10)32, April 1962–April 1967.

(354) V. S. R. Rao and J. F. Foster, *J. Phys. Chem.*, **69**, 656 (1965).

(355) L. Mester, E. Moczar, G. Vass, and A. Schimple, *Tetrahedron Letters*, 2943 (1967).

Table XXX

Chemical Shifts[a] of Some Oxazones during Mutarotation in Deuteriopyridine and *p*-Dioxane

Compound	*Deuteriopyridine*						*p-Dioxane*					
D-Glycerosazone	12.71	11.36	8.22	11.36	10.97	7.99	11.88	8.84	7.40	9.46	8.62	7.21
							11.94[b]	9.69	7.46	9.50	9.23	7.28
D-Xyloxazone							11.89	8.90	7.40	9.95	8.53	*c*
							11.98[b]	9.68	7.40	9.98	9.15	*c*
L-Rhamnosazone	12.74	11.18	8.69	11.44	10.73	8.04						
D-Glycosazone	12.53	11.40	8.36	11.40	10.57	7.77						

[a] Chemical shifts (ppm) relative to internal TMS at 60 MHz. [b] $+H_2O$. [c] Signals included with the benzene peaks.

Table XXXI

Chemical Shifts[a] and Spacings[b] of Ring Protons of Anomeric Pentopyranoses in D_2O

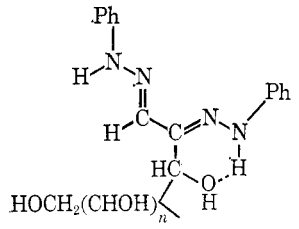
		Xylose		*Lyxose*		*Arabinose*		*Ribose*	
a	*b*	α	β	α	β	α	β	α	β
H-1	(H_1–H_2)	5.26 (3.1)	4.65 (7.4)	5.08 (4.2)	4.94 (1.5)	5.34 (2.7)	4.60 (7.2)	4.91 (2.1)	4.99 (6.4)
H-2	(H_2–H_3)	3.63 (–)	3.26 (8.5)	3.90 (–)	4.04 (3)	3.93 (–)	3.58 (9.9)	3.85 (–)	3.60 (3.1)
H-3	(H_3–H_4)	...	3.49 (8.5)	...	...	3.93 (3.4)	3.75 (3.6)	...	4.13 (3)
H-4	(H_4–H_{5a})	...	3.58 (10)	...	...	4.07 (–)	4.04 (–)	...	3.79 (10)
H-5a	(H_3–H_{5e})	...	3.36 (4.3)	...	3.32 (5)	...	...	...	...
H-5e	(H_{5a}–H_{5e})	...	4.00 (10.4)	...	4.09 (10)	...	...	...	...

[a] Chemical shifts given in ppm relative to external TMS. [b] Approximate coupling constants (cps) given in parentheses.

The addition of water to the *p*-dioxane solutions produced a downfield shift of the nonchelated N–H proton signal (1 ppm), due to solvation of the N–H group, thus distinguishing it from the imino protons which are N or O chelated. A chelated imino group in addition to a nonchelated group were found in the final forms of the sugar osazones given in Table XXX.[350] The N-chelated N–H proton signals were shifted further downfield, and the chelated imino protons in the final form of the osazones were O rather than N chelated.

Mester also observed long-range coupling characteristic of the *syn* (*cis* C-1–H and N–H–Ph) geometric isomers of aldehydophenylhydrazones in the final form of the osazones in mutarotation.

The final structure proposed for the osazones resembles the initial form and is a restricted rotational isomer about the C-1–C-2 bond in the structure proposed by Chapman.



Lemieux and Stevens[839] have determined chemical shifts of ring protons of aldopentoses in D_2O, using double irradiation and specific deuteration techniques. The spectrum of the sugar sample freshly dissolved in D_2O was determined at 60 Mcps (TMS external reference), and at equilibrium the 100-Mcps spectra of the mutarotated sugar solution was determined.

The spectrum of a freshly prepared solution of α-D-xylopyranose revealed a sharp doublet at 5.26 ppm and a narrow signal in the 3.5–3.9-ppm region. At equilibrium, the anomeric proton signals at 5.26 and 4.65 ppm (spacings of 3.1 and 7.4 cps, respectively) were assigned to the α and β anomers, respectively. Integration of the anomeric proton signals indicated the mixture contained 67% of the β anomer.

By spin decoupling, the shift of the α-anomeric H-2 signal was 3.63 ppm. A quartet at 4.00 ppm (4.3- and 10.4-cps spacings) was assigned to the equatorial 5 proton of the β anomer which was confirmed by examination of the spectrum of 5-deuterio-D-xylose. The intensities of the signals at field higher than 3.5 ppm corresponded to three protons relative to the intensity of the anomeric proton signal of the β anomer, and the signals due to H-2, H-3, and the axial proton, H-5, were assigned to this region of the spectrum. Irradiation of the β-anomeric proton indicated a shift of 3.26 ppm for the H-2 signal, and the triplets for the H-3 signal were displayed at 3.49 ppm.

The assignments of the ring protons of β-D-xylopyranose indicated that the compound existed in a "near" C1 conformation. Using similar spin-decoupling experiments, assignments were made for the ring protons of β-D-arabinose, α-D-xylopyranose, and β-D-ribose (Table XXXI).

β-D-Ribose underwent rapid mutarotation to produce a spectrum which had four signals of total intensity one. At 80°, no additional signals were noted and integration showed the total intensities for anomeric protons were one-fifth the intensities of other protons in the molecule. On the basis of spectral assignments for 5-*O*-methyl-D-ribose, the signals at 5.30 and 5.42 ppm in the D-ribose spectrum were assigned to the anomeric protons of the β- and α-furanose forms, respectively. After mutarotation, the ribose solution contained 6% α-furanose, 18% β-furanose, and 56% β-pyranose, and the signal at 4.91 ppm was attributed to the α-D-pyranose form present at 20% in the anomeric mixture. The chemical shift of this anomeric proton indicated that 1C is the preferred conformation of α-D-ribopyranose under these conditions.

