(CON). *Anal.* Calcd for $C_{60}H_{81}N_5O_6Mg$: N, 7.06. Found: N, 6.94.

Pyrochlorophyll b (2b). This compound was prepared from chlorophyll b following the procedure for the preparation of pyrochlorophyll a. It was purified by means of powdered sugar chromatography: λ_{max}^{ether} 644 and 451 mμ, ratio blue:red 2.97; ν_{max}^{THF} 1737 (CO_2R), 1694 (CO), and 1661 cm^{-1} (CHO).

Pheophytin a and isochlorin e_4 6-carboxypropylamide were prepared from the corresponding magnesium complexes by treating ethereal solutions of **1a** and **3a** with 1:1 hydrochloric acid for several seconds. Water was used to wash out the acid. The ether solutions were dried (Na_2SO_4) and evaporated, and the residue was chromatographed on powdered sugar. Isochlorin e_4 6-carboxypropylamide (λ_{max}^{ether} 662 and 441 mμ), prepared in this way, was chromatographically identical with that prepared from pheophytin a and propylamine.

Procedures for Following the Reaction of Chlorophylls with Amines. A. Visible Spectrum Investigations. Ethyl ether solutions (about 40–70 μl) of chlorophyll were injected into quartz or Pyrex cells containing 3.5 ml of redistilled dry amine. A Beckman DK-2A spectrophotometer was used for the measurements with the cell compartment thermostated at 26°. The first spectrum was determined as soon after mixing as possible, and further measurements were made at appropriate intervals, depending on the rate of the reaction. The equation derived by Weller and Livingston[3b] was used for the rate constant calculations with E_0 taken as an extrapolated value.

$$\frac{\text{concn of chlorophyll at time } t}{\text{initial concn of chlorophyll}} = \frac{(E_{obsd} \text{ at time } t) - E_\infty}{E_0 - E_\infty}$$

For $k' = k(\text{concn amine})$, where k' is the pseudo-first-order rate constant

$$k't = \log \frac{(E - E_\infty)}{(E_0 - E_\infty)}$$

For those reactions in which methanol, ethanol, or water were added, amine solutions containing varying amounts of the solvent were prepared, and the procedure above was followed. The 0.01% (v/v) ethanol in piperidine gave a mole ratio of chlorophyll to alcohol of about 1:1.

B. Nmr Spectrum Investigations. (1) Nmr Spectrum of Chlorophyll Used in Kinetic Studies. The chlorophyll (40 mg) was dissolved in chloroform-*d* (*ca.* 0.3 ml) and the spectrum determined. It proved to be virtually the same as that previously reported.[5]

(2) Reaction of Chlorophyll with Amines in Tetrahydrofuran. The chlorophyll (*ca.* 35 mg) was dissolved in about 250 ml of tetrahydrofuran. The amine (20–25 ml) was added and the solution sealed on a vacuum line after degassing. The reaction was usually complete after 1 day. The tube was opened, the contents transferred, and solvent and excess amine were removed *in vacuo*. Chromatography on powdered sugar as described above gave the amides.

Preparation and Properties of 10-Hydroxychlorophylls a and b[1]

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Abstract: Green substances produced by enzymatic oxidation of chlorophylls a and b are shown by chemical analysis, chromatography, nmr, and infrared spectroscopy to be 10-hydroxychlorophylls. These compounds are also shown to be major products of the allomerization reaction of the chlorophylls in CH_3OH. Similar allomerization of the fully deuterated chlorophylls, with H substituted at C-10 by normal exchange, provides the corresponding fully deuterated oxidation products with the C-10 H replaced by OH, as shown by nmr. These results provide critical evidence for the molecular structure of the oxidized chlorophylls and strong support for the structure of the methoxy lactones that are also formed in the allomerization reactions.

