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Proton Magnetic Resonance Spectra of Some Proteins

I Ribonuclease, Oxidized Ribonuclease, Lysozyme and Cytochrome C

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FACILITY FORM 602

N65-29650
(ACCESSION NUMBER)

(THRU)

22
(PAGES)

1
(CODE)

CR 58642
(NASA CR OR TMX OR AD NUMBER)

04
(CATEGORY)

GPO PRICE $ _____

CFSTI PRICE(S) $ _____

Hard copy (HC) 1.00

Microfiche (MF) .50

ff 653 July 65

[redacted]

REPORTS CONTROL No.

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[redacted]

Proton Magnetic Resonance Spectra of Some Proteins

Introduction

In 1957, Saunders, Wishnia and Kirkwood (1) observed the first proton magnetic spectrum of a protein, pancreatic ribonuclease, in D_2O. Their spectrum, obtained at a frequency of 40Mc, consisted of four broad peaks and was interpreted by Jardetsky and Jardetsky (2) on the basis of the n.m.r. spectra of the individual constituent amino acids. The author has examined the proton spectra of ribonuclease, oxidized ribonuclease, lysozyme (3) and cytochrome c at the higher resolution and sensitivity afforded by a 100 Mc apparatus. Since the n.m.r. spectra of many of the 20 common L-amino acids dissolved in H_2O had heretofore not been observed because of sensitivity limitations (4), it was decided to obtain the spectra of all 20 amino acids referred to a common standard, which had been used in the protein spectra.

A number of dipeptides (5) and tripeptides were investigated to explore the effect of the peptide bond on the chemical shifts* of the amino acid protons.

Materials and Methods

All spectra were obtained on a Varian Associates H100 spectrometer operating at a fixed frequency of 100 Mc. For calibration purposes the spectra were also run on a HA60 spectrometer with its calibrated magnetic field sweep. Because of the low solubility of tyrosine, a computer of average transients (CAT) was used in conjunction with the A60 spectrometer to obtain its spectrum. Sodium 2,2-dimethyl-2 sila pentane-5 sulfonate

(DSS) was used as an internal reference standard for all chemical shift measurements. DSS is always on the high field side of the spectrum.

The amino acids and peptides used were of the highest quality manufactured by Mann Research Laboratories. The proteins, ribonuclease, lysozyme and cytochrome c were supplied by Sigma Chemical Company and the oxidized ribonuclease was from Mann Research Laboratories. Solutions were prepared in D_2O obtained from Bio-Rad Labs. All solutions were evaporated several times to reduce the H_2O content to a minimum. The protein concentration was about 5% in 0.5 ml of D_2O. Unless otherwise stated, the proton magnetic resonance spectra were observed at a temperature of $30 \pm 3°C$.

Results and Discussion

Most of the amino acid spectra can be understood on the basis of first order effects, as the chemical shift is generally much larger than the spin-spin couplings. To illustrate this effect we show in Figure 1a the spectrum of an aqueous solution of alanine in D_2O. The CH resonance is split into a quartet by the magnetic field of the CH_3 protons, which can assume discrete values proportional to the allowable values of angular momentum, $I = 3/2, 1/2, -1/2, -3/2$, of the CH_3 group. The CH_3 resonance is split into a doublet by the CH proton whose angular momentum in the presence of an external field can only take on the values $\pm 1/2$ (in units of $\hbar$). The chemical shift between these groups is much larger than the splitting caused by spin-spin interactions.

In those amino acids where the chemical shift is not large compared to the

spin-spin couplings, a very complex pattern of resonance lines is observed, as shown in Figure 1b for serine. The presence of the oxygen atom decreases the shielding of the neighboring CH_2 and therefore its resonance moves down field where it overlaps with the resonance of the CH proton. In this situation the chemical shift and spin-spin interaction are comparable. Between these extreme cases there are many amino acids, part of whose spectrum is first order and part second order. In Figure 1c we see the spectrum of glutamine where the CH resonance is first order, i.e., split into a triplet by the neighboring CH_2 group. Although the two CH_2 groups are separable, their resonance patterns are quite complicated because their chemical shift difference is not large compared to the spin-spin couplings.

As higher magnetic fields become available, these patterns will simplify as the chemical shift is proportional to the field strength but the spin-spin coupling remains constant. A comparison of the glutamine spectrum in Figure 1c taken at 100 Mc with one taken at 60 Mc (figure 2) shows very nicely the advantages of higher magnetic field operation.

In all cases, there is a rapid exchange of the hydrogens attached to nitrogen; thus all the peaks seen in D_2O can be attributed to hydrogens bonded to carbon atoms. Previous work on proton spectra of amino acids (4) indicates that these chemical shifts are independent of pH in the range pH2 to pH9 and independent of concentration.

The chemical shift of the center of the pattern for each chemical group is given in Table 1.

