sible, however, that the equilibrium constant for thermal cleavage may be insignificantly small at 0°. This would imply that the equilibrium constant is salt-dependent and that the number of essential H-bonds (N) may not be. Thus the protective effect of salt might be exerted on the surrounding water, which is involved in the thermal equilibrium, possibly by disrupting its semi-crystalline structure in the vicinity of the DNA molecule.[37]

Denaturation is, by definition, an irreversible process. Thus, as has been shown here (Table II) and elsewhere,[5,31] the molecular collapse and the pK'_a shifts which accompany it are permanent. Whenever this appears not to be so, one must suspect that a variable such as temperature has not been controlled, and that denaturation did not actually occur. The optical density rise, however, may appear under some conditions to be reversible, since the extinction coefficient at 260 mμ, which is constant before denaturation, afterward becomes salt, *p*H and temperature dependent. Therefore optical density cannot validly be used as an indicator for denaturation unless the measurement is carried out at the temperature or *p*H of the experiment. Cooling to room temperature, or neutralizing, will not reverse the collapse of the molecule, but, as Lawley[38] has shown, it will decrease the optical density rise.

Denaturation and degradation appear to be separate phenomena, although caused in many cases by the same agent. For example, the formation of apurinic acid at low *p*H does not proceed to any significant extent[39] under the conditions and durations of the experiments reported here, although denaturation does. A number of investigators[40] have reported a decrease in molecular weight, usually followed by aggregation[40d] or thixotropic[40a–c] behavior, after heat treatment of DNA. The conditions requisite for this effect need not be the same as those which bring about denaturation. There are indications that, at least in lower salt concentrations, denaturation occurs under milder acid and heating conditions than degradation.[40d] Our observations suggest that denaturation is a rapid reaction, while degradation by either acid or heat is undoubtedly slower.

Acknowledgment.—The authors wish to thank Dr. George B. Brown for continued interest and helpful discussions.

---

[37] B. Jacobson, W. A. Anderson and J. T. Arnold, *Nature*, **173**, 772 (1954).

[38] P. D. Lawley, *Biochim. Biophys. Acta*, **21**, 481 (1956).

[39] C. Tamm, M. E. Hodes and E. Chargaff, *J. Biol. Chem.*, **195**, 49 (1952).

[40] (a) G. Goldstein and K. G. Stern, *J. Polymer Sci.*, **5**, 687 (1950); (b) C. A. Dekker and H. K. Schachman, *Proc. Natl. Acad. Sci.*, **40**, 894 (1954); (c) C. Sadron, "Proc. 3rd International Congress Biochim.," Brussels, 1955, p. 125; (d) M. Rosoff, D. Baturay and L. F. Cavalieri, unpublished data.

NEW YORK 21, N. Y.

[CONTRIBUTION FROM THE CHEMICAL LABORATORY, FACULTY OF SCIENCE, TOKYO UNIVERSITY]

Near Infrared Spectra of Compounds with Two Peptide Bonds and the Configuration of a Polypeptide Chain. VI. Further Evidence of the Internal Hydrogen Bonding and an Estimation of its Energy

BY SAN-ICHIRO MIZUSHIMA, TAKEHIKO SHIMANOUCHI, MASAMICHI TSUBOI AND TETSUTARO ARAKAWA

RECEIVED MAY 25, 1957

Near infrared spectra of acetylsarcosine N-methylamide and acetyl-N-methyl-DL-norleucine N-methylamide have been measured in carbon tetrachloride and chloroform solutions. Even at high dilutions both the free and bonded NH vibrations have been observed and have been explained as arising from the extended and folded molecular forms, respectively. The dependence of the intensity of the two NH bands upon temperature has been determined and the energy difference between the extended and folded forms have been calculated.

