# *Nuclear Magnetic Resonance Spectra of Amino Acids and Their Derivatives. III*

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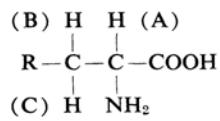
The proton magnetic resonance of NH and CH groups of amino acids has been studied by a number of workers.[1-5] Takeda and Jardetzky[1] measured the resonance positions of NH protons in strongly acidic solutions and those of CH protons of such simple amino acids as alanine and glycine. O. and C. D. Jardetzky[2] published additional measurements of the chemical shifts of CH and NH groups, but they did not include any information concerning the spin coupling constants. Bovey and Tiers[3] obtained the spectra of amino acids in trifluoroacetic acid, where the NH signal is observable. They also presented the spectra with a fine structure due to spin coupling and reported the values of several spin coupling constants obtained by first order analyses.

Generally, the NH proton signal cannot be observed in aqueous solutions because of rapid proton exchange with the solvent. However, it was found that the 2, 4-dinitrophenyl (DNP) derivatives of amino acids dissolved in dry dioxane exhibit signals due to NH protons and that their resonance positions are useful for the identification of molecules.[4]

The CH protons give spectra which are more complex than those due to NH protons. For example, the spectra due to the three protons of the type:




where "R" has no protons interacting with those under investigation, were measured and analyzed. Some of these results have been previously reported.[5] In many cases the three protons, A, B, and C, are non-equivalent to each other. However, in the case of amino acids with small R groups, such as cysteine dissolved in an acid medium, $H_B$ and $H_C$ are equivalent.

The three non-equivalent protons show spectra of the ABC type.[6] This type of spectra has been investigated by a number of groups of workers.[7-13] Iterative methods have commonly been used for the analyses of these spectra. The method of least-squares proposed by Shimizu and the present authors[13] has been successfully applied to acrylic acid and its related compounds[13] and to amino acids.[5] This paper will present the results of the application of this method to L-cystine, L-cysteine, L-serine, L-phenylalanine, and L-aspartic acid.

A chief drawback of the iterative method is that it is always accompanied by ambiguities, whether the molecular constants (chemical shifts and spin coupling constants) obtained by analyses are unique or not. In fact, it has been found that, in analyzing the spectra of acrylic acid and ethyl acrylate, two sets of molecular constants were obtained which gave exactly the same line positions, fitting the observed data satisfactorily.[13] These ambiguities can be avoided when the relative intensities are used as a criterion. A correct set of constants may be obtained by a careful comparison of the relative intensities of the calculated spectra with those observed. The results may be further confirmed by measuring the spectra at different spectrometer frequencies; the correct molecular constants must explain the observed spectra at different frequencies.

The relative signs of spin coupling constants can also be determined conventionally by the iterative method outlined above, utilizing

1) M. Takeda and O. Jardetzky, *J. Chem. Phys.*, **26**, 1346 (1957).
2) O. Jardetzky and C. D. Jardetzky, *J. Biol. Chem.*, **233**, 383 (1958).
3) F. A. Bovey and G. V. D. Tiers, *J. Am. Chem. Soc.*, **81**, 2780 (1958).
4) S. Fujiwara, Y. Arata, N. Hayakawa and H. Momoi, This Bulletin, **35**, 1658 (1962).
5) S. Fujiwara and Y. Arata, ibid., **36**, 578 (1963).
6) H. J. Bernstein, J. A. Pople and W. G. Schneider, *Can. J. Chem.*, **35**, 65 (1957).
7) R. A. Hoffman and S. Gronowitz, *Arkiv Kemi*, **15**, 45 (1957); R. A. Hoffman, *J. Chem. Phys.*, **33**, 1256 (1960).
8) C. A. Reilly and J. D. Swalen, ibid., **32**, 1378 (1960); **33**, 1257 (1960); **34**, 980 (1961).
9) S. Alexander, ibid., **32**, 1700 (1960).
10) E. O. Bishop and R. E. Richards, *Mol. Phys.*, **3**, 114 (1960).
11) W. Brugel, T. Ankel and F. Kruckenberg, *Z. Elektrochem.*, **64**, 1121 (1960).
12) S. Castellano and J. S. Waugh, *J. Chem. Phys.*, **34**, 295 (1961); **35**, 1900 (1961).
13) Y. Arata, H. Shimizu and S. Fujiwara, *J. Chem. Phys.*, **36**, 1951 (1962).

various sets of spin coupling constants with different relative signs. As is well known, the determination of the relative sign can best be accomplished by using the double resonance technique.

