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A fourier transform proton magnetic resonance study of the molecular conformation of S-adenosyl-L-methionine

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A FOURIER TRANSFORM PROTON MAGNETIC RESONANCE STUDY OF THE MOLECULAR CONFORMATION OF S-ADENOSYL-L-METHIONINE

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ABSTRACT

Contrary to a previous report, S-adenosyl-L-methionine (SAM) affords stable solutions in D_2O and the ^1H NMR spectrum can be determined. Comparison with the spectra of the model compounds adenosine, L-methionine and L-methionine-S-methyl sulfonium iodide allows complete assignment of the proton resonances. Coupling constants were determined by homonuclear decoupling and graphical analysis and were confirmed by computer simulation.

Details of the molecular conformation were determined by application of the Karplus equation and calculation of relative rotational isomer populations. Evidence indicates that the ribose ring is puckered preferentially in the C_3'-exo conformation and that the C_4'-C_5' bond is constrained to a rotamer in which the sulfonium center is gauche to H_4'. No conformational constraints were detected for the C_α-C_β and C_β-C_γ bonds of the methionine side chain. The purine ring was shown to be oriented preferentially <u>anti</u> by intermolecular association studies with adenosine 5'-phosphate in the presence of Mn(II).

Spectra of samples of (-)S-adenosyl-L-methionine of biological origin, differing in activity, counter ion and commertial source, have consistently revealed the presence of a small amount of the (+) sulfonium diastereomer. Arguments are presented to explain the failure of previous workers to detect (+)S-adenosyl-L-methionine in biological preparations.

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INTRODUCTION

The biochemical significance of S-adenosylmethionine can be appreciated from the fact that it is utilized in the chemical modification of members of every major class of biomolecule including nucleic acids, proteins, lipids and carbohydrates. It is the usual cofactor to transmethylation enzymes.

The elucidation of its structure and function was an outgrowth of investigations into the metabolic fate of methionine. The role of methionine as methyl donor was first suggested by the observations of Borsook and Dubnoff,[1] Handler and Dann[2] and Perlzweig, Berheim and Berheim.[3] A detailed investigation was subsequently undertaken by Cantoni,[4] in conjunction with a study of the transmethylation of nicotinamide. Biosynthesis of N^1-methylnicotinamide was shown to proceed from L-methionine and nicotinamide in the presence of Mg^{++}, adenosinetriphosphate (ATP) and the appropriate enzyme system. Evidence that the presence of methionine specifically increased dephosphorylation on ATP in the absence of a methyl acceptor[5,6] led to the proposal of an "active methionine" intermediate. Following an earlier suggestion by Toennies,[7] Cantoni proposed that methionine was involved as a sulfonium derivative.

Purification of the methionine-activating enzyme led to the isolation of the "active methionine" intermediate.[8,9]

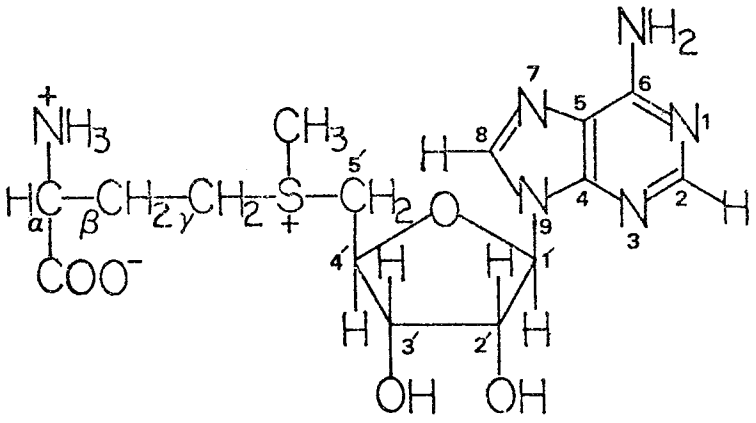


Figure 1. S-adenosyl-L-methionine (SAM)

This intermediate was shown to be S-adenosylmethionine (SAM), Figure 1, because its hydrolysis in neutral solution affords homoserine and 5'-methylthioadenosine. Baddiley and Jamieson[10] corroborated the structure by organic synthesis.

Synthetic S-adenosylmethionine, resulting from the addition of racemic α-amino-γ-butyrolactone to 5'-methylthioadenosine, possesses about half the enzymatic activity of the compound of biological origin. De La Haba, et al.[11] undertook an investigation relating the configuration about the sulfonium center of SAM to its activity with S-adenosylmethionine cleaving enzyme and two methyltransferases. Examination of the reactivity of diastereomers resulting from the enzymatic resolution of mixtures racemic about the sulfonium center indicated that (-)S-adenosyl-L-methionine was the most biologically active compound.

The specificity of the methionine-activating enzyme for L-methionine had been demonstrated previously.[4] An analogous study by Zappia, Zydek-Cwick and Schlenk[12] involving four methyltransferases not previously investigated provided similar results. Cornforth, et al.[13] have determined the absolute configuration at the sulfonium center of a pair of diastereomeric S-carboxymethyl-(S)-methionine salts by X-ray crystallography. Degradation of S-adenosyl-L-methionine to S-carboxymethyl-(S)-methionine under experimental conditions designed to retain the configuration at the sulfonium center, has shown it to possess the physical properties of the diastereomer with the S configuration.

Details of the stability of S-adenosylmethionine under various experimental conditions were ascertained by Parks and Schlenk.[14] Heating at 100°C in the presence of acid over the pH range 4-7 results in the formation of 5'-methylthioadenosine and α-amino-γ-butyrolactone; the latter being subsequently converted to homoserine. In the presence of 0.1 N mineral acid, hydrolysis of the glycosidic bond occurs in conjunction with the above reactions resulting in the formation of adenine, 5'-methylthioribose and homoserine.[15] Hydrolysis in the presence of 0.1 N sodium hydroxide is rapid, but confined to the glycosidic bond; adenine and S-ribosylmethionine result.[14] The greatest stability of SAM is exhibited in moderately acidic solution at low temperature.

The mechanism of the methionine-activating enzyme from various sources has been studied in some detail. The kinetics

of the reaction are complex and authors differ in their interpretation.[16,17] Stoichiometry of the reaction can be expressed as follows:

$$\text{L-methionine} + \text{ATP} \xrightarrow{Mg^{++}} \text{S-adenosylmethionine} + H^{+} + PP_i + P_i$$

Evidence of enzyme bound tripolyphosphate as an intermediate in the reaction was provided by Mudd.[18] The origin of inorganic phosphate from the hydrolysis of ATP was investigated by Cantoni and Durell,[17] who concluded that pyrophosphate was derived from the alpha and beta phosphates of ATP, and orthophosphate from the terminal phosphate. Divalent magnesium is an absolute requirement for the reaction and a requirement for a monovalent cation has been demonstrated for the enzyme from baker's yeast. In the above reaction, utilization of ATP represents a biologically unique dual function, i.e., ATP serves as both adenosine donor and source of energy for the biosynthesis of SAM.[16]

The role of S-adenosylmethionine as a biological cofactor was specifically studied in conjunction with the methylation of guanidinoacetic acid in the biosynthesis of creatine.[19] Isolation of S-adenosyl-L-homocysteine (SAH), Figure 2, as a product of the reaction led Cantoni[20,21,22] to propose that the cofactor is in general employed as follows:

$$\text{SAM} + \text{substrate} \longrightarrow \text{SAH} + \text{methylated substrate}$$

The specificity of various methyltransferases toward the cofactor was investigated in binding studies involving

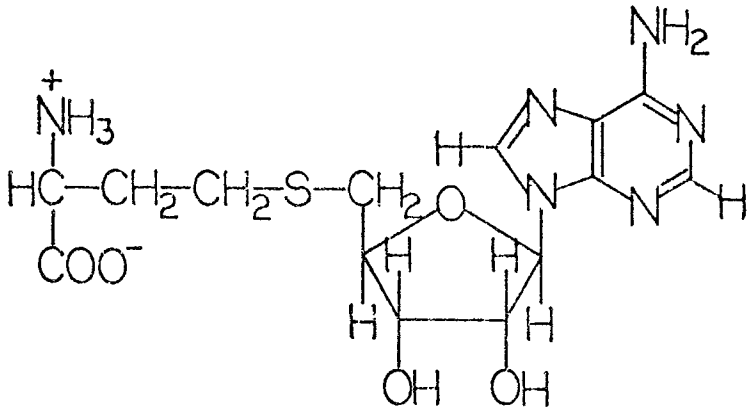


Figure 2. S-adenosyl-L-homocysteine (SAH)

various analogs by Zappia, Zydek-Cwick and Schlenk[23] and in inhibition studies employing S-adenosylethionine by Parks[24] and Selenomethionine by Mudd and Cantoni.[25] Zappia, et al.[23] have proposed that the cofactor binds to enzymes via interactions of the 6-amino group of the purine ring, the sulfonium ion and the α-amino and α-carboxyl groups of the methionine side chain.

Details of the conformation of S-adenosylmethionine are presently limited. Attempts at X-ray crystallographic studies have been unsuccessful, although a model sulfonium compound with labile methyl groups has been examined.[26] A common feature often investigated in nucleotide-like compounds is the orientation of the purine or pyrimidine ring system with respect to the ribose moiety. Donohue and Trueblood[27] have proposed the use of the terms anti and syn, Figure 3, to describe two relatively stable conformations

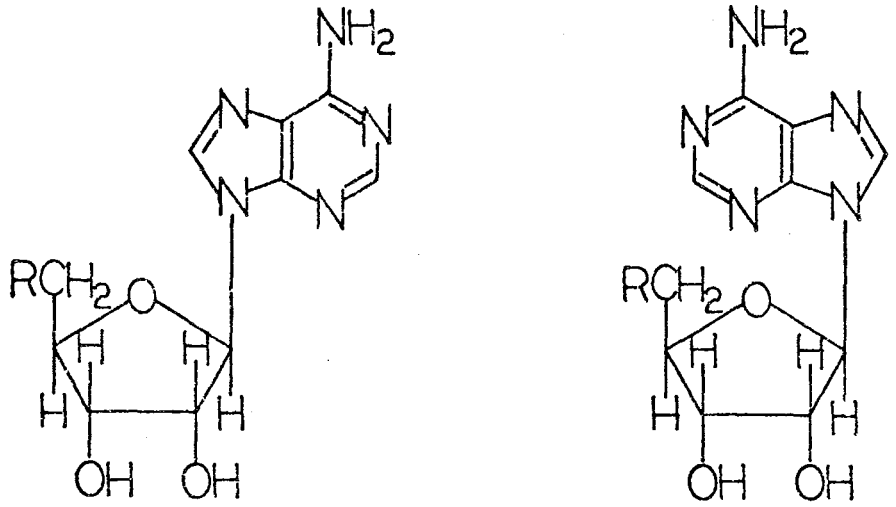


Figure 3. Sugar-Base Torsional Description

that are often observed. Defining zero degrees when the ribose ring oxygen is anti-coplanar to C_2 of the purine or pyrimidine ring, these conformations correspond to angles of ca -30° and ca $+150^{\circ}$, respectively.

Klee and Mudd have examined S-adenosylmethionine by Optical Rotatory Dispersion to ascertain details of its conformation.[28] Measurements of the ORD curves in the range of 220-360 nm for S-adenosylmethionine, S-adenosylhomocysteine, 5'-methylthioadenosine, adenosyldimethylsulfonium, adenosine, adenosine 2'-phosphate and adenosine 3',5'-cyclic phosphate have led to an interesting observation. Whereas the sign of the Cotton effect centered about 260 nm is generally negative when purine nucleosides or nucleotides are examined,[29] all compounds substituted at the 5'-carbon with sulfur exhibited a positive Cotton effect. Samples in this study were racemic about all asymmetric centers except those of the ribose; thus

(±)S-adenosyl-DL-methionine was employed. Klee and Mudd have interpreted changes in the Cotton effect in terms of the orientation of the transition dipole of the base with respect to the asymmetric centers of the ribose moiety. A positive Cotton effect centered about 260 nm is also associated with pyrimidine nucleosides and nucleotides.[30] Model building indicates pyrimidines are generally anti due to steric hindrance in the syn conformation. In light of these observations, Klee and Mudd reasoned that since purines exhibit a Cotton effect of opposite sign to that of the pyrimidines, they must assume an opposite base orientation. The introduction of a sulfur atom at the 5'-carbon, as in S-adenosylmethionine, results in steric interference, driving the molecule into the anti conformation and explaining the positive value of the Cotton effect.

Alternative explanations have been offered to account for changes in the Cotton effect. Jardetzky and Jardetzky[31] studied purine and pyrimidine nucleosides by nuclear magnetic resonance spectroscopy. Purine nucleosides were found to exhibit higher H_1 coupling constants than do the corresponding pyrimidines, indicating differing conformations of the ribose ring. Jardetzky[32] suggested that this might explain the differences in optical rotation. Emerson, et al.[33] have examined the ORD curves of the model compounds 2',3'-isopropylidene, N^3,5'-cycloadenosine and C^8,5'-cycloadenosine, which are sterically constrained to assume the syn and anti conformations, respectively, by ring formation.

Contrary to the interpretation of Klee and Mudd, the negative Cotton effect was here found to be associated with the anti conformation, but the possible instability of these model compounds prompted Klee and Mudd to discount these observations. Reasoning along similar lines as those employed in the assignment of the conformation for S-adenosylmethionine, Klee and Mudd have proposed that adenosine 3',5'-cyclic phosphate assumes the syn conformation. Subsequent X-ray crystallographic and nuclear magnetic resonance studies have shown this compound to be predominately anti.[34]

The discrepancies apparent in the above discussion, and the absence of further details of the molecular conformation, have prompted us to investigate S-adenosyl-L-methionine by nuclear magnetic resonance techniques.

EXPERIMENTAL

(-)S-adenosyl-L-methionine iodide, (-)S-adenosyl-L-methionine chloride, L-methionine-S-methyl sulfonium iodide, L-methionine, S-adenosyl-L-homocysteine and adenosine were purchased from Sigma. The activity of the SAM chloride and iodide salts was reported by the manufacturer as 70% and 85-90%, respectively, when assayed enzymatically according to the method of Axelrod and Tomchick.[35] (-)S-adenosyl-L-methionine hydrogen sulfate and disodium adenosine 5'-phosphate were purchased from Boehringer Mannheim. (±)S-adenosyl-L-methionine was prepared by reaction of S-adenosyl-L-homocysteine with methyl iodide as described by De La Haba, et al.[11]

100 MHz ^{1}H NMR spectra were recorded with a Varian XL-100-15 spectrometer equipped with a Nicolet TT-100 Fourier transform accessory. The spectrometer was locked to the internal resonance of D_2O. An exponential weighting factor of 0.2 was applied to all accumulated free induction decays prior to Fourier transformation. Data points were 4119 or 8419. Resonance frequencies, peak intensities and chemical shifts were obtained as digital print-outs from the Nicolet computer. All signal positions were measured vs. internal TSP (sodium 3 (trimethylsilyl) tetradeutero propionate). The spectrometer probe was maintained at ca 25°C.

360 MHz ^{1}H NMR spectra were recorded with a Brucker HXS-360 spectrometer interfaced with a Nicolet 1180 computer.

Data points were 8419. Further details of data aquisition were as described for the 100 MHz experimentation.

Routine spectra for the determination of chemical shifts and coupling constants were obtained from materials twice lyophilized from D_2O. Solutions were 0.025 M in D_2O with the pD (pD = pH + 0.40[36]) adjusted to ca 3.4 by the addition of DCl (20% in D_2O). Samples were centrifuged for three minutes to remove suspended materials before being transferred to 5 mm NMR tubes (Wilmad 507-PP or 528-PP). Spectra were inspected for evidence of acid hydrolysis by comparison to a spectrum of the hydrolysis products, obtained by heating a sample at 100°C for one hour.