Introduction

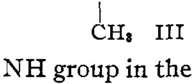
In this series of researches we have studied the structure of molecules of the type

$CH_3CONHCHRCONHR'$ I

which can be considered as a structure unit of a polypeptide chain.[1] From the measurement of the NH absorption bands we have concluded that such molecules exist in both the extended and folded forms shown in Fig. 1. They show the free NH absorption at about 3460 cm.$^{-1}$ and the bonded NH absorption at about 3360 cm.$^{-1}$ in dilute carbon tetrachloride solution and in chloroform solution. In contrast to the band due to the *intermolecular* hydrogen bond, the 3360 cm.$^{-1}$ band shows no dependence on concentration in a dilute solution which shows the band arises from the *intramolecular* hydrogen bond[2] of the folded form of Fig. 1A. This is compatible with the result of our measurement on acetylamino acid ester

$CH_3CONHCHRCOOR''$ II

for which we found no absorption due to the intramolecular hydrogen bond.[3] It would be interesting to see whether we can observe the bonded NH absorption at high dilution for a molecule of the type



III

$$CH_3CO\underset{\displaystyle CH_3}{\underset{|}{N}}CHRCONHR'$$

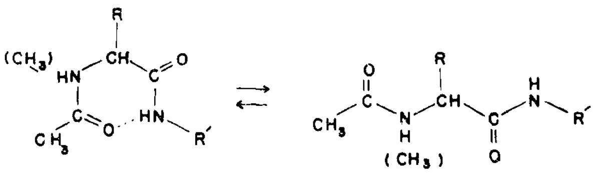
with only one NH group in the molecule, the hydrogen atom of the other NH group being replaced by a methyl group.

---

[1] S. Mizushima, M. Tsuboi, T. Shimanouchi and M. Asai, THIS JOURNAL, **76**, 6003 (1954).

[2] S. Mizushima, T. Shimanouchi, M. Tsuboi, T. Sugita, K. Kurosaki, N. Mataga and R. Souda, *ibid.*, **74**, 4639 (1952).

[3] S. Mizushima, T. Shimanouchi, M. Tsuboi, K. Kuratani, T. Sugita, N. Mataga and R. Souda, *ibid.*, **75**, 1863 (1953).



A, folded form B, extended form

Fig. 1.—Two forms of the monomer of $CH_3CONHCHR\text{-}CONHR'$.

The existence of the intramolecular hydrogen bond of a molecule of this type can be proved more easily than that of a molecule of type I, since the former forms an *intermolecular* hydrogen bond much weaker than the latter. This forms double hydrogen bond as we have shown previously,[3] whereas a molecule of type III can form only single *intermolecular* hydrogen bond. Consequently in this case we can get rid of associated molecules at not too low concentrations.

Furthermore, the population ratio of the extended and the folded forms of the molecule of type III can be determined more easily than that of the molecule of type I. For this molecule the 3460 cm.$^{-1}$ band arises from the free NH vibration of the folded form as well as from those of the extended form, whereas for the molecule of type III the same band arises merely from a single NH vibration of the extended form. (Actually we could resolve the 3460 cm.$^{-1}$ band of a molecule of type I into three bands with an instrument of higher resolving power. The result will be published elsewhere.)

As examples of molecules of type III we have taken acetyl-N-methyl-DL-norleucine N-methylamide ($R = (CH_2)_3CH_3$, $R' = CH_3$) and acetylsarcosine N-methylamide ($R, = H$, $R' = CH_3$). The result of the measurements will be reported in the present paper. (For the sake of convenience these two molecules are designated as AMnLMA and ASMA, respectively, in the part of Result and Discussion.)

Experimental

Acetyl-N-methyl-DL-norleucine N-Methylamide.—N-Methyl-DL-norleucine ethyl ester hydrochloride was prepared according to the method of Cherbuliez and Plattner[4] from N-methyl-DL-norleucine. The ethyl ester hydrochloride was then mixed with sodium acetate and acetic anhydride and extracted by ether to obtain acetyl-N-methyl-DL-norleucine ethyl ester. This was then dissolved in methanol, cooled by Dry Ice and mixed with methylamine. The mixture was allowed to stand for weeks until the colorless crystal of acetyl-N-methyl-DL-norleucine N-methylamide was obtained; m.p. 40°.

Anal. Calcd. for $C_{10}H_{20}O_2N_2$: C, 59.97; H, 10.07; N, 13.99. Found: C, 60.53; H, 9.45; N, 13.88.

Acetylsarcosine N-Methylamide.—Sarcosine hydrochloride was suspended in absolute ethanol and dry hydrogen chloride gas was passed through the suspension. Thus crystalline sarcosine ethyl ester hydrochloride was obtained. The crystal mixed with sodium acetate and acetic anhydride was heated at 70° to obtain acetylsarcosine ethyl ester. The ester was mixed with methylamine and was allowed to stand for several weeks at room temperature.

