longing to (IV) does not point to the O-atom of the C=O group (see Fig. 5). This configuration does not result in a strong hydrogen bond. If the N–C bond (IV) is in the *trans* position with respect to the N–C bond (II), the configuration cannot at all form an internal hydrogen bond. However, if the C–C bond (III) is a polar bond of the piperidine ring, we may again expect the formation of the N–H . . . O internal hydrogen bond which can be concluded to be very weak from the inspection of the relative position of N- and O-atoms. In short we cannot consider the formation of a strong internal hydrogen bond in all the conceivable configurations of acetylpiperidine α-carboxylic acid N-methylamide in which the N–C bond (I) cannot be in the gauche position with respect to the C–C bond (III).

Besides internal hydrogen bond acetylpiperidine α-carboxylic acid N-methylamide differs from acetylproline N-methylamide in *intermolecular* hydrogen bond. As referred to above, the former substance begins to show association at 0.005 mole/l. in carbon tetrachloride solution and at 0.05 mole/l. in chloroform solution, while the latter substance shows no association even at 0.05 mole/l. in these two solutions. This is due to the fact that in the latter substance the N–H group is involved in strong internal hydrogen bonding and almost no N–H group (in carbon tetrachloride solutions) or few N–H groups (in chloroform solutions) are left free to form *intermolecular* hydrogen bond.

Summarizing the results obtained with regard to the hydrogen bonding, all the acetylaminoacid N-methylamides of the type of $CH_3CONHCHRCONHCH_3$ (where R = H, CH_3, $CH(CH_3)_2$, $CH_2CH(CH_3)_2$ and $CH_2CH_2CH_2CH_3$) so far studied in this series of researches[1-5] showed the NH bands involved in internal hydrogen bonding in carbon tetrachloride and chloroform solutions, when these molecules are in the folded configurations. The shift in frequency from the free NH band was found to amount to about 100 cm.$^{-1}$ just as in the case of the bonded NH band of acetylproline N-methylamide. The formation of this fairly strong hydrogen bond is due to the gauche relation between the N–C bond (I) and the C–C bond (III). For the corresponding amino acid residues contained in the folded polypeptide chain (*i.e.*, glycine, alanine, valine, leucine and norleucine residues), we can consider configurations similar to this. However, as we have seen from the different behavior between acetylpiperidine α-carboxylic acid N-methylamide and acetylproline N-methylamide, a change in the internal rotation state will affect considerably the *intramolecular* and *intermolecular* hydrogen bonds of a polypeptide chain and hence also the configuration of the chain.

Acknowledgment.—The authors thank Prof. J. Noguchi of Kanazawa University for the preparation of piperidine α-carboxylic acid. Their thanks are also due to Prof. K. Kozima for the loan of the LiF prism and to Mr. Ishino and Miss Mitsui, Faculty of Agriculture, for the performance of the microanalysis here recorded.

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[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, BROOKHAVEN NATIONAL LABORATORY]

A Chlorophyll Substance Possessing a Spectrum Very Similar to that of Chlorophyll-b[1]

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RECEIVED APRIL 28, 1954

A chlorophyll substance is obtained along with chlorophyll-*b'* when chlorophyll-*b* has been heated in solvents and also without solvent, in the absence of oxygen and water. Its spectrum, and its fluorescence and solvation properties are almost identical with those of chlorophyll-*b*. Despite the fact that it does not undergo the Molisch phase test, reasons are advanced that the three substances, chlorophyll-*b*, chlorophyll-*b'* and the new substance, may constitute the three, long-discussed possible tautomers of chlorophyll-*b* whose structures may be written for the chlorophyll molecule by bonding magnesium atom in turn to three different pairs of pyrrol nitrogen atoms. There are indications that a corresponding substance exists in the chlorophyll-*a* series also and it is tentatively proposed that these substances be known as chlorophyll-*b''* and chlorophyll-*a''*.

In the preparation[2] of chlorophyll-*b'* from chlorophyll-*b* it was observed during the chromatographic analyses that in addition to the two zones of chlorophylls-*b* and -*b'*, a distinct third zone appeared. The order of the zones, from the top of the column consisted of the substance X under consideration, chlorophyll-*b* and finally chlorophyll-*b'*, roughly in the proportions 1:4:2, respectively.

