

from the excitations $\psi_b \rightarrow \psi_b$ and $\psi_1 \rightarrow \psi_a$ are allowed for. It would not be surprising if the predicted B_b and B_a bands of benzaldehyde and benzonitrile (Table IV) deviate appreciably from the observed spectra. Any deviation of the calculated result from the observed data should be attributed to the use of second order perturbation theory and to the assumption that δ and γ are fixed in all interactions. Moreover, we have assumed the inductive perturbations only on the atom attached to the substituent. In view of large values of δ for the nitrile and aldehyde groups, perturbations should be extended to the other atoms.

Now some comments should be made on the large values of δ for the nitrile and aldehyde groups. The π -ionization potentials of a substituted benzene is increased due to the inductive effect of an electron attracting substituent and decreased by the conjugative effect of the substituent. These two opposing effects should therefore be considered in the determination of the π -ionization potentials of substituted benzenes. In view of higher observed ionization potentials of benzonitrile and benzaldehyde than that of benzene, large values of

δ in these two cases are therefore not surprising. Although large values of δ in benzonitrile and benzaldehyde compared to that in chlorobenzene and phenol² seem to be incompatible with the empirical evidence conventionally quoted, some support of these results may be had from the discussions of dipole moments. It should be mentioned in this connection that the values of δ as are shown in Table IV may involve appreciable error since they are very much sensitive to any error in the observed ionization potentials of the substituted benzenes.

Several works^{1,12} have been done on the spectra of substituted benzenes. They have chosen to deal with this problem either using perturbation method or by the straightforward solution of the secular determinant but not including higher order configuration interaction in either case. It should be noted that the present paper and the references^{2,11} show that two electron excited configurations play the important role in the spectra of substituted benzenes.

(12) I. Goodman and H. Shull, *J. Chem. Phys.*, **27**, 1388 (1957), and I. Goodman, I. G. Ross and H. Shull, *ibid.*, **26**, 474 (1957).

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Evidence for Exchangeable Hydrogen in Chlorophyll¹

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RECEIVED JUNE 2, 1961

Both chlorophylls a and b undergo slow exchange of hydrogen with methanol in carbon tetrachloride solution. Both compounds possess one enolizable (or exchangeable) hydrogen atom and exist predominantly in the keto form.

Introduction

Whether chlorophyll possesses any labile or readily exchangeable hydrogen has long been the subject of discussion. From the structural formula it can be anticipated that the hydrogen atom at position 10 is in a favorable position for enolization and should, therefore, be both labile and exchangeable. Fischer and Goebel² observed that ethyl chlorophyllide has one hydrogen atom that reacts with Grignard reagent and showed that this reaction occurs at carbon 10; these experiments were carried out in pyridine solution. Direct attempts to observe hydrogen exchange in chlorophyll, however, have in the past been uniformly unsuccessful. The early experiments of Norris, Ruben and Allen³ indicated less than 5% exchange with a mixture of chlorophylls a and b in aqueous-ethanol solution in a 30 minute period; these workers concluded that no hydrogen exchange occurs. Weigl and Livingston⁴ made a detailed search for exchangeable hydrogen in chlorophyll. No detectable hydrogen-deuterium exchange was observed in neutral organic solvents containing an

excess of deuterium oxide; a reaction time of about 2 hr. was usually employed. Although in one experiment the exchange observed in 24 hr. was about 0.2 atom per molecule of chlorophyll, nevertheless, Weigl and Livingston concluded that no exchangeable hydrogen was present in chlorophyll. More recently, Kutyria,⁵ in connection with a study of possible photochemical exchange of hydrogen in chlorophyll, found a small amount of hydrogen exchange between chlorophyll and aqueous acetone in a 3-hr. period. Kutyria did not exclude the possibility that chlorophyll does in fact have a labile hydrogen atom.