The solvent HOD resonance was suppressed from the spectrum when necessary by application of a 180°-τ-90° pulse sequence, varying τ such that the observing pulse is applied just as the solvent magnetization passes through zero.[37]

Serial dilution of a 0.05 M solution of (-)S-adenosyl-L-methionine chloride in D_2O provided samples used to determine the concentration dependence of chemical shifts. Spectra were recorded over the concentration range 0.01-0.04 M. The initial solution was at pD of ca 3.8 and the pD of subsequent dilutions was not adjusted.

Paramagnetic shifts and line broadening resulting from the presence of Mn(II) in solution was investigated by recording the spectrum of the intermolecular complex of 0.02 M (-)S-adenosyl-L-methionine chloride and 0.004 M disodium

adenosine 5'-phosphate in the presence of 2.5×10^{-4} M, 5.0×10^{-4} M, 7.5×10^{-4} M and 1.0×10^{-3} M $MnCl_2 \cdot 4H_2O$ at pD of ca 6.0.

Determination of Nuclear Overhauser Effects is greatly facilitated by elimination of all sources of intermolecular relaxation.[38] The large magnetic moment associated with the HOD molecult makes D_2O an inapropriate choice of solvent for NOE experiments. (-)S-adenosyl-L-methionine chloride was lyophilized twice from D_2O and subsequently dissolved in dimethyl sulfoxide-D_6 at a concentration of 0.25 M. One ml of the solution was centrifuged for three minutes and then transferred to a constricted 5 mm NMR tube. The solution was degassed by at least three freeze-pump-thaw cycles at ca 10^{-5} torr before the tube was sealed. Protons of interest were irradiated to saturation level. Nuclear Overhauser enhancements were measured in terms of percent increase in peak intensities.

RESULTS AND DISCUSSION

We here propose a model for the molecular conformation of S-adenosyl-L-methionine in aqueous solution, derived from the complete analysis of the 100 MHz and 360 MHz Fourier transform proton magnetic resonance spectra. Fourier transform techniques have allowed us to overcome the difficulties encountered by Danyluk[39] in an earlier attempt to study this molecule by nuclear magnetic resonance.

A. Spectral Assignment and Interpretation

Analysis of the proton resonances associated with SAM and SAH resulted from considerations of chemical shift and spin multiplicity and was aided by comparison with the spectra of the model compounds adenosine, L-methionine and L-methionine-S-methyl sulfonium iodide.

The 100 MHz spectrum was initially obscured by a broad HOD resonance even after two lyophilizations of the compound from D_2O. A useful repositioning of the HOD resonance was unattainable by the addition of acid or dilute alkali. Water Eliminated Fourier Transform (WEFT)[37] NMR spectroscopy was successfully employed to resolve this problem. This technique capitalizes on the large difference in spin-lattice relaxation time between the HOD resonance and the remaining resonances of interest. A 180°-τ-90° pulse sequence is applied and τ adjusted such that the HOD resonance attains

zero net longitudinal magnetization just as the observing 90° pulse is applied. The 100 MHz spectrum with the HOD line eliminated is illustrated in Figure 4.

WEFT techniques allowed for the unambiguous assignment of all except the H_4', H_5', H_5'' and H_α multiplets which were insufficiently resolved at 100 MHz. The proximity of these resonances prevented their analysis by homonuclear decoupling and necessitated the examination of the spectrum at higher magnetic field. Sufficient resolution to complete the analysis was attained upon examination at 360 MHz.

Assignments for the cofactor and related model compounds are given in Table 1. Chemical shifts and coupling constants were determined by analysis of the 360 MHz spectrum. Chemical shifts associated with first-order multiplets were determined by center of gravity approximation and coupling constants by direct measurement. Homonuclear decoupling was employed to confirm the assignment of all first-order spectral features. Chemical shifts and coupling constants associated with higher-order spectral features were determined by graphical analysis as described by Bible,[40] and Johnson and Bible.[41]

The ribose protons constitute a six spin system of the spectral type ABKMNX where A = H_5'', B = H_5', K = H_4', M = H_3', N = H_2' and X = H_1'. As indicated, only the H_1', H_2' interaction is of a strictly first-order nature. The methionine side chain protons constitute a five spin system of the spectral type A_2MXZ where $A_2 = 2H_\beta$, M = H_γ', X = H_γ

and Z = H_α. Interactions are of a first-order nature although the appearence of the H_γ and H_γ' multiplets are complicated by the chemical shift nonequivalence of these protons which results from their location adjacent to the asymmetric sulfonium center. The remaining major spectral features include the adenine ring and sulfonium methyl protons, which are uncoupled and appear as singlets.

Close examination of the spectrum baseline reveals evidence of the presence of impurities. Inspection of a spectrum of the acid hydrolysis products reveals no correlation with the impurity resonances indicating the impurities were present initially and did not result from decomposition of the cofactor. Comparison with the spectra of the model compounds listed in Table 1 indicate the possible identity of these impurities to be adenosine and L-methionine.

Computer simulation of the spectrum provided final confirmation of the chemical shift and coupling constant assignments. Theoretical spectra were calculated according to the method of Castellano and Bothner-By[42] utilizing the LOACN3 algorithm. Subsequently, the theoretical transitions were interated by least squares criteria to fit the experimentally observed spectral lines. Theoretical spectra corresponding to the five and six spin systems were calculated independently and are illustrated in Figure 6. In each case they were found to converge with the experimentally observed spectral lines to within a RMS deviation of 0.03.

Figure 4. 100 MHz ^{1}H WEFT NMR spectrum of (-)S-adenosyl-L-methionine iodide. Solution was 0.025 M in D_2O, pD ≈ 3.4

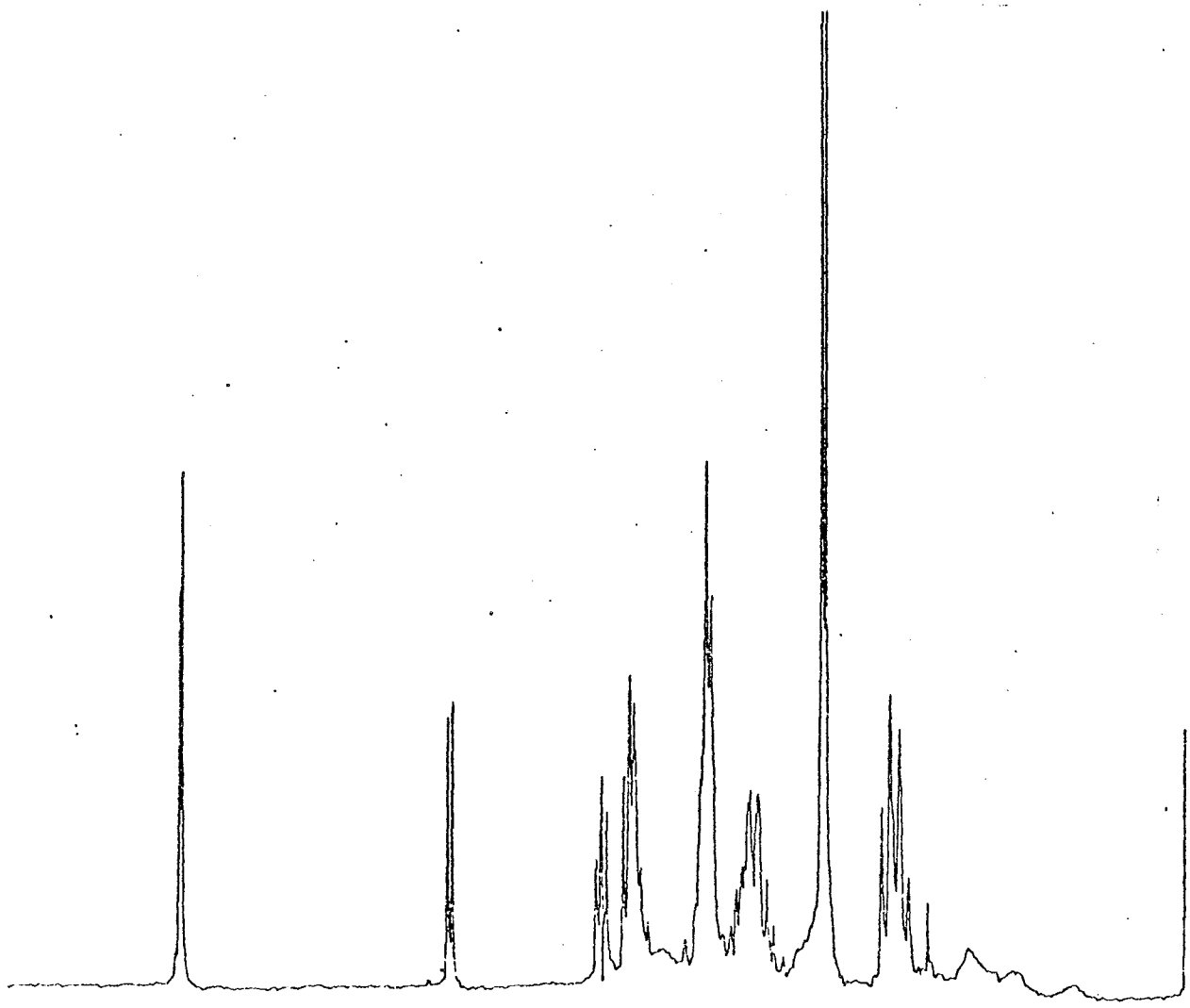


Figure 5. 360 MHz ^{1}H FT NMR spectrum of (-)S-adenosyl-L-methionine iodide. Solution was 0.025 M in D_2O, pD ≈ 3.4

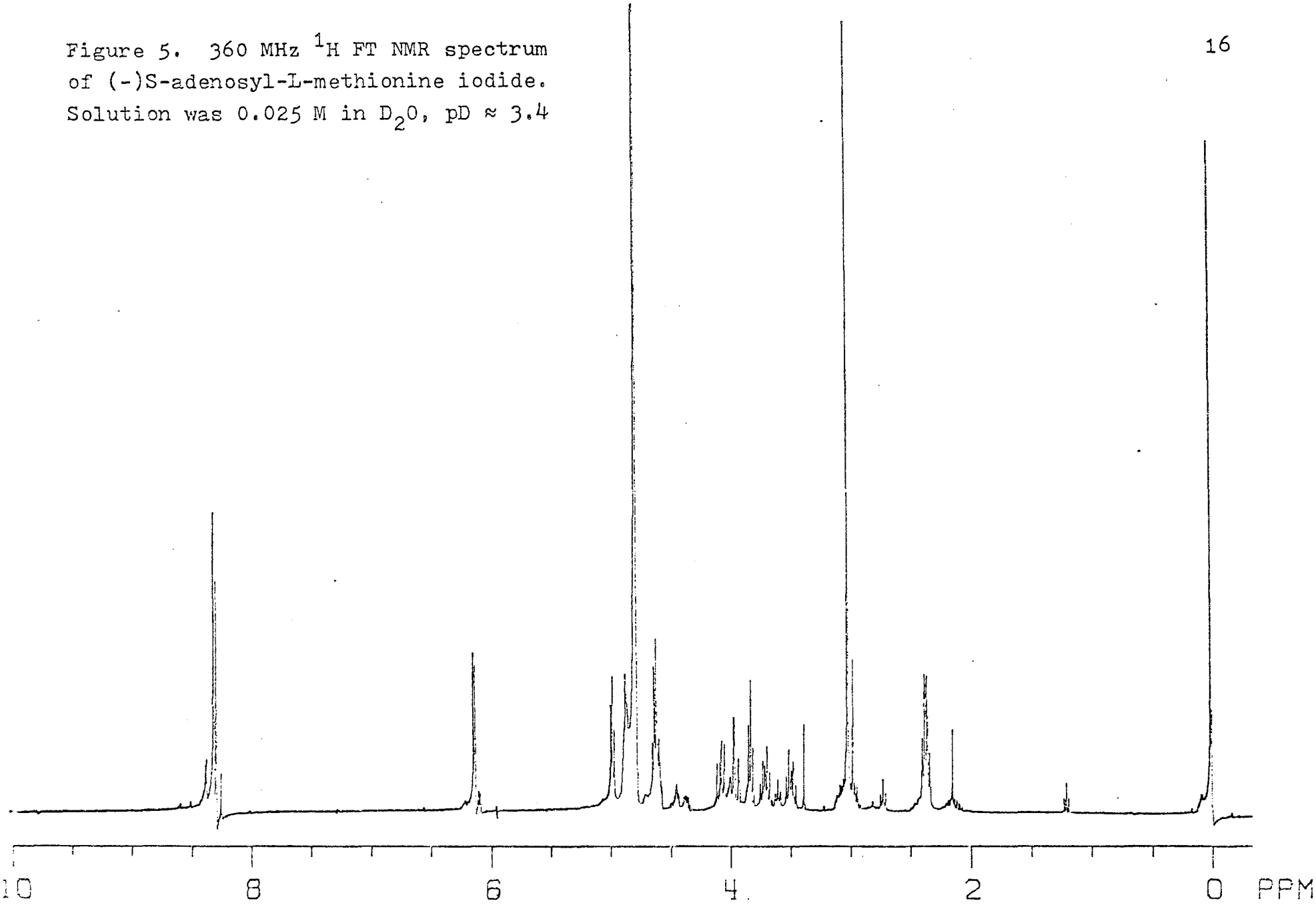


Table 1a. Characteristic Chemical Shifts (δ) of S-adenosyl-L-methionine, S-adenosyl-L-homocysteine and Related Model Compounds

	H_α	H_β	H_γ	H_{SCH_3}	$H_{5',5''}$	$H_{4'}$	$H_{3'}$	$H_{2'}$	$H_{1'}$	H_2	H_8
Met	3.86	2.16	2.65	2.19	-	-	-	-	-	-	-
S-methyl-Met I^-	3.89	2.46	3.41 3.62	2.97	-	-	-	-	-	-	-
Adenine HCl	-	-	-	-	-	-	-	-	-	8.19	8.13
Adenosine	-	-	-	-	3.89	4.32	4.44	4.80	6.05	8.18	8.30
SAH	3.85	2.19	2.73	-	3.06	4.38	4.45	4.87	6.09	8.25	8.35
SAM I^-	3.81	2.36	3.48 3.70	2.96 3.01	3.92 4.06	4.60	4.86	4.97	6.12	8.27	8.29
SAM Cl^-	3.80	2.35	3.47 3.69	2.96 3.00	3.93 4.06	4.60	4.84	4.96	6.12	8.29	8.36
SAM HSO_4^-	3.89	2.32	3.48 3.70	2.97 3.01	3.92 4.04	4.63	4.82	4.88	6.18	8.46	8.48

Table 1b. Characteristic Coupling Constants (Hz) of S-adenosyl-L-methionine. Values for all counter ions are equivalent within experimental error

$J_{\alpha\beta}$	$J_{\alpha\beta'}$	$J_{\beta\gamma}$	$J_{\beta'\gamma}$	$J_{\beta\gamma'}$	$J_{\beta'\gamma'}$	$J_{1'2'}$	$J_{2'3'}$	$J_{3'4'}$	$J_{4'5'}$	$J_{4'5''}$	$J_{\gamma\gamma'}$	$J_{5'5''}$
6.6	6.6	7.4	7.4	7.4	7.4	4.4	5.3	0.0	9.5	2.4	-11.2	-12.8

Values for S-adenosyl-L-methionine were determined at 360 MHz. All others were determined at 100 MHz. Samples were 0.025 M in D_2O with the pD adjusted to ca 3.4.