The data on the chemical shifts of the amino acid groups in peptides are presented in Table II. Although this list includes only a few of the total number of possible dipeptides and tripeptides, some general conclusions about the effects of the peptide bond on the chemical shift of amino acid protons are given below. In the following discussion, by "chemical shift" we mean the change in chemical shift from that of the free amino acid.

1. α-carbon protons, which include the CH_2 group in glycine, undergo the largest chemical shifts. In the dipeptides observed this varied from 0.15 p.p.m. for the α-proton of histidine in the dipeptide L-histidylglycine to 0.48 p.p.m. for the α-proton of leucine in the dipeptide leucyl-L-glycine.

2. From data on the oligopeptides of glycine it appears that the chemical shift of the α-protons is effected mainly by nearest and next nearest neighbors for short chains.

3. In tripeptides the chemical shifts for the α-protons of the middle amino acid are roughly additive. In glycyl-L-leucine the chemical shift for the α-proton of leucine is -.48 p.p.m. and in L-leucylglycine it is -.30 p.p.m.. In the tripeptide glycyl-L-leucylglycine, where leucine is involved in two peptide bonds, the chemical shift for its α-proton is -.70 p.p.m.. In the tripeptide L-leucylglycylglycine we find that the two glycine CH_2 groups have chemical shifts of -.21 and -.46 p.p.m.. Since the chemical shift of the CH_2 group of glycine in L-leucylglycine is -.24 p.p.m. and in glycylglycine is -.27 and -.31 p.p.m. we would, on the basis of this additivity rule, assign the chemical shift of -.46 p.p.m.

to the glycine bonded to leucine. Other evidence for this assignment based on steric hindrance to free rotation will be given later.

4. In the tripeptides examined the resonance of the α-proton of the N-terminal amino acid shifts to slightly lower fields (more negative chemical shift) and the C-terminal α-proton shifts to slightly higher fields compared to the corresponding dipeptides. For example, in L-leucylglycine we find the α-proton of leucine shifted -.30 p.p.m. and in L-leucylglycylglycine the shift is -.33p.p.m..

5. Methylene group shifts seem to be quite variable. For leucine in both dipeptides and tripeptides the shift is small, of the order of $\pm$0.1 p.p.m.. In histidine and proline dipeptides the shift in the methylene resonance ranges from +0.21 to -.30 p.p.m. respectively. For a given amino acid in a dipeptide the shift is more negative when it is the C-terminal end.

6. Methyl groups are the most shielded (occur at highest fields) in the amino acids and seem to be least effected by the peptide bond with shifts of the order of $\pm$0.04 p.p.m..

7. Imidazole protons can undergo sizable shifts both up and down field.

In addition to the chemical shift effects there are some interesting steric conformational changes which are illustrated in Figure 3. In the L-leucylglycine spectrum (Figure 3a), the leucine is essentially unchanged from its amino acid spectrum except for the chemical shift, but the free rotation of the CH_2 protons of glycine is hindered, making the two protons non-equivalent and each one has its own chemical shift. In the spectrum of glycyl-L-leucine (Figure 3b) the glycine resonance is unchanged except for the shift, but the free rotation

of the CH_2 and CH_3 groups of leucine becomes hindered. In Figure 3c we see the spectrum of L-leucylglycylglycine where the low field glycine CH_2 resonance is split because of the non-equivalence of its two protons. We assign this glycine to the mid-position of the tripeptide.

In Figure 4 we see the proton spectra of ribonuclease, oxidized ribonuclease, lysozyme and cytochrome c (horse heart). Allowing for the chemical shift due to peptide bond formation, the spectra of these proteins can be correlated with the chemical shifts of their constituent amino acids. This correlation for ribonuclease is presented in Table III. Using the data in Tables I and II, this correlation can be made for the other proteins. A correlation of this kind requires a knowledge of the amino acid composition and some rough ideas of the effects of peptide bonds. As the resolution of our spectra increases, it will require even more detailed knowledge of the amino acid sequences and of the effects of peptide bonds and protein conformation on chemical shifts.

The real usefulness of high resolution n.m.r., however, lies in the fact that because of the chemical shift specific amino acids can be identified and isolated in the protein spectra. In ribonuclease, peak II arises solely from one of the imidazole protons of histidine. Histidine residues are thought to be involved in the active site. This provides us with a window to observe the active site under various conditions. Peak III is due mainly to the aromatic rings of tyrosine (24 protons) and phenylalanine (15 protons) and an imidazole proton of histidine (4 protons). The effect

of temperature denaturation of this peak will be discussed in a forthcoming publication. In comparing the spectra of ribonuclease and oxidized ribonuclease, we see that the spectral lines in the latter are much narrower. The disulfide bridges of ribonuclease play a key role in maintaining structure; their cleavage by oxidation (7) results in a loosening of the structure and allows for increased motion of the side chains of the unfolded protein. It is well known in n.m.r. theory (8) that motion (rotation and translation) reduces the width of a resonance line. It is this "motional narrowing" which is the basis for high resolution n.m.r. spectroscopy.