X has a spectrum very similar to those of chlorophyll-*b* and -*b'*, just distinguishable from them by the slight displacements of the corresponding maxima. From the table and the figures it may be noted that at room temperature X in solution has the principal features of its spectrum closer to those of chlorophyll-*b* than does chlorophyll-*b'*

TABLE I

WAVE LENGTHS OF ABSORPTION MAXIMA OF SOLUTIONS OF CHLOROPHYLL SUBSTANCES

Wave lengths were reproducible to ±3 Å. At 300°K. the solvent was 10% *n*-propyl ether in methylcyclohexane and at 75°K. it was 10% *n*-propyl ether in 1:1 propane, propene.

Substance	75°K.	300°K.	75°K.	300°K.
Chlorophyll-*b*	4765	4512	6436	6411
Chlorophyll-*b'*	4756	4535	6447	6427
X	4736	4506	6443	6411
Allomerized chlorophyll-*b*		4530		6600
Ethyl chlorophyllide-*b*		4523		6425
Pheophytin-*b*		3950		. .

(1) Research performed under the auspices of the U. S. Atomic Energy Commission.

(2) H. H. Strain and W. M. Manning, *J. Biol. Chem.*, **146**, 275 (1942).

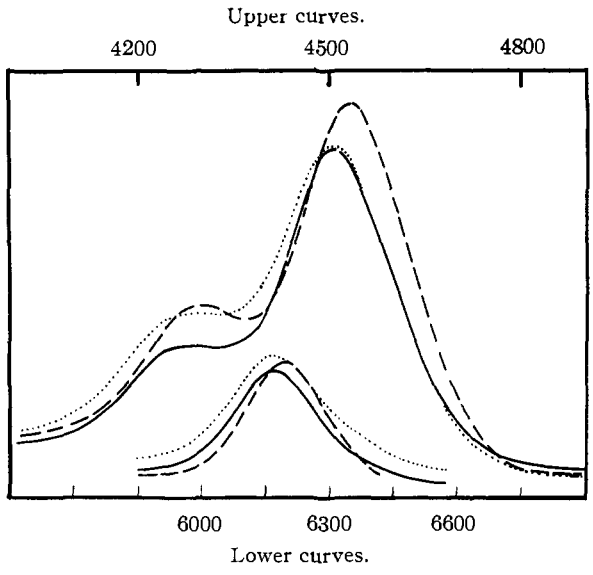

Fig. 1.—300°K., spectra of solutions in 10.0% di-*n*-propyl ether in methylcyclohexane: chlorophyll-*b*, ——; chlorophyll-*b'*.------; substance X, ...

while at 75°K. the relative closeness of their spectra is reversed.

Substance X and also chlorophyll-*b'* were prepared by heating chlorophyll-*b* dissolved in *n*-propanol at 95–100°C. for about an hour. The amount of X produced seemed to be little affected by substituting *n*-propyl ether or methylcyclohexane for the *n*-propanol. All solvents were carefully dried. However, when calcium hydride was added directly to the solutions of chlorophyll in *n*-propyl ether and also in the methylcyclohexane to remove traces of water, the yield of X seemed to increase and the degree of decomposition seemed less. When, however, the chlorophyll was heated in absence of solvent X formed to a lesser degree. The heating was done in the dark. To exclude oxygen, all solutions were prepared on a vacuum line; the chlorophyll film was pumped free of previous solvent and the new one was distilled on the film by condensation at the temperature of Dry Ice. Argon or nitrogen filled the gaseous space of the vessel undergoing the heating. In one instance the volume of the solution was kept to a minimum and the glass container was sealed off in a high vacuum. The volume of the solution in methylcyclohexane was about one milliliter containing about 5×10^{-8} mole chlorophyll-*b* while the total volume of the sealed off tube was about two milliliters. In view of the low pressures at which distillation and pumping occurred, we estimate that the content of oxygen was considerably less than 10^{-10} mole, which is much less than would be required for a 1:1 oxidation of the chlorophyll.

The chromatographic column consisted of sucrose and the developer of 0.5% propanol in *n*-hexane. In most of our work we employed one-dimensional ascending chromatography on paper impregnated with sucrose.[3] Chromatography yielded a brown spot at the origin followed by a succession of spots

(3) A. H. Sporer S. Freed and K. M. Sancier, *Science*, **119**, 68 (1954).

