[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. IV.

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Near infrared spectra of acetylglycine-N-cyclohexylamide, acetyl-L-valine-N-methylamide and acetylnorleucine-N-methylamide have been measured in chloroform and carbon tetrachloride. The spectra of some other acetylaminoacid-N-methylamides previously measured in carbon tetrachloride have also been measured in chloroform. These measurements show that the molecular association of these compounds and the equilibrium ratio of different molecular configurations depend on the solvent. This will be related to the configurational change of a polypeptide chain with environment.

In this series of researches we have studied the near infrared spectra of various acetylaminoacid-N-methylamides in carbon tetrachloride solutions and have discussed the molecular configurations in relation to the configuration of a polypeptide chain.[1-4] Recently we have added three new compounds, acetylglycine-N-cyclohexylamide $CH_3CONHCH_2CONHC_6H_{11}$, acetyl-L-valine-N-methylamide $CH_3CONHCHCONHCH_3$ and acetylnorleucine-N-methylamide $CH_3CONHCHCONHCH_3$ to this series and have measured the spectra in chloroform as well as in carbon tetrachloride. The spectra of some other acetylaminoacid-N-methylamides previously measured in carbon tetrachloride have also been measured in chloroform. The results are important in the understanding of the change of the polypeptide-chain configuration with environment.

Experimental

Acetylglycine-N-cyclohexylamide.—A mixture of acetylglycine ethyl ester and cyclohexylamine was heated on a water-bath for four hours. The product was cooled and was shaken with ether to remove the excess of cyclohexylamine. The sample used for the measurement was recrystallized from the mixture of ethanol and ethyl acetate; m.p. 178.5°.

Anal. Calcd. for $C_{10}H_{18}O_2N_2$: C, 60.58; H, 9.15; N, 14.13. Found: C, 60.65; H, 9.03; N, 13.95.

Acetylnorleucine-N-methylamide.—An excess of methylamine was added to the cooled methanol solution of acetylnorleucine ethyl ester. The liquid allowed to stand overnight was found to be solidified. After the solid mass was kept at room temperature for two weeks, the excess of methylamine and the solvent were removed and the sample was recrystallized from the mixture of ethanol and ethyl acetate; m.p. 170°.

Anal. Calcd. for $C_9H_{18}O_2N_2$: C, 58.03; H, 9.74; N, 15.04. Found: C, 58.23; H, 9.25; N, 14.96.

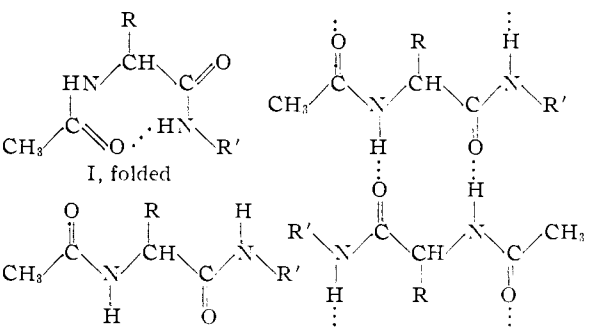
Acetyl-L-valine-N-methylamide.—L-Valine was isolated from the mixture of amino acid esters prepared from casein by the Fischer method.[5] To the solution of L-valine dissolved in an excess of 2 *N* NaOH kept at temperatures lower than 5°, acetic anhydride was added to obtain acetyl-L-valine without racemization. By the action of diazomethane on this substance in ether solution the methyl ester was prepared. Finally by adding methylamine to the cooled methanol solution of acetyl-L-valine methyl ester the N-methylamide was obtained and was recrystallized from ethyl acetate; m.p. above 250°, sublimes at 200°; $[\alpha]^{13.6}D$ −40.5° (in H_2O, *c* 1.66).

Anal. Calcd. for $C_8H_{16}O_2N_2$: C, 55.86; H, 9.37; N, 16.28. Found: C, 56.27; H, 9.50; N, 16.30.

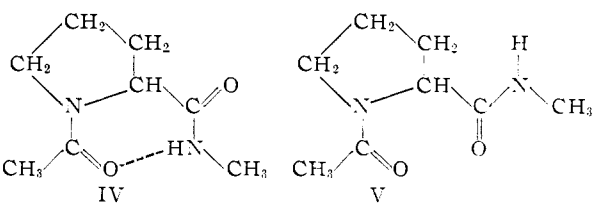
The absorption measurements were made using the same reflection monochromator used previously[1] except for the use of the LiF prism in place of the CaF_2 and quartz prisms.