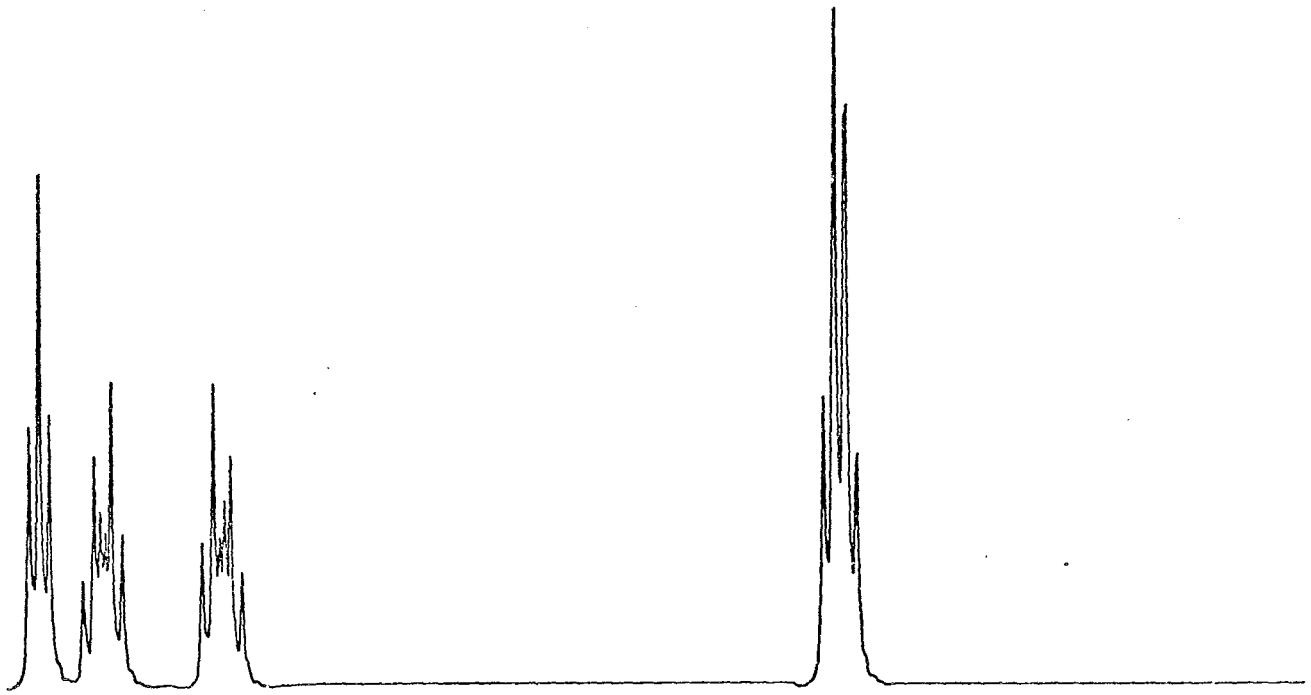


Figure 6a. Computer Simulation of the Methionine Side Chain Protons

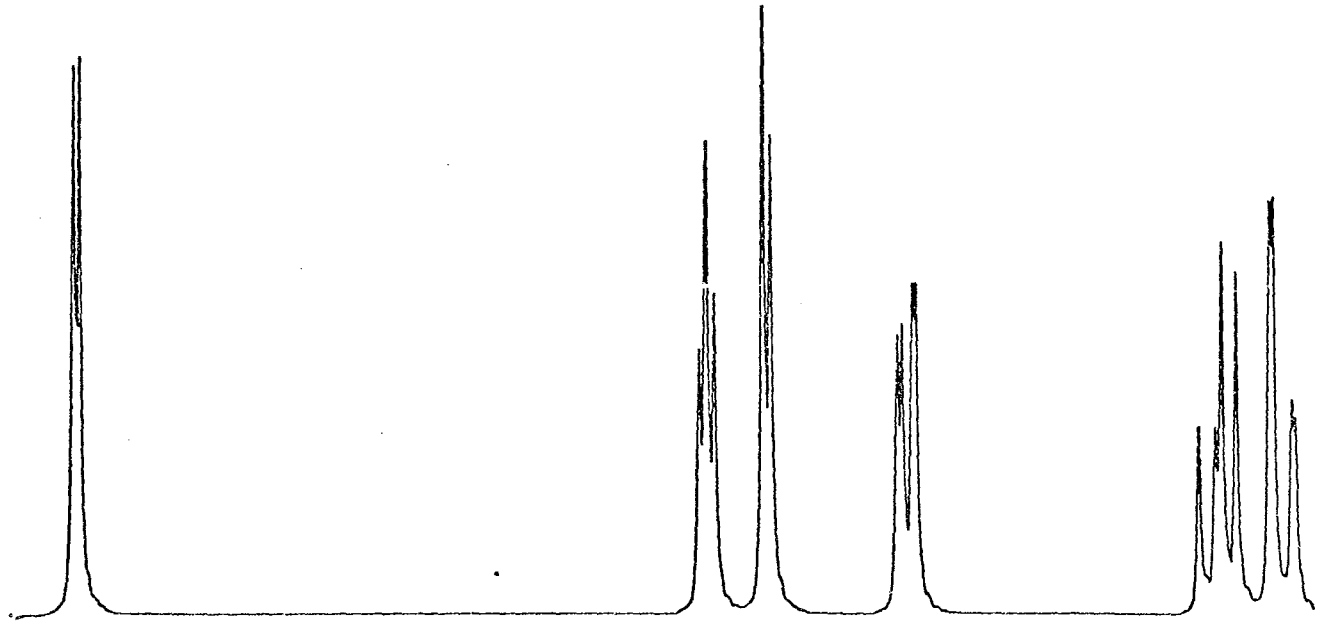


Figure 6b. Computer Simulation of the Ribose Ring Protons

B. Concentration and pH Dependent Phenomena

The concentration dependent, intermolecular association of purine and pyrimidine nucleosides in aqueous solution was systematically investigated by Ts'o, Melvin and Olson,[43] Ts'o and Chan,[44] Chan, Schweizer, Ts'o and Helmkamp,[45] and Schweizer, Chan and Ts'o.[46] This interaction has been characterized as a vertical stacking of the nucleoside bases and has important implications for the structure of nucleic acids.[44] These compounds can be arranged in a series of increasing association equilibrium constants: purine-purine purine-pyrimidine pyrimidine-pyrimidine.[43] Evidence of the presence of dimeric, trimeric and higher-order complexation has been reported to correspond to increases in nucleoside concentration.[45]

In the NMR spectrum, the magnetic anisotropy of the associated bases causes progressive shielding of protons in proximity to the interactive site. Table 2 summarizes the chemical shift variation observed over the concentration range 0.01-0.04 M. The chemical shift variation of the adenine ring protons is illustrated in Figure 7. We interpret the displacement of the proton resonances to higher magnetic field with increasing concentration as evidence of the formation of the intermolecular complex. The magnitudes of the observed displacements are largest for the base and H_1' protons and decrease with increasing distance from the glycosidic bond.

Severe limitations posed by the acidic and alkaline hydrolysis of S-adenosyl-L-methionine have precluded a quanti-

FIGURE 7. CONCENTRATION DEPENDENCE OF CHEMICAL SHIFTS

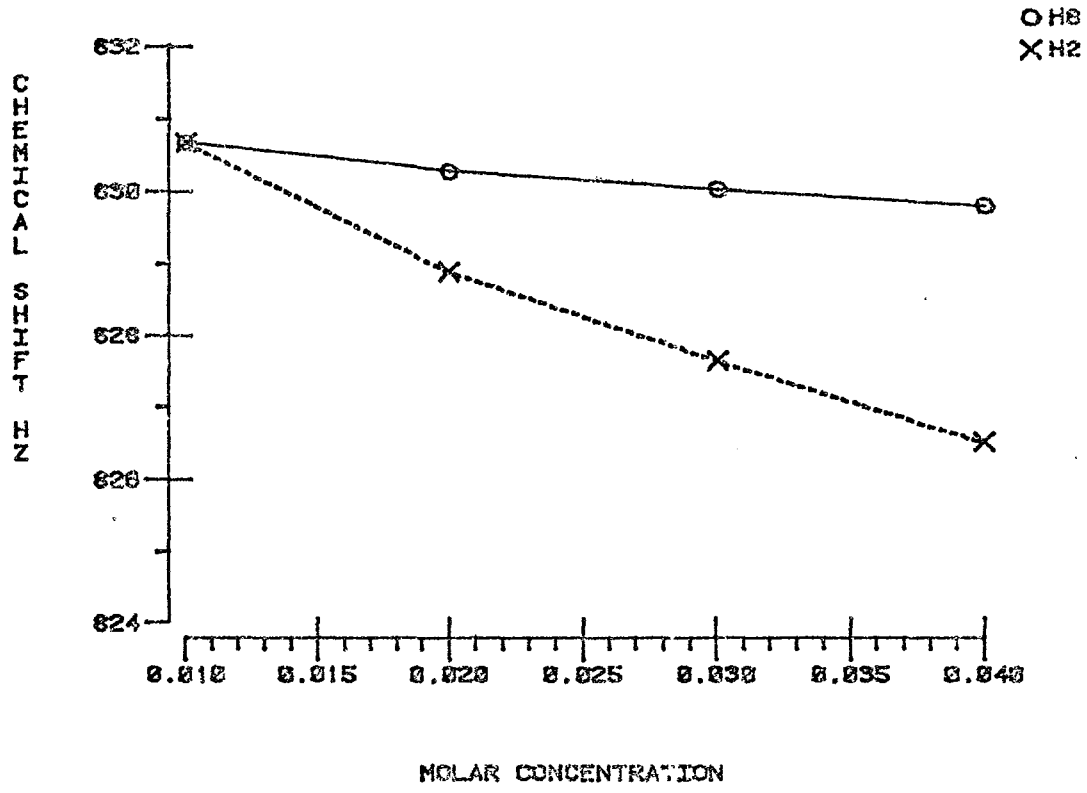
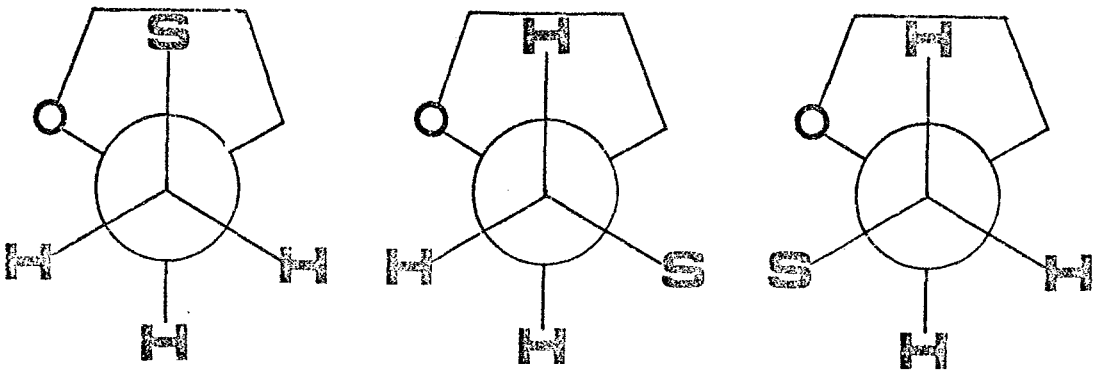


Table 2. Concentration Dependence of Chemical Shifts for (-)S-adenosyl-L-methionine Chloride

Concentration	H_8	H_2	H_1'	H_2'
0.01	830.655	830.655	616.065	497.962
0.02	830.271	828.867	615.133	497.140
0.03	830.009	827.634	614.624	496.742
0.04	829.778	826.506	614.303	495.944

tative investigation of pH effects. However, our attempts to reposition the HOD resonance by the addition of acid have resulted in a downfield shift of the purine ring protons. Danyluk and Hruska,[47] have reported quantitative details of the effect of pH upon the NMR spectrum of adenosine and note that a deshielding of the purine ring protons with increasing acidity results from the protonation of the 6-amino position of the adenine base (pK_a = 3.2). Klee and Mudd,[28] have determined the pK_a of the adenine base in S-adenosyl-L-methionine to be 3.4, suggesting that the observed shift may similarly be ascribed to the protonation of the adenine base.



I (gauche-gauche) II (anti-gauche) III (gauche-anti)

Figure 8. Rotational Isomers About the C_4'-C_5' Bond

C. Conformation About the Exocyclic C_4'-C_5' Bond

An estimate of the relative populations of rotational isomers corresponding to the energy minima which occur upon rotation about the C_4'-C_5' bond, Figure 8, is obtainable from the analysis of the appropriate coupling constants. We have employed the method of Blackburn, et al.[48] The coupling constants associated with the fragment of interest are assumed equivalent to the weighted average of the coupling constants in the three rotamers. This is expressed algebraically as equations (1) and (2), where P_I, P_{II} and P_{III} are the mole fractions of each rotamer and J_I', J_{II}', etc. are the coupling constants associated with each rotamer. Equation (3) expresses the conservative nature of the molar fractions.

$$(1) \qquad J_{4'5'} = P_I J_I' + P_{II} J_{II}' + P_{III} J_{III}'$$

$$(2) \qquad J_{4'5''} = P_I J_I'' + P_{II} J_{II}'' + P_{III} J_{III}''$$

$$(3) \qquad 1 = P_I + P_{II} + P_{III}$$

The values of J_I', J_{II}', etc. are derived from the analysis of the Karplus equation,[49,50] which relates the vicinal coupling constant, J_{HH}', to the dihedral angle, θ, between the HCC' and CC'H' planes in the fragment HCC'H'. This relationship assumes the form of equation (4).

$$(4) \qquad J_{HH}' = J_o \cos^2\theta - 0.28 \text{ Hz}$$

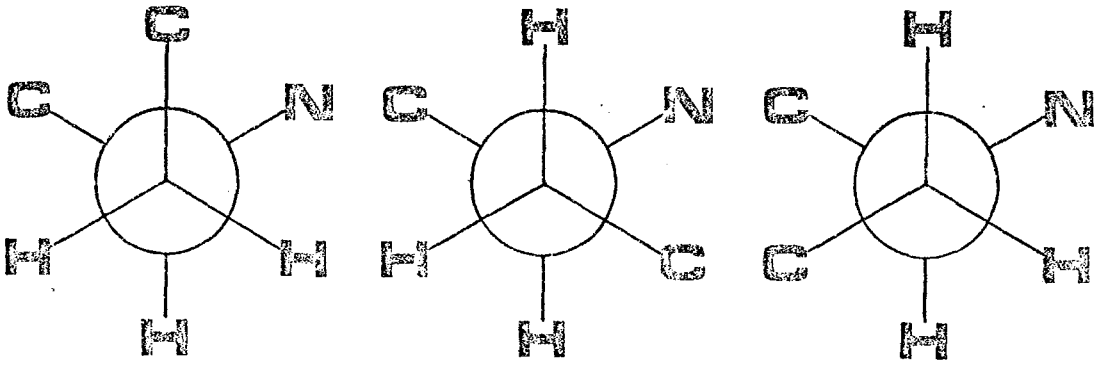
Although the form of equation (4) is believed to be correct, values of J_o from 8 to 16 Hz have been applied to the analysis

of various systems. In the present study we will adopt the values suggested by Abraham, et al.[51] for a number of carbohydrate ring systems: J_o = 9.27 Hz for $0^\circ \leq \theta \leq 90^\circ$ and J_o = 10.36 Hz for $90^\circ \leq \theta \leq 180^\circ$. Abraham's values of J_o are those most often employed in the analysis of nucleosides and provide the standard for the comparison of conformations.

The H_4', H_5' and H_5'' protons constitute a three spin system of the ABX spectral type with vicinal couplings $J_{4'5'}$ = 9.5 Hz and $J_{4'5''}$ = 2.4 Hz. Substitution of these values into equations (1) and (2), followed by simultaneous solution of equations (1), (2) and (3) results in the following estimate of the relative rotamer populations: P_I(gauche-gauche) ≈ 2%, P_{II}(anti-gauche) ≈ 93% and P_{III}(gauche-anti) ≈ 5%. We must emphasize that the individual 5'-CH_2 proton resonances are experimentally indistinguishable and hence cannot be assigned a particular position in a given rotamer. Consequently, rotamers II and III are experimentally indistinguishable, although the pronounced preference for a rotamer in which the sulfonium center is gauche to H_4' is unaffected. Examination of a molecular model indicates that in rotamer II the sulfonium methyl group is in close proximity to the ribose and purine ring systems, whereas, in rotamer III it protrudes from the main body of the molecule. Borchardt and Wu,[52] have investigated the geometric requirements of the enzymatic binding site for S-adenosyl-L-methionine and have concluded that the cofactor binds to enzymes in a conformation which is consistent with the orientation of the sulfonium center in rotamer III.

