model for *p*H effects, permits definition of *p*H independent lower limits for the rate constants in terms of these variables. The Haldane relation, equation 13, is satisfied by both the primed and unprimed variables.

Four "acid dissociation constants" are defined in the above equations, *e.g.*

$$K_{A1} = \frac{\sum\limits_{i=1}^{n}\sum\limits_{s=i}^{n}\frac{K_i{}^sK_{i1}}{k_{(s+1)}}}{\sum\limits_{i=1}^{n}\sum\limits_{s=i}^{n}\frac{K_i{}^s}{k_{(s+1)}}}$$

These quantities are usually readily obtainable from an analysis of the *p*H dependence of the maximum velocities in the forward and reverse direction (see ref. 23–26). However, it is only in the case of a system described in terms of a single intermediate that these parameters can be identified with dissociation constants. When $i = n = 1$, $K_{A1} = K_{B1} = K_{11}$ and $K_{A2} = K_{B2} = K_{12}$. In general these parameters will be complex aggregates of rate constants and dissociation constants.

It is evident from equations 27 that plots of either $V_A/K_A$ or $V_B/K_B$ *vs.* *p*H yield the dissociation constants for the free enzyme, *i.e.*, $K_{01}$ and $K_{02}$ irrespective of the number of intermediates in the system. *Hence for an enzyme which catalyzes the reaction of several substrates the pH dependence of* $V_A/K_A$ *should be the same provided that the substrates all interact with the same groups in the enzyme.* This is very clear from studies by Smith and coworkers of the hydrolysis of a number of synthetic substrates by the enzyme papain. Plots of the quantity $V_A/(E)_0K_A$ *vs.* *p*H yield values of the *p*K's of the two dissociable groups which are identical for both charged and neutral substrates while the *p*H dependence of $V_A/(E)_0$ may be markedly different.[28,29] Smith identifies $V_A/K_A(E)_0$ with the bimolecular rate constant for a one intermediate hydrolytic mechanism.[30] From the preceding discussion this quantity is always a lower limit for such a constant, but studies of its variation with *p*H are of course consonant with any interpretation of its significance. For an enzyme which reacts with many substrates, it is tempting to propose the superimposability of $V_A/K_A$ or $V_B/K_B$ *vs.* *p*H plots for the various substrates as a diagnostic test for the involvement of the same *acidic* or *basic* groups in all its reactions.

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# Infrared Spectra of Acetylglycine N-Methylamide and the Assignment

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The infrared spectra of acetylglycine N-methylamide and its N-deuterated compound have been measured in the crystalline state by using a grating spectrophotometer. Based on the experimental results and on our previous analysis of the normal vibrations of N-methylacetamide, a complete assignment has been made of the fundamental vibrational frequencies of acetylglycine N-methylamide observed in the 1800–400 cm.$^{-1}$ region.

## Introduction

Acetylglycine N-methylamide, $CH_3CONHCH_2$-$CONHCH_3$, can be regarded as a unit of the polypeptide chain containing two peptide bonds. The infrared spectra of this molecule were measured in detail in the 3 μ region in our laboratory.[1–6] The measurement of the infrared spectra in the NaCl region also have been made.[7] From the results we could draw some important conclusions on the molecular structure of the polypeptide chain. Since progress has been made recently in the analysis of the vibrational spectra of the molecules containing peptide bonds,[8–10] further researches on this molecule have been made in our laboratory. An exact measurement of infrared spectra of acetylglycine N-methylamide in solid state were made with a grating spectrophotometer. The variation in the intensity of the observed absorption bands with the direction of the incident beam was measured. Furthermore, the measurement on the N-deuterated molecule was made. Based on the result of the measurements and on our analysis of the normal vibrations of simpler molecules, a complete assignment of the observed frequencies was made. The results are reported in the present paper.

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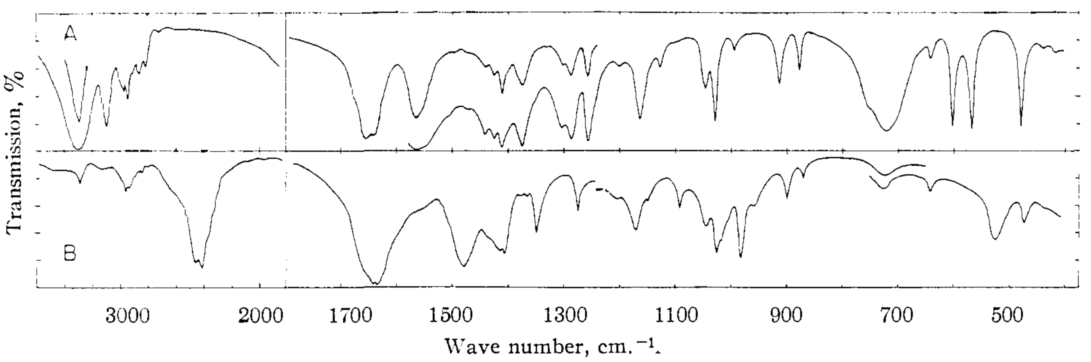


Fig. 1.—Infrared absorption spectra of acetylglycine N-methylamide (A) and its deuterated (ND) product (B).