The data in Table XXXI for arabinose do not distinguish between the 1C and C1 conformations. The anomeric proton

occurs at low field, and the other protons are not well shifted from one another. The 1C conformation has been attributed to β-D-arabinopyranose, however.[340]

From the data for the ring protons of aldopyranoses, the chemical shift of equatorial protons at a given position is independent of configurational changes at other ring proton positions. The shielding of an axial proton is affected by the axial or equatorial orientation of the hydroxyl group at neighboring positions, and an axial O–H group has a deshielding effect (*ca.* 0.35 ppm) on an opposing axial proton.[339,356,357]

Integration of the pmr signals of the isomeric forms of solutions of D-allose, D-altrose, D-talose, D-arabinose, and 3-deoxy-D-ribohexose has been used to determine the α- and β-furanose and pyranose composition of the aldose mixtures at equilibrium. Furanose signals are not detected in equilibrated solutions of D-glucose, D-mannose, D-galactose, or 2-deoxy-D-arabinohexose.[358]

Pmr spectral studies (60 Mcps) of D-glucose in D_2O and KOD have demonstrated the C1 conformation of the D-glucopyranose ring in neutral and alkaline solutions. Changes in the optical rotation of D-glucose in alkaline solution are ascribed to a shift in favor of the β anomer. Methyl β-L-arabinopyranoside produces similar spectra in alkaline and neutral solution, and the changes in rotation are explained by slight distortions in the ring due to the ionization of the axial O–H group.[359]

D-Gluconic acid and related compounds have been investigated by pmr in D_2O at 60 and 100 Mcps. The spectra were recorded as a function of pH to allow the effect of protonation of the carboxylate group on the chemical shifts of neighboring protons to be observed.[69] Tetramethylammonium chloride (TMA) was used as internal standard of reference, its constant chemical shift was independent of solution pH and composition, and its shift relative to the C–H protons of the compounds provided smaller and more sensitive sweep widths than TMS.

Pmr studies on the oxidative glycol scission of specifically deuterated D-glucose and its 3-*O*-methyl ether have been carried out in D_2O at 100 Mcps.[360] Structural assignments, based upon deuteration effects of the signal of the proton located α to the formyloxy group in the undeuterated compound, allowed the determination of the positions of formyl ester groups in the oxidation products of specifically deuterated D-glucose and the 3-*O*-methyl ether. The product of lead tetraacetate oxidation of 3-*O*-methyl-D-glucose-5-*d* displayed a modified signal, while the signal was absent in the spectrum of the monoformate obtained by periodate oxidation, suggesting the 3- and 4-formate, respectively. The furanose form of the methyl ether is involved in the lead tetraacetate reaction, while periodate oxidation degrades the pyranose form of the sugar directly. Lead tetraacetate oxidation of D-glucose-6-6′-d_2 yielded 2,3-di-*O*-formyl-D-erythrose-4-4′-d_2, and it was suggested that this degradation involves two successive pyranose to furanose interconversions.

The spectra of 1,4:3,6-dianhydro-D-glucopyranose, isolated from the vacuum pyrolysis products of amylose and 3,6-anhydro-D-glucose, and 1,4:3,6-dianhydro-D-mannopyranose in D_2O were examined at 60 Mcps and assignments confirmed by 100-Mcps double irradiation. The ring protons displayed a wide range of chemical shifts, the H-4 and H-1 signals almost coinciding, which was attributed to ring strain. A conformation in which the 3,6-anhydro ring has an "envelope" conformation with C-5 displaced from the plane of the four ring atoms was suggested.[361]

The ^{19}F and ^{1}H nmr spectra of a series of aldopyranosyl fluorides of known ring conformation and fluorine configuration with respect to the ring have provided measured fluorine chemical shifts and vicinal ^{19}F–^{1}H coupling constants and identification of the anomeric configurations of these compounds.[362,363] The 100- and 220-MHz spectra of similar fluoride derivatives indicates that α- and β- (D or L) pentopyranosyl fluorides assume a preferential conformation in which the fluorine substituent is in the axial orientation.[364]

Vicinal coupling constants obtained from the spectrum (100 MHz) of 2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl fluoride in acetone-d_6 were inconsistent with the C1 conformation, and indicated the derivative assumed the 1C conformation. Similarly, the 100- and 220-MHz spectra of 2,3,4-tri-*O*-benzoyl-β-xylopyranosyl fluoride also indicated the 1C conformation which requires the axial orientation of the bulky substituents. The anomeric effect of other halogen substituents has been noted; the 1-chloro derivative of 2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl fluoride also favors the 1C conformation.[365–367]

Long-range couplings between protons having 1,3-axial, equatorial ($^4J_{a,e}$), and possibly 1,3-diaxial ($^4J_{a,a}$) orientations have been noted.[368] With the compounds investigated, (2,3,4-tri-*O*-acetyl-1,6-anhydro-β-D-glucopyranose, 2,3,4-tri-*O*-acetyl-1,6-anhydro-β-D-mannopyranose, and 2,3,4-tri-*O*-acetyl-1,6-anhydro-β-D-iodopyranose), $^4J_{ee}$ was found to be positive and $^4J_{ea}$ negative. Derivatives undergoing conformational inversion at room temperature gave intermediate ^4J couplings. 1,2,3,4-Tetra-*O*-acetyl-β-D-ribopyranose, which exists in rapid conformational equilibrium at room temperature[357,369] had $J_{2,4} = +0.8$ Hz. ^4J couplings across the ring oxygen atom ($J_{1,5}$) were also observed, $^4J_{ee}$ couplings through the ring oxygen being smaller in magnitude than $^4J_{e,a}$ counterparts. ^5J coupling also is noted in these derivatives.