In the course of previous work on the nuclear magnetic resonance and infrared spectroscopy of the chlorophylls,[3-5] we tried to prepare the methyl chlorophyllides by enzymatic methanolysis, using a procedure similar to the one used by Holt and Jacobs.[6] The plant material available to us, however, contained an active oxidative enzyme in addition to the chlorophyllase required for the *trans* esterification and we isolated a product, different from both chlorophyll and methyl chlorophyllide, which we decided after brief study must be an oxidized chlorophyll, probably formed by the introduction of an hydroxyl group at C-10.[7] The compounds corresponding to both the a and b series were obtained.[8] Another green compound that appeared to be closely related to the oxidized chlorophylls was also encountered by Barrett and Jeffrey[9] in the extracts of diatoms. Although their substance has not been definitely characterized, it seemed to be an hydroxy derivative of the free acid (chlorophyllide a).

On the basis of further work, we now believe we have definitely established the structure of the "oxidized" chlorophylls prepared enzymatically as 10-hydroxychlorophylls a and b and have shown them to be structurally identical with major products of the allomerization reaction of chlorophylls in methanol. The allomerization of the ordinary chlorophylls and of the fully deuterated chlorophylls thus becomes a practical procedure for the preparation of 10-hydroxychlorophylls.

(1) This work was performed under the auspices of the U. S. Atomic Energy Commission.

(2) Resident Research Associate, from Coe College 1962–1963, under the Associated Colleges of the Midwest–Argonne Semester Program.

(3) G. L. Closs, J. J. Katz, F. C. Pennington, M. R. Thomas, and H. H. Strain, *J. Am. Chem. Soc.*, **85**, 3809 (1963).

(4) J. J. Katz, G. L. Closs, F. C. Pennington, M. R. Thomas, and H. H. Strain, *ibid.*, **85**, 3801 (1963).

(5) J. J. Katz, R. C. Dougherty, and L. J. Boucher in "The Chlorophylls," L. P. Vernon and G. R. Seely, Ed., Academic Press Inc., New York, N. Y., 1966, pp 186–251.

(6) A. S. Holt and E. E. Jacobs, *Am. J. Botany*, **41**, 710 (1954).

(7) F. C. Pennington, H. H. Strain, W. A. Svec, and J. J. Katz, *J. Am. Chem. Soc.*, **86**, 1418 (1964).

(8) H. H. Strain, *J. Agr. Food Chem.*, **2**, 1222 (1954).

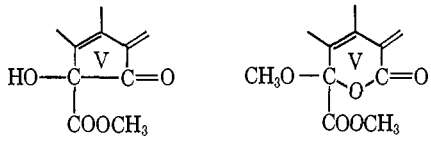
(9) J. Barrett and S. W. Jeffrey, *Plant Physiol.*, **39**, 44 (1964).

In the course of this work, we have also made some nmr observations on other allomerization products of the ordinary chlorophylls and of the fully deuterated chlorophylls that support their previously suggested structures as 10-methoxy lactones.

Results

Allomerization and Oxidation Products.[8] Chlorophyll allowed to stand in alcohol in contact with air undergoes a very complex reaction that yields a variety of products. The most recent investigations of the allomerization reactions of chlorophyll have been reported by Johnston and Watson,[10] Holt,[11] and Gilman and Linschitz.[12] The detailed review by Seely of the allomerization and oxidation reactions of chlorophyll may be consulted for a key to the earlier literature.[13]

It has been suggested that two of the products of the chlorophyll allomerization reaction are the 10-hydroxy derivative **1** and the purpurin 7-lactonem ethyl ether methyl ester (**2**), which originate by attack on the carbocyclic ring V of chlorophyll.



1 **2**

Our nmr and analytical data establish the formation of 10-hydroxychlorophyll by enzymatic oxidation and confirm its identity with one of the allomerization products[7,8] (Johnston and Watson's product Y).[10] Our nmr data are also consistent with, even though they do not prove, Holt's conclusion that another one of the allomerization products is a lactone.

Our allomerization results are somewhat different from those reported by Johnston and Watson[10] in that we observed two rather than three major products. We attribute this difference to the fact that the chlorophyll concentrations we used were different from those of Johnston and Watson.[10] In all our allomerization experiments, several minor components could be detected in the reaction products by chromatography, but we did not investigate these extensively. Separation of the two major components could be more readily accomplished in the case of the chlorophyll a reaction products than with products derived from methyl chlorophyllide a or chlorophyll b.