## Experimental

**Materials.**—The amino acids studied were L-cysteine, L-cystine, L-phenylalanine, L-aspartic acid, and L-serine, all commercial materials (Ajinomoto Co.). They were dissolved in heavy water of various acidities. The acidity was adjusted by adding concentrated hydrochloric acid or powdered sodium peroxide to heavy water. One-molar solutions were used for all measurements. Heavy water was purchased from the Showa Denko Co. and had a purity of 99.5 mol.%.

**Spectrometer.**—A Varian DP-60 NMR spectrometer operating at 60.00 Mc./sec. was employed for all measurements.

**Calibration of the Spectra.**—Measurements were performed at about 28°C. The frequency separations were determined by the usual side-band technique. Numerical data for the line positions used for the analyses were obtained by averaging the results of ten determinations. The numerical data for line positions are listed in Table I.

## Numerical Calculations

Analyses were carried out through the use of the Parametron digital computer PC-2 of the Physics Department of the University of Tokyo. The flow diagram of the computation is given in Fig. 1. If molecular constants are adequately assumed, after four or five iterations, within one minute, the refined molecular constants and the corresponding spectrum are printed out.

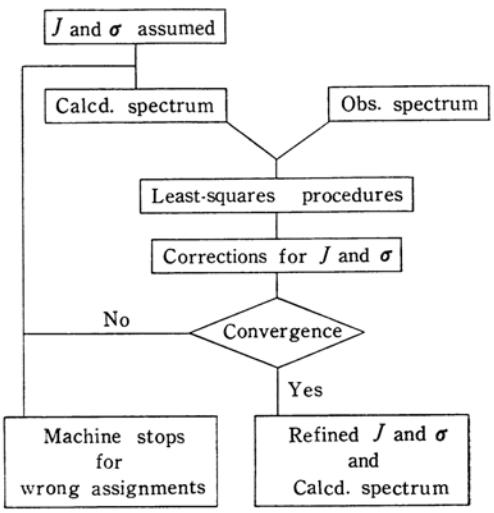



Fig. 1. The flow diagram of computation.

## Results

**Analyses of the Spectra and the Relative Sign of the Spin Coupling Constants.**—As an

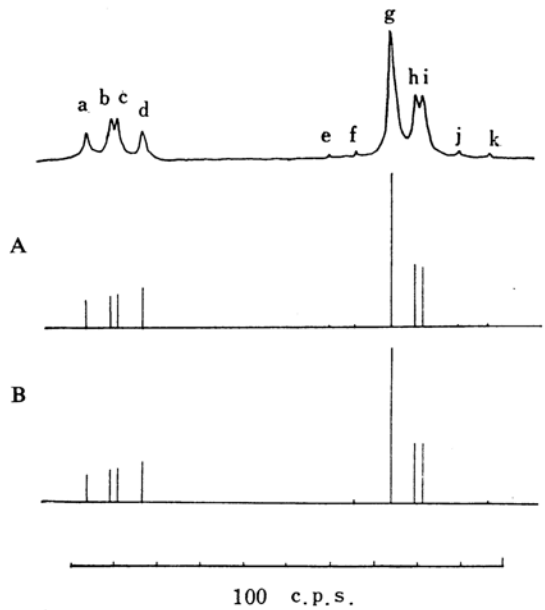



Fig. 2a. The observed and calculated spectra of phenylalanine in 2 N HCl at 60 Mc./sec. A and B were calculated from the molecular constants given in Table II. The magnetic field increases from left to right.

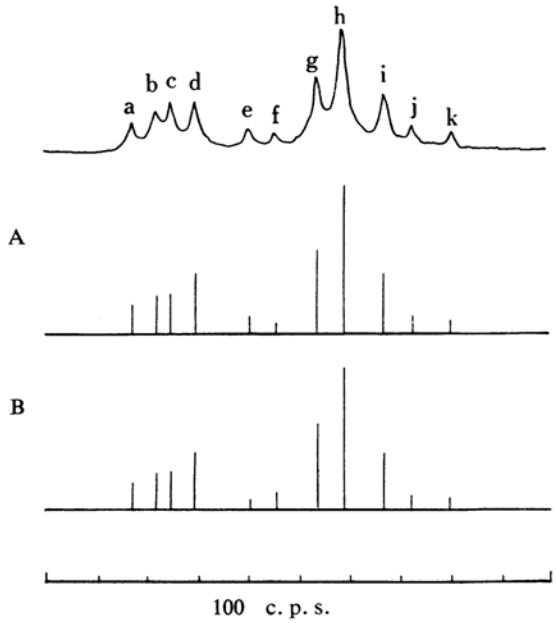



Fig. 2b. The observed and calculated spectra of phenylalanine in 2N NaOD at 60 Mc./sec. A and B were calculated from the molecular constants given in Table II. The magnetic field increases from left to right.