Our interest in the subject originated in observations we have made of the infrared spectra of ordinary and fully deuteriated (hereafter referred to as deuterio-) chlorophylls a and b.⁶ Holt and Jacobs,⁷ who have made the most detailed study of the infrared spectra of chlorophylls a and b, interpreted the absorption peaks in the 1600–1700 cm.⁻¹ region to indicate that chlorophyll a exists as the enol, but that chlorophyll b is in the keto-form. The hydrogen at carbon atom 10 was assumed to be the enolized hydrogen atom in chlorophyll a. The reasons for the existence of chlorophyll a as the enol while

(1) Based on work performed under the auspices of the U. S. Atomic Energy Commission.

(2) H. Fischer and S. Goebel, *Ann.*, **522**, 168 (1936).

(3) T. H. Norris, S. Ruben and M. B. Allen, *J. Am. Chem. Soc.*, **64**, 3037 (1942).

(4) J. W. Weigl and R. Livingston, *ibid.*, **74**, 4160 (1952).

(5) V. M. Kutyria, *Fiziologia Rastenii*, **7**, 133 (1960).

(6) H. H. Strain, M. R. Thomas, H. L. Crespi, M. I. Blake and J. J. Katz, *Ann. New York Acad. Sci.*, **84**, 617 (1960).

(7) A. S. Holt and E. E. Jacobs, *Plant Physiol.*, **30**, 553 (1955).

the very similar chlorophyll b retained the keto configuration were not clearly stated. If chlorophyll a does exist as the enol, then rapid exchange should be detectable in a but not in b. We have carried out such studies. Both chlorophyll a and b are found quite conclusively to possess at least one enolizable (or exchangeable) hydrogen atom, and both substances are observed to exist predominantly in the keto form.

Experimental

Since chlorophyll a contains 72 hydrogen atoms, it did not appear feasible to detect the replacement of one hydrogen atom by deuterium by a direct examination of the infrared spectrum of the exchanged chlorophylls. Consequently, an indirect procedure, also depending on infrared spectrophotometry, was employed. Chlorophyll was treated with a large excess of CH_3OD ; if exchange occurs, then deuterium is introduced into the chlorophyll. If this chlorophyll is allowed to exchange with an equivalent amount of ordinary methanol, CH_3OH , exchange will result in a mixture of CH_3OH and CH_3OD . The ratio of CH_3OH to CH_3OD is easily determined in the infrared and the extent of exchange thus readily deduced. Although simple in principle, this is not a simple experiment to carry out. The principal problems result from the disparity in molecular weight of chlorophyll and methanol, the absolute necessity for making certain that no impurities with exchangeable hydrogen accompany the chlorophyll, and the requirement that the CH_3OD initially needed to introduce deuterium be rigorously removed before measuring the subsequent exchange. By careful attention to detail, however, these difficulties can be surmounted, and we believe that reliable results can be secured. Model experiments have been carried out that indicate this to be the case.

Apparatus and Procedure.—The exchange studies were carried out in a vacuum line that could be evacuated to a pressure of 1×10^{-6} mm. as measured by a Phillips ion gauge. The reaction vessel (B, in Fig. 1) was designed with a wide flat bottom to accommodate a glass jacketed stirrer. This was essential to provide smooth evaporation of the solvent. The chlorophyll samples were introduced through a long tube extending to the bottom of the vessel; this avoided adhesion of statically charged chlorophyll to the walls.

Carbon tetrachloride rinses and the carbon tetrachloride-methanol exchange mixtures were introduced at A with a positive pressure of dry nitrogen. The solvents were degassed and then distilled into the reaction vessel. The solvents were transferred by distillation to a tube at A and removed for analysis. The apparatus is shown in Fig. 1.