The 100-MHz spectra of 1-thioaldopyranoses having the β-gluco, β-galacto, β-xylo, and β-ribo configurations have been analyzed by comparing the ring proton signals in chloroform-*d*, acetone-d_6, and benzene-d_6 solvents.[370] The 220-MHz spectrum of 1-thio-α-L-arabinopyranose tetraacetate in chloroform indicates a C1 conformation for this compound.[371]

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Table XXXII[881]

Chemical Shifts[a] and Coupling Constants[b] of Some Di-, Oligo-, and Polysaccharides in DMSO

Compound	O-1-H doublet: Eq	O-1-H doublet: Axial	C-1-H doublet: Eq	C-1-H doublet: Axial	OH's under 5 τ: Doublets	Other OH's
α,α′-Trehalose [1-*O*-(α-D-glucopyranosyl)-α-D-glucose]			5.15 (3.0)			5.33 (4.5), doublet 5.48 (6.0), doublet 5.70 (4.0), triplet
α-Maltose [4-*O*-(α-D-glucopyranosyl)-α-D-glucose][d]		3.70 (4.5)	*c*		4.75 (c)	*c*
β-Maltose [4-*O*-(α-D-glucopyranosyl)-β-D-glucose]	3.40 (6.5)		5.01 (3.0)	5.70[e] (6.5, 6.5[e]) pair of doublets	4.63 (<2); 4.66 (6.5)	[5.07, 5.15, 5.21, 5.57][e]
α-Cellobiose [4-*O*-(β-D-glucopyranosyl)-α-D-glucose][d]		3.72 (4.5)	*c*	*c*	*c*	*c*
β-Cellobiose [4-*O*-(β-D-glucopyranosyl)-β-D-glucose]	3.42 (7.0)			5.60[e] (7.0, 7.0[e]) pair of doublets 5.71 (6.5)[e]	4.82 (3.5)	[5.06, 5.12, 5.39, 5.46, 5.54][f]
Maltotriose			5.00 (−)		~4.60 (−), ~4.68 (−)	[~5.15, ~5.60][f]
Isomaltotriose			~5.10 (−)			[~5.30, ~5.65][f]
Maltotetraose			~5.00 (−)		~4.60 (−)	[~5.20, ~5.50][f]
Maltopentaose			~5.00 (−)		~4.60 (−)	~5.50 (−)
Maltohexaose			~5.00 (−)		~4.60 (−)	~5.50 (−)
Amylose			4.90 (3.0[g])		~4.55 (−), 4.61 (−)	5.51 (−)
α-Cyclodextrin			5.19 (3.0[g])		4.55 (6.0), 4.59 (<2.0)	5.57 (−)
β-Cyclodextrin			5.17 (3.0[g])		4.33 (6.0), 4.38 (<2.0)	5.62 (−)
Laminaran				~5.56[g] (7.0[g])	4.75 (−)	[5.31, ~5.50][f]
Linear dextran			5.40[g] (2.5[g])			[~5.20, ~5.55][f]
Branched dextran			~5.10[g] (−) ~5.35[g] (−)			[~5.60, ~5.70][f]

[a] Chemical shifts given as τ values (ppm) relative to internal TMS at 60 Mcps. [b] Coupling constants (cps) given in parentheses. [c] Values not obtainable from the anomeric mixture. [d] Product obtained by mutarotation and examined in mixture with anomer. [e] Value obtained from deuterated product. [f] Prominent peaks from complex resonances. [g] Value obtained from 80 to 120° 100-Mcps spectrum.

C. OLIGOSACCHARIDES AND POLYSACCHARIDES

Oligo- and polysaccharides exhibit DMSO spectra in which the relative positions and intensities of the proton signals are similar to those present in the spectra of pyranoside monosaccharides. A difference, however, in the position of the nonanomeric hydroxyl signals has been noted in the 100-Mcps spectra of oligomers and polymers of glucose.[881] Thus, while α,α-trehalose, α-melibiose, and isomaltotriose display these nonanomeric signals between the usual τ 5 and 6 range, maltose, cellobiose, and maltotriose produce these O–H signals below τ 5 (Table XXXII).

A trend in the position of the nonanomeric O–H signals is apparent in the maltosaccharide series, in that a further downfield displacement below τ 5 occurs as the degree of polymerization increases (α-maltose, τ 4.75; β-maltose, τ 4.63 and 4.66; maltotriose, τ 4.60; amylose, τ 4.55).[881,872] Similarly, the Schardinger dextrins, α- and β-cyclodextrin (cyclohexaamylose and cycloheptaamylose), exhibit the nonanomeric signals further downfield (τ 4.55 and 4.59 for α-cyclodextrin, τ 4.33 and 4.38 for β-cyclodextrin) (Figure 18).

The nonanomeric O–H signal of maltose corresponds to two protons in intensity or two O–H groups per molecule. These low-field signals have been assigned by Reggiani to the protons of the internal O-2–H and O-3′–H groups which face one another in adjacent α-1,4-linked glucose units. Spin-decoupling experiments on β-maltose[853] have confirmed this assignment of the low-field O–H signals to the internal O-2–H (doublet at τ 4.66) and O-3′–H (singlet at τ 4.63), while amylose and the cyclodextrins have the O-2–H doublet at field lower than the O-3′–H signals. Similarly, the intensity of these signals in the maltotriose spectrum corresponds to four O–H groups per molecule. Measurements of maltotetraose, maltopentaose, and maltohexoase do not provide accurate evaluation of the number of hydroxyl groups corresponding to this low-field absorption, but the intensity of these signals indicates a trend which approaches a hydroxyl ratio of 2:3 proceeding from tetramer to polymer.

Linear (NRRL B-512F, 95%, 1,6 linkages) and branched

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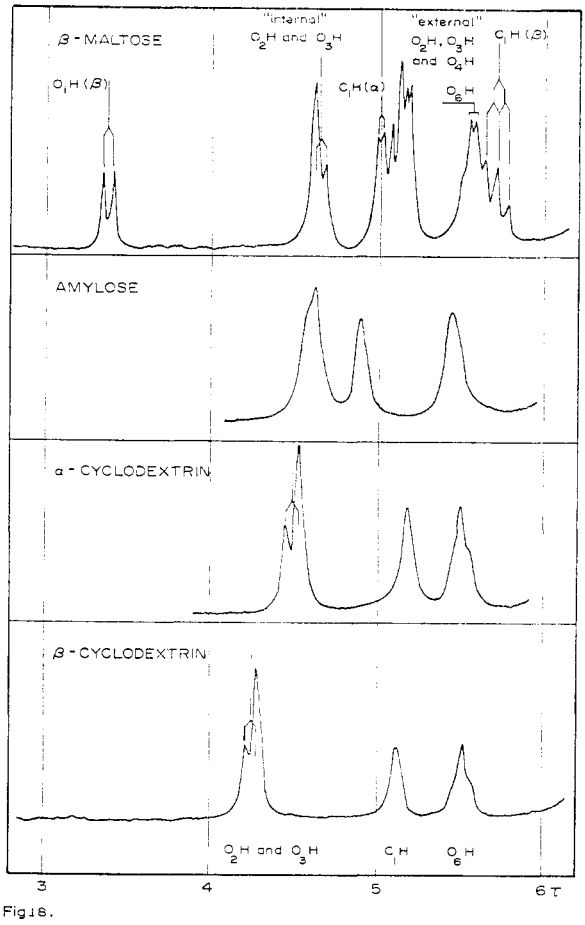

Fig 18.