Anal. Calcd. for $C_6H_{12}O_2N_2$: N, 19.43. Found: N, 19.55.

The infrared absorption measurements were made with a Perkin-Elmer 112 spectrometer with a LiF prism. The slit width was kept 0.10 mm. throughout this measurement. The samples were placed in cells made of silica glass with an electric heating device. The absorption intensity of a band is expressed as

$$\kappa = \frac{1}{cd}\log_{10}\frac{I_0}{I}$$

where c is the concentration (in mole/l.) of the solution, d, the optical path length (in cm.) in the solution, I, the intensity of the radiation transmitted by the solution, and I_0, the intensity of the radiation transmitted by the solvent of the equal optical path length to that of the solution.

Result and Discussion

(1) Carbon Tetrachloride Solution of AMnLMA.—In Fig. 2 are shown the absorption curves of crystalline AMnLMA and of its carbon tetrachloride solution at 30°. The crystal shows bonded NH absorption bands at 3080 and 3319 cm.$^{-1}$. The corresponding two bands are also observed at 3071 and 3336 cm.$^{-1}$, respectively, in concentrated carbon tetrachloride solution, but as the concentration is lowered, the 3071 cm.$^{-1}$ band soon disappears and the 3336 cm.$^{-1}$ band decreases in intensity and is shifted toward higher frequency region. At the same time the free NH band appears at 3452 cm.$^{-1}$. The result is similar to that we obtained for N-methylacetamide[5] and can be explained as due to the fact that the *intermolecular* hydrogen bond N–H· · · ·O=C is broken on dilution of the solution.

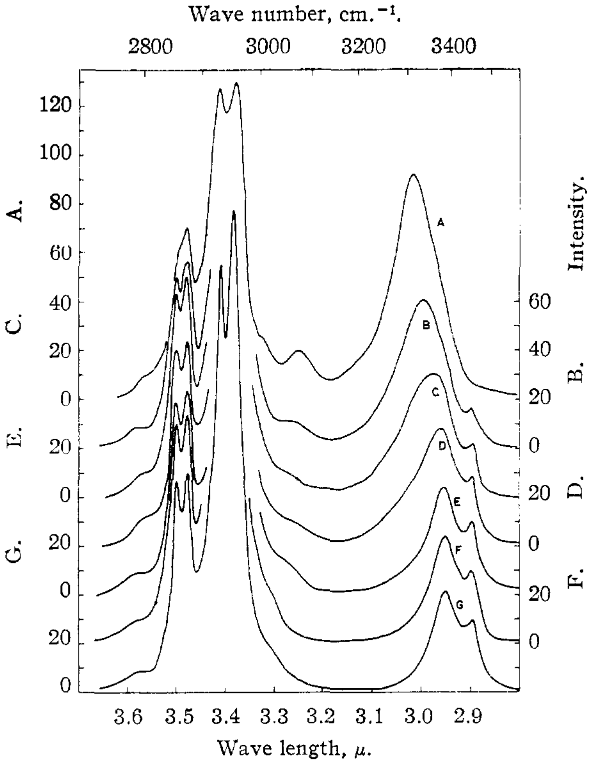


Fig. 2.—Infrared absorption curves of AMnLMA in the crystal (A) and in carbon tetrachloride solutions of 0.100 mole/l. (B); 0.025 (C); 0.0098 (D); 0.0030 (E); 0.00100 (F); and 0.00050 (G) at 30°.

---

[4] E. Cherbuliez and Pl. Plattner, *Helv. Chim. Acta*, **12**, 317 (1929).

[5] S. Mizushima, T. Shimanouchi, S. Nagakura, K. Kuratani, M. Tsuboi, H. Baba and O. Fujioka, This Journal, **72**, 3490 (1950); M. Tsuboi, *Bull. Chem. Soc. Japan*, **22**, 215 (1949).