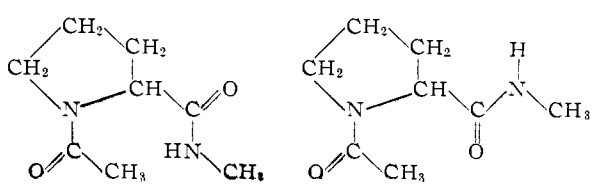
Results and Discussions

I. Carbon Tetrachloride Solutions.—Our previous investigations[1-4] have shown that acetylaminoacid-N-methylamides of the type $CH_3CONHCHR CONHR'$ exhibit two NH bands at 2.90 μ and at 3.00 μ in dilute carbon tetrachloride solutions. The fact that the molar absorption coefficients of these two bands are almost independent of concentration at high dilutions (concentration lower than 10^{-4} mole/l.) has led to the assignment of the 3.00 μ band to the vibration of the hydrogen-bonded NH group of configuration I and the 2.90 μ



Configurations of $CH_3CONHCHRCONHR'$





(1) S. Mizushima, T. Shimanouchi, M. Tsuboi, T. Sugita, E. Kato and E. Kondo, THIS JOURNAL, **73**, 1330 (1951).

(2) S. Mizushima, T. Shimanouchi, M. Tsuboi and R. Souda, *ibid.*, **74**, 270 (1952).

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

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

(5) E. Fischer, *Z. physiol. Chem.*, **33**, 151 (1901).

band to the vibration of the free NH group in both configurations I and II.[1-3] The absorption band due to the *intermolecular* hydrogen bond appears at almost the same wave length as that of the *intramolecular* hydrogen bond of configuration I and, therefore, the 3.00 μ absorption becomes stronger in intensity than the 2.90 μ band, as the concentration is raised. The associated molecule has configuration shown in III.[3,4] In the case of acetylproline-N-methylamide only one NH band is observed in the 3 μ region and the molar absorption coefficient remains practically constant in the concentration range 0.0001 to 0.1 mole/l. and in the temperature interval from 30 to 60°.[3] This indicates that almost all the molecules of this substance are in the folded configuration shown in IV.[6]

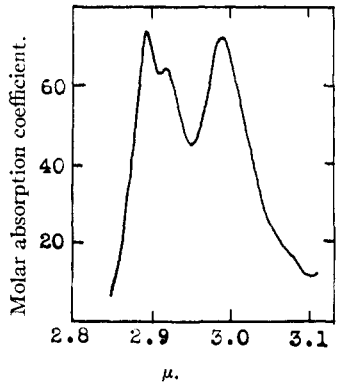


Fig. 1.—Acetylglycine-N-methylamide 0.0002 mole/l. in CCl_4 at 30°.

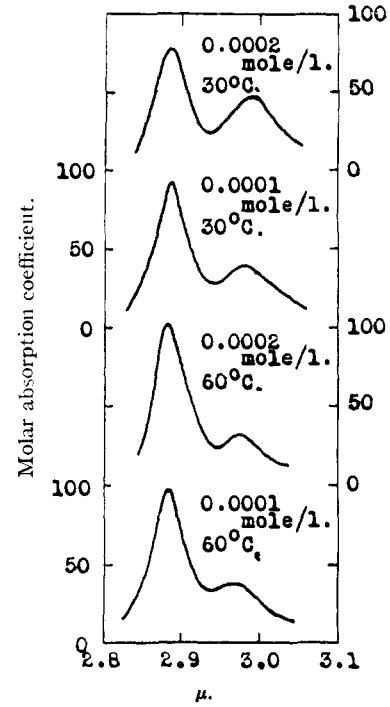


Fig. 2.—Acetylvaline-N-methylamide in CCl_4.

These conclusions have been confirmed by the present experiments. However, with the LiF prism, the 2.90 μ band of acetylglycine-N-methylamide and acetylleucine-N-methylamide (0.0002–0.001 mole/l. in CCl_4) has been resolved into two bands with peaks at 2.89 μ and at 2.92 μ (see Fig. 1).