Figure 18. Pmr spectra of β-maltose, amylose, and α- and β-cyclodextrin in DMSO at 100 Mcps at 28°.

(NRRL-B-742, 70% 1,6, 12% 1,4, and 18% 1,3 linkages) dextrans and 1,6-linked saccharides gentiobiose, melibiose, and isomaltotriose do not exhibit nonanomeric O–H signals below τ 5, while laminarin gives a signal at τ 4.75 of one proton in intensity.

Considering representative alcohols in order to determine environmental effects on O–H groups in the DMSO solvent, Reggiani determined that O–H groups free from the influence of electron-withdrawing groups absorb in DMSO between τ 5 and 6. Steric hindrance from other constituent groups (*e.g.*, the methyl groups in 2,3-trimethyl-2-butanol) partially prevents the association of the O–H function with the DMSO molecule, thus causing absorption at higher field than τ 6.

In an ir study of the formation of hydrogen-bond complexes of DMSO with cyclohexanol and each of the conformationally homogeneous 4-*t*-butylcyclohexanols in dilute CCl_4, the free O–H band absorbancy change upon addition of DMSO was used to qualitatively determine the degree of hydrogen bonding. For the dilute alcohol the sharp free O–H stretching band (ν_{max} 3624 cm^{-1}) was the only band in this region of the spectrum. Addition of DMSO gave rise to a broad symmetrical band at 3418 cm^{-1}, which was ascribed to the hydrogen-bonded O–H stretch of the alcohol–DMSO complex. Additional DMSO caused an increase in the intensity of the hydrogen-bonded O–H stretch band, and a decrease in that of the free O–H stretch.[333] The equilibrium constant (K_{eq}) for the formation of a 1:1 alcohol–DMSO complex was found to be constant within experimental error over a fivefold concentration variation of both donor and acceptor. The resulting data showed no significant difference in the strengths of the R–OH–DMSO hydrogen bonds of the three alcohols (cyclohexanol, *cis*-4-*t*-butylcyclohexanol, *trans*-4-*t*-butylcyclohexanol) contrary to the findings of Ouellette.[373]

In CCl_4 solution, axial and equatorial epimers form with DMSO hydrogen bonds of comparable strength, and factors other than hydrogen bonding appear to influence the chemical shift differences of axial and equatorial O–H protons in DMSO. The difference in O–H proton shielding of the rotational conformers (two axial and three equatorial) may result from the magnetic anisotropy of the C–C bonds, which affects the relative chemical shifts of axial and equatorial carbinol protons bonded directly to the ring. Axial and equatorial epimers can donate a proton to form a hydrogen bond, while the equatorial alcohol can accept a hydrogen-bonding proton more readily than its epimer.[333,374]

O–H groups which are intra hydrogen bonded in nonpolar solvents are replaced by solvent bonding with the DMSO molecule, with the product of signals in the usual range of between τ 5 and 6. In some cases, the intra hydrogen bond of a molecule is not broken by the solvent, and the signal for the O–H group occurs at low field (below τ 3).

Application of these findings to the O–H signals observed for oligo- and polysaccharides in DMSO indicated that those O–H functions producing signals between τ 5 and 6 are bonded or are available for bonding with the DMSO solvent, while O–H signals which resonate below τ 5 are involved in a stronger intra hydrogen bonding which is less affected by the DMSO solvent. From the linear relationship which was observed between the ir ν_{O-H} band and the chemical shifts of the O-2–H and O-3′–H signals of maltose, amylose, and α- and β-cyclodextrin, the stronger hydrogen bonding is considered to be intra hydrogen bonding since the accessibility of the O–H groups to DMSO is thought to be reduced rather than increased with an increase in the degree of polymerization. The ir band broadens in increasing order from maltose, maltotriose, amylose, and α- and β-cyclodextrins, while no downfield shift of the O–H signal or ir band broadening is observed in the spectra of oligosaccharide and polysaccharide molecules which do not display a close proximity of O–H groups on adjacent units (*e.g.*, α,α′-trehalose and the dextrans).[375]

Such intra hydrogen bonding will involve O-2–H and O-3′–H groups on adjacent units, and the proximity of these groups and the strength of the internal hydrogen bond will vary with the conformation of the monomeric units and the conformation of the polymeric chain which results from the rotation of the units about the glycosidic bonds.[331,376]

The C1 conformation of the glucose units in the cyclodextrins and amylose[377] has been supported by the chemical shifts and splittings of the C-1–H signals (Table XXXII). Monomeric units in this conformation are consistent with strong intramolecular hydrogen bonding between O-2–H and O-3′–H groups, and this internal association should

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promote stabilization of the chain conformation.[381] The 3B conformation of the units would also produce this same intra hydrogen bonding but would be inconsistent with the splitting values of the C-1–H signals and with the internal hydrogen bonding. The proposed conversion[387] of the B1 to 3B conformation from cyclic to linear dextrin indicates an increase in the C-1–H/C-2–H dihedral angle and a closer approach of the O-2–H and O-3′–H groups. The dihedral angle (Table XXXII) of the cyclodextrins and amylose are very nearly the same, and the internal hydrogen bond is stronger in the cyclodextrins than in amylose as indicated by the greater downfield shift of the nonanomeric O–H signals. Models of C1 units show the internal hydrogen bonding more likely to be stronger in cyclodextrins than in maltose, maltosaccharides, or amylose.[381]