However, there is an essential difference in the behavior of the bonded NH band at high dilution between N-methylacetamide and AMnLMA. For the latter the bonded NH band does never disappear and at any concentration less than 0.003 mole/l., the relative intensities of the bonded band at 3387 cm.$^{-1}$ and the free band at 3452 cm.$^{-1}$ as well as their frequencies remain constant. (See the last three curves E, F and G of Fig. 2.) This fact shows that in this range of concentration there should be no appreciable amount of associated molecules and, therefore, the bonded band at 3387 cm.$^{-1}$ must be considered to arise from a single molecule with *intramolecular* hydrogen bond (Fig. 1A). Consequently we conclude that there is an equilibrium between the folded and extended molecules in carbon tetrachloride solutions as we have seen in many other acetylamino acid N-methylamide.[1–8]

Measurements similar to those stated above also have been made at 45 and 60°. Frequencies and intensities of the free and the bonded NH bands are shown in Table I. It is seen that the ratio of the intensity of the bonded NH band to that of the free NH band becomes independent of concentration at high dilution and that this stage is reached at higher concentrations as the temperature is raised. Consequently, the relative number of associated molecules becomes smaller at higher temperatures.

TABLE I

POSITIONS AND INTENSITIES OF ABSORPTION MAXIMA OF THE NH BANDS OF ACETYL-N-METHYL-DL-NORLEUCINE N-METHYLAMIDE

	Temp., °C.	Concn., mole/l.	Bonded NH band Cm.$^{-1}$	Bonded NH band Intensity	Free NH band Cm.$^{-1}$	Free NH band Intensity	Int. ratio
In CCl_4	30	0.100	3336	60	3450	16	3.8
		.025	3357	50	3450	22	2.3
		.0098	3374	47	3451	28	1.7
		.0030	3383	44	3452	30	1.5
		.00100	3387	41	3452	30	1.4
		.00050	3387	40	3452	29	1.4
	45	.098	3338	57	3450	16	3.6
		.025	3361	45	3450	23	2.0
		.0096	3382	38	3451	27	1.4
		.0029	3385	38	3452	28	1.4
		.00098	3387	38	3452	29	1.3
		.00049	3387	35	3452	28	1.3
	60	.096	3341	53	3450	17	3.1
		.024	3367	41	3450	25	1.6
		.0094	3383	35	3451	25	1.4
		.0029	3387	35	3452	27	1.3
		.00096	3387	37	3452	28	1.3
		.00048	3387	34	3452	26	1.3
In $CHCl_3$	30	.152	3393	32	3448	46	0.7
		.051	3397	34	3450	54	.6
		.0152	3398	34	3450	54	.6
		.0030	3398	34	3450	60	.6
	45	.149	3393	29	3448	43	.7
		.050	3397	32	3450	51	.6
		.0030	3398	29	3450	52	.6
	60	.146	3393	27	3448	41	.7
		.049	3398	30	3450	48	.6
		.0146	3398	28	3450	48	.6
		.0029	3398	23	3450	45	.5

In order to study the temperature dependence of the NH absorption intensity, we first showed that the CH absorption bands appearing in the region of 2800–3000 cm.$^{-1}$ remain constant in frequency and in absorption intensity at 30, 45 and 60° and, therefore, these can be used as reference bands. By comparing the behavior of the NH bands with that of the CH band at different temperatures, it was shown that the frequencies of the NH bands remain constant but their intensity depends considerably on temperature even at high dilution as shown in Fig. 3.

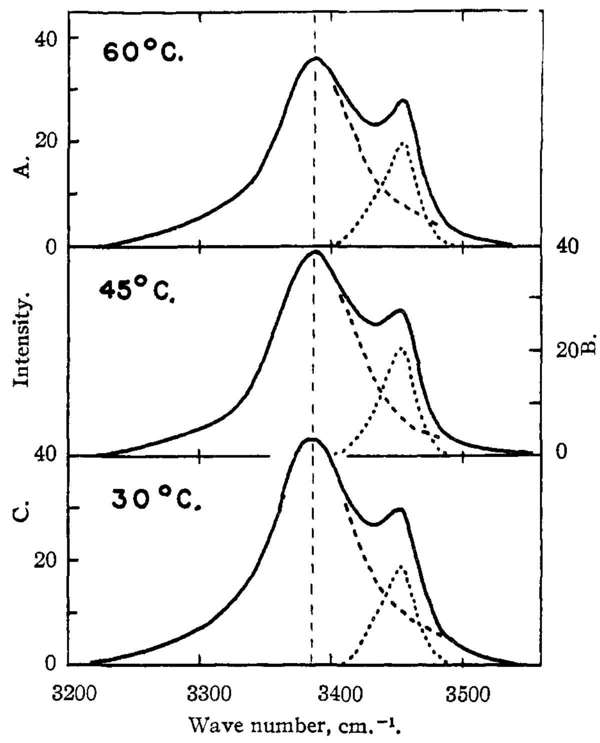


Fig. 3.—Infrared absorption curves of AMnLMA in carbon tetrachloride solutions of 0.00096 mole/l. at 60° (A); 0.00098 mole/l. at 45° (B); 0.00100 mole/l. at 30° (C).