In lysozyme, the aromatic protons again are well isolated from the rest of the spectrum as shown in peak I (Figure 4c). Peak Ia is due to 24 tryptophan protons; peak Ib arises from 18 tryptophan protons and 15 phenylalanine protons and peak Ic from 12 tyrosine protons.

Summary

Proton magnetic resonance spectra of 20 common amino acids and some representative di-and tripeptides are presented. Some general conclusions about the effect of peptide bonds on chemical shifts are given. The spectra of ribonuclease, oxidized ribonuclease, lysozyme and cytochrome c are shown and the significance of their high resolution n.m.r. spectra indicated.

Acknowledgements

The author would like to thank Eugene Pier of Varian Associates and Dr. Lois Durham of the Stanford Chemistry Department for their technical assistance in

obtaining the spectra. This work was supported by grants from the ~~U.S.~~ National Institutes of Neurological Diseases and Blindness grant NB-04270, National Institutes of Health Linc Computer Evaluation grant FR-00151-01, and from N.A.S.A. grant NsG 81-60.

Footnote

*The chemical shift in this paper is defined as $\frac{H\ \text{sample} - H\ \text{reference}}{H\ \text{reference}} \times 10^6$ and is given in parts per million. H is the magnetic field required for resonance.

References

1. Saunders, M., A. Wishnia, and J. G. Kirkwood, J Am Chem Soc 79: 3289 (1957).

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3. Saunders, M. and A. Wishnia, Ann NY Acad Science 70: 870 (1958).

4. Jardetsky, O. and C. D. Jardetsky, J Biol Chem 233: 383 (1958).

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6. Wishnia, A. and M. Saunders, J Am Chem Soc 84: 4235 (1962).

7. Harrington, W. F. and M. Sela, Biochim et Biophys Acta, 31:427 (1959).

8. Bloembergen, N., E. Purcell and R. V. Pound, Phys. Rev. 73:679 (1948).

Captions

Figure 1: Proton magnetic resonance spectra of an aqueous solution of alanine, serine and glutamine in D_2O taken at 100 Mc in a magnetic field of 23,000 gauss. DSS is an internal reference standard.

Figure 2: Proton magnetic resonance spectra of an aqueous solution of glutamine in D_2O taken at 60 Mc in a magnetic field of 13,900 gauss.

Figure 3: Proton magnetic resonance spectra of aqueous solutions of the dipeptides, L-leucylglycine and glycyl-L-leucine and of the tripeptide L-leucylglycylglycine in D_2O taken at 100 Mc.

Figure 4: Proton magnetic resonance spectra of ribonuclease, oxidized ribonuclease, lysozyme and cytochrome c observed at a frequency of 100 Mc. The proteins (5% by weight) were dissolved in .5 ml of D_2O and were at a pH of 4.5, 3.4, 5.4, and 9.5 respectively.

Table I

Chemical shifts of amino acid protons in D_2O

(All shifts are negative, i.e., to lower magnetic field, with an accuracy of ± .04 p.p.m.)

Amino acid	CH	CH_2	CH_3	Other	
Glycine		3.56			
Alanine	3.78		1.49		
Valine	α 3.62 2.29		1.06		
Leucine	α 3.70 1.71	1.71	.96		
Isoleucine	α 3.66 1.97	1.35	1.00		
Serine	3.94	3.94			
Threonine	α 3.58 4.23		1.32		
Tyrosine	n.o.	n.o.		Aromatic ring	6.95
Phenylalanine	3.97	3.20		Aromatic ring	7.36
Tryptophan	4.04	3.38		Aromatic ring	7.25 7.48 7.65
Aspartic acid	4.08	3.01			
Glutamic acid	3.75	2.49 2.12			
Asparagine	4.00	2.90			
Glutamine	3.77	2.43 2.16			
Lysine	3.37	2.95 1.65 1.65 1.65			
Arginine	3.18	3.18 1.63 1.63			
Histidine	3.97	3.16		Imidazole	7.03 7.72
Cysteine	3.82	2.93			
Methionine	3.80	2.60 2.12	2.12		

	α 3.66 1.97	1.35	1.00		
rine	3.94	3.94			
Threonine	α 3.58 4.23		1.32		
Tyrosine	n.o.	n.o.		Aromatic ring	6.95
Phenylalanine	3.97	3.20		Aromatic ring	7.36
Tryptophan	4.04	3.38		Aromatic ring	7.25 7.48 7.65
Aspartic acid	4.08	3.01			
Glutamic acid	3.75	2.49 2.12			
Asparagine	4.00	2.90			
Glutamine	3.77	2.43 2.16			
Lysine	3.37	2.95 1.65 1.65 1.65			
Arginine	3.18	3.18 1.63 1.63			
Histidine	3.97	3.16		Imidazole	7.03 7.72
Cystoine	3.82	2.93			
Methionine	3.80	2.60 2.12	2.12		
roline	4.08	3.34 2.06 2.06			