The internal O-2–H and O-3′–H groups of maltose seem sufficiently accessible to DMSO if a slight rotation of the glucose units about the glycosidic linkage is assumed to relieve the nonbonding interactions of the C-1–H and C-4′–H bonds. Such rotational freedom about the linkage as well as the accessibility of the internal hydroxyl groups to the solvent would be progressively reduced going from dimer to polymer.[381,872]

Maximum closeness of the internal hydroxyls is predicted for the cyclodextrins due to the cyclic structure which reduces the rotational freedom about the glycosidic linkage. The strength of solvent–solute association with the O–H groups was studied using β-cyclodextrin because of its large downfield shift from τ 5 and lack of reducing groups. The 100-Mcps spectra of the cyclodextrin at 28 and 80° in DMSO-d_6 showed an upfield shift of the O-6–H signal (0.43 ppm) and the O-3–H and O-2–H signals (0.33 and 0.39 ppm, respectively) with increase in temperature owing to diminished solvent–solute association. This caused the O–H signals to broaden and coalesce with weakened hydrogen bonding. Similar behavior was not observed with the anomeric proton signal, which occurred at nearly the same field at both temperatures. At 80°, the splitting of the C-1–H signal (3.0 cps), as predicted from the 60 Mcps spectra at 38°, was identical with that observed for amylose and α-cyclodextrin. Only the O–H groups were affected by the temperature increase, indicating an effect of weakened hydrogen bonding with the solvent. Dextran and laminarin spectra at high temperature showed the splitting of the anomeric proton as 2.5 and 7.0 cps, respectively.[381]

Nmr and ir data suggest for linear dextran a chain conformation with no proximity of the glucose units, while for cellobiose and laminarin, internal hydrogen bonds involving one O–H group and an acceptor nonhydroxyl group is indicated. Reggiani has suggested models of cellobiose and laminarin in which the internal hydrogen bonds form six-membered rings joining successive glucose units, an arrangement which gives a linear chain together with a close proximity of the intra-hydrogen-bonded O–H group and the acceptor oxygen atom. The conformation for cellobiose implies a O-3′–H–O-5 intra hydrogen bond, while laminaran chain conformation indicates an internal hydrogen bond between an O–H group and the ring oxygen of the successive glucose units.

Intra hydrogen bonding between adjacent glucose units is regarded as a stability factor of cyclic structures or large helices (as in "V" amylose) composed of C1 units.[381,853,877,878] The occurrence of a O-2–H—O-3′–H internal hydrogen bond in DMSO suggests that a helix or a coil composed of short helical segments is substantially retained by amylose in this solvent. No information of the geometry or regularity of the helix is obtained from these findings.

Upon methylation at O-2 of α-1,4-linked di- and polyglucoses, the C-1–H proton of the ring bearing the glycosidic linkage shifts to lower field (Table XXXIII). This shift is not shown by the β-1,4-linked hepta-*O*-methylcellobioses. The C-1–H peak splitting is of the order of 3 cps for α-1,4- and 7.0 cps for β-1,4-linked products in both DMSO and $CDCl_3$.[381,884] The chemical shift difference between the anomeric proton signals in DMSO and $CDCl_3$ indicates that only in *O*-methylated methyl maltosides and amylose is the shift within the range observed for *O*-methylated glucoses (+0.17 to 0.29 ppm). Di- and tri-*O*-methylated α-cyclodextrin and tri-*O*-methylated β-cyclodextrin show a shift consistently lower (*ca.* +0.08 ppm). No solvent effect is shown by di-*O*-methylated β-cyclodextrin or by *O*-methylated cellobioses.

Upon methylation of adjacent hydroxyl groups, shifts in the hydroxyl signals occur similar to those observed for the monosaccharides: amylose (degree of substitution = 2.3) = −0.21 and +0.33 ppm, 2,6-di-*O*-methyl-β-cyclodextrin = 0.78 ppm, and 2,6-di-*O*-methyl-α-cyclodextrin = +0.68 ppm with respect to the O-3–H of unsubstituted products. Diglucoses hepta-*O*-methylcellobioses and methyl hepta-*O*-methylmaltosides give two separate signals for the O-6–Me and O′-6–Me groups. When the anomeric center is α-1,4- linked with another glucose residue, the O-2–Me signal shifts about 0.10 ppm downfeld with respect to the range (τ 6.64–6.67 in DMSO-d_6) of the O-2–Me signal.

As with unsubstituted cyclodextrin, the anomeric proton signal of O-methylated derivatives occurs upfield from the anomeric proton signals of O-methylated amylose and maltose. This has been attributed to the magnetic anisotropy effect associated with the O-1–C′-4 bond which produces a larger shielding of the anomeric proton in the cyclic structures. With steric restrictions in the macrocycles, the glucopyranose units of cyclodextrin are considered to closely approach a staggered conformation in which the C-1–H bond eclipses the O-1–C′-4 bond of the continuous unit, producing a shielding of the C-1–H proton.[384,879]

The areas under each pmr peak are proportional to the protons responsible for the peak, and since the area of the anomeric proton peak is not affected by methylation, the degree of substitution of partially O-methylated amylose and the cyclodextrins can be found by measuring the total area under the hydroxyl signals (A_{OH}) and referring it to the area under the anomeric proton peak (A_{C_1H}). The degree of substitution (DS) is then given as [353]

$$DS = 3 - (A_{OH}/A_{C_1H}) \quad (45)$$

The areas of the O–H and C-1–H peaks are readily measurable if the peaks do not overlap. When superposition occurs, as with O-methylated amylose, the O–H and C-1–H peaks can be graphically extrapolated. The hydroxyl absorptions are distinguished from the C-1–H peaks by taking the spectrum at higher temperature.