## Experimental

Acetylglycine N-methylamide (hereafter designated as AGMA) was prepared as previously described.[1] The infrared absorption measurements were made with KBr disks, Nujol mulls, hexachlorobutadiene mulls and a thin single crystal. The crystal was formed by cooling the molten sample placed between two KCl plates.

The N-deuterated sample ($CH_3CONDCH_2CONDCH_3$) was prepared by dissolving the amide in an excess of $D_2O$ in a dry box and pumping off the heavy water. The procedure was repeated twice so that an almost completely deuterated product was obtained. The product was mulled with Nujol or with hexachlorobutadiene in a dry box, to avoid the rehydrogenation due to the atmospheric water vapor.

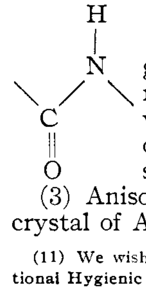
The measurements of infrared absorptions were made with a Perkin-Elmer model 112G grating spectrophotometer with a 75 line/mm. grating (4000-530 cm.$^{-1}$), a Koken DS-301 double-beam triple-pass spectrophotometer with two NaCl prisms (4000–650 cm.$^{-1}$)[11] and a Hilger H-800 spectrophotometer with a KBr prism (800–400 cm.$^{-1}$).

## Results

The spectra of AGMA and its N-deuterated compound are given in Fig. 1. In Table I are listed the observed frequencies. The assignments given in Table I will be discussed below. In Fig. 2 are shown the absorptions by the single crystal and those by the powder in Nujol and hexachlorobutadiene mulls of AGMA.

**Bases of Assignments.**—The assignments given in Table I are based on these three points

(1) The positions of the absorption bands of AGMA and their shift upon deuteration (Fig. 1).

(2) The results of the analyses of the normal vibrations of the *trans* peptide. In the molecule of AGMA the C=O and N–H bonds are considered to be in the *trans* position to each other with respect to the C–N axis.[4] Also, it may be considered that in a solid state AGMA takes an extended form (shown in Fig. 3) and a chain-polymer or network of the associated molecules is formed by the N–H . . . O=C hydrogen bonds between molecules.[4] A detailed analysis of the characteristic frequencies of  group in such a state has already been made in this Laboratory.[8,9] This is a very important basis for the assignment of the bands of AGMA observed in the solid state.

(3) Anisotropy of the absorptions in a single crystal of AGMA. According to an X-ray examination of Dr. Y. Iitaka,[12] the crystal of AGMA is triclinic, but it is very much like hexagonal crystal, its unit cell dimensions being $a$ = 4.8 Å., $b$ = 4.8 Å., $c$ = 17.6 Å., $\alpha \doteqdot 90°$, $\beta \doteqdot 90°$, $\gamma \doteqdot 120°$. The crystal is tabular and the $c$-axis is nearly perpendicular to the plate. Based upon the dimensions of the unit cell (which should contain two molecules) and the dimensions of the molecule (see Fig. 4), it is concluded that all the molecules are oriented with the axis of zig-zag chain nearly parallel to the $c$-axis. Our absorption measurement of the single crystal (Fig. 2A) was made with the infrared beam almost perpendicular to the plate (*ab* plane), namely, almost parallel with the $c$-axis (that means probably almost parallel with the axis of the zig-zag chain of the molecule). The electric vector of such a beam is in the plane perpendicular to the $c$-axis. Therefore, if a normal vibration of the AGMA molecule has the transition moment nearly parallel to the axis of the zig-zag chain, the corresponding absorption band would be observed with