TABLE I. OBSERVED AND CALCULATED SPECTRA OF AMINO ACIDS AT 60.00 Mc./sec.

The line positions are given in c. p. s. and the relative intensities are normalized to unity.
O: observed, A and B: calculated from the molecular constants given in Table II.

(1) L-Phenylalanine in 2 N HCl

| | Line position | | | Relative intensity | | |
|---|---|---|---|---|---|---|
| | O | A | B | O | A | B |
| a | 50.7 | 50.7 | 50.7 | 0.064 | 0.068 | 0.068 |
| b | 44.6 | 44.8 | 44.8 | 0.092 | 0.080 | 0.077 |
| c | 43.5 | 43.4 | 43.4 | 0.092 | 0.084 | 0.079 |
| d | 37.5 | 37.5 | 37.5 | 0.070 | 0.101 | 0.101 |
| e | − 4.0 | − 4.0 | − 4.0 | 0.013 | 0.003 | 0.000$_0$ |
| f | −10.0 | −10.0 | −10.0 | 0.020 | 0.006 | 0.011 |
| g | −18.8 | −18.6<br>−18.9 | −18.6<br>−18.9 | 0.315 | 0.179<br>0.182 | 0.184<br>0.183 |
| h | −24.4 | −24.6 | −24.6 | 0.148 | 0.146 | 0.142 |
| i | −26.2 | −26.1 | −26.1 | 0.158 | 0.143 | 0.140 |
| j | −33.5 | −33.5 | −33.5 | 0.015 | 0.003 | 0.000$_0$ |
| k | −40.8 | −40.7 | −40.7 | 0.014 | 0.005 | 0.006 |

(2) L-Phenylalanine in 2 N NaOD

| | Line position | | | Relative intensity | | |
|---|---|---|---|---|---|---|
| | O | A | B | O | A | B |
| a | 30.2 | 30.2 | 30.2 | 0.057 | 0.057 | 0.058 |
| b | 25.1 | 25.2 | 25.2 | 0.081 | 0.076 | 0.077 |
| c | 22.5 | 22.4 | 22.4 | 0.101 | 0.081 | 0.079 |
| d | 17.5 | 17.4 | 17.4 | 0.102 | 0.118 | 0.117 |
| e | 7.3 | 7.3 | 7.3 | 0.043 | 0.031 | 0.022 |
| f | 2.4 | 2.3 | 2.3 | 0.034 | 0.022 | 0.033 |
| g | − 6.1 | − 6.1 | − 6.1 | 0.148 | 0.164 | 0.174 |
| h | −11.2 | −11.1<br>−11.3 | −11.1<br>−11.3 | 0.242 | 0.117<br>0.170 | 0.107<br>0.171 |
| i | −19.3 | −19.1 | −19.1 | 0.108 | 0.110 | 0.109 |
| j | −24.7 | −24.7 | −24.7 | 0.047 | 0.029 | 0.026 |
| k | −32.6 | −32.6 | −32.6 | 0.036 | 0.023 | 0.026 |

(3) L-Cystine in 2 N HCl

| | Line position | | | Relative intensity | | |
|---|---|---|---|---|---|---|
| | O | A | B | O | A | B |
| a | 50.3 | 50.4 | 50.4 | 0.078 | 0.069 | 0.070 |
| b | 45.4 | 45.5 | 45.4 | 0.081 | 0.078 | 0.075 |
| c | 43.6 | 43.5 | 43.5 | 0.078 | 0.083 | 0.079 |
| d | 38.6 | 38.5 | 38.5 | 0.077 | 0.099 | 0.099 |
| e | − 4.2 | − 4.1 | − 4.1 | 0.013 | 0.001 | 0.000$_0$ |
| f | − 9.1 | − 9.1 | − 9.1 | 0.020 | 0.010 | 0.013 |
| g | −19.2 | −19.2<br>−19.3 | −19.2<br>−19.3 | 0.347 | 0.178<br>0.181 | 0.182<br>0.181 |
| h | −24.1 | −24.2 | −24.1 | 0.143 | 0.146 | 0.144 |
| i | −26.3 | −26.3 | −26.3 | 0.130 | 0.142 | 0.140 |
| j | −34.4 | −34.4 | −34.4 | 0.016 | 0.002 | 0.000$_0$ |
| k | −41.4 | −41.3 | −41.3 | 0.017 | 0.007 | 0.008 |