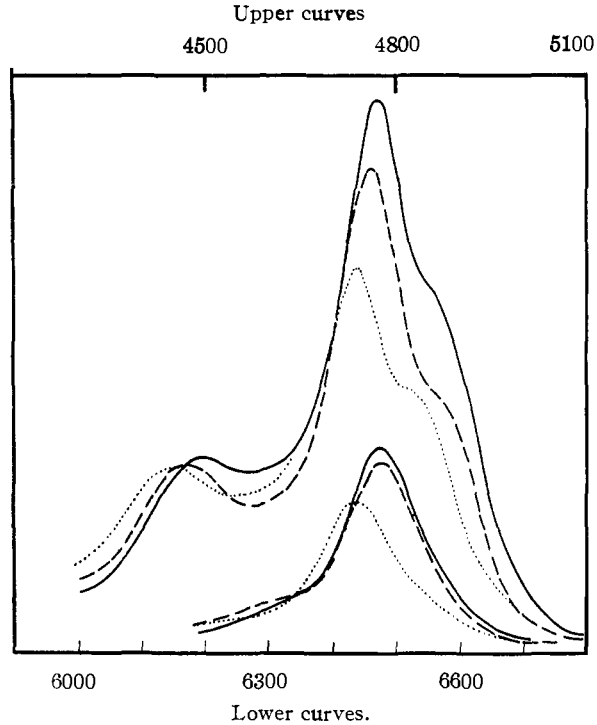

Fig. 2.—75°K., spectra of solutions in 10.0% di-*n*-propyl ether in 1:1 propane, propene: chlorophyll-*b*, ——: chlorophyll-*b'*. ------: substance X, ...

of the chlorophyll substances in the same order as on the sucrose column. A solution of X heated at 100° about four hours produced only a single spot on the paper, that of the original substance with no evidence of a brown spot or decomposition. Other instances of heating resulted in small brown spots at the origin. However we observed no chlorophyll-*b* or -*b'* produced by heating X at 100°.

When dissolved in dry hydrocarbon, X showed no fluorescence but the addition of a trace of polar substance such as ether or water activated[4] the solution to bright fluorescence, to the same degree as occurs in solutions of the chlorophylls-*b* and -*b'*. Also X has the same characteristic temperature of solvation as chlorophyll-*b* and -*b'* and presumably the same equilibrium constant for the solvation process.[5]

In one important respect, X differs from the chlorophyll-*b* and -*b'*. It does not show the Molisch phase test, the classical color test for

(4) R. Livingston, W. F. Watson and J. McArdle, THIS JOURNAL, **71**, 1542 (1949).

(5) At the characteristic temperature associated with a given solvent which we have called the transit temperature (S. Freed and K. M. Sancier, *ibid.*, **76**, 198 (1954)) the two main peaks in the blue originating in the co-existing mono- and di-solvates are the same height. The transit temperatures of chlorophyll-*b* and of chlorophyll-*b'* in di-*n*-propyl ether, hydrocarbon solvent were found originally (S. Freed and K. M. Sancier, *Science*, **114**, 275 (1951)) to differ by about forty degrees. These transit temperatures were also reproduced with chlorophylls prepared again several months later in the same laboratory, those of Professor Robert Livingston of the University of Minnesota. However, all later preparations from that laboratory as well as those made in this Laboratory, from corn and from cinnamon fern, gave chlorophyll-*b* and -*b'* which had the same transit temperatures (±3°). We have endeavored to repeat in all detail the purification of solvents previously followed but we have been unable to obtain the former discriminations by means of transit temperatures.

chlorophylls. The test was carried out in the usual way and also by adding a drop of alcoholic potassium hydroxide to the spots on paper. That is, the enol–keto couple at carbon atoms 9 and 10 does not seem available for reaction, presumably salt formation, upon addition of a base. The first impulse is to infer that X is allomerized chlorophyll-*b* in which this couple no longer exists. However (1) its spectrum differs greatly from that of allomerized chlorophyll-*b*, as may be seen in the table; (2) it does not fluoresce in dry hydrocarbons whereas allomerized chlorophylls do; (3) allomerized chlorophyll-*b* is developed on the chromatographic column more rapidly even than chlorophyll-*b'* so that the order on the column would be different from the actual one.

In the table are listed the maxima of two other substances which might in some way have been formed during the heating process. Propyl chlorophyllide-*b* actually represented here by the corresponding ethyl chlorophyllide-*b* shows the Molisch phase test and its maximum in the blue differs from that of X in being at longer wave lengths rather than at shorter wave lengths than that of chlorophyll-*b*. Pheophytin-*b* which possesses the enol–keto couple at carbon atoms 9 and 10 but in which the magnesium atom is replaced by hydrogen atoms does not undergo the Molisch phase test but its spectrum cannot possibly be mistaken for that of X.