The pmr spectra of hepta-*O*-methylmaltoside (Figure 19) indicates that the chemical shift of the C-1–H protons be-

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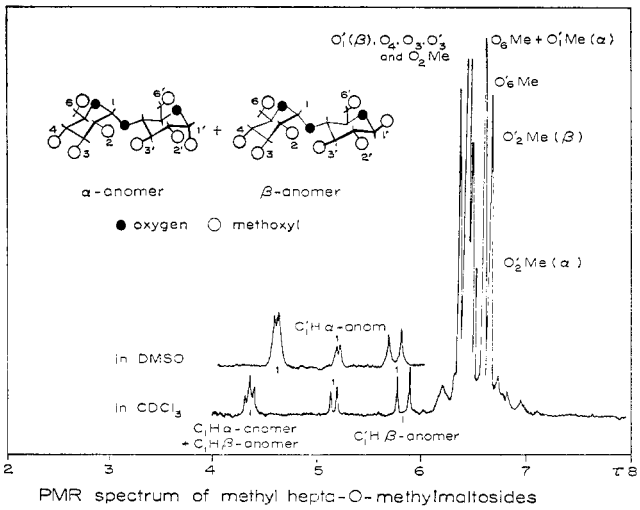
Table XXXIII[334]

Chemical Shifts[a] and Coupling Constants[b] of Some O-Methylated Glucosides in DMSO-d_6 at 38°

Compounds	*C_1H doublets*	*Nonanomeric OH's*[i]	*Assign.*	*O–CH_3 singlets*	*Assign.*
Methyl hepta-*O*-methyl-α-maltoside[g]	4.62 (3.0)[c]			6.52	O_2Me
	5.20 (3.0)[d]			6.52	O_3Me
				6.55	O_3Me
				6.59	O_4Me
				6.64	O_2Me
				6.71	O_1Me
				6.71	O_6Me
				6.77	O_6Me
Methyl hepta-*O*-methyl-β-maltoside[g]	4.62 (3.0)[c]			6.52	O_2Me
	5.77 (7.5)[e]			6.52	O_2Me
				6.55	O_3Me
				6.58	O_3Me
				6.59	O_4Me
				6.59	O_1Me
				6.71	O_6Me
				6.77	O_6Me
O-Methylamylose, DS = 2.3	4.61 (~3)	4.40[h]		6.55	O_2Me
		4.94[h]		6.59	O_3Me
				6.74	O_6Me
Tri-*O*-methylamylose	4.61 (~3[f])	...		6.55	O_2Me
				6.59	O_3Me
				6.74	O_6Me
2,6-Di-*O*-methyl-α-cyclodextrin	5.04 (~3)	5.37 (<2.0)	O_3H	6.52	O_2Me
				6.78	O_6Me
Tri-*O*-methyl-α-cyclodextrin	5.03 (3.0[f])	...		6.52	O_2Me
				6.62	O_3Me
				6.78	O_6Me
2,6-Di-*O*-methyl-β-cyclodextrin	5.03[f,h]	5.06[h]	O_3H	6.52	O_2Me
				6.76	O_6Me
Tri-*O*-methyl-β-cyclodextrin	4.97 (3.0[f])	...		6.52	O_2Me
				6.63	O_3Me
				6.76	O_6Me

[a] Chemical shift given as τ values relative to internal TMS, 60 Mcps. [b] Coupling constants (ppm) given in parentheses. [c] Equatorial C_1H or ring bearing the glycosidic linkage. [d] Equatorial C_1H of ring bearing the O_1Me group. [e] Axial C'_1H of ring bearing the O_1Me group. [f] Value observed after addition of 5% CF_3COOH and/or temperature variation. [g] Values obtained from an anomeric mixture. [h] Broad absorption. [i] Secondary = doublets; primary = triplet.



PMR spectrum of methyl hepta-O-methylmaltosides

Figure 19. Pmr spectrum of methyl hepta-*O*-methylmaltoside at 60 Mcps.

longing to the rings methylated at O-1 are little affected by the solvent. The ring bearing the glycosidic linkage in β-1,4-linked α- and β-cellobiose (linked through an equatorial glycosidic bond) exhibits no solvent effect, while α-1,4-linked α- and β-maltoside and amylose (linked through an axial glycosidic bond) display a consistent solvent effect in $CDCl_3$. The shift of the anomeric proton in DMSO with respect to $CDCl_3$ for methylated glucoses containing unsubstituted O–H groups is explained in terms of hydrogen bonding, but the solvent dependence of the anomeric proton signal of O-methylated α-1,4-linked linear di- and polyglucoses is considered a solvent-induced change of the chain conformation.[334] The shift is well reduced in O-methylated α-cyclodextrins which are assumed only moderately free to depart from the eclipsed chain conformation and is zero for O-methylated β-cyclodextrins which have a more tightly constrained conformation.

Molecular models of 2,6-di-O-methylated cyclodextrins built with C1 units suggest the O–H at C-3 of each unit is hydrogen bonded to the O'-2 of the contiguous unit. From pmr data, the O-3–H proton of dimethylated cyclodextrins are not clearly indicative of an intramolecular hydrogen bonding, since these signals in DMSO fall within the range of hydroxyls essentially hydrogen bonded to the solvent. The low-field signals of dimethylated cyclodextrins in $CDCl_3$ (τ 5.09 for α- and τ 4.95 for β-cyclodextrin derivatives) indicate a stronger hydrogen bonding, and the low frequency

and the solvent and concentration independence of the ir ν_{OH} band of 2,6-di-O-methylated cyclodextrins suggest an intramolecular hydrogen bond not broken by polar solvents or replaced by molecular self-association. The slightly lower frequency and the higher intensity of the ν_{OH} band of the β-cyclodextrin with respect to the α-cyclodextrin indicates a stronger hydrogen bond for the β-cyclodextrin derivative.

A similar internal hydrogen bonding is indicated in partially O-methylated amylose, from the ν_{OH} absorption in DMSO in the 3450–3410-cm^{-1} region and the two low-field pmr O–H signals attributed to residual O-2–H and O-3–H groups hydrogen bonded to the oxygen atoms at C-1 and C-2 in adjacent residues. The internal hydrogen bond in amylose is probably similar to that found in the dimethylated cyclodextrins, which stabilizes a helical conformation of the amylose chain as suggested for the unsubstituted polymer.

Internal hydrogen bonding may effect the relative reactivities of the hydroxyls of amylose and cyclodextrins during methylation. The O–H at C-3 of amylose is resistant to methylation with dimethyl sulfate. The low reactivity at C-3 of amylose is explained by the intramolecular hydrogen bond involving the hydroxyls at C-3 and C-2 in amylose and cyclodextrins with C1 units and the resistance to methylation, and the intramolecular hydrogen bond strength increases from amylose to α- to β-cyclodextrins.