D. Conformation About the C_α-C_β Bond

The H_α, H_β and H_β' protons constitute a three spin system of the A_2X spectral type. Experimentally it is observed that $J_{\alpha\beta} = J_{\alpha\beta}' = 6.6$ Hz. The appearence of equivalent coupling constants is indicative of equivalent populations of the three rotational isomers, Figure 9. Spectral behavior of this type results from free rotation about the C_α-C_β bond which is rapid on the NMR time scale. Bovey,[53] and Pople, et al.[54] have investigated details of differential rotamer populations of the above type and their effect upon vicinal couplings.

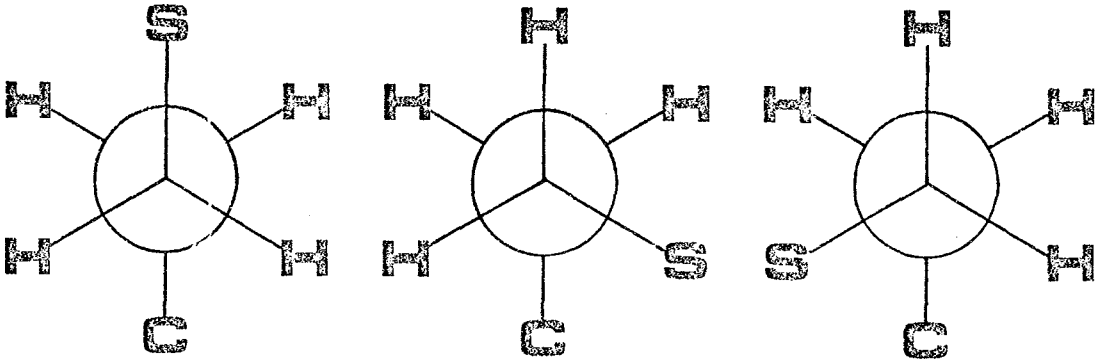


I (gauche-gauche) II (anti-gauche) III (gauche-anti)

Figure 9. Rotational Isomers About the C_α-C_β Bond

E. Conformation About the C_β-C_γ Bond

Rotational isomers about the C_β-C_γ bond are illustrated in Figure 10. The H_β, H_β', H_γ and H_γ' protons constitute a four spin system of the A_2MX spectral type. Experimentally it is observed that $J_{\beta\gamma} = J_{\beta\gamma'} = J_{\beta'\gamma} = J_{\beta'\gamma'} = 7.4$ Hz, indicating that the three rotamers are equally populated and that there is free and rapid rotation about the C_β-C_γ bond. It is prudent to note that the H_β and H_β' as well as the H_γ and H_γ' protons are experimentally indistinguishable, as was the case for the 5'-CH_2 protons described above.



I (gauche-gauche) II (anti-gauche) III (gauche-anti)

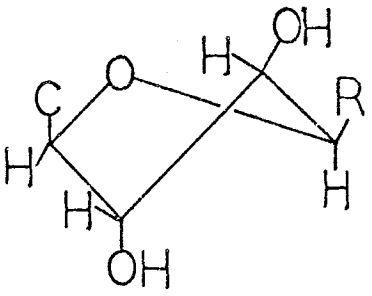
Figure 10. Rotational Isomers About the C_β-C_γ Bond

F. Conformation of the Ribofuranose Ring

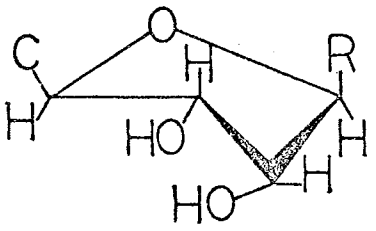
NMR conformational analysis of a variety of purine and pyrimidine nucleosides and nucleotides has resulted in reports of considerable variation among the individual ribose ring coupling constants. Investigators have interpreted these results as consistent with the presence of various conformers is solution. Sundaralingam[55] has calculated the energies associated with conformational interconversions to be near equivalent to normal hydrogen bond energies. In 1968, Smith and Jardetzky[56] employed the Karplus equation to predict a table of coupling constants associated with twenty possible bucklings of the D-ribose ring. Table 3 is an abbreviated form of the aforementioned table containing data for those conformers observed in X-ray crystallagraphic and nuclear magnetic resonance studies during subsequent years. Figure 11 illustrates four conformers contained in Table 3 which are representative of extremes among the possible ring bucklings.

Table 3. Estimated Dihedral Angles and Coupling Constants for D-Ribose Conformations[56]

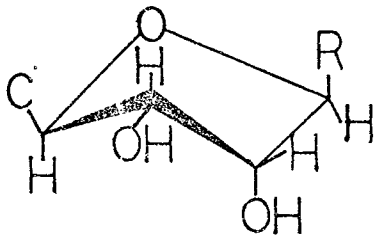
	θ1'2'	J1'2'	θ2'3'	J2'3'	θ3'4'	J3'4'
C_2'-endo	165	8.6	45	3.9	105	0.4
C_2'-exo	75	0.2	45	3.9	135	4.6
C_3'-endo	105	0.4	45	3.9	165	8.6
C_3'-exo	135	4.6	45	3.9	75	0.2
C_2'-endo-C_3'-exo	165	8.6	60	1.7	75	0.2
C_2'-exo-C_3'-endo	75	0.2	60	1.7	165	8.6
C_3'-endo-C_4'-exo	105	0.4	45	3.9	180	9.2
C_3'-exo-C_4'-endo	135	4.6	45	3.9	60	1.7



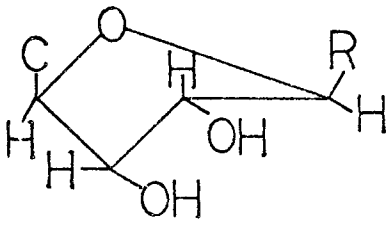
C_2'-endo



C_2'-exo



C_3'-endo



C_3'-exo

Figure 11. Representative Ribose Ring Conformations

Experimentally we observe the following vicinal couplings for the ribose ring protons in SAM: $J_{1'2'}$ = 4.4 Hz, $J_{2'3'}$ = 5.3 Hz and $J_{3'4'}$ = 0 Hz. Inspection of Table 3 reveals these results to be most consistent with the C_3'-exo conformation. As authors have varied in their choice of dihedral angles employed to approximate the ribose ring conformers, considerable variations among theoretical estimates of coupling constants have appeared in the literature. The estimated dihedral angles and associated coupling constants for the exo conformation from three references are given in Table The correlation of our data appears to remain, regardless the source of theoretically predicted angles and couplings

Table 4. Estimated Dihedral Angles and Coupling Constants for the Ribose C_3'-exo Conformation[56,57,58]

θ1'2'	J1'2'	θ2'3'	J2'3'	θ3'4'	J3'4'
135	4.6	45	3.9	75	0.2
140	5.8	28	7.0	95	0
145	6.7	40	5.0	100	0
Observed	4.4		5.3		0

Recently Hruska,[59] and Sarma and Mynott[60] have suggested that the error (≈ 10%) associated with the use of the Karplus equation to estimate coupling constants for small angles is too great to differentiate between various ring conformers with certainty. Alternatively, they have proposed that the conformation of the ribose ring might better be described qualitatively as an equilibrium between the ^2E (C_2'-endo) and ^3E (C_3'-endo) conformers. As estimate of the relative populations of these two conformers can be calculated by analogy to the calculation of rotational isomer populations. The $J_{1'2'}$ coupling constant and the theoretical values suggested by Hruska, Grey and Smith[57] give rise to the following estimate: ^2E, 44% and ^3E, 56%. The absence of a marked preference for either conformer is suggestive of a considerable amount of conformational flexibility.

G. Sugar-Base Torsional Angle

Analysis of the sugar-base torsional angle in adenosine, adenosine 5'-phosphate, adenosine 5'-diphosphate, adenosine 5'-triphosphate, adenosine 3',5'-cyclic phosphate and

adenosine diphosphoglucose have revealed that the purine bases in these compounds are oriented preferentially in the anti conformation.[61] Only compounds substituted at the 8 position of the purine ring such as 8-bromoadenosine and 8-iodo-adenosine have been shown to be oriented preferentially in the syn conformation. The Optical Rotatory Dispersion studies of Klee and Mudd[28] have suggested that (±)S-adenosyl-DL-methionine and all other nucleoside derivatives substituted at the 5'-carbon with sulfur are oriented preferentially anti. We thus expect that the purine ring in S-adenosyl-L-methionine is oriented preferentially anti and have sought evidence in the spectrum to confirm this anticipated result.

The sugar-base torsional preference in purine nucleotides is easily determined by observing the deshielding of the H_8 proton which results from the ionization of the α-phosphate. Additionally, the binding of increasing concentrations of Mn(II) to the phosphate anion serves to intensify spectral evidence of the anti conformation as considerable paramagnetic shifts of the H_8 proton result. However, determinations of the above type are inapplicable with respect to SAM due to absence of an anionic site in proximity to the purine ring.

Evans and Sarma[62] have devised an elegant determination of the sugar-base torsional preference which utilizes the tendency of nucleotide-like molecules to form base-stacked complexes when present in solution at concentrations greater than ca 0.025 M. Figure 12 illustrates the purine proton

Table 6. Anticipated Paramagnetic Shifts of the Complex of SAM and 5'-AMP in the Presence of Mn(II)

SAM	5'-AMP	H_8(SAM)	H_2(SAM)	H_8(AMP)	H_2(AMP)
<u>syn</u>	<u>syn</u>		shifted		shifted
<u>syn</u>	<u>anti</u>		shifted	shifted	
<u>anti</u>	<u>syn</u>	shifted			shifted
<u>anti</u>	<u>anti</u>	shifted		shifted	

displacements associated with the intermolecular complex of S-adenosyl-L-methionine and adenosine 5'-phosphate in the presence of increasing concentrations of Mn(II). Table 5 summarizes the corresponding data. Four modes of association of the purine bases in these two molecules can be envisioned: SAM <u>anti</u>-5'-AMP <u>syn</u>, SAM <u>syn</u>-5'-AMP <u>syn</u>, SAM <u>syn</u>-5'-AMP <u>anti</u> and SAM <u>anti</u>-5'-AMP <u>anti</u>. The anticipated experimental results of the modes of association of S-adenosyl-L-methionine and adenosine 5'-phosphate with Mn(II) are shown in Table 6. Adenosine 5'-phosphate has been shown to be rigidly in the <u>anti</u> conformation,[62] eliminating two of the aforementioned possibilities. Experimentally we note the progressive paramagnetic shifts of both H_8 protons with increasing Mn(II) concentration. Chan and Nelson[63] have encountered a similar situation in their investigation of the intramolecular conformation of adenylyl (3'-5')-adenosine. They have presented arguments which indicate that progressive paramagnetic shifts of the H_8 protons in the absence of significant shifts of the H_2 protons is consistent only with an intermolecular complex in which both purine bases are oriented preferentially <u>anti</u>.

FIGURE 12. MN(II) CONCENTRATION VS. CHEMICAL SHIFT (SAM-AMP)

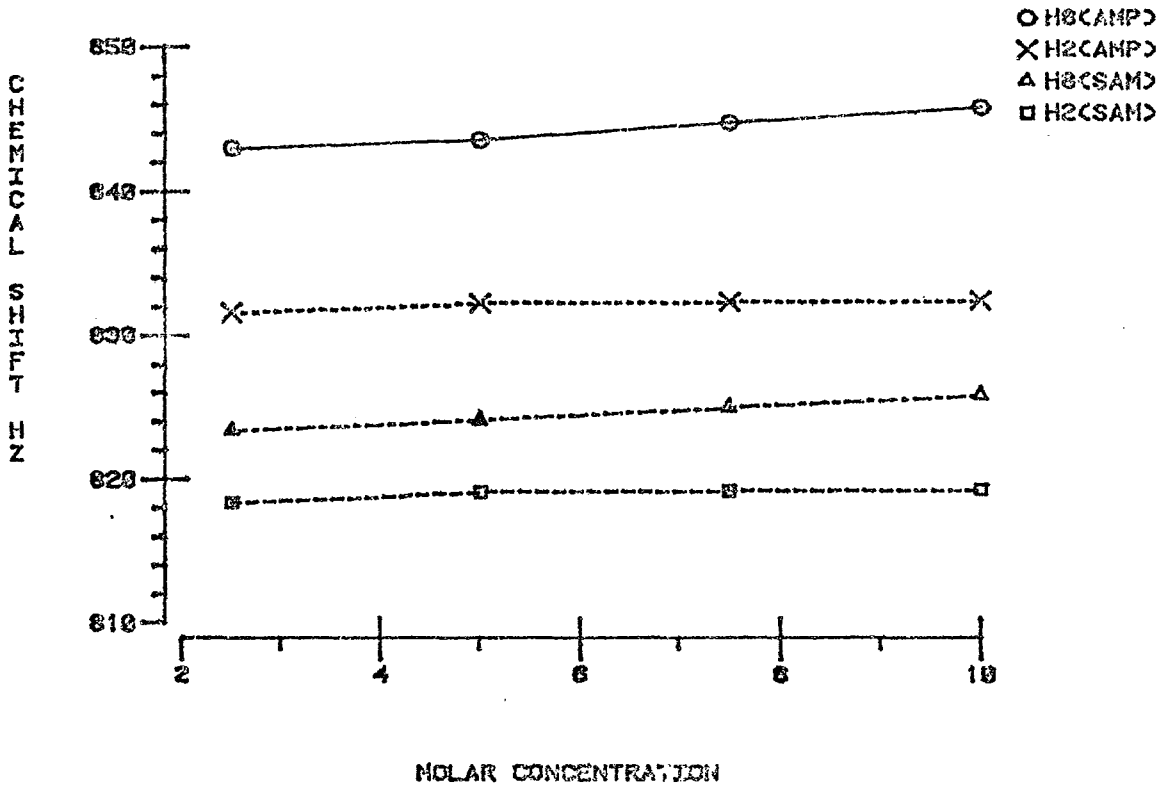


Table 5. Chemical Shifts (Hz) of the Intermolecular Complex of SAM and 5'-AMP with Increasing Mn(II) Concentration

(Mn(II))	H_8(AMP)	H_2(AMP)	H_8(SAM)	H_2(SAM)
2.5×10^{-4}	842.969	831.519	823.381	818.363
5.0×10^{-4}	843.546	832.149	824.139	819.119
7.5×10^{-4}	844.684	832.264	825.006	819.174
1.0×10^{-3}	845.714	832.342	825.789	819.232

H. Sulfonium Diastereomerism

The NMR spectra of samples of (-)S-adenosyl-L-methionine, differing in activity, counter ion and commercial source, have always revealed a second smaller singlet upfield of the sulfonium methyl group, Figure 13. Careful consideration of the origin of this secondary resonance has revealed no correlation with spectra of model compounds corresponding to known degradation products or sample impurities. The proximity of this resonance relative to the sulfonium methyl group in Hz is dependent upon the field strength of the spectrometer, indicating that it does not arise from a long range coupling of the sulfonium methyl protons. Additionally, the relative intensity of this resonance was found to vary among samples of varying activity. Having accounted for all other observable spectral features, we are led to associate this resonance with the sulfonium methyl group of opposite configuration, indicating the presence of a small amount of (+)SAM in all samples we have investigated to date. We have encountered no reports of the spontaneous racemization of (-)SAM and note that racemization of the structurally related S-carboxymethyl-(S)-methionine was shown to proceed only after prolonged heating in acidic solution ($t_{\frac{1}{2}}$ = 24 h, 60°C, 5 N acetic acid).[13] We therefore interpret these results as evidence of the natural occurance of (+)S-adenosyl-L-methionine. In an attempt to confirm this conclusion we have examined the spectrum of (±)S-adenosyl-L-methionine iodide, Figure 14, prepared

by reaction of S-adenosyl-L-homocysteine with methyl iodide. The appearence of the two expected singlets at the appropriate chemical shifts supports our assignments for the diastereomeric sulfonium methyl groups.