A distinct isotope effect appears to be operative in the allomerization of deuteriochlorophyll a. The major fractions were indeed obtained from the allomerization reaction, but one of these was found on further chromatography to contain two additional minor components that do not correspond to any of the products obtained from chlorophyll a itself. The major components consisted of 10-hydroxydeuteriochlorophyll a and the corresponding 10-methoxy lactone. The presence of additional products from the allomerization of deuteriochlorophyll a suggests that the course of kinetic events is sufficiently different with deuteriochlorophyll to affect the product distribution, and this is consistent with Holt's view that the allomerization reaction products are formed concurrently rather than sequentially.[11]

Allomerization of deuteriochlorophyll in ordinary methanol makes it easy to determine by proton magnetic resonance spectroscopy where and how much hydrogen has been introduced into the molecule during the reaction. It illustrates the utility of fully deuterated compounds in the study of the chemical behavior of complicated natural products.

Nmr Spectra. The nmr spectra summarized in Tables I and II establish unequivocally that the green allomerization product and oxidized chlorophyll are identical and that they are both 10-hydroxy derivatives. The high-field regions of 10-hydroxychlorophyll and chlorophyll itself are essentially identical; the methyl regions show the same number of methyl and methoxy groups; the low-field methine regions are likewise very similar. The most prominent difference is the disappearance of the C-10 proton resonance at 6.21 ppm, characteristic of the chlorophyll a spectrum, and its replacement by a somewhat broader peak at 6.38 ppm, which we assign to the proton of an hydroxy group. The hydrogen giving rise to this resonance is instantaneously exchanged by CH_3OD, unlike the C-10 proton resonance, which requires hours for complete exchange.[14] A similar situation is provided by the b series, where the C-10 proton of chlorophyll b, which appears at 6.05 ppm, is replaced by a 10-hydroxy peak at 6.51 ppm in oxidized or allomerized chlorophyll.

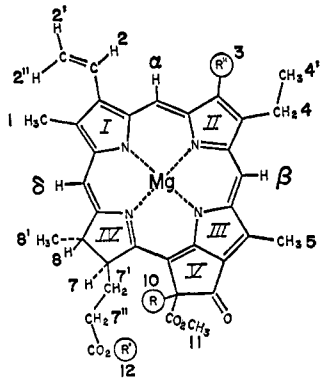
The allomerization reaction of deuteriochlorophyll a[15] yields a fraction chromatographically identical with 10-hydroxychlorophyll. Its nmr spectrum shows only the 10-hydroxy proton resonance; no new methyl groups are introduced into this product. The only other hydrogen present in the 10-hydroxydeuteriochlorophyll a is that introduced by exchange into the δ-methine position. This result and a corresponding experiment with deuteriochlorophyll b provide strong confirmatory evidence for the proposition that oxidized chlorophyll and the corresponding allomerization fraction arise from chlorophyll only by oxidation at the C-10 carbon atom.

A major product of the allomerization reaction of chlorophyll a is a blue-green product, which has been assigned the structure of a 10-methoxy lactone (**2**). Our data from the allomerization of chlorophyll a and methyl chlorophyllide a are consistent with this formulation. The C-10 proton in this substance is no longer visible, no resonance from an hydroxy group can be seen, and a new methyl resonance at 3.86 ppm in the a compound and 3.89 ppm in the b compound appears. The introduction of one, and only one, new methyl group is clearly demonstrated by the results of the experiments with deuteriochlorophylls a and b. These results indicate the presence of a 10-CH_3O group, and this is consistent with the proposed structure of the compound as a methoxy lactone. However, our data do not prove the lactone structure.