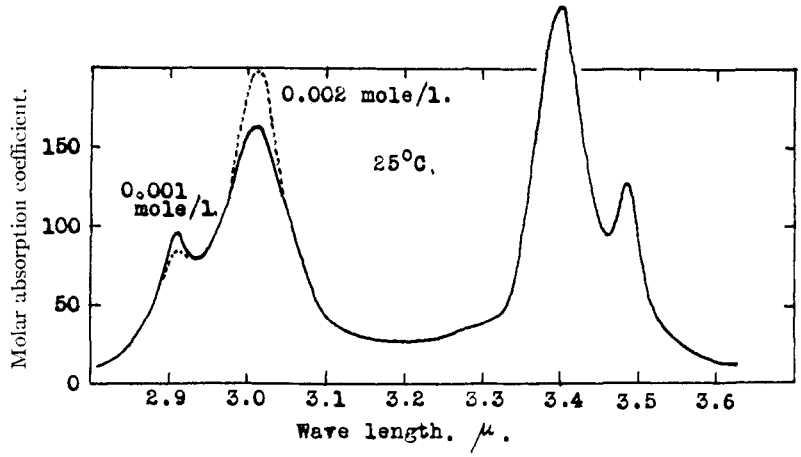


Fig. 3.—Acetylnorleucine-N-methylamide in CCl_4.

Acetylvaline-N-methylamide and acetylnorleucine-N-methylamide in carbon tetrachloride solutions show near infrared absorptions quite similar to acetylleucine-N-methylamide. They show the free NH bands at 2.88–2.92 μ and the hydrogen-bonded NH bands at 2.98 μ. At higher concentrations the bonded NH band is shifted to 3.02 μ (see Figs. 2 and 3).

II. Chloroform Solutions.—Acetylaminoacid-N-methylamides dissolve in chloroform much more readily than in carbon tetrachloride. For example, the solubilities of acetylvaline-N-methylamide and acetylnorleucine-N-methylamide in carbon tetrachloride are, respectively, about 0.0002 and 0.005 mole/l., while the concentrations of the corresponding chloroform solutions can go up as high as 0.02 and 0.3 mole/l. It is interesting that the absorption spectrum in the 3 μ region of a fairly concentrated chloroform solution is quite similar to that of a carbon tetrachloride solution at a high dilution. The chloroform solutions show two absorption peaks, one at 2.88–2.90 μ assigned to the free NH vibration and the other at 2.98–3.02 μ assigned to the bonded NH vibration in H—N ··· O=C (see Figs. 4, 5 and 6).

In the case of the carbon tetrachloride solution of acetylvaline-N-methylamide, a strong and sharp band at 2.88 μ arising from the free NH vibration can be observed at the concentration of 0.0001 mole/l. At the same time a weak and broad band at 2.98 μ arising partly from the *intramolecularly* bonded NH and partly from the *intermolecularly* bonded NH is observed. The intensity ratio of these two NH bands, at 2.88 μ and at 2.98 μ,

(6) Formulas show the projections of the molecular configurations on the plane of the paper. As to the spatial configurations see, for example, S. Mizushima, T. Shimanouchi and M. Tsuboi, *Bull. Chem. Soc. Japan*, **23**, 176 (1950).

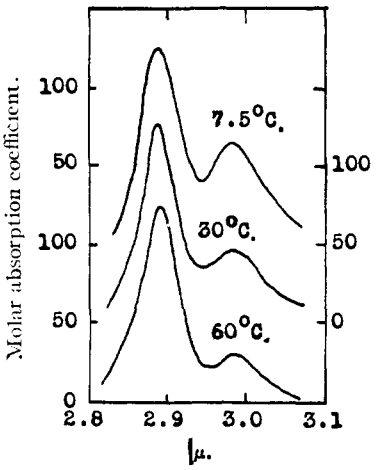


Fig. 4.—Acetylvaline-N-methylamine, 0.01 mole/l. in $CHCl_3$.

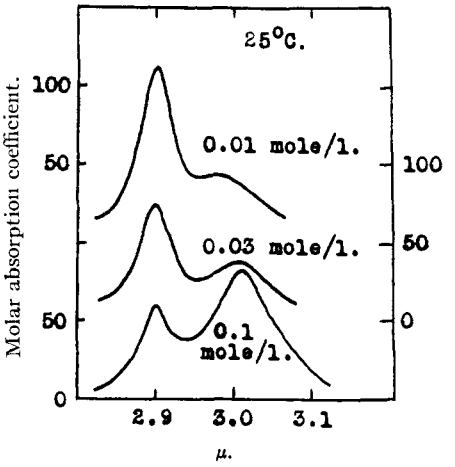


Fig. 5.—Acetylnorleucine-N-methylamide in $CHCl_3$.