For the exchange experiments 100 mg. of chlorophyll was introduced into the reaction vessel and the system evacuated. Carbon tetrachloride, degassed on the line at A, was distilled onto the chlorophyll and after a short interval, distilled away. This procedure was repeated several times with fresh carbon tetrachloride and proved effect in removing water held by the chlorophyll. When the chlorophyll was free of moisture, the reaction vessel was closed, and the line pressurized to slightly above atmospheric pressure with dry nitrogen. A mixture of 2 ml. of CH_3OD and 0.5 ml. of CCl_4 was then placed on the line. The $\text{CH}_3\text{OD}-\text{CCl}_4$ mixture was thoroughly degassed by repeated freezing with liquid nitrogen, evacuating and thawing. The degassed mixture was distilled into the reaction vessel, and the resulting solution was stirred with the magnetic stirrer to effect solution and exchange. After the desired reaction period, the solvents were distilled back to A, and the residue washed several times with carbon tetrachloride. Each rinse was examined by infrared after removal from the line to detect the presence of methanol. Generally three or four rinses with CCl_4 were adequate to remove all the methanol from the chlorophyll. When the CCl_4 rinse was ascertained to be free of methanol, a weighed portion of a solution of 0.5% of CH_3OH in CCl_4 was degassed and distilled onto the chlorophyll. The amount of CH_3OH was equimolar with the chlorophyll sample. The $\text{CH}_3\text{OH}-\text{CCl}_4$ was allowed to remain in contact with the chlorophyll for the desired time interval and then removed by distillation and used to determine the extent of exchange. A concentration of 0.5% methanol was especially useful; enough CCl_4 is

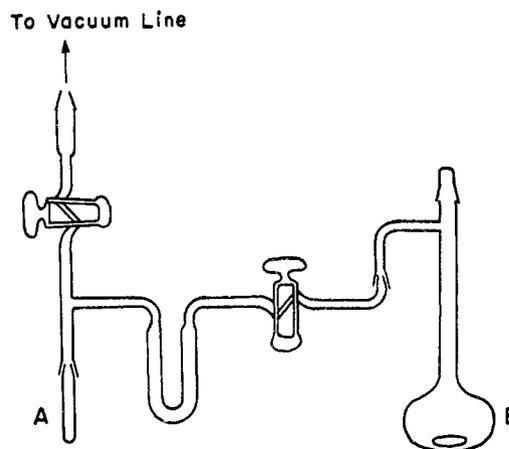


Fig. 1.—The vacuum line exchange apparatus: A, solvent introduction; B, reaction vessel with magnetic stirrer.

present to dissolve the chlorophyll and thus bring the CH_3OH in contact with all the chlorophyll. Further, a methanol concentration of about 0.5% is suitable for analysis in the infrared in a cell with a 1 mm. light path. All operations were carried out in dim light, and the reaction mixture was kept in the dark during exchange.

Model experiments with lauryl alcohol and carbethoxycyclopentanone were carried out in very similar fashion. Since methanol is miscible with these substances, no carbon tetrachloride solvent is required during the initial deuteration.

The vacuum system itself was checked for exchangeable hydrogen by running a blank. The apparatus was allowed to remain in contact with CH_3OD for a protracted period, the apparatus was then evacuated and ordinary methanol allowed to remain in contact with the glass for at least 48 hr. This procedure was repeated each time a new reaction vessel was used. No retention of deuterium by the apparatus was found.

Analytical Procedure.—A Beckman IR-4 infrared spectrophotometer fitted with a beam condenser was utilized for analysis. Ultra micro liquid cells with a light path of 1 mm. requiring about 20 μ of solution were used. The spectrum of the methanol-carbon tetrachloride recovered from an exchange experiment was recorded in the range 2000–4000 cm^{-1} . The OH, OD and CH peaks are in this region. Since the methanol is measured in CCl_4 solution at a low concentration, unassociated OH and OD are found at 3644 and 2711 cm^{-1} , respectively; these peaks can be used more accurately than the broad polymeric associated OH and OD bands for measuring the concentration of the respective alcohols. Since Beer's Law is obeyed, a calibration curve can be used to convert the absorbancy readings to a $\text{CH}_3\text{OH}/\text{CH}_3\text{OD}$ ratio and thus measure the extent of exchange.