Infrared Spectra. The infrared spectra of 10-hydroxychlorophyll are entirely compatible with the pre-

(10) L. G. Johnston and W. F. Watson, *J. Chem. Soc.*, 1203 (1956).
(11) A. S. Holt, *Can. J. Biochem. Physiol.*, **36**, 439 (1958).
(12) S. Gilman, Ph.D. Thesis, Syracuse University, Oct 1956; *Dissertation Abstr.*, **19**, 1929 (1959); University Microfilms (Ann Arbor, Mich.), Publication No. 20821.
(13) G. R. Seely, ref 5, p 91 ff.
(14) R. C. Dougherty, H. H. Strain, and J. J. Katz, *J. Am. Chem. Soc.*, **87**, 104 (1965).
(15) (a) H. H. Strain, M. R. Thomas, H. L. Crespi, M. I. Blake, and J. J. Katz, *Ann. N. Y. Acad. Sci.*, **84**, 617 (1960); (b) H. F. DaBoll, H. L. Crespi, and J. J. Katz, *Biotechnol. Bioeng.*, 4, 281 (1962).

Table I. Nmr Assignments, Chlorophyll a Series[a,b]



Compound	R	R′	R″	Ring V
Chlorophyll a	H	Phytyl	CH_3	Present
10-OH-Chlorophyll a	OH	Phytyl	CH_3	Present
10-CH_3O-Lactone from chlorophyll a	OCH_3	Phytyl	CH_3	Absent
10-OH-Methyl chlorophyllide a	OH	CH_3	CH_3	Present
10-CH_3O-Lactone from methyl chlorophyllide a	OCH_3	CH_3	CH_3	Absent
10-OH-Deuteriochlorophyll a	OH	Phytyl-d_{39}	CD_3	Present
10-CH_3O-Lactone from deuteriochlorophyll a	OCH_3	Phytyl-d_{39}	CD_3	Absent

	α	β	δ	10-H	10-OH	10-CH_3O	12-CO_2CH_3	11-CO_2CH_3	1-CH_3	3-CH_3	5-CH_3
Chlorophyll a (0.07 *M*)	9.36	9.61	8.45	6.21	...	...	...	3.73	3.33	3.29	3.61
10-OH-Chlorophyll a (0.07 *M*)	9.35	9.60	8.43	...	6.38	...	...	3.55	3.26	3.22	3.34
10-CH_3O-Lactone from chlorophyll a (0.04 *M*)	9.40	9.65	8.49	...	...	3.86	...	3.71	3.28	3.24	3.31
10-OH-Methyl chlorophyllide a (0.06 *M*)	9.37	9.62	8.45	...	6.35	...	3.46	3.56	3.28	3.24	3.36
10-CH_3O-Lactone from methyl chlorophyllide a (0.05 *M*)	9.41	9.62	8.50	...	...	3.88	3.47	3.70	3.28	3.25	3.31
Deuteriochlorophyll a	...	...	...	6.21	...	...	...	...	...	...	...
10-OH-Deuteriochlorophyll a (0.06 *M*)	...	...	8.44	...	6.35	...	...	...	...	...	...
10-CH_3O-Lactone of deuteriochlorophyll a (<0.01 *M*)	...	...	8.48	...	...	3.87	...	...	...	...	...

[a] Blank positions in the table mean that no resonance can be observed for the indicated proton position. [b] All spectra were taken in tetrahydrofuran-d_8.

Table II. Nmr Assignments, Chlorophyll b Series.[a,b]

Compound	R	R′	R″	Ring V
Chlorophyll b	H	Phytyl	CHO	Present
10-OH-Chlorophyll b	OH	Phytyl	CHO	Present
10-CH_3O-Lactone from chlorophyll b	OCH_3	Phytyl	CHO	Absent
10-OH-Deuteriochlorophyll b	OH	Phytyl-d_{39}	CDO	Present

	CHO	α	β	δ	10-H	10-OH	10-CH_3O	11-CO_2CH_3	1-CH_3	5-CH_3
Chlorophyll b (0.09 *M*)	11.18	10.09	9.65	8.25	6.05	...	...	3.66	3.19	3.50
10-OH-Chlorophyll b (0.07 *M*)	11.20	10.16	9.72	8.32	...	6.51	...	3.56	3.23	3.40
10-CH_3O-Lactone from chlorophyll b (0.06 *M*)	11.19	10.10	9.65	8.32	...	...	3.89	3.66	3.19	3.33
Deuteriochlorophyll b	...	...	...	...	6.05	...	...	...	...	...
10-OH-Deuteriochlorophyll b (<0.01 *M*)	...	...	...	8.32	...	6.42	...	...	...	...
10-CH_3O-Lactone from deuteriochlorophyll b (0.06 *M*)	...	...	...	8.31	...	...	3.89	...	...	...