.o. = not observed

Table II

Chemical shifts of amino acid protons in peptides with respect to the corresponding chemical group in the free amino acid

Peptide	Amino Acid	Chemical Group CH	CH_2	CH_3	Other
Glycyl-L-leucine	Glycine		α-.27		
	Leucine	α-.48 β-.11	-.11	+.04	
L-Leucylglycine	Leucine	α-.30 β-.02	-.02	0.00	
	Glycine		α-.24		
Glycyl-L-proline	Glycine		α-.39		
	Proline	-.21	-.30 0.00		
L-Prolylglycine hydrate	Proline	-.33	-.08 -.05		
	Glycine		α-.24		
L-Histidylglycine	Histidine	-.15	-.04		Imidazole -.11 -.26
	Glycine		α-.22		
Histidylhistidine	Histidine	-.45	+.14		Imidazole +.20 +.13 -.01 +.16
		-.05	+.21		
L-Leucyl-L-phenylalanine	Leucine	n.o.	n.o.	+.05	
	Phenylalanine	n.o.	n.o.		Aromatic ring +.06
Glycylglycine	Glycine		-.27 -.31		
Glycylglycylglycine	Glycine		-.22 -.34 -.49		
L-Leucylglycylglycine	Leucine	α-.33 β-.03	-.03	0.00	
	Glycine		-.21 -.46		
Glycyl-L-leucylglycine	Glycine		-.20 -.31		
	Leucine	-.70	+.07	+.04	
Tetraglycine	Glycine		-.21 -.34 -.42 -.49		