It has often been conjectured that the three molecular structures which may be written for chlorophyll, with magnesium bonded to three different pairs of pyrrol nitrogen atoms are tautomers rather than resonance hybrids. That is, potential barriers may exist against their interconversion. It is then natural to ask whether the three substances we are discussing may be identified with the possible tautomers. It should be recalled that we have not yet succeeded in transforming X back into chlorophyll-*b*. However, this may be a matter of activation energy. The transition, chlorophyll-*b*–chlorophyll-*b'*, requires a temperature of about 100° to reach convenient mobility and it may well be that the transitions X–chlorophyll-*b* and X–chlorophyll-*b'*, require still higher temperatures. The failure of the phase test also creates doubt that X may be one of the tautomers since it indicates the absence of the enol–keto couple at carbon atoms 9 and 10. On the other hand the presence of the couple in X is indicated by the fluorescence properties. The activation of fluorescence of the chlorophylls by polar substances has been assigned by Livingston, Watson and McArdle[4] in their comprehensive investigation to an intermediate formed at the enol–keto couple with the polar substance. A clue to the resolution of this dilemma may be given by the observation that in one of the structures A of chlorophyll,[6] the magnesium atom is not bonded to the nitrogen of ring III which is fused with the pentenone ring containing the active enol–keto couple. This absence of attachment of magnesium atom recalls the situation in pheophytin which does not undergo the Molisch phase test. The pheophytin structure contains no magnesium atom but it does contain the enol–keto couple. Hence, the fact that substance X does not give the color test need not exclude it from being one of the tautomers. Clearly more work is required to decide this possibility.

There are indications that analogous to the formation of X from chlorophyll-*b* or -*b'*, a corresponding substance may be formed in the chlorophyll-*a* series. We are then inclined tentatively to call these substances chlorophyll-*b''* and chlorophyll-*a''*, respectively.

(6) E. I. Rabinowitch, "Photosynthesis," Vol. 1, Interscience Publishers, Inc., New York, N. Y., 1945, p. 422.

Further Experimental Notes

Solvents. Di-*n*-propyl Ether.—Eastman Kodak White Label ether was refluxed over sodium potassium for two hours before distillation at atmospheric pressure through a 30-plate fractionating column. The ether was stored in a glass flask under vacuum over calcium hydride and was protected from the light by metal foil encasing the flask.

***n*-Propyl Alcohol.**—C.P. distilled from calcium hydride.

Methylcyclohexane.—Eastman Kodak White Label distilled from calcium hydride.

Diethyl Ether.—C.P. distilled from sodium and stored over iron away from light.

Hexane.—Matheson Company Hexane washed free of olefins with concentrated sulfuric acid and distilled in an all glass still.

Preparations. Chlorophyll-*b*.—This was prepared from corn and cinnamon fern by the method of Zscheile and Comar (*Botan. Gaz.*, **102**, 463 (1941)).

Chromatographic Analysis.—The analyses were performed both on sucrose columns and on sucrose impregnated paper.[3] In these columns, chromatographic tubes of varying sizes were used ranging in inside diameter from 9 to 35 mm. The tubes were usually packed to a height of 150–200 mm. with a slurry of Jack Frost 6X confectioner's sugar in hexane. Dry nitrogen pressure was used throughout the chromatographic analyses. The column was first prewashed with diethyl ether and the appropriate quantity of sample in 20% diethyl ether in hexane was added followed by development with a 0.5% *n*-propyl alcohol in hexane solution.[3] When a clear separation of the zones was obtained, the column was post-washed with hexane to wash out the remaining developer. Each zone was carefully removed from the column to avoid contamination of one zone by the other. In addition, compound X was removed from the top of the column while chlorophyll-*b* and -*b'* were removed from the bottom of the column. The top and bottom sections of all zones were discarded; only the central section of a zone was placed in a clean sintered-glass filter and eluted with a minimum of diethyl ether. Solutions that were used within a few days were kept in the freezing compartment of the refrigerator (0°); all other solutions were stored in a special compartment in the Dry Ice chest.

The ascending paper chromatograms were run in the manner described in reference 3.

Apparatus.—Most of the small scale reactions were run in the specially built cells described in reference 5, to permit the spectra of the reaction systems to be taken directly and to simplify the necessary manipulations on the high vacuum line to which these cells are specially suited.

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