[a] Blank positions in the table mean that no resonance can be observed for the indicated proton position. See Table I for structural formula. [b] All spectra were taken in tetrahydrofuran-d_8.

sumed structure (Table III). In CCl_4 solution, 10-hydroxychlorophyll a shows an "extra" band in the carbonyl region comparable to the band shown by chlorophyll a, although the intensity of this absorption peak seems somewhat lower in the 10-hydroxy compound. Katz and co-workers[4] have concluded that this absorption peak in chlorophyll a arises from coordination of the ketone carbonyl oxygen with mag-

Table III. Comparison of the Infrared Spectra of Chlorophylls a and b and 10-Hydroxychlorophylls a and b

Compound	Solvent	Carbonyl, cm^{-1}	Ketone carbonyl (uncoordinated), cm^{-1}	Ketone carbonyl[a] (coordinated), cm^{-1}	Aldehyde carbonyl (uncoordinated), cm^{-1}	Aldehyde carbonyl (coordinated) cm^{-1}
Chlorophyll a	CCl_4	1736	1695	1653		
Chlorophyll a	Tetrahydrofuran	1739	1696			
10-Hydroxychlorophyll a	CCl_4	1734	1693	1666		
10-Hydroxychlorophyll a	$CHCl_3$[b]	1736	1690	1640–1675		
10-Hydroxychlorophyll a	Tetrahydrofuran	1732	1690			
Chlorophyll b	CCl_4	1735	1695	1652	1652	1608
10-Hydroxychlorophyll b	CCl_4	1737	~1704	1682	~1670	1643
10-Hydroxychlorophyll b	$CCl_4 + 5\% C_2H_5OH$	1743	1707		1669	
10-Hydroxychlorophyll b	$CHCl_3$[b]	1739	1702		1660	
10-Hydroxychlorophyll b	Tetrahydrofuran	1743	1703		1661	

[a] The ketone aggregation peak. [b] The ethanol added as a stabilizer was removed by washing repeatedly with water and drying the extracted chloroform.

nesium and have designated it as an "aggregation peak." In polar solvents, self-aggregation by coordination interaction with magnesium does not occur, and in agreement with this, neither chlorophyll a nor 10-hydroxychlorophyll a exhibits an aggregation band in tetrahydrofuran solution. In $CHCl_3$ the coordinated carbonyl band is not clearly resolved but appears as a shoulder in the 1640–1675-cm^{-1} region. The absence of a well-defined aggregation based on this solvent is consistent with the disaggregating effect shown by this solvent in molecular weight studies. Broad absorption at 3250–3550 cm^{-1} in the hydroxyl region of the infrared spectra of 10-hydroxychlorophyll is observed, consistent with the presence of an OH group.

Molecular Weights and Self-Aggregation in Nonpolar Solvents. Molecular weight measurements (Table IV) in CCl_4 and in destabilized chloroform show 10-hydroxychlorophyll to be aggregated in nonpolar solvents. Aggregation in CCl_4 is, as expected, much more pronounced than in $CHCl_3$. Although the aggregation peak of 10-hydroxychlorophyll appears less intense than that of chlorophyll a, nmr methanol titration data[3] confirm the self-aggregation of 10-hydroxychlorophyll and suggest that the extent of self-aggregation is greater for 10-hydroxychlorophyll than for chlorophyll a itself in CCl_4 solution.

Table IV. Molecular Weights and Aggregation of 10-Hydroxychlorophyll

	10-Hydroxychlorophyll a molarity			10-Hydroxychlorophyll b molarity		
Solvt	Calcd	Obsd	R[a]	Calcd	Obsd	R[a]
CCl_4	0.066	0.027	2.4	0.044	0.010	4.4
	0.045	0.018	2.5	0.022	0.006	3.7
$CHCl_3$[b]	0.052	0.038	1.4			
	0.043	0.032	1.3			
	0.026	0.020	1.3			

[a] Ratio of calculated to the observed molarity. [b] Destabilized by washing with water and drying.