It is to be noted that the ratio of the intensity of the bonded NH absorption to that of the free NH absorption decreases as the temperature is raised. If, therefore, we assume that temperature dependence of the molar absorption coefficient of the two NH bands to be the same,[6] the change of intensity ratio referred to above is due to the change of the ratio of the population of the folded form to that of the extended form with temperature.

(2) **Chloroform Solution of AMnLMA.**—Figure 4 shows the absorption curves of chloroform solutions of AMnLMA at various concentrations observed at 30°. Table I lists the frequencies and intensities of the NH absorption peaks measured at 30, 45 and 60°. It is seen that the chloroform solution of AMnLMA also shows both the bonded and free NH vibrations and the ratio of the intensity of

---

(6) It has been found by our measurements (*e.g.*, those for N-methylacetamide), that the molar absorption coefficient of the CH absorption does not depend on temperature, whereas that of the NH absorption does to some extent. However, we can assume that the temperature dependence of the free and bonded NH absorptions are the same.

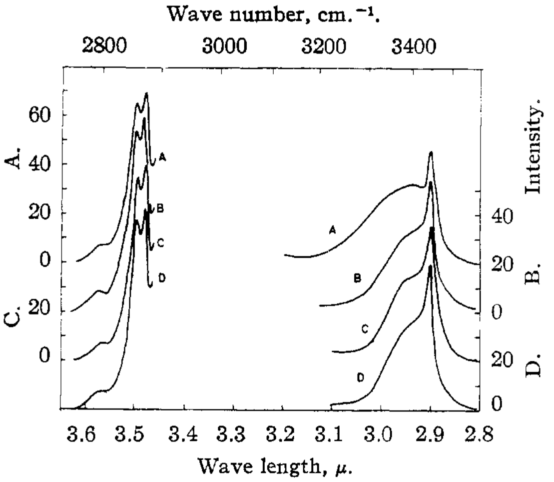


Fig. 4.—Infrared absorption curves of AMnLMA in chloroform solutions of 0.152 mole/l. (A); 0.051 mole/l. (B); 0.0152 (C); 0.0030 (D) at 30°.

the bonded NH band to that of the free NH band becomes constant at higher dilution. Therefore, we can conclude the existence of the folded and extended forms in this case also.

It is to be noted that both the *intramolecular* and *intermolecular* hydrogen bond become weaker in chloroform solution than in carbon tetrachloride solution. In chloroform solution the *intermolecular* hydrogen bond disappears at a concentration as high as 0.01 mole/l. The ratio of the intensity of the bonded NH band to that of the free NH band at high dilution, which corresponds to the ratio of the population of the folded form to that of the extended form, is much smaller than that in carbon tetrachloride solution. Furthermore, the frequency difference between the bonded and free NH bands amounts to 65 cm.$^{-1}$ in carbon tetrachloride solution, whereas it is 52 cm.$^{-1}$ in chloroform solution.

(3) **Carbon Tetrachloride Solution of ASMA.**—Figure 5 shows the absorption curve of the carbon tetrachloride solution of ASMA at about 25° and Table II lists the frequencies and intensities of the NH bands at different concentrations observed at different temperatures. As in the case of AMnLMA the ratio of the intensity of the bonded NH band to that of the free NH band becomes independent of concentration at a concentration lower than 0.003 mole/l. Therefore, we can conclude the existence of the folded form in this case also.