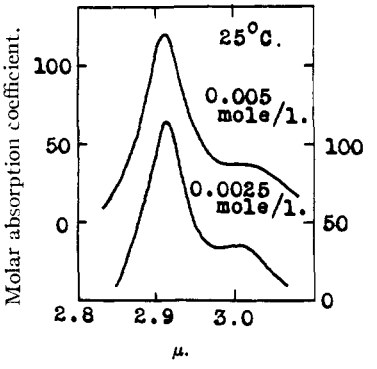


Fig. 6.—Acetylglycine-N-cyclohexylamide in $CHCl_3$.

changes slightly with concentration and temperature as shown in Fig. 2. In the case of the chloroform solution the same behavior can be observed at a concentration of 0.01 mole/l. which is one hundred times as high as that of the corresponding carbon tetrachloride solution (see Fig. 4).

For acetylnorleucine-N-methylamide in carbon tetrachloride the intensity of the associated NH band is twice as strong as that of the free NH band even at such a low concentration as 0.001 mole/l. (Fig. 3), while such a strong absorption due to the associated NH band can only be observed in chloroform solution at a concentration as high as 0.1 mole/l. (Fig. 5).

Figure 6 shows the absorption curves for chloroform solutions of acetylglycine-N-cyclohexylamide at concentrations of 0.005 mole/l. and 0.0025 mole/l. Chloroform solutions of acetylglycine-N-methylamide and acetylnorleucine-N-methylamide of the same concentrations give absorption curves quite similar to those shown in Fig. 6. For all these solutions the intensity ratio of the free and the bonded NH bands is independent of concentration up to 0.005 mole/l., while for the carbon tetrachloride solutions of these substances this is the case only at very high dilutions (concentration lower than 0.0001 mole/l.).

The experimental results described above show that although the configurations of single molecules in chloroform are essentially the same as those in carbon tetrachloride, molecular association in chloroform is much weaker than that in carbon tetrachloride.

In connection with the intensity problem referred to above we should like to note that the absolute values of the absorption intensity of the NH bands in chloroform solutions are greater than those in carbon tetrachloride solutions. This can be explained by the increased polarity of the NH bond in chloroform over that in carbon tetrachloride.

In the 3 μ region acetylproline-N-methylamide shows only one absorption band at 3.00 μ in carbon tetrachloride, but it shows two absorption bands at 2.90 and 3.00 μ in chloroform (see Fig. 7). The

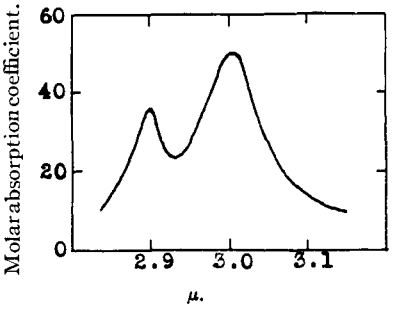


Fig. 7.—Acetylproline-N-methylamide in $CHCl_3$.

intensity ratio of these two bands is about two to three and is independent of temperature from 10° to 40° in the concentration range 0.0009–0.03 mole/l. This result shows that in chloroform acetylproline-N-methylamide exists not only in configuration IV, but also in one or more of the configurations, V, VI and VII. The stability of these three configurations will not be much different from that of configuration IV from the viewpoint of the internal rotation potential, but the *intramolecular* hydrogen bond makes configuration IV much more stable than the other three in carbon tetrachloride, so that only configuration IV is spectroscopically detectable. However, in chloroform the *intramolecular* hydrogen bonding is reduced and the relative number of molecules in the configurations V, VI or VII increases with the result that the free NH band can be observed together with the bonded band. We shall show in a following paper how far we have

to take into account the existence of the folded configurations without internal hydrogen bonds in other acetylaminoacid-N-methylamides.

As shown above the equilibrium ratio of different configurations of acetylaminoacid-N-methylamides depends on the nature of the solvent. This result suggests that the configuration of a polypeptide chain changes with environment. However, it should be realized that this change is essentially due to the change in *intramolecular* and *intermolecular* hydrogen bonds and not due to the change of the stable positions of the internal rotation potential in which the steric repulsion between the rotating groups plays the most important part.[7]

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(7) S. Mizushima, Y. Morino and T. Shimanouchi, *J. Phys. Colloid Chem.*, **56**, 324 (1952).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

Studies of the Enzyme Fumarase. II.[1] Isolation and Physical Properties of Crystalline Enzyme

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A method is described for the isolation and crystallization of fumarase from pig heart muscle which differs from methods which have previously been reported. Based on the muscle extract, a 2600-fold increase in specific activity and a recovery of 20% are obtained. This protein yields a single boundary in the electrophoresis apparatus and in the velocity ultracentrifuge. The ultraviolet absorption is characteristic of a protein, and it has not been possible to detect an enzyme–substrate complex spectrophotometrically.