Further consideration of Figure 14 illustrates several points of interest. Comparison of the relative intensities of the diastereomeric sulfonium methyl resonances of (±)SAM reveals a marked preference for the formation of the biologically inactive diastereomer. Also, methylation of S-adenosyl-L-homocysteine proceeds over a five day period in acidic solution, accordingly, some hydrolysis of the cofactor is to be expected. In the presence of excess methyl iodide, methylation of both hydrolysis products and appropriate sample impurities results. The extraneous resonances in Figure 14 have tenatively been assigned to the sulfonium methyl groups of L-methionine-S-methyl sulfonium and adenosyldimethylsulfonium, resulting from the methylation of L-methionine and 5'-methylthioadenosine, respectively.

De La Haba, et al.[11] and Zappia, Zydek-Cwick and Schlenk[12] have investigated the relative activities of preparations of (-)SAM and (±)SAM. Assuming that (±)SAM was a racemic mixture of sulfonium diastereomers, the experimental observation that (±)SAM exhibited ca 50% the activity of samples of biological origin led these investigators to conclude that only the (-) sulfonium diastereomer was present in biological preparations. The nonracemic nature of (±)SAM raises questions as to the

validity of this conclusion. It is interesting to note that if samples of biological origin contained a small amount of the (+) sulfonium diastereomer, as is indicated by Figure 13, and if the activities of these samples were compared to the activities of diastereomeric mixtures in which the (+) sulfonium diastereomer were present in slight excess, as is indicated by Figure 14, the relative activities of the diastereomeric mixtures would still appear to be ca 50%.

These findings are corroborated by the observations of Cornforth, et al.[13] Degradation of ($^{14}CH_3$)S-adenosyl-L-methionine to S-carboxymethyl-(S)-methionine under experimental conditions designed to retain the configuration at the sulfonium center resulted in the repeated elution of two radioactive peaks from an amino acid analyzer. A minor peak accounting for at least 10% of the radioactivity was eluted at the retention time corresponding to the sulfonium diastereomer of opposite configuration. Cornforth's group have explained the presence of this minor peak as possibly, "resulting from the presence of some radioactive impurity, chiral at sulfur and resulting in the formation of the degradation product of the opposite sulfonium configuration."

Although it is difficult to anticipate the possible biological significance of this finding, it is interesting to note the report of at least one enzyme, homocysteine S-methyl transferase, which shows no specificity with respect to the sulfonium diastereomers of S-adenosyl-L-methionine.

Figure 13. (-)SAM I^-

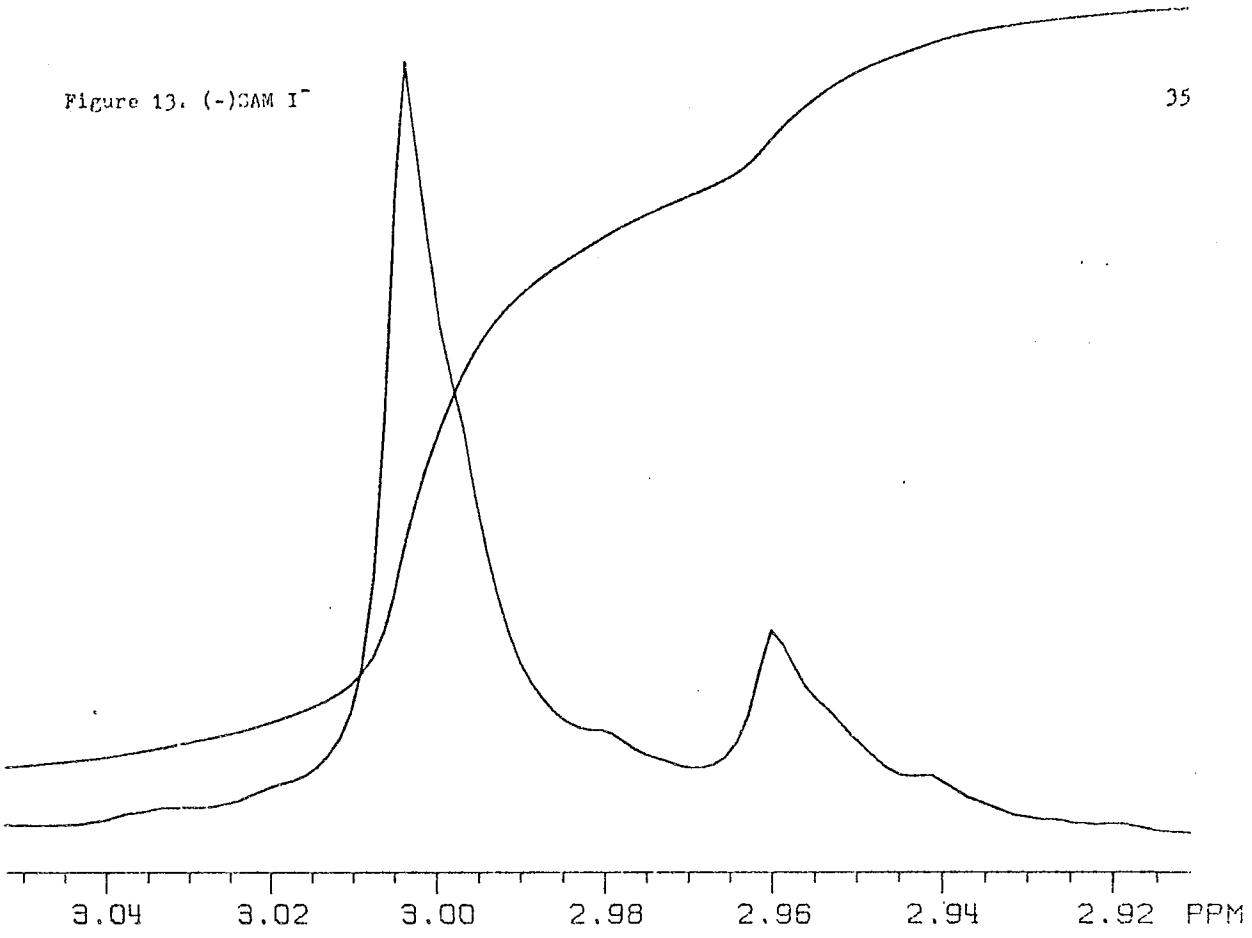
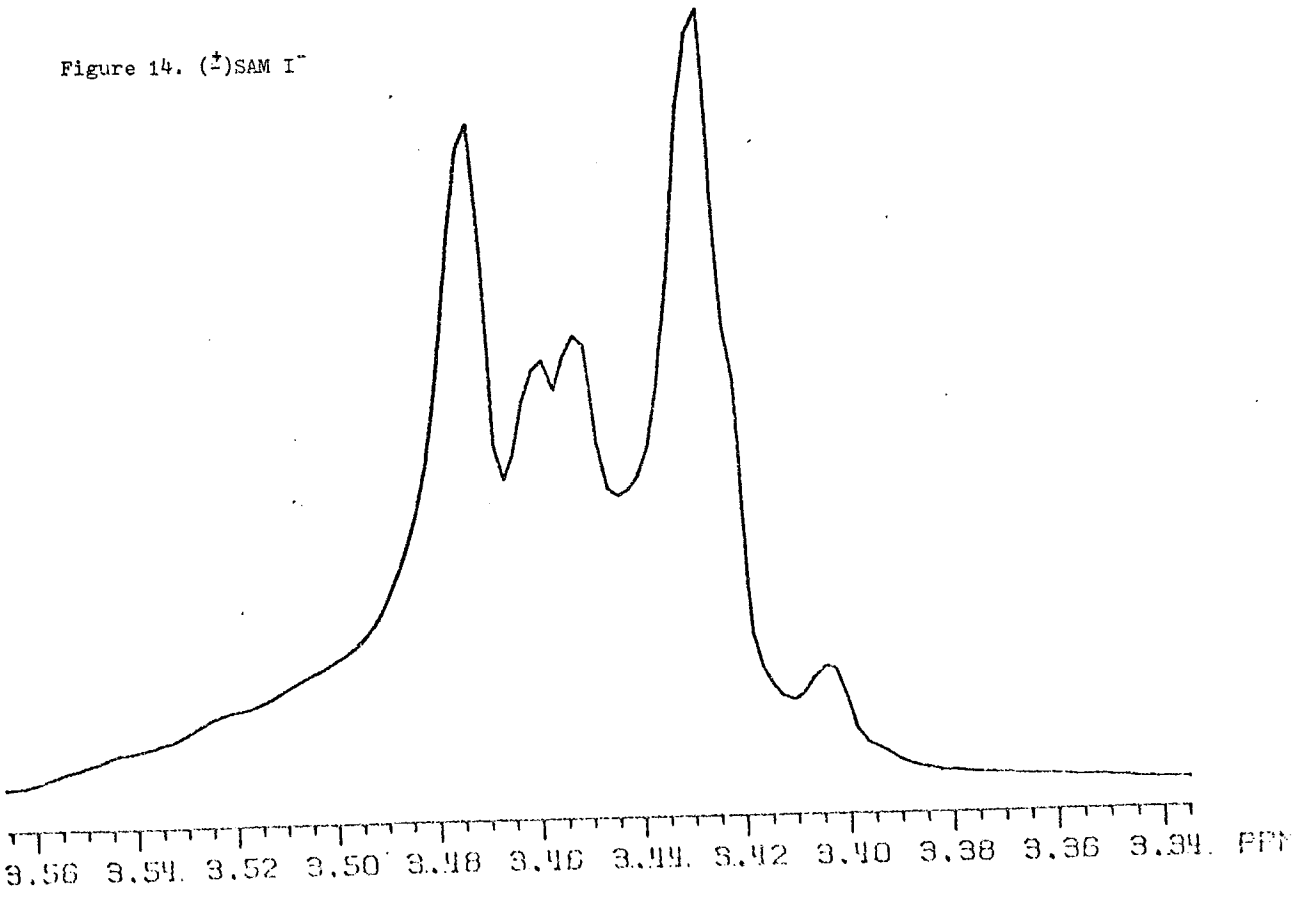