(4) L-Aspartic acid in 2 N NaOD

| | Line position | | Relative intensity | |
|---|---|---|---|---|
| | O | A | O | A |
| a | 50.2 | 50.2 | 0.052 | 0.068 |
| b | 45.6 | 45.7 | 0.066 | 0.078 |
| c | 41.2 | 41.2 | 0.079 | 0.086 |
| d | 36.8 | 36.7 | 0.080 | 0.101 |
| e | 0.4 | 0.4 | 0.028 | 0.027 |
| f | − 4.2 | − 4.1 | 0.034 | 0.032 |
| g | −15.1 | −15.1 | 0.153 | 0.153 |
| h | −19.5 | −19.6 | 0.160 | 0.122 |
| i | −21.5 | −21.6 | 0.172 | 0.158 |
| j | −30.7 | −30.6 | 0.114 | 0.117 |
| k | −37.0 | −37.1 | 0.034 | 0.030 |
| l | −46.1 | −46.1 | 0.028 | 0.029 |

(5) L-Cysteine in 2 N NaOD

| | Line position | | Relative intensity | |
|---|---|---|---|---|
| | O | A | O | A |
| a | 28.5 | 28.5 | 0.052 | 0.058 |
| b | 24.0 | 24.0 | 0.088 | 0.076 |
| c | 21.1 | 21.1 | 0.120 | 0.083 |
| d | 16.4 | 16.6 | 0.116 | 0.117 |
| e | 7.0 | 6.9 | 0.020 | 0.026 |
| f | 2.3 | 2.4 | 0.030 | 0.021 |
| g | − 6.4 | − 6.2 | 0.146 | 0.166 |
| h | −10.6 | −10.4<br>−10.7 | 0.261 | 0.174<br>0.119 |
| i | −17.7 | −17.8 | 0.107 | 0.112 |
| j | −23.4 | −23.5 | 0.024 | 0.026 |
| k | −30.7 | −30.8 | 0.013 | 0.022 |

example of spectra of the ABC type, the observed spectra of phenylalanine in 2 N hydrochloric acid and 2 N sodium deuteroxide are shown in Fig. 2. The numerical data of the observed line positions and relative intensities are listed in Table I. In the table the observed data of other amino acids exhibiting spectra of the ABC type are also included.

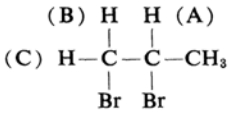
When spin coupling constants are initially assumed to have the same relative sign, one obtains the refined constants shown in Table II, a portion of which has already been reported.[5] These results seem quite satisfactory with regard to the positions of the spectral lines. Calculated spectra obtained from these constants are compared with the observed ones in Table I and in Fig. 2, which show good coincidence. A careful comparison of the relative intensities of the observed spectra with those calculated shows that these constants are unsatisfactory. For instance, in the calculated spectra of phenylalanine the "e" and "f" lines are reversed in the ratio of intensities (in an alkaline solution) and the "e" and "j" lines

TABLE II. THE RESULTS OF ANALYSES IN C. P. S. AT 60.00 Mc./sec.

| | | | $\delta_{AC}$ | $\delta_{BC}$ | $J_{AB}$ | $J_{BC}$ | $J_{CA}$ |
|---|---|---|---|---|---|---|---|
| Phenylalanine | 2 N HCl | A | 68.1 | 4.9 | 5.5 | −14.6 | 7.7 |
| | | B | 67.4 | 3.7 | 3.2 | 14.6 | 10.0 |
| | 2 N NaOD | A | 42.1 | 14.6 | 5.1 | −13.5 | 7.9 |
| | | B | 42.1 | 14.5 | 4.0 | 13.4 | 8.9 |
| Cystine | 2 N HCl | A | 68.9 | 5.3 | 3.7 | −15.1 | 8.2 |
| | | B | 68.3 | 4.3 | 2.0 | 15.1 | 9.8 |
| Aspartic acid | 2 N HCl | | 76.4 | 0 | 5.3 | — | 5.3 |
| | 2 N NaOD | A | 73.8 | 18.4 | 4.1 | −15.5 | 9.4 |
| Cysteine | 2 N HCl | | 74.2 | 0 | 5.0 | — | 5.0 |
| | $D_2O$ | | 55.5 | 0 | 4.9 | — | 4.9 |
| | 2 N NaOD | A | 39.5 | 13.0 | 4.5 | −13.2 | 7.5 |