Fumarase catalyzes the reversible hydration of fumarate to *l*-malate. It has been found in numerous animal and plant tissues, and in 1941, Laki and Laki[2] reported a method for the isolation and crystallization of fumarase from ox heart. The protein obtained, however, was shown in 1948[3] and 1950[4] to be impure, and in 1951,[5] Massey reported that he had crystallized fumarase from pig heart muscle extract with about a tenfold increase in specific activity over the preparation of Laki and Laki. The procedure developed in this Laboratory differs substantially from that of Massey.[6] However, side by side comparisons of the two crystalline enzyme preparations show that kinetic constants for the enzyme prepared by the two different methods are the same within experimental error as is the specific activity, and that the ultraviolet absorption spectra are superimposable. Both preparations yield a single boundary in the electrophoresis apparatus and ultracentrifuge.

Experimental

Assay.—*Fumarase activity* is determined spectrophotometrically[1] using ultraviolet light at 250 mμ, in a solution of 0.05 *M* sodium phosphate buffer at *p*H 7.3 and 25° with an *l*-malate concentration of 0.05 *M*. The number of units of activity for an aliquot of enzyme solution in 3 ml. of the buffer is arbitrarily defined as the initial rate of change in optical density per 10 sec. × 10^3 due to the formation of fumarate.

The *specific activity* is defined as the ratio of the activity for a given aliquot of enzyme solution to the optical density at 250 mμ of the same aliquot in 3 ml. of 0.05 *M* phosphate buffer of *p*H 7.3 and at 25°.

(1) I in this series, R. M. Bock and R. A. Alberty, THIS JOURNAL, **75**, 1921 (1953).

(2) E. Laki and K. Laki, *Enzymologia*, **9**, 139 (1941).

(3) E. M. Scott, *Arch. Biochem.*, **18**, 131 (1948).

(4) E. Racker, *Biochem. Biophys. Acta*, **4**, 211 (1950).

(5) V. Massey, *Nature*, **167**, 769 (1951).

(6) V. Massey, *Biochem. J.*, **51**, 490 (1952).

Preparation.—The procedure given by Massey is similar to that followed originally by Laki and Laki in that the fumarase remains in the supernatant of the initial extract after the *p*H is lowered and calcium phosphate gel is used to adsorb the enzyme in the purification procedure. In the present method, fumarase is precipitated from the initial extract by lowering the *p*H and the temperature, and calcium phosphate gel is not used.

The following procedure has been used in obtaining the results summarized in Table I. Twenty fresh pig hearts are freed of fat and connective tissues and ground in a meat grinder. The meat is washed 6 times with 6–7 liters of cold (5°) distilled water per wash and strained in cheesecloth. The meat is then suspended in 6 l. of 0.01 *M* phosphate buffer of *p*H 7.3 which is warm enough (about 60°) to make

TABLE I

ISOLATION OF CRYSTALLINE FUMARASE

Step			Volume (*V*) in ml.	Activity (*A*) for 0.1 ml. enzyme solution	Total units $AV \times 10^{-5}$	Specific activity	Purity, %
Washes 1–6			45,000	30	13.5	40	
Original extract			5,000	70	5.3	130	0.04
Re-extraction			3,000	65			
*p*H 6.0, S[a]			8,000	57	4.5	190	.06
	P[b]			0			
*p*H 5.3, S			8,000	10	0.8	50	
	P		200	1500	3.0	440	.13
$(NH_4)_2SO_4$ fractionation							
(1)	0–35%	P	125	300	0.37	108	
	35–65%	P	50	3500	1.4	3500	1.0
	65%	S	260	110	0.29	265	
(2)	15–45%	P	20	1000	0.2	800	
	45–60%	P	35	3800	1.3	6100	1.8
	60%	S	20	196	0.04	680	
(3)	50%	P (A)	12	1000	0.12	2000	
	50%	P (B)	15	6000	1.1	50000	15
	50%	S	25	252	0.05	500	
B suspended in 15% satd. $(NH_4)_2SO_4$		S	25	956	0.24	4000	
		P	14	8500	1.1	330000	
Repeated crystallization			10	11000	1.1	336000	100

[a] Supernatant. [b] Precipitate.