Figure 14. ($\pm$)SAM I^-



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APPENDIX A

SPECTRA OF MODEL COMPOUNDS

Figure 14, Adenosine

Figure 15, L-methionine

Figure 16, L-methionine-S-methyl Sulfonium Iodide

Figure 17, S-adenosyl-L-homocysteine

Figure 18, S-adenosyl-L-methionine Chloride

Figure 14. Adenosine

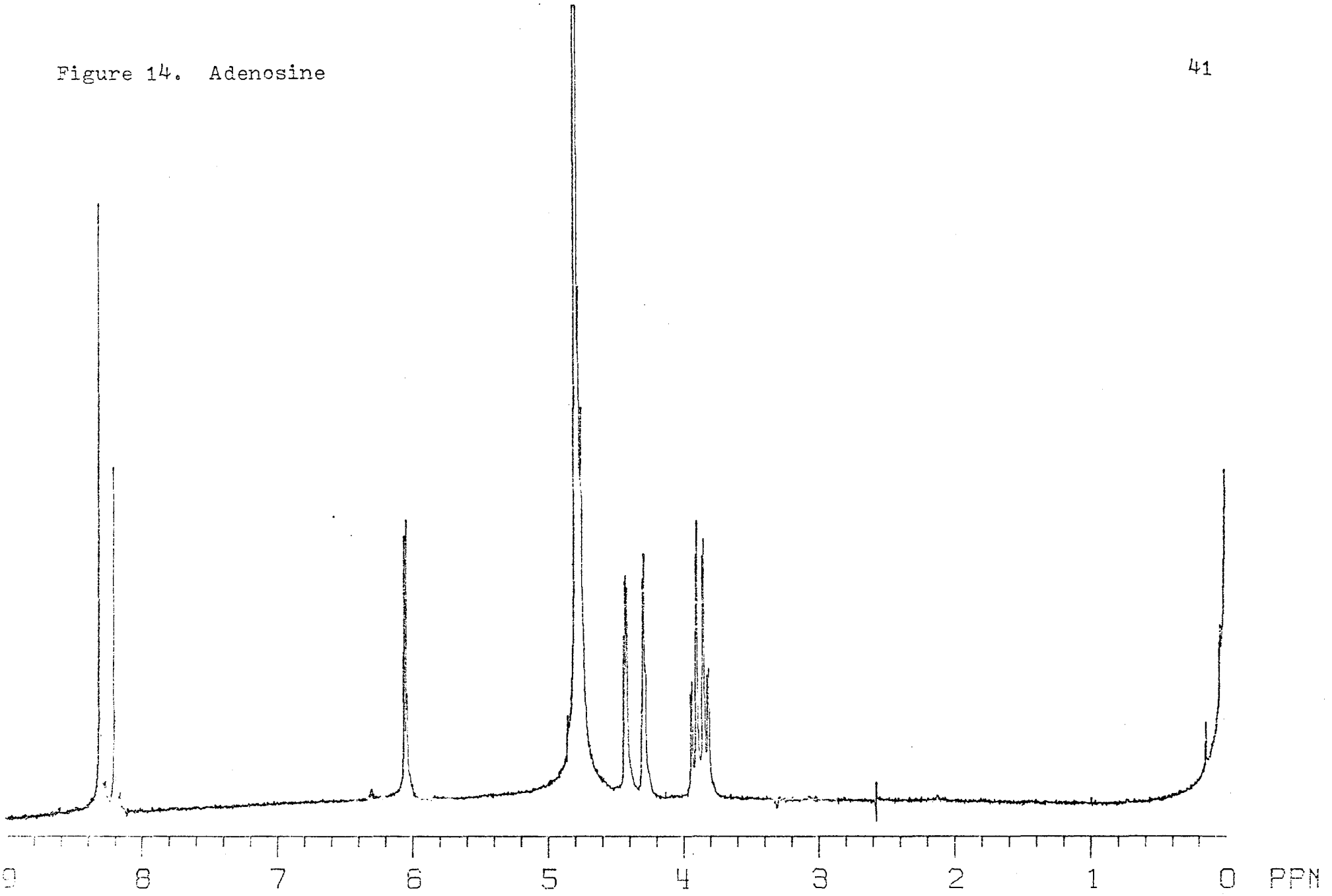


Figure 15. L-methionine

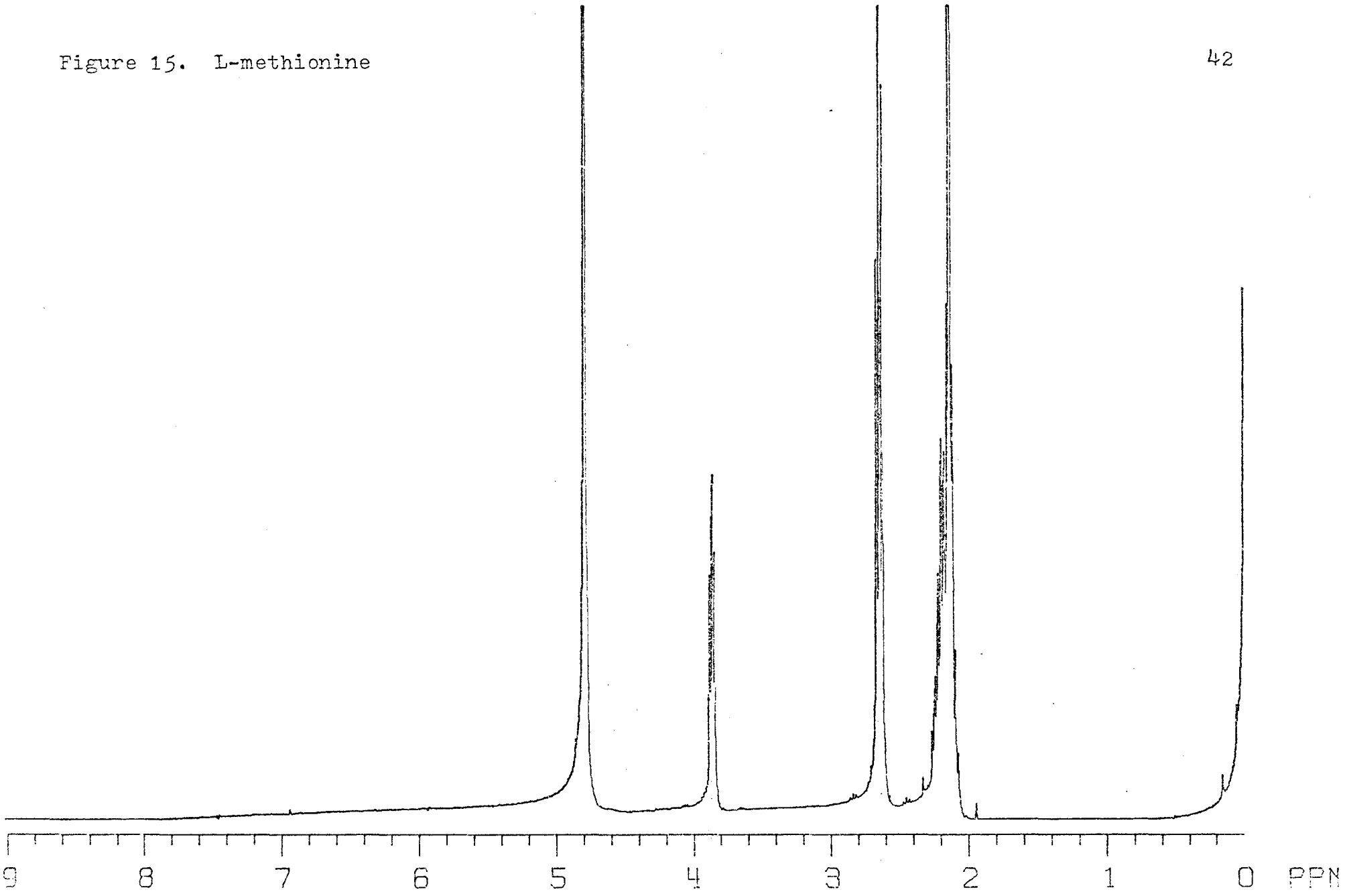


Figure 16. L-methionine-S-methyl Sulfonium Iodide

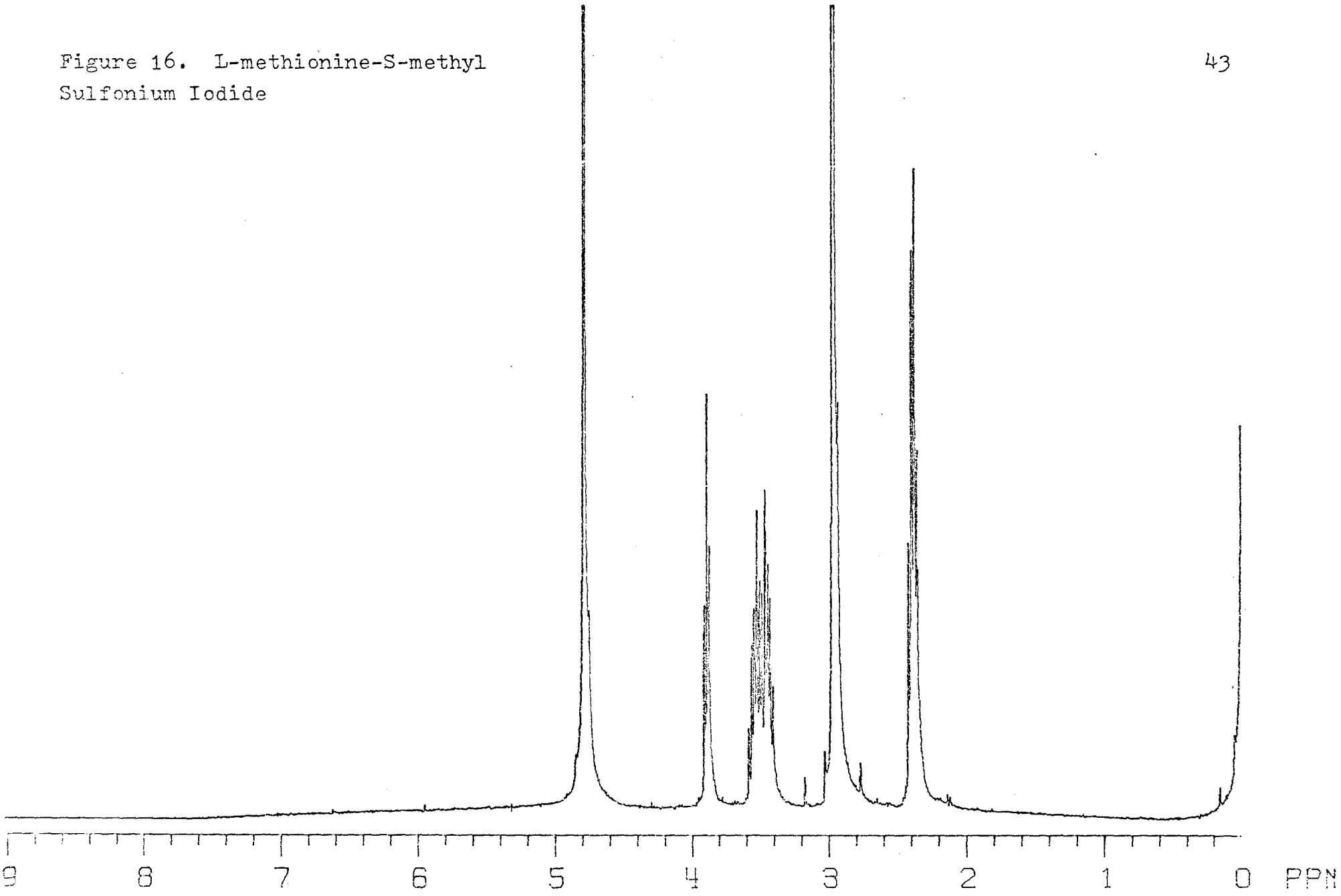


Figure 17. S-adenosyl-L-homocysteine

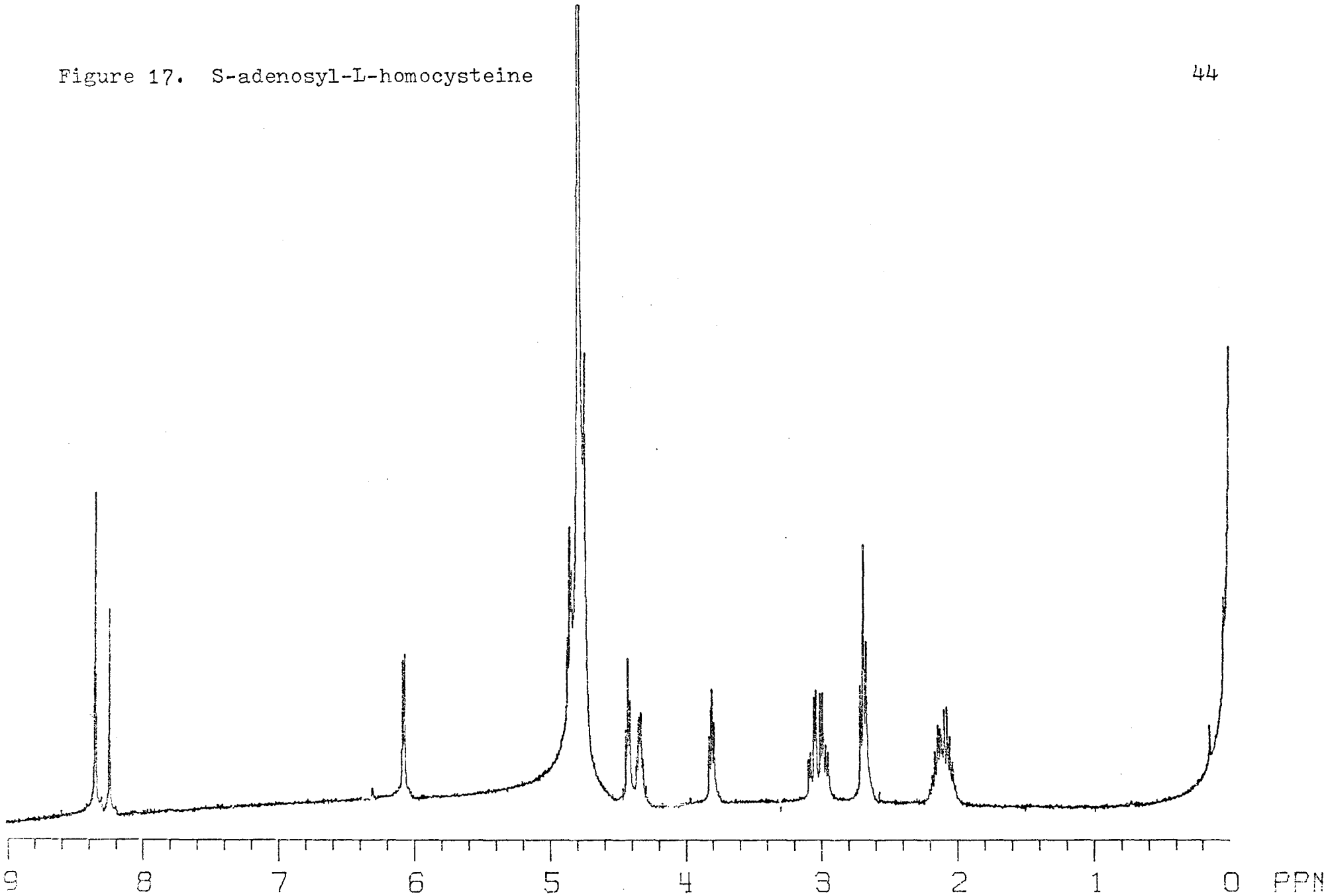
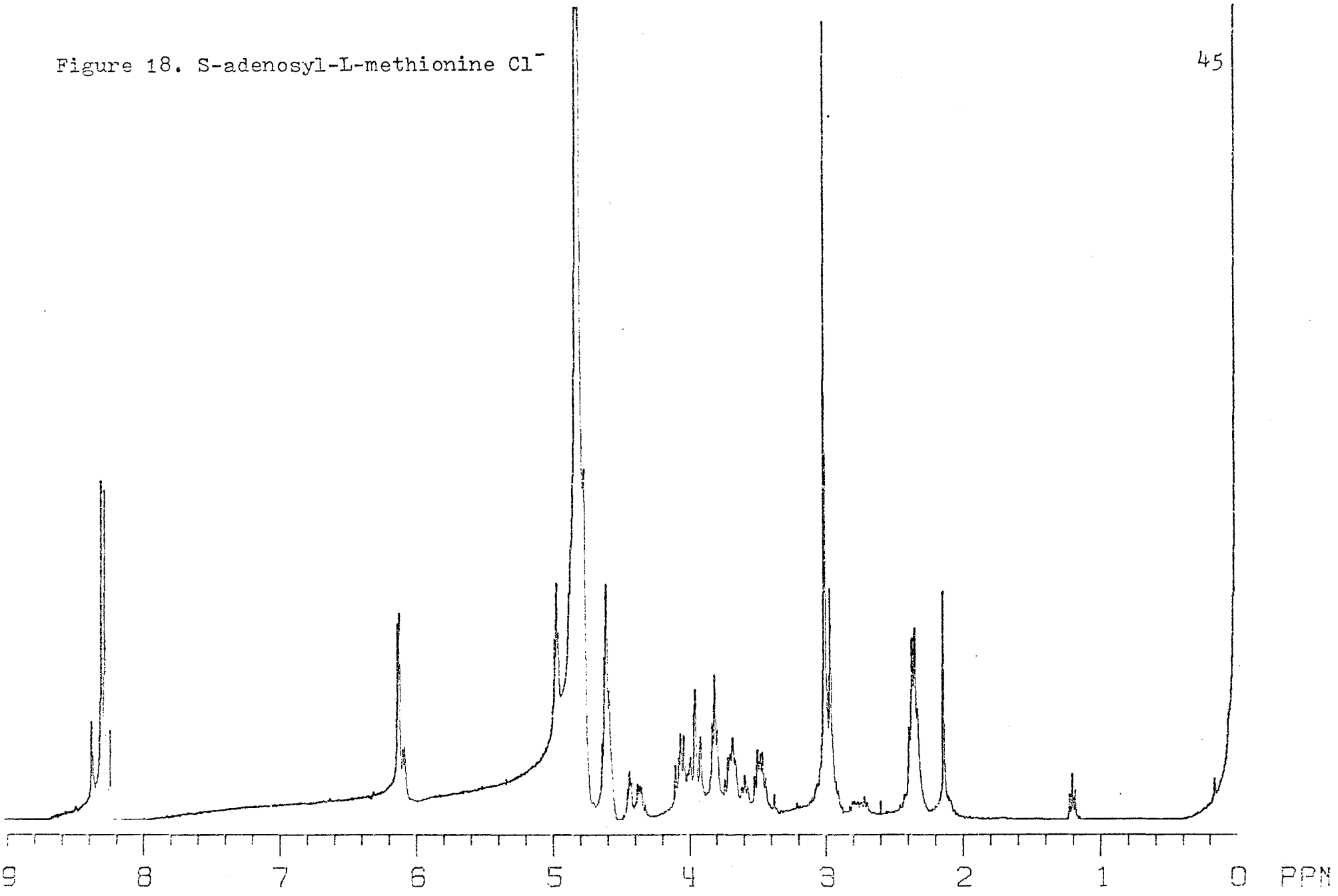