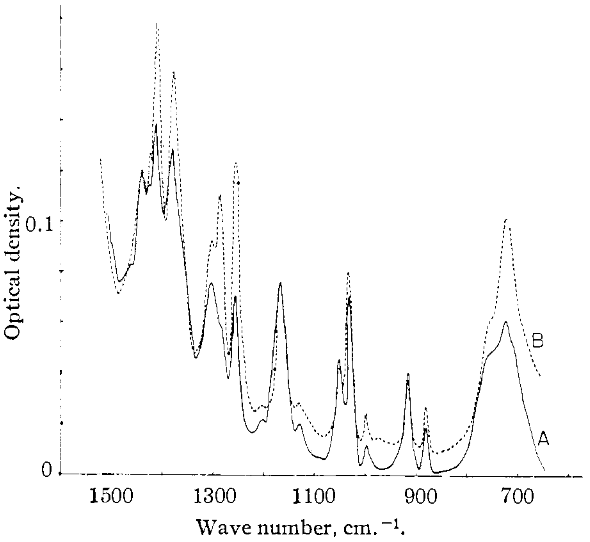


Fig. 2.—Infrared absorption curves of acetylglycine N-methylamide in crystalline state: A, a single crystal is placed with the cleavage face (*ab*-plane) perpendicular to the incident beam; B, small crystals are oriented at random in a Nujol or hexachlorobutadiene mull.

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[11] We wish to express our sincere thanks to Mr. T. Oba of National Hygienic Laboratory for our use of this instrument.

[12] Department of Mineralogy, Faculty of Science, Tokyo University. We wish to express our sincere thanks for his X-ray examination.

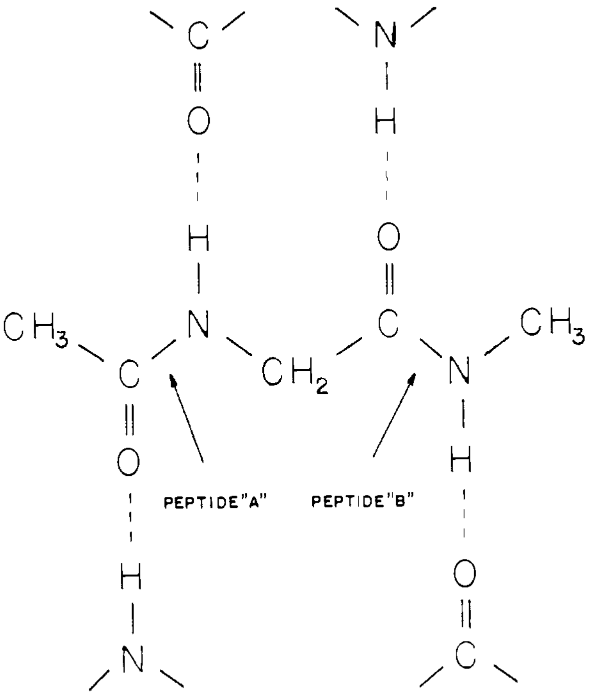

Fig. 3.—Molecular configuration of acetylglycine N-methylamide in its crystal.

Table I[a]

Infrared Frequencies in Cm.$^{-1}$ of Acetylglycine N-Methylamide and its N-Deuterated Compound with Their Assignments

| AGMA | N-Deut. | Assignment | |
|---|---|---|---|
| 1654(s) | 1640(s) | C=O str. | (Amide I) |
| 1641(s) | 1633(s) | | |
| 1563(m) | | NH in-plane def. + C–N str. | (Amide II) |
| | 1479(s) | C–N str. | (Amide II′) |
| 1441(m) | 1417(m) | $CH_3$ deg. def. | |
| 1424(m) | | | |
| 1410(s) | 1406(m) | $CH_2$ bend. | |
| 1375(s)[c] | 1376(w) | $CH_3$ sym. def. | |
| | 1369(w) | | |
| | 1349(s) | $CH_2$ wag. | |
| 1302(m) | 1276(m) | $CH_2$ twist. | |
| 1287(s)[c] | | C–N str. + NH in-plane def. in "peptide b" | (Amide IIIb) |
| 1257(s)[c] | | C–N str. + NH in-plane def. in "peptide a" | (Amide IIIa) |
| 1204(w) | 1206(w) | | |
| 1165(s) | 1172(m) | $CH_3$(–N) rock. | |
| 1129(w)[c] | 1092(m) | $CH_3$–N str. | |
| 1046(m) | 1045(m) | $CH_3$(–C) rock. | |
| 1029(s)[c] | 1025(s) | N–($CH_2$)–C out-of-phase str. | |
| | 980(s) | ND in-plane def. | (Amide III′) |
| 995(w) | 960(w) | $CH_3$(–C) rock. | |
| 914(m) | 899(m) | $CH_2$ rock. | |
| 877(m)[c] | 869(w) | C–$CH_3$ str. | |
| 750(mb) | | NH out-of-plane def. | (Amide V) |
| 721(s)[c] | 733(m) | C=O in-plane bend. | (Amide IV) |
| 642(w) | 643(w) | | |
| 603(s) | | C=O out-of-plane def. | (Amide VI) |
| 569(s) | | | |
| | 524(mb)[c] | ND out-of-plane def. | (Amide V′) |
| 480(s) | 471(w) | | |