Pmr studies of oligo- and polysaccharides in D_2O solution have produced results similar to those obtained from later DMSO spectra. Because of mutarotation in this solvent, the anomeric protons of reducing units appear in different regions of the spectrum, and the signals due to the O–H protons are not observed due to hydrogen–deuterium exchange. From the signals produced by the anomeric protons, the apparent coupling constants (measured spacing) have been used to differentiate the diaxial and axial–equatorial orientation of the C-1 and C-2 protons in oligomer and polymers of glucose.[358,360] From these data, the C1 conformation has been suggested for the glycopyranose units of maltose, cellobiose, and α- and γ-Schardinger dextrins and other polyglucoses (Table XXXIV).

Table XXXIV[380]

Chemical Shifts[a] and Coupling Constants[b] of the Anomeric Protons of Oligomers and Polymers of Glucose in D_2O

	J_{H1-H2}	Anomeric H_e	Anomeric H_a	*Electronegativity of O-1 substituent*
Hydrolyzed starch (α-1,4-)	3.0	4.62		2.48
Dextran B1355 (α-1,3-)	*c*	4.73		2.47
(α-1,6-)	*c*	5.07		2.41
Dextran B512E (α-1,6-)	2.3	5.09		2.41
Methyl α-D-glucopyranoside	3.3	5.25		2.34
Methyl α-D-isomaltoside	2.8	5.17		...
	3.0	5.10		
Crown gall polysaccharide (β-1,2-)			5.12	2.54
Laminarin (β-1,3-)	6.7		5.25	2.47
Methyl β-D-glucopyranoside	7.4		5.65	2.34

[a] Chemical shifts given as τ values relative to HOD signal (τ 5.30). [b] Coupling constants (measured spacings), cps, given as J_{H1-H2}. [c] Methyl signal; insufficient resolution.

The variation in the chemical shift values of the α- and β-linked series indicates a superposition of the equatorial and axial anomeric proton ranges which has been interpreted by the differences in the electronegativity of the substituent at C-1 of the anomeric series.[380] Thus, as the chemical shift values within a series increase, the electronegativity of the anomeric substituent decreases (Table XXXIV).

As in the DMSO solvent, anomeric proton signals of α- and γ-Schardinger dextrins (τ 4.95, 2.7 cps, and τ 4.93, 3.5 cps, respectively) occur at fields higher than the anomeric proton signals of maltose (τ 4.80, 3.3 cps) and starch (τ 4.62, 3.0 cps). The chemical shift of the anomeric proton of α-cyclodextrin is intermediate between the shifts of these signals of acid-hydrolyzed starch and laminarin (τ 4.62 and 5.25, respectively), and this is explained by the possible orientation that the entire cyclodextrin can assume relative to the magnetic field which makes the pyranose rings magnetically equivalent. The anomeric proton signals of α-cyclodextrin are assumed at a field typical of similar signals of protons free from ring current effects.[380] Alternatively, the magnetic anisotropy effect of O-1–C-4′ bond produces a larger shielding of the anomeric proton in cyclic structure than in linear ones.[384]

Acetoxy signals occur as sharp singlets in the spectral region of τ 7.8–8.0 in $CDCl_3$ with equatorial signals appearing in the τ 7.8–8.0 range and axial signals in a more restricted region of the spectrum.[327,353] Exceptions to this generalization have been reported.[292,357] The spectra (60 Mcps) of α-cyclodextrin and amylose acetates display a signal within the axial region, while cellulose triacetate gives this signal at field greater than τ 8.[353,381]

The deshielding effect of the acetyl group at C-1 on the anomeric proton is not apparent in acetylated polysaccharides, and the anomeric proton signal occurs at higher field and may be superimposed on the nonanomeric proton signals at higher field.[353]

Ir spectral data of acetylated amylose and the cyclodextrin provide limited information due to the dominating absorption by the acetoxy groups.[353] The lack of polarization of the C=O and C—O acetyl absorptions in an oriented sample of amylose acetate is explained by an average alignment of these bonds along the polymer axis.[353] The ir polarization of linear cellulose acetate, which displays a strong perpendicular dichroism of the C=O and C—O acetyl bands and parallel dichroism of the C—O glycosidic band, is suggested as consistent with a substantially linear polymer having acetyl groups projected in a plane normal to the chain axis.[353]

Nmr studies on the viscosity of cellulose in urea and the effects of crystallinity and water content on polysaccharide pmr signals have been reported.[382,383]

The spectra of isomaltotriose and linear (NRRL B-512, 96% α-1,6, 4% α-1,3 linkages) and branched (NRRL B-742, 70% α-1,6, 18% α-1,3; and 12% α-1,4 linkages) dextrans have been examined in D_2O.[384]

In the isomaltotriose spectrum, the nonanomeric C–H signals are displayed at −3.70 and −3.90 ppm relative to

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Table XXXV

Pmr Spectral Data of Isomaltotriose, Dextrans, and Dextran Sulfates in D_2O[a]

Compound	*Chemical shift*[b] *C-5, C-6*	*C-2, C-3, C-4*	*C-1*[c]	*C-1*[d]	*C-1*[e]	*DOH*
Isomaltotriose	−3.69	−3.88	−5.05			−4.78
Linear dextran (B512)	−3.70	−3.96	−5.05			−4.80
Linear dextran sulfate	−3.70	−3.95	−5.06	−5.35		−4.80
Branched dextran (B742)	−3.70	−3.90	−5.05		−5.40	−4.80
Branched dextran sulfate	−3.70	−3.91	−5.06	−5.38	−5.38	−4.80

[a] 60 Mcps. [b] Chemical shifts relative to external TMS (ppm). [c] Anomeric carbon bearing the 1,6 linkage. [d] Anomeric carbon bonded to the sulfate-bearing C-2. [e] Anomeric carbon of the "non-1,6-linkage."

external TMS standard, while the anomeric proton signals of the two carbons bearing the α-1,6 linkage appear at −5.05 ppm. The protons of the reducing end group produce signals at −4.67 and −5.29 ppm for the α and β anomers, respectively.