Figure 18. S-adenosyl-L-methionine Cl^-



APPENDIX B

HOMONUCLEAR DECOUPLING

Figure 19, (-)SAM HSO_4^- H_1' Irradiated

Figure 20, (-)SAM HSO_4^- H_2' Irradiated

Figure 21, (-)SAM HSO_4^- H_3' Irradiated

Figure 22, (-)SAM HSO_4^- H_4' Irradiated

Figure 23, (-)SAM HSO_4^- H_5' H_5'' Irradiated

Figure 24, (-)SAM HSO_4^- H_α Irradiated

Figure 25, (-)SAM HSO_4^- H_γ H_γ' Irradiated

Figure 26, (-)SAM HSO_4^- H_β Irradiated

Figure 19. (-)SAM HSO_4^- H_1' Irradiated

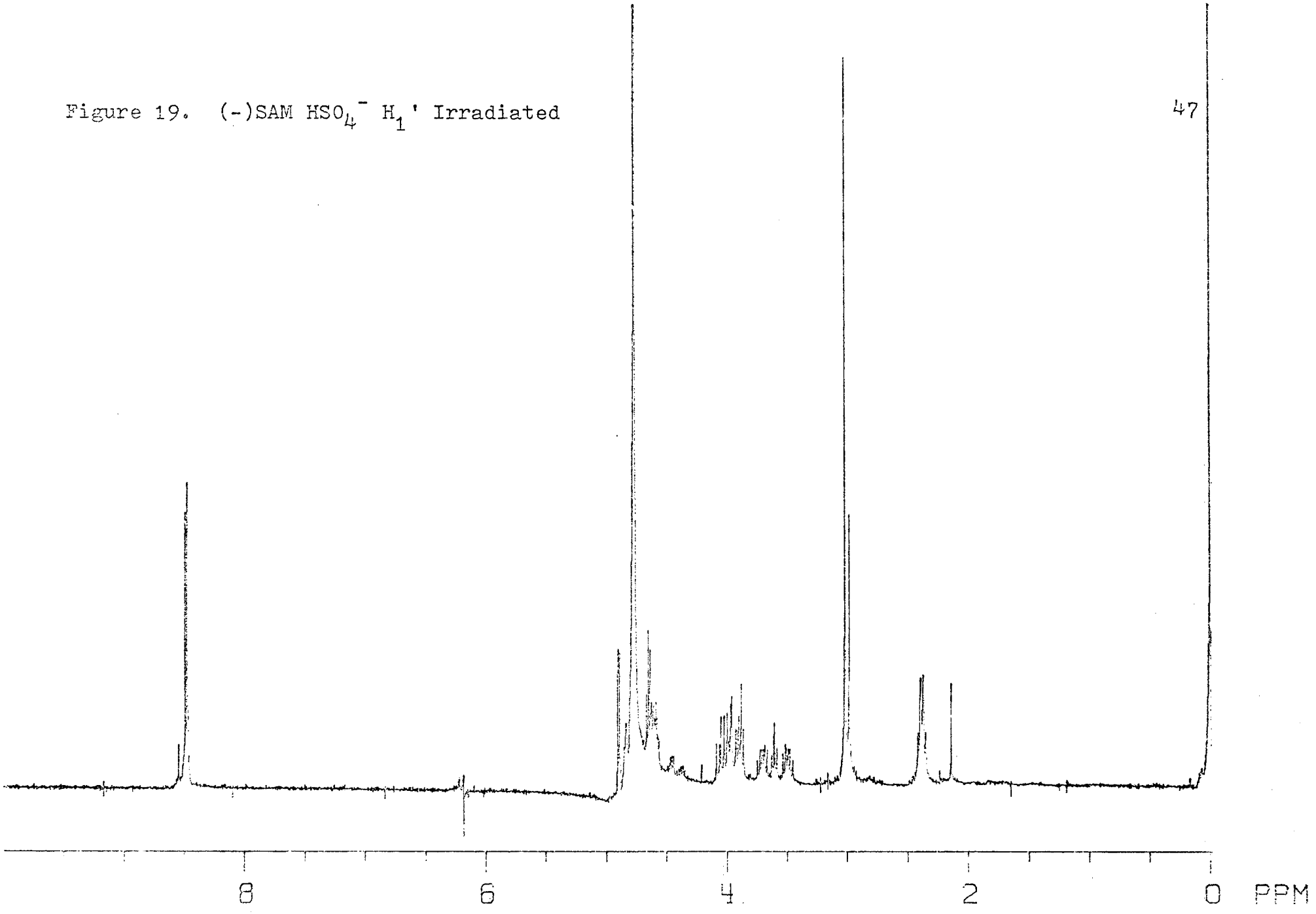


Figure 20. (-)SAM HSO_4^- H_2' Irradiated

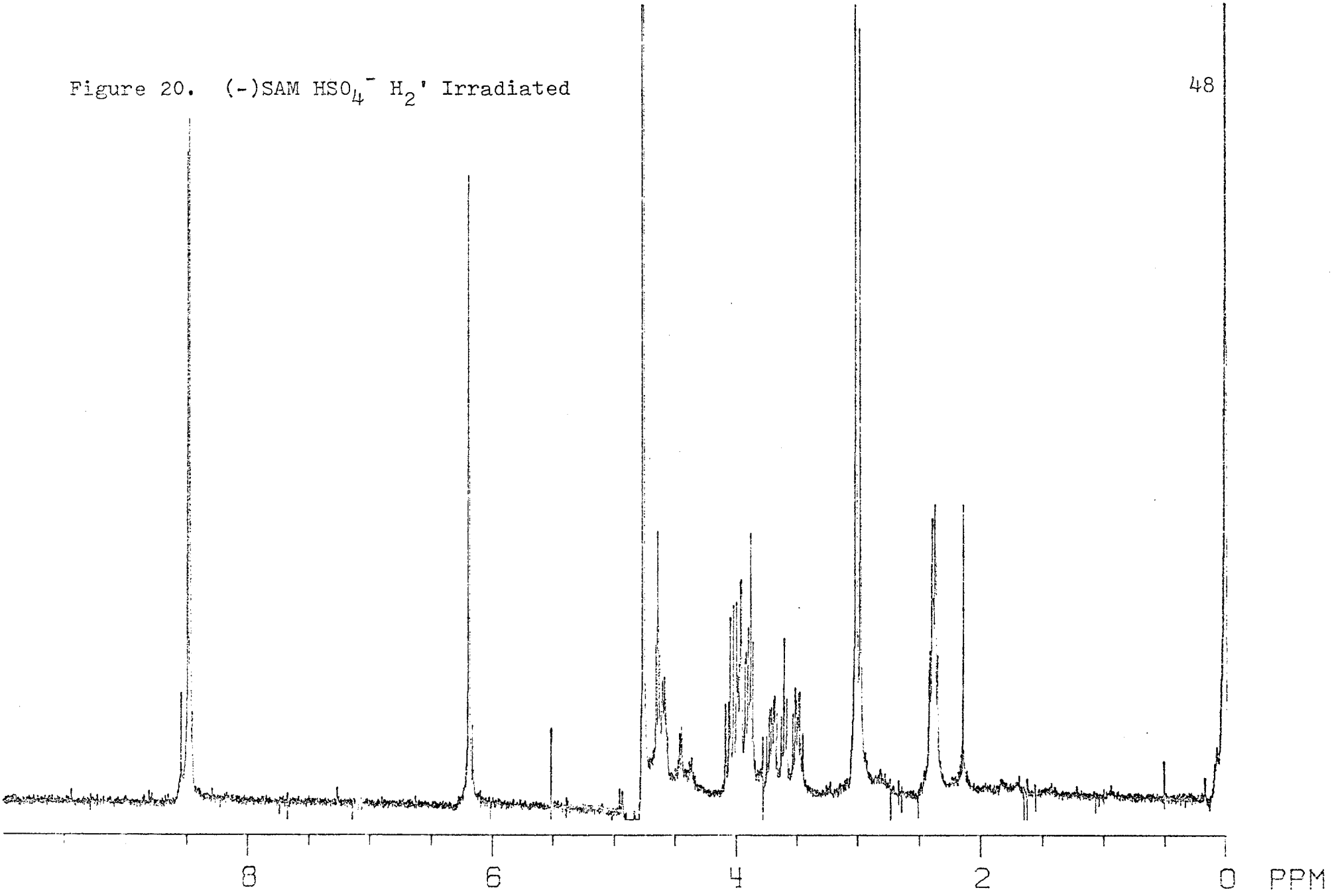


Figure 21. (-)SAM HSO_4^- H_3' Irradiated

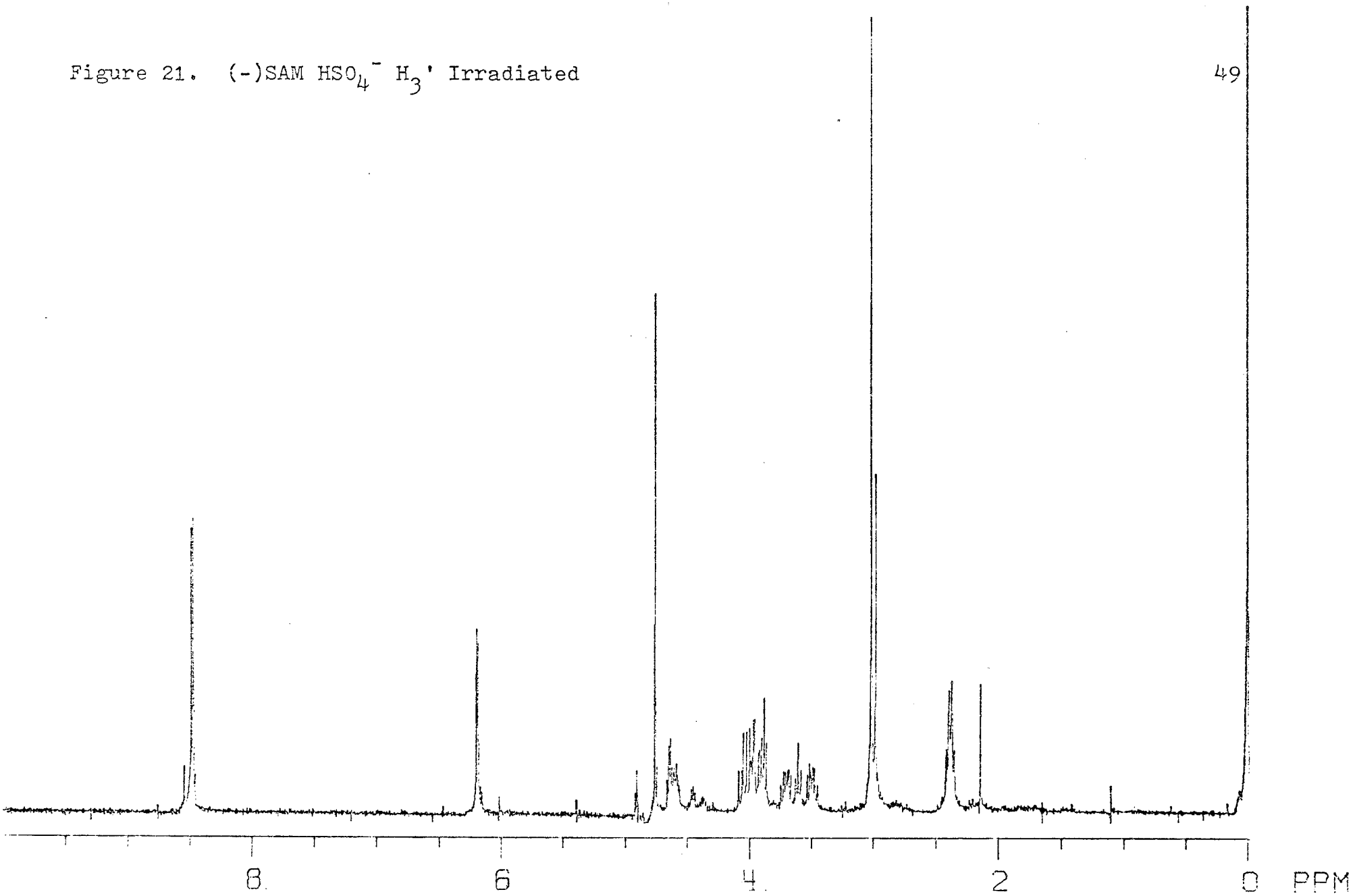


Figure 22. (-)SAM HSO_4^- H_4' Irradiated

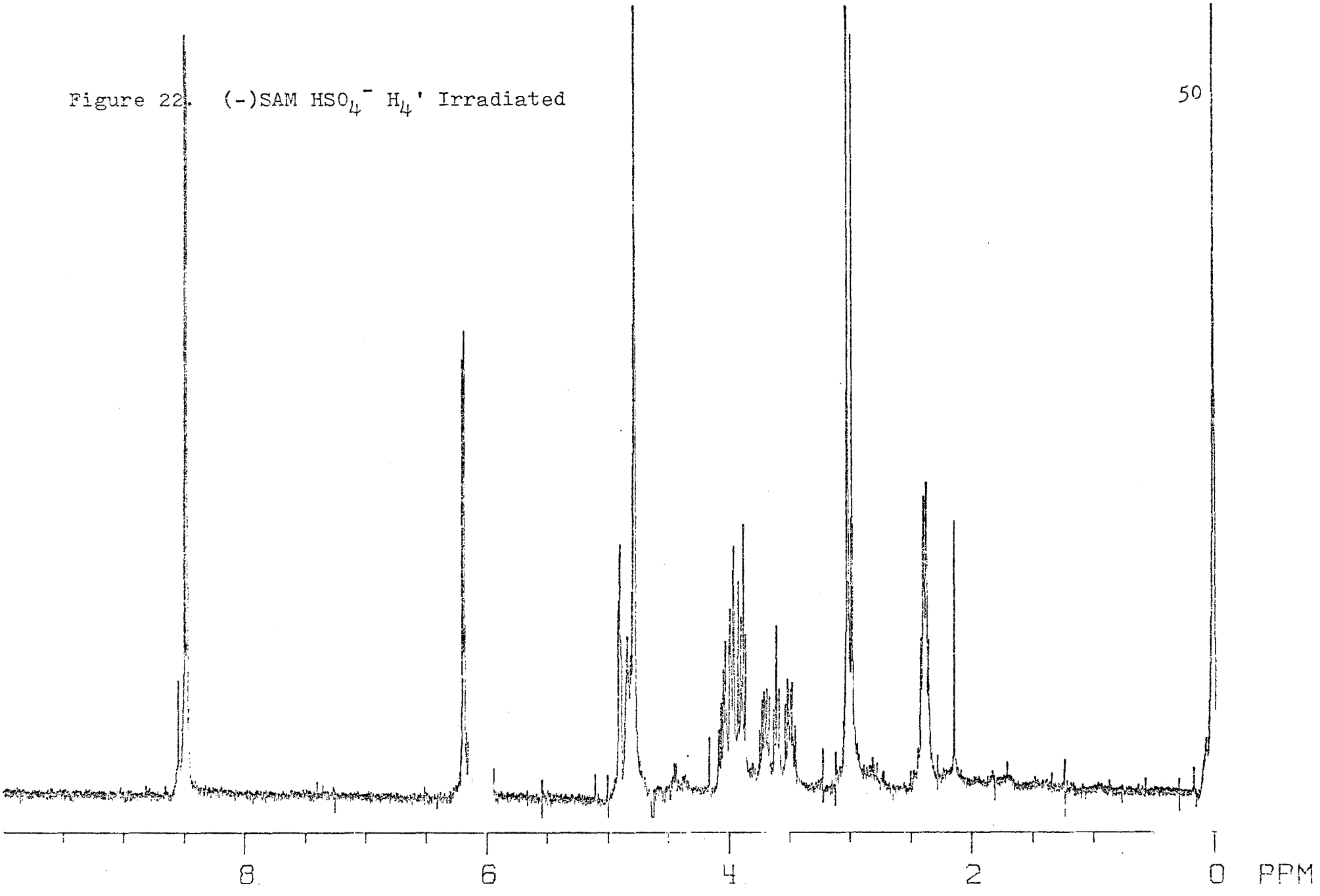


Figure 23. (-)SAM HSO_4^- H_5' H_5'' Irradiated

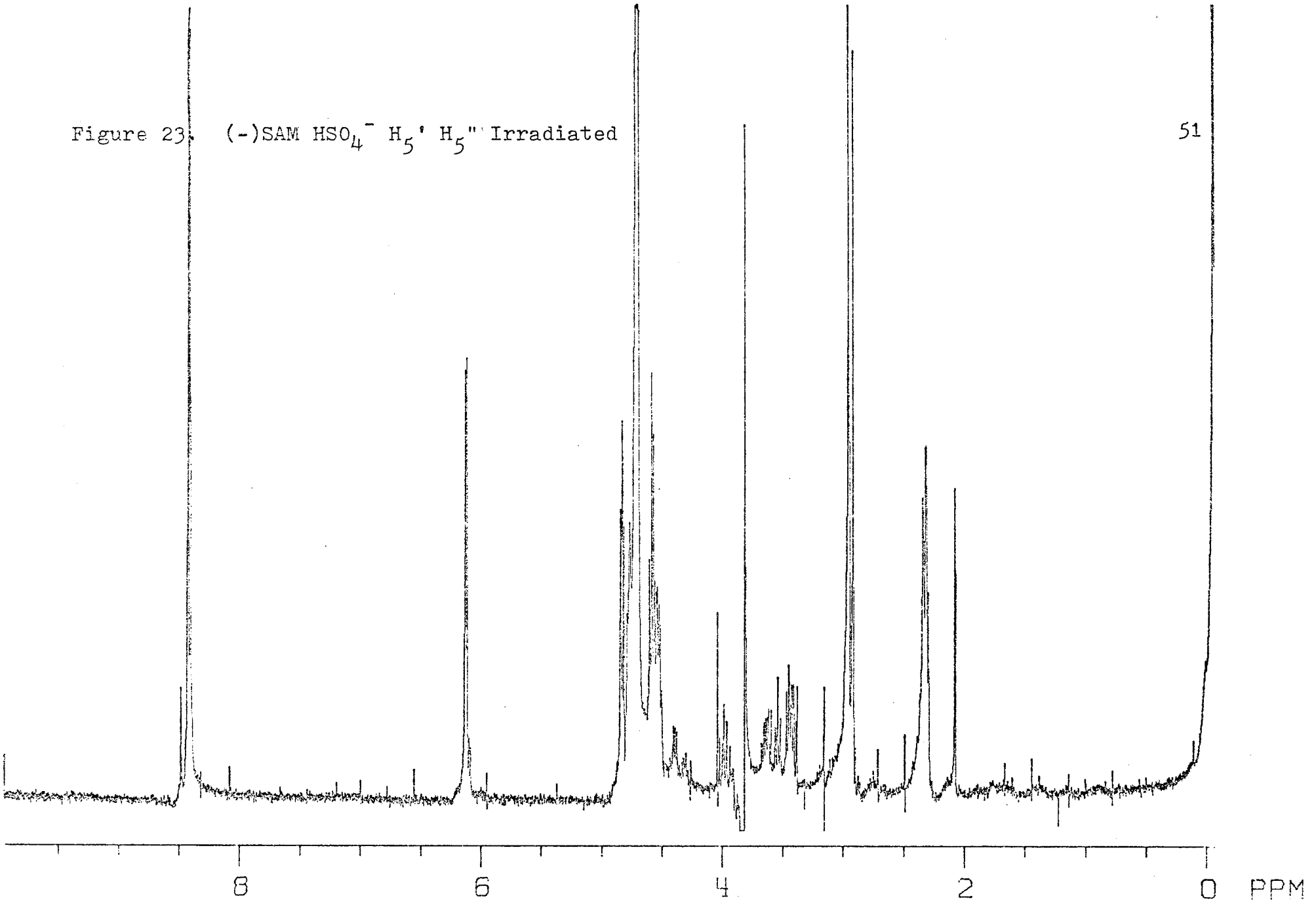


Figure 24 (-)SAM HSO_4^- H_α Irradiated

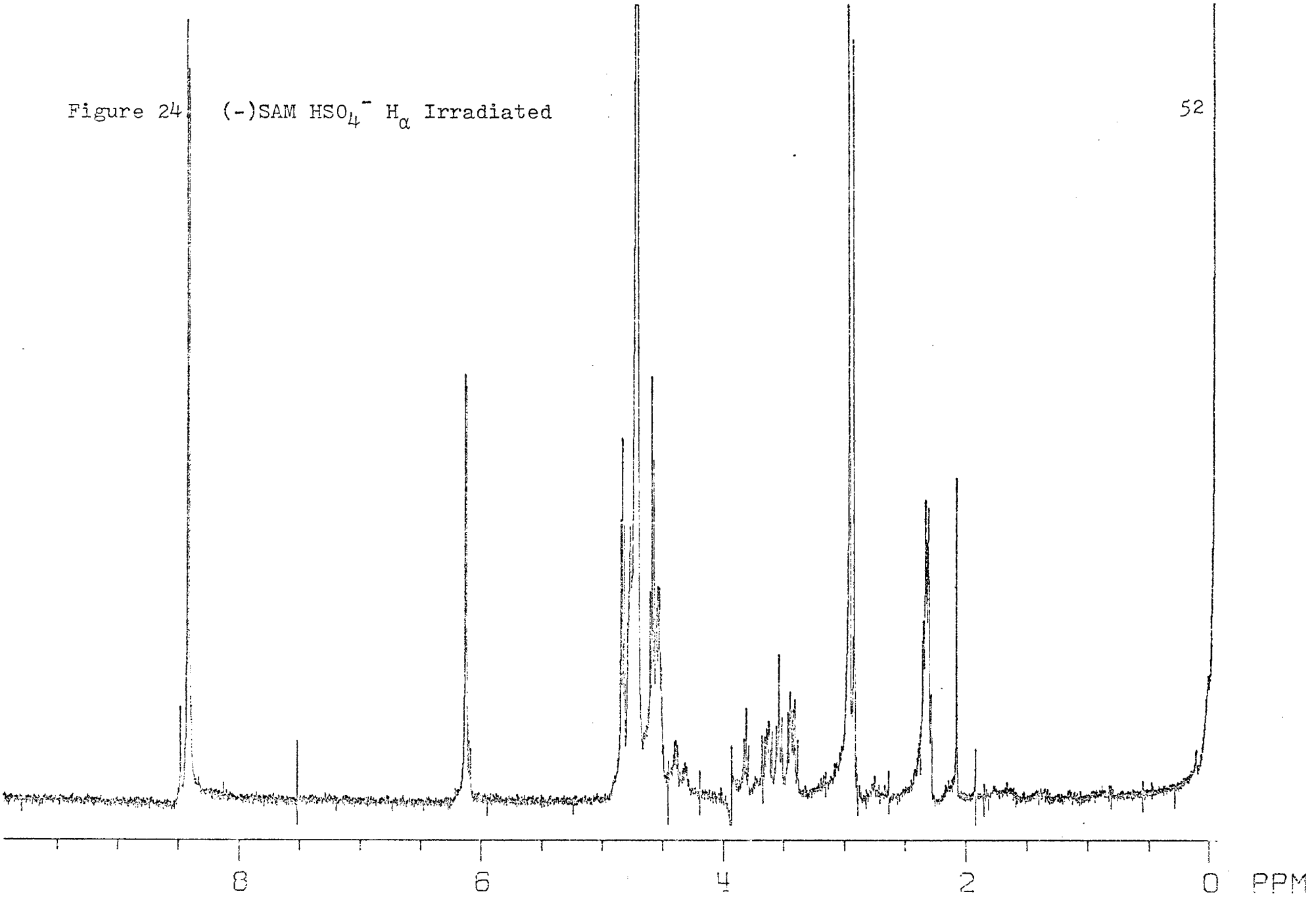


Figure 25. (-)SAM HSO_4^- H_γ H_γ' Irradiated

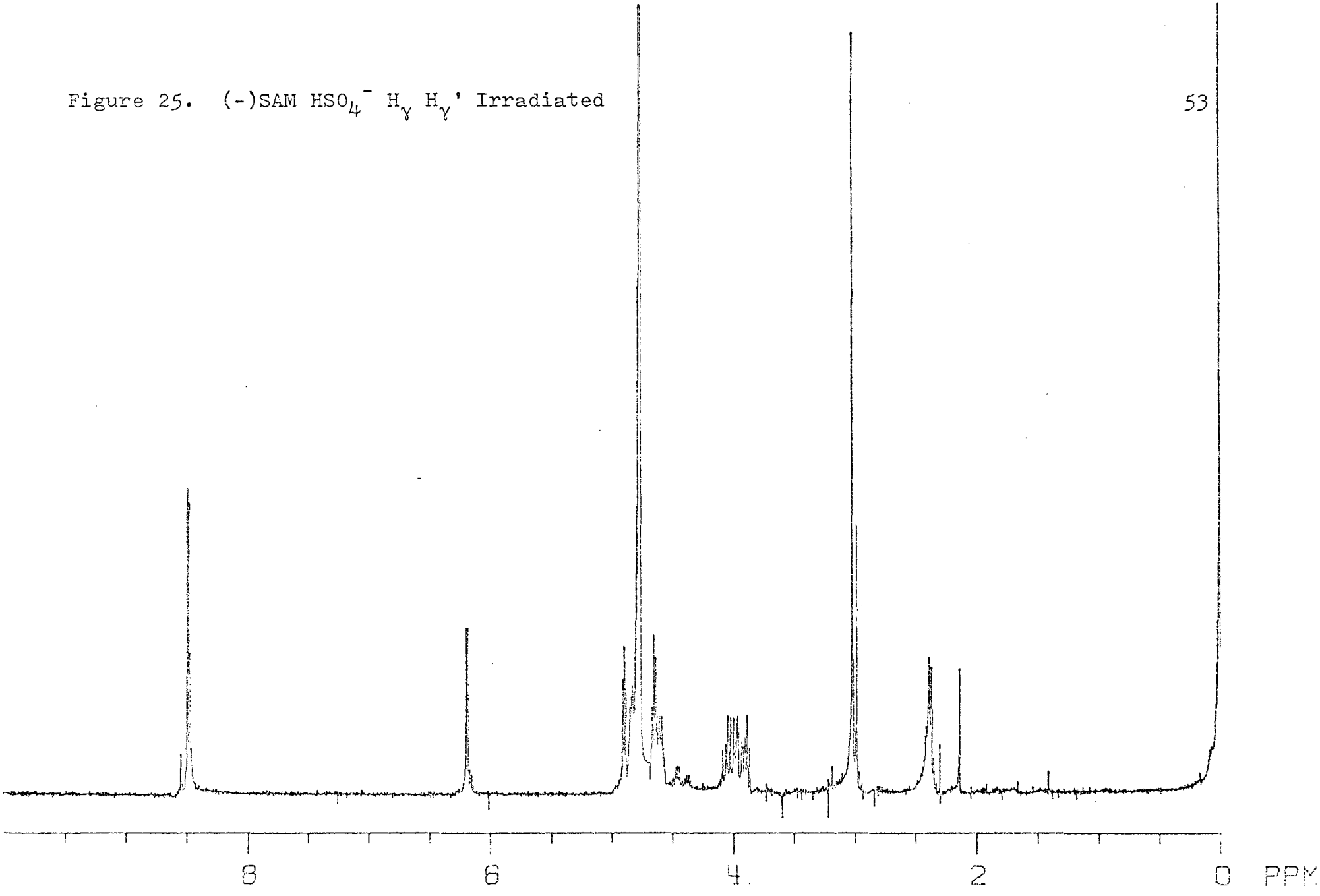
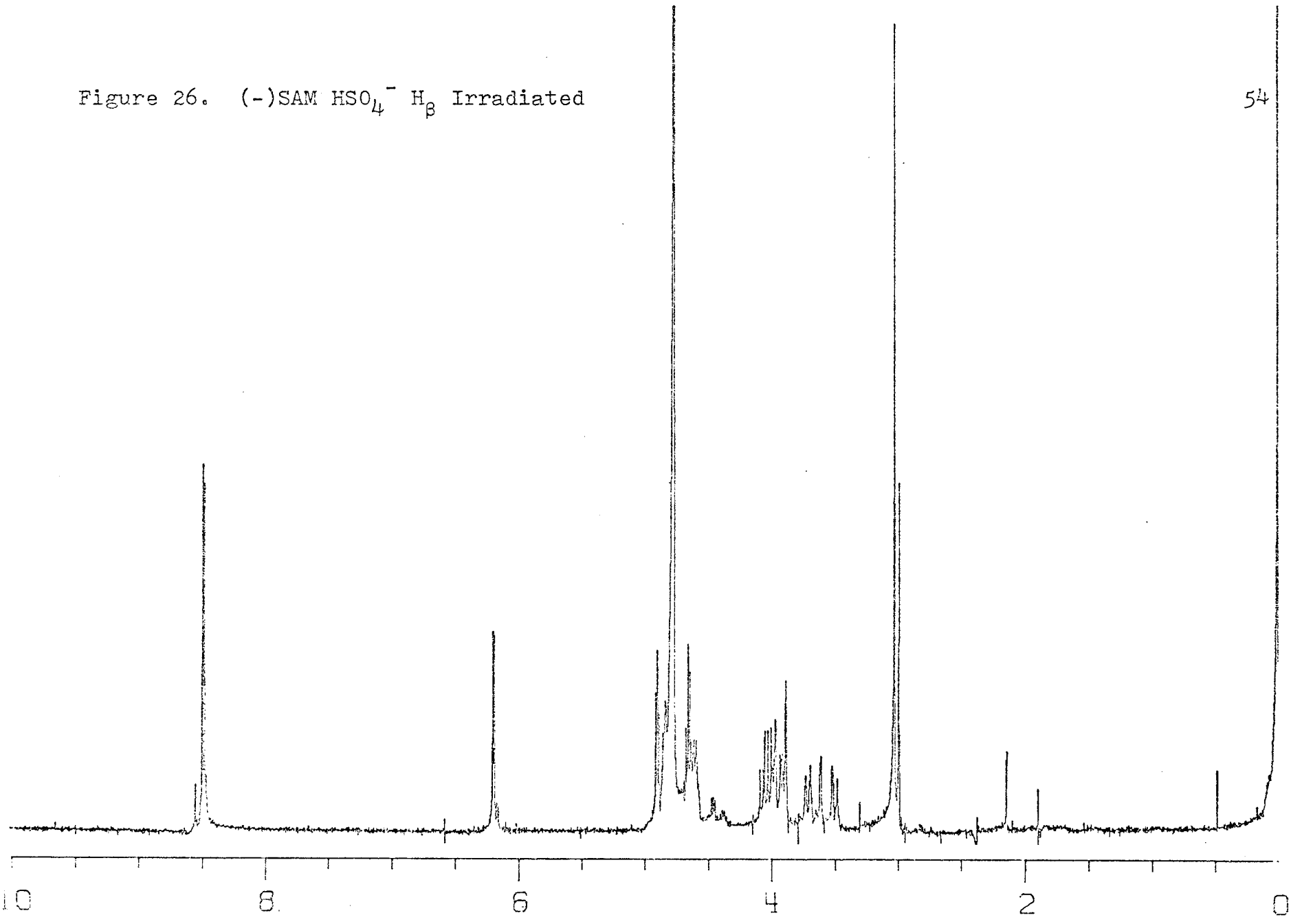


Figure 26. (-)SAM HSO_4^- H_β Irradiated



APPENDIX C

NUCLEAR OVERHAUSER ENHANCEMENTS

The nuclear Overhauser effect (NOE) is the change in the integrated intensity of an NMR absorption which results from the concurrent saturation of another NMR absorption. An increase in an observed integrated intensity is termed a nuclear Overhauser enhancement. A dipolar intramolecular spin-lattice relaxation mechanism, this effect is radially dependent upon the internuclear distance between the enhanced and concurrently saturated nuclei. The magnitudes of Overhauser enhancements may therefore be utilized to infer the relative proximity of nuclei in a given conformation. NOE determinations have been employed extensively in recent years to ascertain both qualitative and quantitative information concerning the sugar-base torsional angle in purine and pyrimidine nucleosides and nucleotides.