[a] Abbreviations: (s) = strong; (m) = medium; (w) = weak; (b) = broad. [c] These bands are polarized along the *c*-axis.

relatively weak intensity. On the other hand, if its transition moment is nearly perpendicular to the axis of the zig-zag chain, the corresponding band would be observed with considerable intensity. The weakness of the relative intensity of the band in the former case should be detected by comparing the absorption curve obtained with the single crystal (Fig. 2A) with that obtained with the powdered sample (Nujol or hexachlorobutadiene mull, Fig. 2B), in which the crystals are oriented at random. As can be seen from the figure, the relative intensities of the bands at 1375, 1287, 1257, 1129, 1029, 877 and 721 cm.$^{-1}$ are weaker in Fig. 2A than in Fig. 2B. These bands are indicated by (*c*) in Table I and may be assigned to the vibrations with the dipole oscillations nearly along the axis of the zig-zag chain of the molecule.

**Assignment of the Characteristic Bands of Amide.**—We can easily identify Amide I, II and II′ bands[8,9] from their positions and intensities. Since this molecule has two peptide groups, "a" and "b" (*cf.* Fig. 3), some of these bands may split into two peaks. In fact, the amide I band has two peaks both in the undeuterated and the N-deuterated forms.

Amide III band is expected to appear around 1300 cm.$^{-1}$. In AGMA three strong bands at 1257, 1287 and 1302 cm.$^{-1}$ are observed in this region. Two of these bands disappear upon deuteration and only one band at 1276 cm.$^{-1}$ remains. This may be assigned to the $CH_2$ twisting vibration. One of the above-mentioned three bands of undeuterated AGMA is to be assigned to the $CH_2$ twisting vibration, it probably being the one at 1302 cm.$^{-1}$. The other two bands may be assigned to the amide III bands, namely, the 1257 cm.$^{-1}$ band may be assigned to that of peptide "a" and the 1287 cm.$^{-1}$ band to that of peptide "b." The amide III vibration of peptide "a" is considered to be coupled with the $CH_2$ wagging vibration and slightly lowered in frequency. For this reason the $CH_2$ wagging band is not at its usual position, 1340 cm.$^{-1}$, and is shifted to higher frequency so that it overlaps the $CH_3$ symmetric deformation band at 1370 cm.$^{-1}$. Upon deuteration the amide III band disappears, and so the $CH_2$ wagging vibration band appears at its usual position (1349 cm.$^{-1}$).

As shown in Fig. 2, the intensity of the three bands at 1375, 1287 and 1257 cm.$^{-1}$ becomes considerably weaker when the incident beam is parallel to the *c*-axis. This fact is compatible with the assignment of these bands, respectively, to the $CH_2$ wagging, the amide IIIb and the Amide IIIa vibrations in all of which the dipole moments oscillate in the direction of the zig-zag chain of the molecule.

The amide III′ band is expected to appear in the region from 950 to 990 cm.$^{-1}$. The strong band of N-deuterated AGMA at 980 cm.$^{-1}$ can be assigned to the amide III′ band, since the undeuterated form has no corresponding band in this region. The 995 cm.$^{-1}$ band of undeuterated AGMA can be considered to correspond to the band of the N-deuterated form at 960 cm.$^{-1}$ which arises from the $CH_3$ rocking vibration of the $CH_3$–C group.

The amide V band arising from the NH out-of-plane deformation vibration is expected to appear as a strong and broad band in the region from 650 to 800 cm.$^{-1}$. A broad band centered at 750 cm.$^{-1}$ can be assigned to this vibration. The band seems to be overlapped by a comparatively sharp band (Fig. 1, 2) at about 721 cm.$^{-1}$, which persists after deuteration (Fig. 1). For this reason and from the frequency value the latter can be assigned to the amide IV band (C=O in-plane bending). Since this band becomes very weak for the incident beam parallel to the *c*-axis (Fig. 2), the corresponding dipole oscillation may be considered to occur in the direction of the zig-zag chain of the molecule. This is in agreement of our conclusion that the sharp band at 721 cm.$^{-1}$ is the amide IV band but not the amide V band. The band at 642 cm.$^{-1}$ which is not shifted on deuteration, might be another amide IV band arising from the other of the two peptide groups. The broad absorption band at about 524 cm.$^{-1}$ observed for the N-deuterated AGMA can be assigned without doubt to the amide V′ band arising from the ND out-of-plane deformation vibration.