The visible spectrum of chlorophyll samples recovered from exchange experiments were examined to detect possible chemical changes in the pigments during the course of exchange. The only spectral change detected in any of the exchange experiments was associated with the formation of chlorophylls a' and b'. This is expected since a' and b' normally exist in equilibrium with chlorophylls a and b. In all other respects, including chromatographic behavior, the recovered chlorophylls appeared to be identical with the starting material.

Materials.—Chlorophylls a and b were prepared by the improved procedure of Strain, *et al.*⁶ Deuteriated methanol, CH_3OD , was obtained from Volk Radiochemical Co. of Chicago. The isotopic composition of the methanol was 97.2% CH_3OD by analysis of the water of combustion. 2-Carbethoxycyclopentanone was obtained from Arapahoe Chemical Co., lauryl alcohol was Eastman grade. The solvents used were Eastman spectrograde.

Results and Discussion

Model experiments with lauryl alcohol showed, as anticipated, exchange of hydrogen between the

TABLE I

EXCHANGE OF HYDROGEN IN 2-CARBETHOXYCYCLOPENTANONE AND LAURYL ALCOHOL WITH METHANOL IN CARBON TETRACHLORIDE SOLUTION

2-Carbethoxy-cyclopentanone (mM)	Amount of CH ₃ OH (mM)	Time ^a (hr.)	Hydrogen atoms exchanged
0.0359	0.0341	4	0.74-0.755
.0180	.0178	18.5	0.85-0.925
.0264	.0263	48	1.0
Lauryl alcohol			
0.0286	0.0274 ^b	0.16	1.0

^a Temperature 20-21°. ^b 0.45% CH₃OD in CCl₄ used for this experiment.

hydroxyl groups of lauryl and methyl alcohols proceeded very quickly, and that equilibrium distribution of hydrogen and deuterium was attained in a few minutes or less in a solvent of low dielectric constant. Experiments with 2-carbethoxycyclopentanone are summarized in Table I. Extensive exchange is observed in a few hours, but more than 24 hr. are required for complete exchange under the present reaction conditions.

Exchange with chlorophyll in a solvent of low dielectric constant is slow, but unmistakable. The results for chlorophyll exchange are given in Table II. More than 40 hr. is required for complete exchange under the experimental conditions employed here. The results clearly indicate that both chlorophyll a and b have at least one exchangeable hydrogen atom and that there is no qualitative difference in their exchange behavior. The slow rate of

TABLE II

EXCHANGE OF HYDROGEN BETWEEN CHLOROPHYLL AND METHANOL IN CARBON TETRACHLORIDE SOLUTION

Chlorophyll	Quantity (mM)	CH ₃ OH (mM)	Time ^a (hr.)	Hydrogen atoms exchanged
a	0.0173	0.0170	0.16	0
a	.0173	.0174	24	0.83
a	.0079	.0084	48	1.08
a	.0165	.0169	48	0.96
b	.0135	.0139	0.16	0
b	.0135	.0129	48	1.00

^a Temperature 20-21°.

exchange also excludes the possibility that the observed exchange occurs with CH₃OD that may be coordinated to the chlorophyll or that long chain alcohol impurities in the chlorophyll are involved.

Since the exchange is so slow and since exchange with lauryl alcohol is rapid, it can be concluded that both chlorophylls a and b exist in CCl₄ solution predominately in the keto form. The spectral analysis of Holt and Jacobs⁷ is thus open to serious question.

Vishniac and Rose⁸ studied the photochemical incorporation of tritium into chlorophyll in algae performing photosynthesis; interpretation of their results was predicated on the absence of exchangeable hydrogen atoms in chlorophyll in the dark. The fact that hydrogen exchange is very slow implies that the results of Vishniac and Rose are not seriously affected, but a reexamination of the situation may now be desirable.

(8) W. Vishniac and I. A. Rose, *Nature*, **182**, 1089 (1958).