are too weak to be observed (in a hydrochloric acid solution). The situation is almost the same with other amino acids.

Because of the above findings, it seemed advisable to seek another means of choosing a set of spin coupling constants with different relative signs. When $J_{BC}$ was initially assumed to have a sign opposite $J_{CA}$ and $J_{AB}$, a different set of molecular constants was obtained, which gave quite a satisfactory fit between the observed and calculated spectra with respect not only to the line positions but also to the relative intensities. The molecular constants obtained are shown in Table II, while the spectra calculated by using these constants are presented in Fig. 2. The results on the relative sign of spin coupling constants are consistent with those of Freeman and Bhacca,[14] who showed by using the double resonance technique that in 1,2-dibromopropane,




$J_{BC}$ has a sign opposite $J_{AB}$ and $J_{CA}$.

To confirm the results obtained, spectra were measured at 40 Mc./sec. and were compared with the spectra calculated by using two sets of constants with different relative signs. For example, the observed and calculated spectra of cystine in 2 N hydrochloric acid are shown in Fig. 3. Unfortunately, as can been seen from this figure, the calculated spectra were very similar to each other. In addition to this, the signal-to-noise ratio was not satisfactory. Therefore, it was difficult to eliminate a wrong set and to determine the relative sign by this procedure.

14) R. Freeman and N. S. Bhacca, *J. Chem. Phys.*, **38**, 1088 (1963).

TABLE III. THE CHEMICAL SHIFTS AND SPIN COUPLING CONSTANTS IN C. P. S. OF L-SERINE AT 60.00 Mc./sec.

| | | $\nu_A - \nu_B$ | $J$ |
|---|---|---|---|
| 2 N HCl | | 11.1 | 4.2 |
| pH | 2 | − 5.2 | 4.6 |
| | 5 | − 6.9 | 4.8 |
| | 9 | −10.6 | 4.9 |

**The Spectra of Serine and Their pH Dependence.**—The molecular constants and, hence, the patterns of the spectra are sensitively influenced by the acidity of the solutions. An example of this is the case of L-valine, which was previously reported on.[5] Another example will now be presented. The spectra of serine in heavy water with various acidities are given in Fig. 4. The a through d spectra are of the $AB_2$ type[6] and can easily be analyzed to furnish the molecular constants. They are shown in Table III. Serine dissolved in a 2 N sodium deuteroxide solution gives a different type of a spectrum. At first sight it seems to be of the $AB_2$ type. However, it does not give a satisfactory fit to the observed spectrum when $J_{AB}$ and $J_{CA}$, and $\delta_{AB}$ and $\delta_{CA}$, are assumed to be identical. This suggests that the B and C protons are no longer equivalent.

## Concluding Remarks

The values obtained above for chemical shifts and spin coupling constants are actually averages over rotational isomers. The appearance of the spectra is governed by the factors, including the relative energies of the rotational isomers, the potential barriers to internal rotation about the C–C bond, and the chemical shifts and spin coupling