The proton signals of the reducing end groups are not apparent in the spectrum of linear dextran, which consists of the peaks due to the nonanomeric protons and the protons of the carbon atoms bearing the glycosidic linkages (Table XXXV). The branched dextran spectrum is similar to that of the linear polysaccharide, excepting an additional peak at −5.40 ppm which is ascribed to the C-1 proton of the "non-1,6-linkage."

At a given concentration the combined intensities of the nonanomeric C–H signals are the same for both dextrans, and the intensity of the proton signal of the carbon atoms bearing the glycosidic linkages of linear dextran equals the sum of the intensity of this same signal and the signal assigned to the proton of the "non-1,6-linkage" in the branched dextran. The ratio of the peak areas of the 1,6-linkage proton signal and the non-1,6-linkage proton signal was calculated as 2.4 or 7.29, which agrees with earlier periodate oxidation results.[384]

In the pmr spectra of sulfated linear and branched dextrans in D_2O, an additional peak (−5.35 and −5.38 ppm, respectively) is displayed (Table XXXV), whose chemical shift is the same as that of the anomeric proton of the "non-1,6-linkage" in the branched dextran sulfate.[385] The presence of this additional signal in the spectrum of the linear dextran sulfate implicates the sulfate group. In addition, a broad shoulder, more pronounced in the sulfated branched dextran, occurs between −4.0 and −4.5 ppm in both spectra.

These additional peaks are assigned to the protons attached to the C-1 carbons bonded to sulfate-bearing C-2 carbons, while the shoulder peaks are attributed to protons of the sulfate-bearing C-2, C-3, and C-4 carbons. Sulfate groups attached to C-2, C-3, and C-4 of the anhydroglucose units of linear dextran reduce the electron density in the vicinity of these protons causing a decrease in the peak intensity and a concomitant increase in a new signal. The effect of sulfate groups on the adjacent carbon, C-2, on the anomeric protons associated with the 1,6 linkages produces a separate peak shifted from that of the anomeric proton signal.

Preferential substitution of the sulfate groups in linear dextran is indicated from the spectrum. If sulfate groups are assumed equidistant among the C-2, C-3, and C-4 carbons, in partially sulfated linear dextran (0.74 sulfate group per anhydroglucose unit), a third is assumed effective in producing the new peak at −5.35 ppm and reducing the peak due to the proton of the anomeric carbon of the 1,6 linkage. The ratio (R) of these peak areas to the area of the new peak is given in eq 46. The measured value of this peak ratio was 0.84

$$R = \frac{\text{no. of moles of sulfate} \times 1/3}{\text{no. of moles in repeating unit} - \text{no. of moles of sulfate} \times 1/3} \quad (46)$$

in the case of linear dextran, and the sulfate groups were preferentially substituted at the C-2 carbon (62%) by back calculation.

Pmr spectral studies of heparin and related compounds have been made in D_2O.[386–388] Heparin obtained from various sources is similar in that it is composed of partially sulfated units of α-D-glucuronic acid and 2-amino-2-deoxy-α-D-glucose linked by 1,4 bonds.[387,389] A small proportion of N-acetyl groups as well as sugar units and glycosidic linkages other than 1,4 bonds have been noted in the structure of heparin, and variation in composition and biological activity with source occurs.

Examination of the spectra (60 Mcps, DSS internal reference) of chondroitin sulfates A and C isolated from whale cartilage and shark, respectively, in D_2O reveals a signal at −3.8 ppm in the chondroitin sulfate A spectrum which is assigned to the C-6 protons of galactosamine.[386] This signal is shifted downfield in the chondroitin sulfate C spectrum due to the sulfate group at C-6, and a new peak occurs (−4.2 ppm). The signal of the protons of the sulfate-bearing C-4 of galactosamine in chondroitin sulfate A is assigned to the peak at −4.5 ppm.

Heparin samples from bovine lung and whale intestine reveal spectra which contain a signal at −5.4 ppm which is not present in the chondroitin sulfate spectra. This signal is assigned to the equatorial anomeric proton, and the ratio of this peak to the sum of the peak areas in the −3.3 to −5.0 ppm region of the spectrum is 5.2. A peak at 4.9 ppm is assigned to the C-2 protons of N-sulfated glucosamine, and the peak at −4.1 ppm is assigned to the protons on the sulfate-

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bearing C-6 atom. Whale heparin produces a peak at −2.00 ppm which is assigned to the acetyl group on the amino group of glucosamine. The ratio of the peak due to the α linkage (−5.5 ppm) to the area of the acetyl signal was calculated as 8:3; on the basis of these assignments, one acetyl group for every eight α linkages was suggested for whale heparin.

A similar spectral analysis of different heparin samples revealed a signal due to the α-anomeric proton. The molar ratio of acetyl groups to glucosamine was obtained by a comparison of the sum of the τ 5.0–7.2 peak area of the spectrum with the peak area due to the methyl protons (τ 8.0) of the acetyl group.[388]

Perlin[387] has compared the pmr spectra of heparin samples at 100 Mcps in D_2O. Anomeric signals[8] are assigned to the 4.7–5.5-ppm spectral region, on the basis of chemical shift values and relative signal intensities. These signals suggest the presence of three types of sugars in approximately equimolar proportions.[387]

The anomeric proton signal at 5.35 ppm is assigned to H-1 of the N-sulfated 2-amino-2-deoxy-α-D-glucopyranosyl units. A broad signal centered at 3.25 ppm is assigned to the H-2 of this unit. α-D-Glucopyranosyl units sulfated at position 2 are assigned to the anomeric signal at 5.20 ppm and the anomeric signal at 4.75 ppm is suggested for the unsulfated α-D-glucuronsyl unit or alternatively the H-1 of a β-D-uronide sulfated at C-2.[387] It was noted that the latter signal was strongly influenced by pH and is displaced during N-desulfation of the heparin.

Heparitin sulfate produces a spectrum which is similar to that of the whale heparin.

Acknowledgments. In conclusion, the authors express their gratitude to the following for reprints (and preprints): Professor R. U. Lemieux, Professor M. Reggiani (and ref 353), Dr. W. J. O'Sullivan, and Dr. O. Jardetzky.

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We also thank our typist, Miss Diane Johnson, for exceedingly efficient and rapid work.