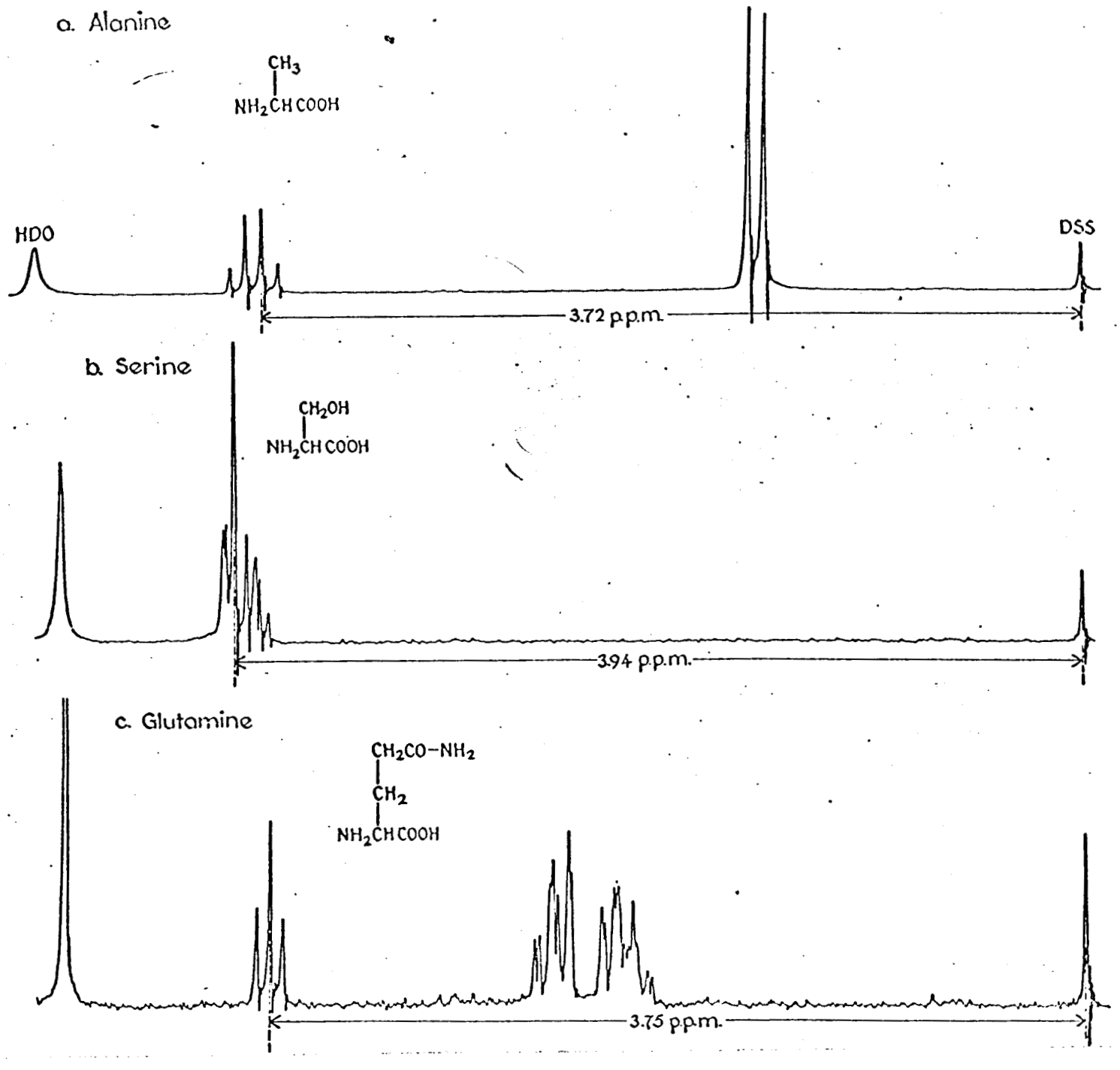
n.o. = not observed

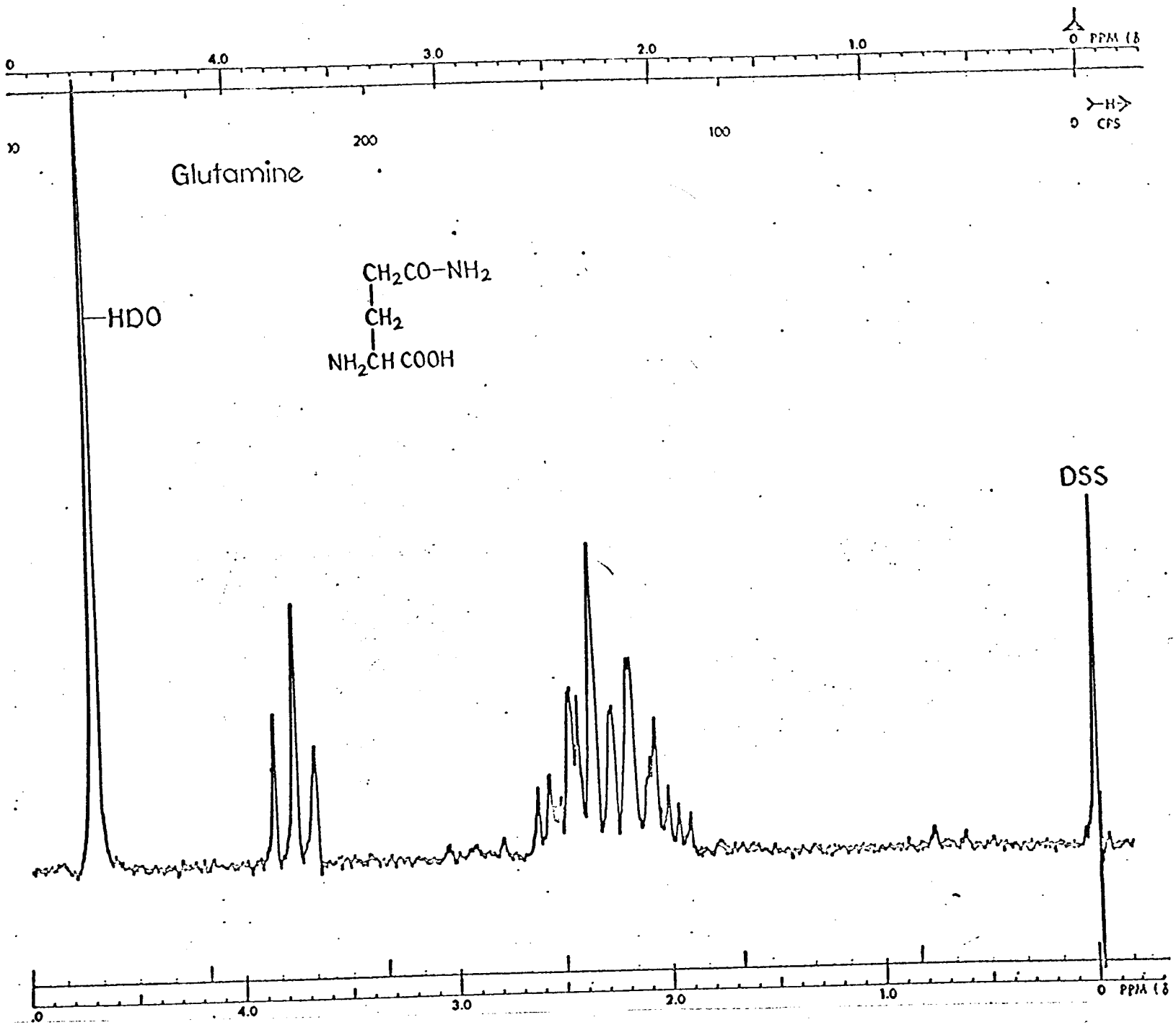
Table III

Correlation of Ribonuclease Spectrum with Amino Acid Protons

Peak	Chemical Shift (p.p.m.)	Amino Acid Protons in Peak
I	-8.85	Protons attached to nitrogen which did not exchange *
II	-7.8	Imidazole proton of histidine
III		
a	-7.30	Aromatic rings of tyrosine and phenylalanine and an imidazole proton of histidine
b	-7.00	
c	-6.78	
d	-6.45	
IV		
a	-5.42	α-protons of all 124 amino acids, one CH_2 of serine and proline and the β-proton of threonine
b	-4.82	
c	-4.4	
d	-4.05	
V	-3.0	A CH_2 group of phenylalanine, aspartic acid, glutamic acid, lysine, asparagine, glutamine, arginine, cysteine and methionine
VI		
a	-2.25	CH_2 of glutamic acid, glutamine, methionine, CH of valine, CH_3 of methionine
b	-2.05	2 CH_2 groups of proline, CH of isoleucine
c	-1.67	2 CH_2 groups of arginine, CH and CH_2 of leucine
d	-1.49	CH_3 of alanine, CH_3 of threonine
e	-1.06	2 CH_3 groups of valine
f	- .87	2 CH_3 groups of leucine, 2 CH_3 groups of isoleucine

* See reference 6


a. Alanine
CH3
NH2CH COOH
HDO
DSS
3.72 p.p.m.
b. Serine
CH2OH
NH2CH COOH
3.94 p.p.m.
c. Glutamine
CH2CO-NH2
CH2
NH2CH COOH
3.75 p.p.m.