The two strong absorption bands at 603 and 569 cm.$^{-1}$ of AGMA can be assigned to the amide VI band from their frequency values. This arises from the C=O out-of-plane deformation vibration. The corresponding amide VI′ bands could not be observed in the spectrum of N-deuterated AGMA. They might possibly be overlapped by the strong and broad band centered at 524 cm.$^{-1}$.

**Assignment of the Other Bands.**—The assignment of the $CH_3$ degenerate deformation vibration, the $CH_2$ bending vibration, the $CH_3$ symmetric deformation vibration and the $CH_2$ wagging vibration can be made from their frequency values as shown in Table I. However, as explained above, the $CH_2$ wagging vibration of the undeuterated form is considered to be coupled with the amide IIIa vibration and is shifted to a higher frequency, overlapping the $CH_3$ symmetric deformation band. In the N-deuterated form two bands arising from the $CH_3$ symmetric deformation vibration are observed at 1376 and 1369 cm.$^{-1}$. The two different frequencies may be due to the fact that one of the $CH_3$ groups is combined with C on one end of the molecule and the other with N on the other end.

The $CH_3$(–N) rocking vibration, $CH_3$(–C) rocking vibration, $CH_3$–N stretching vibration and $CH_3$–C stretching vibration of N-methylacetamide have been assigned, respectively, to the bands observed at 1159, 1040 (and 987), 1096 and 881 cm.$^{-1}$.[8] The corresponding bands of AGMA may be assigned to those at 1165, 1046 (and 995), 1129 and 877 cm.$^{-1}$, respectively. In the N-deuterated form, the corresponding bands are observed at 1172, 1045 (and 960), 1092 and 869 cm.$^{-1}$. From the $CH_2$ rocking frequency of glycine (910 cm.$^{-1}$)[13] the band at 914 cm.$^{-1}$ of AGMA and the one at 899 cm.$^{-1}$ of the N-deuterated form can be assigned to the same vibration. The bands assigned to $CH_3$ rocking vibration and $CH_2$ rocking vibration are expected to be polarized in the direction perpendicular to the *c*-axis, whereas the bands assigned to C–C and C–N stretching vibrations are expected to be polarized in the direction parallel to the *c*-axis. The observed bands shown in Fig. 2 conform very well to this expectation.

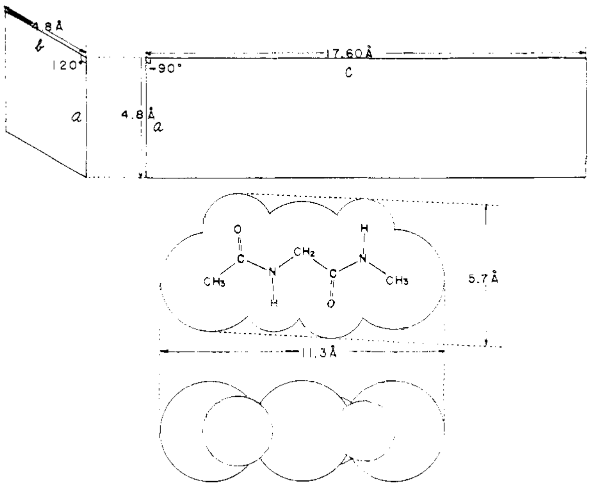

Fig. 4.—Unit cell and molecular dimensions of acetylglycine N-methylamide.

Now, only the strong, sharp bands at 1029 cm.$^{-1}$ of AGMA and at 1025 cm.$^{-1}$ of the N-deuterated form remain to be assigned. These are concluded to arise from the N–($CH_2$)–C skeletal stretching vibration of the middle part of the molecule. The band at 1029 cm.$^{-1}$ is polarized almost in the direction of the zig-zag chain of the molecules (Fig. 2). Therefore it is reasonable to assign it to N–($CH_2$)–C out-of-phase stretching vibration. As pointed out previously, a sharp absorption band at 1015 cm.$^{-1}$ is characteristic of the structure with more than two adjoining glycine residues, and also a sharp absorption band at 965 cm.$^{-1}$ is characteristic of the structure with more than two adjoining alanine residues.[7] The band at 965 cm.$^{-1}$ is polarized in the direction of the extended polypeptide chain as shown in polyalanine and tussah silk.[14,15] Therefore, it is reasonable to assign them to the out-of-phase stretching vibration of N–(CHR)–C in the polypeptide chain.

The absorption band arising from the N–($CH_2$)–C in-phase stretching vibration is probably weaker than that of the out-of-phase stretching vibration and is to be found in the region a little lower than 1000 cm.$^{-1}$. This has not yet been detected.

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