TABLE II

POSITIONS AND INTENSITIES OF ABSORPTION MAXIMA OF THE NH BANDS OF ACETYLSARCOSINE N-METHYLAMIDE IN CARBON TETRACHLORIDE SOLUTIONS

Temp., °C.	Concn., mole/l.	Bonded NH band Cm.$^{-1}$	Bonded NH band Intensity	Free NH band Cm.$^{-1}$	Free NH band Intensity	Int. ratio
∼25	0.094	3338	36	3452	10	3.6
	.0094	3363	28	3455	13	2.2
	.0042	3365	28	3455	15	1.9
	.00094	3367	27	3453	16	1.7
	.00047	3367	26	3453	16	1.6
30	.099	3344	32	3452	8	4.0
	.0099	3365	23	3454	11	2.1
	.00049	3364	22	3454	14	1.6
60	.096	3348	29	3453	13	2.2
	.0096	3369	21	3454	14	1.5
	.00048	3367	27	3454	18	1.5

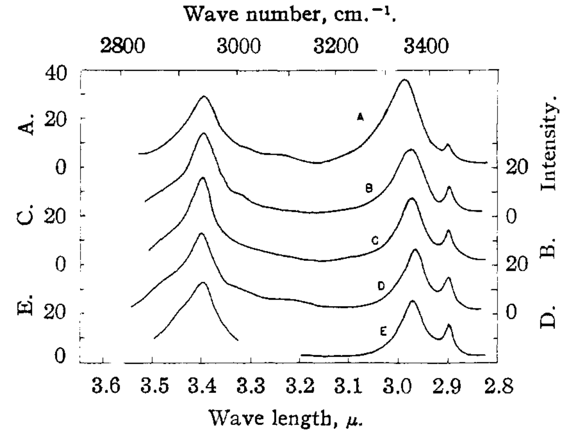


Fig. 5.—Infrared absorption curves of ASMA in carbon tetrachloride solutions of 0.094 mole/l. (A); 0.0094 (B); 0.0042 (C); 0.00094 (D); 0.00047 (E) at about 25°.

However, the frequency difference between the bonded and free NH bands amounts to 86 cm.$^{-1}$ which is much larger than 65 cm.$^{-1}$ of AMnLMA. This shows that the *intramolecular* hydrogen bond of the folded form of ASMA is stronger than that of AMnLMA.

This difference in the strength of the internal hydrogen bond can be explained from the difference in the internal rotation about the N–C axis of the folded form. In AMnLMA the repulsion arising from the interaction between the CH_3 and R (*n*-butyl) groups of part CH_3-N-CH-R (Fig. 1) is so great as to affect considerably the internal rotation about the N–C axis and will considerably reduce the *intramolecular* hydrogen bonding, as in the case of acetylpiperidine-α-carboxylic acid N-methylamide.[1] Such a large repulsive potential is not expected for the folded form of ASMA with the CH_3-N-CH_2 structure. Consequently the folded form of ASMA forms *intramolecular* hydrogen bonds stronger than that of AMnLMA.

Energy Difference between the Folded and Extended Forms of AMnLMA in Carbon Tetrachloride Solution.—As shown above the 3387 and 3452 cm.$^{-1}$ bands of AMnLMA can be assigned, respectively, to the NH stretching vibrations of the folded and extended forms. Assuming the change of ratio of the intensity of the 3387 cm.$^{-1}$ band to that of the 3452 cm.$^{-1}$ band with temperature to be due to the change of the population ratio of these two forms with temperature, we have determined the energy difference between these two forms.

In measuring the intensity of an absorption band we have taken into account the fact that the foot of a peak may overlap that of another. We have assumed that the intensity curve of the 3387 cm.$^{-1}$ band is symmetric to the vertical axis crossing the peak and the foot of the 3452 cm.$^{-1}$ band does not extend to 3387 cm.$^{-1}$ (see Fig. 3).

The half widths of these two bands have been found to be independent of temperature in the range from 30 to 60° at the concentrations 0.001–0.00048 mole/l. Therefore, the integral intensities of the two bands can be considered to be proportional to the maximum intensity and the ratio x of the corrected maximum intensities of the two bands is proportional to the population ratio of the two molecular forms.

Let K be the constant of equilibrium of the two forms and a be a proportional constant independent of temperature and concentration. We have then

$$K = ax$$

The energy difference ΔE of the two forms can be calculated as

$$\begin{aligned}\Delta E &= -RT_i \ln ax_i + T_i\Delta S \\ &= -RT_i \ln x_i + T_i p\end{aligned}$$

where x_i is the intensity ratio observed at the absolute temperature T_i, R the gas constant, ΔS the entropy difference between the two forms and $p = \Delta S - R \ln a$.