The determination of nuclear Overhauser effects is greatly facilitated by elimination of all sources of intermolecular relaxation. The large magnetic moment associated with the HOD molecule results in a prominent dipolar relaxation mechanism making D_2O an inappropriate choice of solvent for NOE investigations. Alternatively, we have elected to determine Overhauser enhancements in dimethyl sulfoxide-D_6.

Values for the observed Overhauser enhancements of the H_8, H_2, H_1', H_2' and H_{methyl} protons resulting from the concurrent saturation of members of the same set are given in the table which follows:

	Observed				
Irradiated	H_8	H_2	H_1'	H_2'	H_{SCH_3}
H_8	-	-	-	-	+1.26
H_2	-	-	-0.26	-	+3.79 +4.36
H_1'	+1.52	+3.29	-	-	-3.37
H_2'	-	+1.18	+0.07	-	-

Any interpretation of the above results must be considered as tenative since the magnitudes of the observed Overhauser enhancements are small as compared to those reported for nucleosides in general (Overhauser enhancements of 10-30% are not uncommon). Additionally, the frequency difference between the purine ring protons in DMSO is insufficient to insure that saturation of either proton does not result in the partial saturation of the neighboring proton. With due consideration of the possible pitfalls we conclude that the above results indicate the possible proximity of the H_1' and H_2 protons which is consistent with the anti orientation of the sugar-base torsional angle. No plausable interpretation for the enhancements of H_{methyl} is apparent.