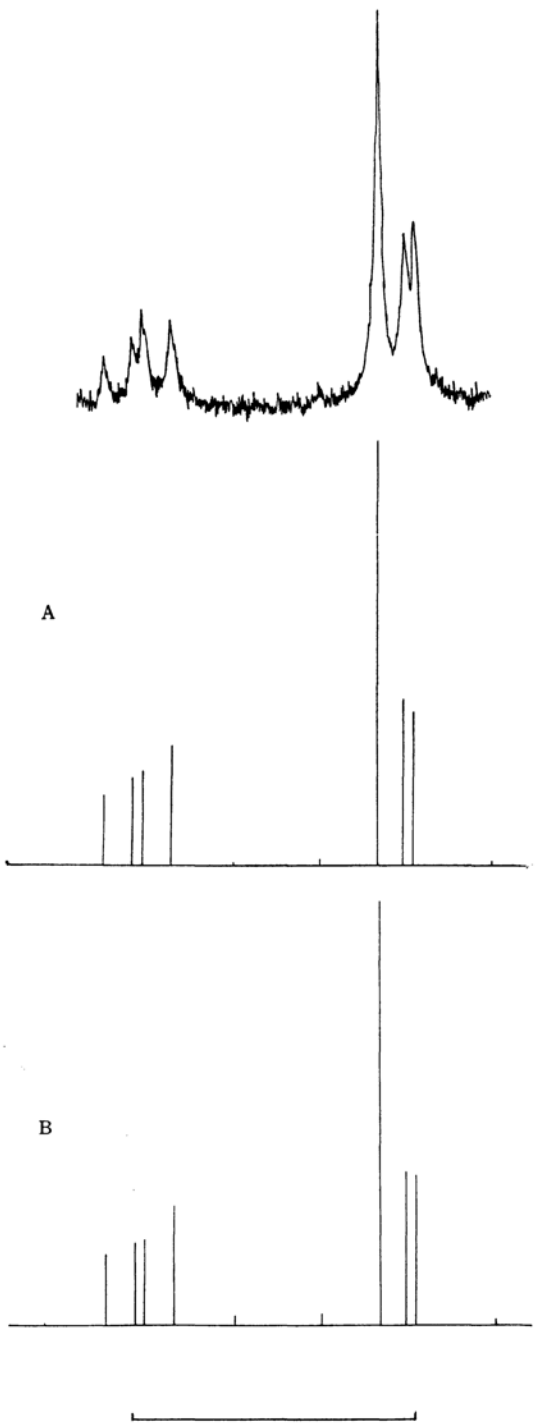



Fig. 3. The observed and calculated spectra of cystine in 2 N HCl at 40 Mc./sec.
A and B were calculated from the molecular constants given in Table II. The magnetic field increases from left to right.

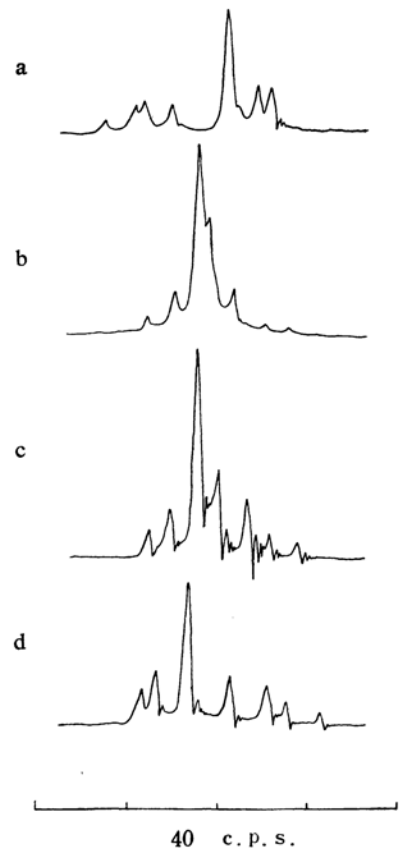



Fig. 4. The observed spectra at 60 Mc./sec. of serine in heavy water of various acidities (a: 2 N HCl, b: pH 2, c: pH 5, d: pH 9). The magnetic field increases from left to right.

constants, characteristic of each isomer.[15,16] Several groups of workers have tried to obtain these quantities by analyzing spectra at various temperatures.[17-19] Gutowsky, Belford, and McMahon[19] studied the temperature dependence of the spectra of polysubstituted ethanes and developed a method including the least-squares procedure. When the spectra of the amino acids studied here show an appreciable dependence upon the temperature, it may be possible to obtain the above quantities utilizing this method.

15) J. A. Pople, W. G. Schneider and H. S. Bernstein, "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Company, Inc., New York (1959).
16) H. S. Gutowsky, "Technique of Organic Chemistry," Ed. by A. Weissberger, Interscience Publishers, Inc., New York (1960).
17) R. W. Fessenden and J. S. Waugh, *J. Chem. Phys.*, **37**, 1466 (1962).
18) D. S. Thompson, R. A. Newmark and C. H. Sederholm, ibid., **37**, 411 (1962).
19) H. S. Gutowsky, G. G. Belford and P. E. McMahon, ibid., **36**, 3353 (1962).

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