Using the experimental data shown in Fig. 6 and Table III (concentrations: 0.001 and 0.0005 mole/l., temperatures: 30, 45 and 60°), the energy difference has been calculated by the method of least squares as

$$\Delta E = -1.7 \pm 0.2 \text{ kcal./mole}$$

TABLE III

DATA USED FOR THE ESTIMATION OF THE ENERGY DIFFERENCE OF THE FOLDED AND THE EXTENDED FORMS OF AMn-LMA IN CCl_4 SOLUTION

Concn., mole/l.	Temp., °C.	Intensity of the 3387 cm.$^{-1}$ band	Intensity of the 3452 cm.$^{-1}$ band (cor.)	Intensity ratio, x (cor.)
0.00100	30	43.1	18.8	2.29
.00098	45	38.9	20.5	1.90
.00096	60	35.9	20.0	1.80
.00050	30	39.2	18.0	2.18
.00049	45	36.3	19.9	1.82
.00048	60	34.3	20.6	1.67

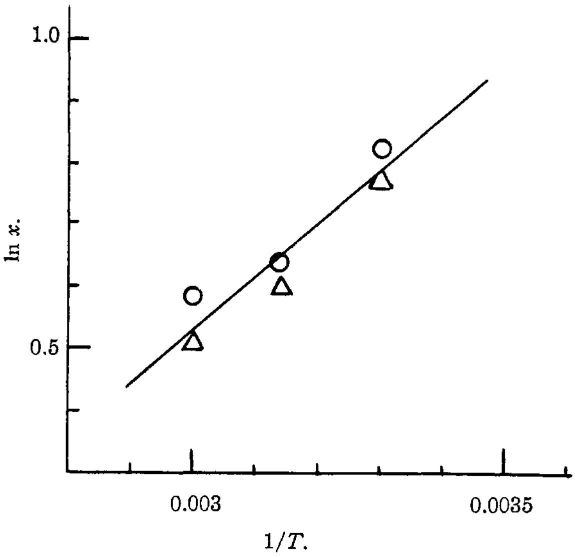


Fig. 6.—Ln x observed at different temperatures: ○, observed at about 0.001 mole/l.; △, observed at about 0.0005 mole/l.; —, determined by the method of least squares.

This difference arises from the energy of the *intramolecular* hydrogen bond of the folded form and also from the energy due to the difference of internal rotation state about the N–C and C–C single bonds.

TOKYO, JAPAN

[CONTRIBUTION FROM THE MALLINCKRODT LABORATORY OF HARVARD UNIVERSITY]

The Nuclear Magnetic Resonance Absorption of Hydrogen in Methyl Groups. The Electronegativity of Substituents

BY A. L. ALLRED[1] AND E. G. ROCHOW

RECEIVED JUNE 3, 1957

The chemical shifts of the hydrogen atoms in the methyl groups of twenty-five compounds, both in the pure state and in infinitely dilute solutions in carbon tetrachloride, are reported herein. Also, chemical shifts are given for methyl chloride, methyl bromide and methyl iodide at varying concentrations in benzene, 2,2-dimethylpropane and carbon disulfide as solvents. Differences in the chemical shifts of these methyl halides (relative to water) in carbon tetrachloride, 2,2-dimethylpropane and carbon disulfide are shown to result largely from the change in bulk magnetic susceptibility of the solvent. The chemical shifts of hydrogen atoms obtained *at infinite dilution* for the methyl halides are found to be linearly related to the electronegativities of the halogens, and a qualitative order of electronegativities of the substituents in 21 other methyl compounds has been obtained by a reverse of this procedure.

Introduction

The basic equation for nuclear magnetic resonance is $\omega = \gamma H$, where ω is the resonance (or Larmor) frequency, γ is the gyromagnetic ratio, and H is the strength of the magnetic field at the nucleus in question. In a given external magnetic field, each nuclear species with a spin quantum number equal to one-half has a single characteristic resonance frequency which is the result not only of the imposed external field but also of perturbations caused by the magnetic shielding due to orbital (bonding) electrons and by the net bulk magnetic susceptibility of the sample. The change in resonant frequency due to changes in orbital electron environment, called the "chemical shift," often is difficult to separate from the change due to altered bulk susceptibility, yet the distinction must be made if conclusions about the polarity of the chemical bond are to be made. This paper describes an effort to separate the effects and, after demonstrating the validity of the method as applied to the

---

[1] Natvar Corporation Fellow at Harvard University 1954–1956.