Glutamine
HDO
DSS
CH_2CO-NH_2
CH_2
$NH_2CH\ COOH$
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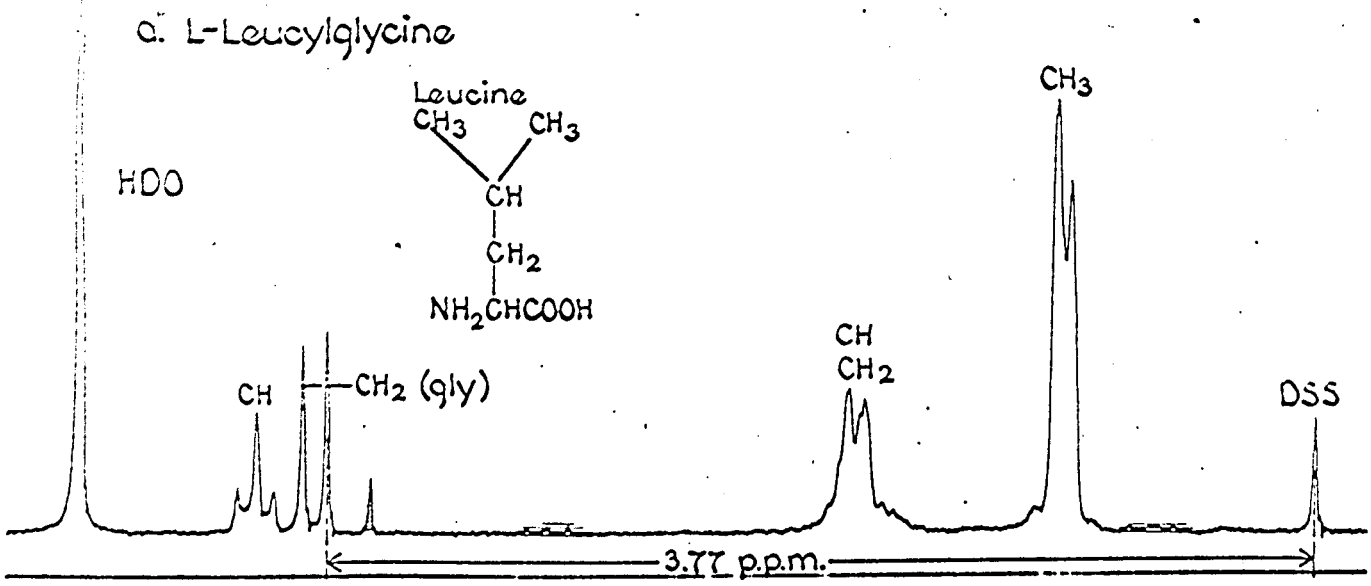
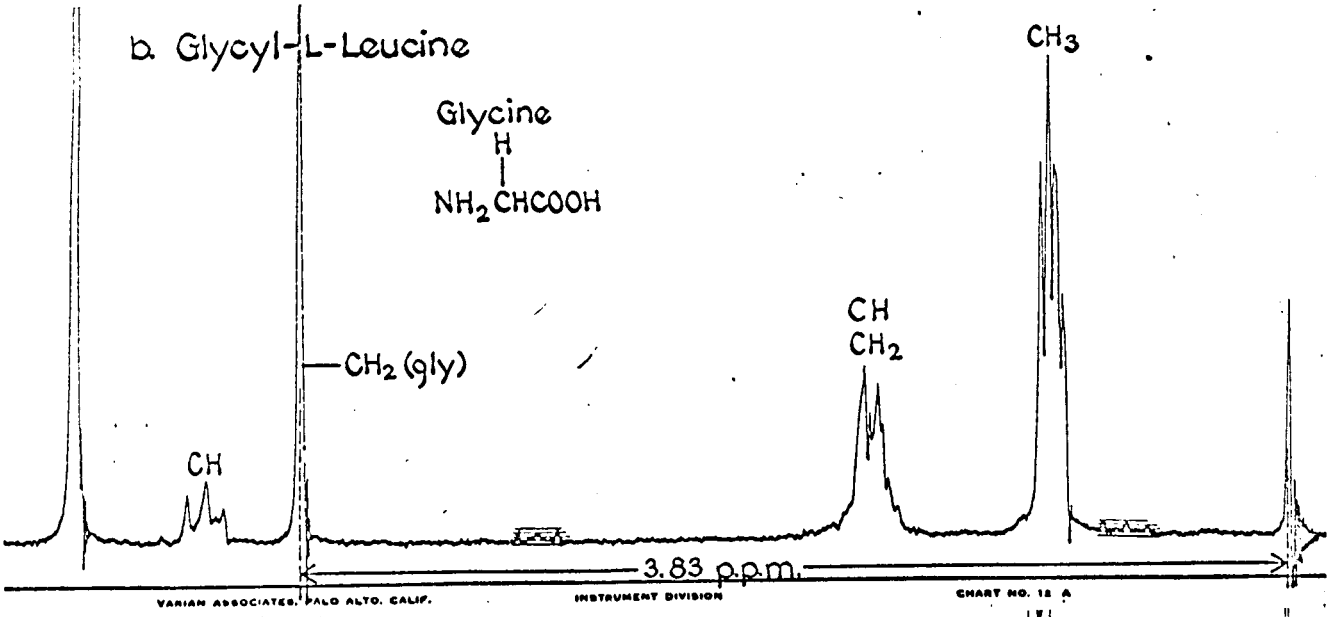
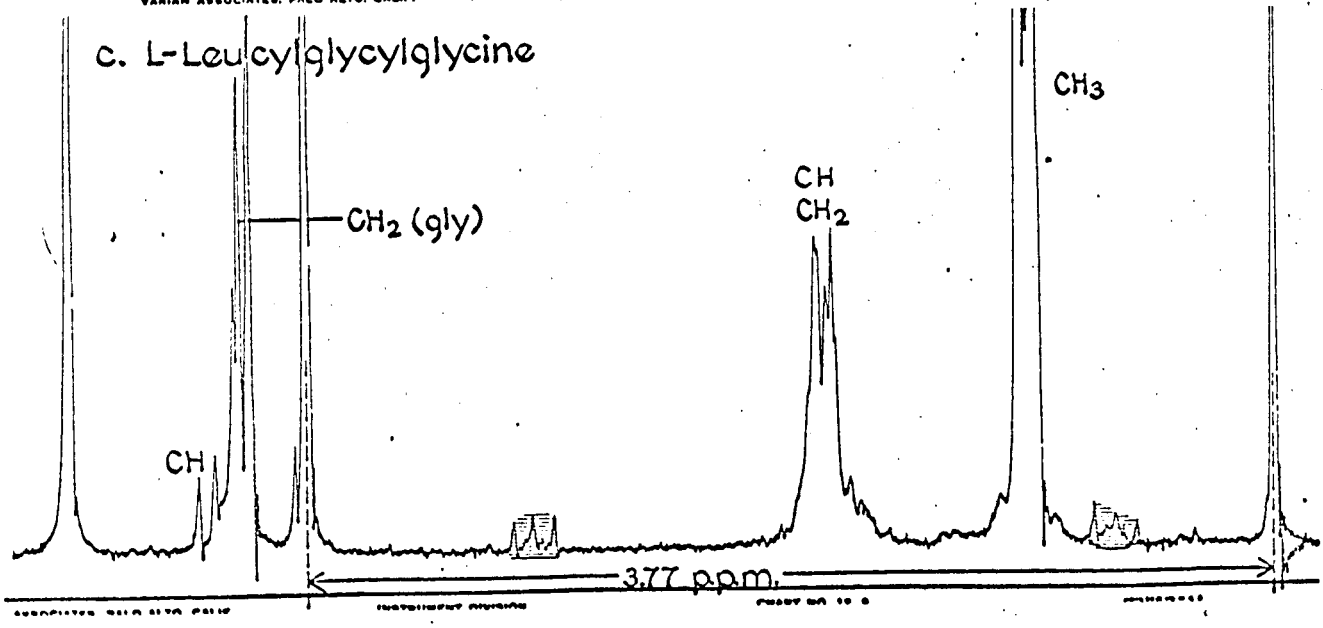
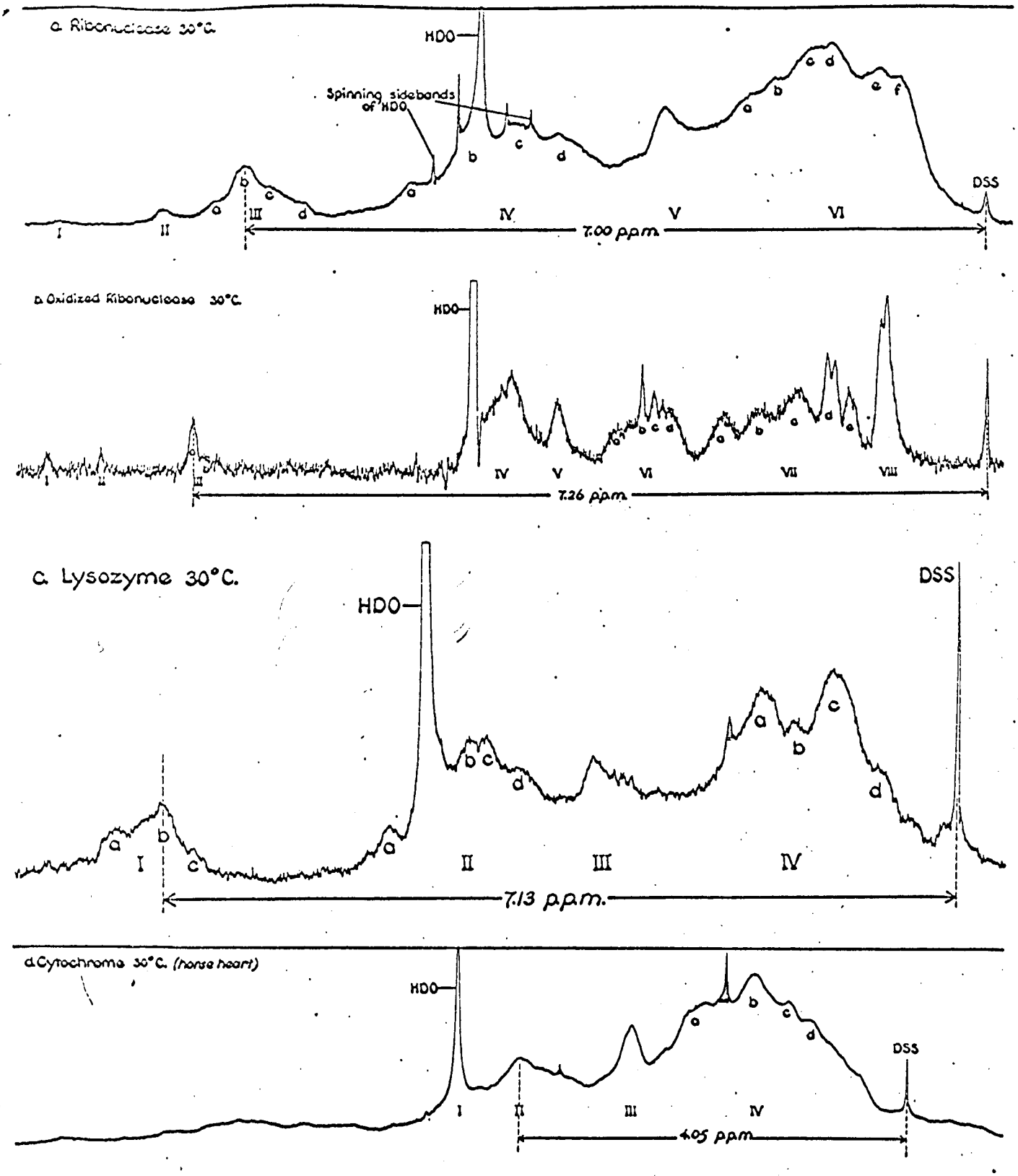
4.0
3.0
2.0
1.0
0 PPM (δ)
200
100
0 CPS


a. L-Leucylglycine
Leucine
CH3
CH3
CH
CH2
NH2CHCOOH
HDO
CH
CH2 (gly)
CH
CH2
CH3
DSS
3.77 p.p.m.


b. Glycyl-L-Leucine
Glycine
H
NH2CHCOOH
CH3
CH
CH2
CH2 (gly)
CH
3.83 p.p.m.
VARIAN ASSOCIATES, PALO ALTO, CALIF.
INSTRUMENT DIVISION
CHART NO. 12 A


c. L-Leucylglycylglycine
CH3
CH
CH2
CH2 (gly)
CH
3.77 p.p.m.


a. Ribonuclease 30°C.
HDO
Spinning sidebands of HDO
DSS
I
II
III
IV
V
VI
7.00 ppm
b. Oxidized Ribonuclease 30°C.
HDO
IV
V
VI
VII
VIII
7.26 ppm
c. Lysozyme 30°C.
HDO
DSS
I
II
III
IV
7.13 ppm.
d. Cytochrome 30°C. (horse heart)
HDO
DSS
I
II
III
IV
4.05 ppm