Absorption Spectra in the Visible Region. The absorption spectra of the oxidized chlorophylls and of the allomerized chlorophylls are compared in Table V. In agreement with our earlier reports, the 10-hydroxy derivatives formed by enzymatic oxidation and by allomerization exhibited essentially identical spectral absorption maxima.[8] Their absorption maxima and their absorption ratios were very similar to those of the parent chlorophylls.[16] By contrast, the methoxy lactones exhibited absorption maxima at wavelengths shorter than those of the parent chlorophylls[8,10] and absorption ratios much greater than those of the corresponding chlorophylls. Spectral properties of the fully deuterated species were identical with those of the ordinary hydrogen compounds.

Table V. Spectral Absorption Properties of the Enzymatic (Enzym) Oxidation and the Allomerization (Allom) Products from Ordinary and Fully Deuterated Chlorophylls; Solvent, Diethyl Ether

Compounds	Preparation	Red λ_{max}, mμ	Blue λ_{max}, mμ	$\frac{E_{blue}}{E_{red}}$
C-10 Hydroxy Derivatives				
Oxidized a	Enzym	660.5	428	1.294
	Allom	660	428	1.29
Oxidized deuterio a	Allom	659.5	427	1.30
Oxidized b	Enzym	641	450.5	2.81
	Allom	641	450	2.83
Oxidized deuterio b	Allom	639	447	2.91
Methoxy Lactones				
CH_3O-Lactone a	Allom	656	416	1.82
CH_3O-Lactone deuterio a	Allom	651	415	1.89
CH_3O-Lactone b	Allom	630	442	4.40
CH_3O-Lactone deuterio b	Allom	630	441	4.60

Chromatographic Sequence. The chromatographic sequence of the oxidized chlorophylls, the chlorophyllides, the methyl chlorophyllides, and the leaf chlorophylls and carotenoids was determined to serve as a guide in the separation and characterization of all these pigments. To this end, mixtures of several of these pigments were dissolved in diethyl ether plus petroleum ether. These solutions were filtered into columns of powdered sugar, and the adsorbed pigments were washed with petroleum ether plus 0.5% 1-propanol. To separate the more strongly sorbed pigments, the column was later washed with petroleum ether plus 1.0% 1-propanol. The resultant chromatographic sequence is shown in Table VI. Overlapping zones are indicated by brackets.

(16) H. H. Strain, M. R. Thomas, and J. J. Katz, *Biochem. Biophys. Acta*, 75, 306 (1963).

Table VI. Chromatographic Sequence of Chlorophyllides, Oxidized Chlorophylls, Methyl Chlorophyllides, Chlorophylls, and Carotenoids of a Leaf Extract

Chlorophyllides b and a (most adsorbed)
Neoxanthin
Oxidized chlorophyll b
Methyl chlorophyllide b
Violaxanthin
Chlorophyll b
Chlorophyll b′
Oxidized chlorophyll a
Methyl chlorophyllide a
Lutein
Chlorophyll a
Chlorophyll a′
Carotenes (not adsorbed)

Experimental Section

Enzymatic Hydroxylation. In exploratory experiments, the formation of the oxidized chlorophylls in leaf material was followed by chromatography of the reaction products in columns of powdered sugar with petroleum ether plus 0.5% 1-propanol as the wash liquid.[8] The extent of the reactions was indicated by the diminution of the chlorophyll zones and by the appearance of more sorbed, green substances.

The alteration of chlorophylls a and b to green products varied with the leaf material, with its treatment, with the solvents required for promotion of the reaction, and with the composition of the gas phase. Leaves of several species yielded the chlorophyll oxidation products, but these leaves also contained chlorophyllase, which removed the phytol from the chlorophyll by exchange with water or with alcohols, depending upon the solvent employed as the reaction medium. The former reaction, with water, yielded the green, strongly sorbed chlorophyllides with a free carboxylic acid group. The latter reaction, with alcohols, provided the respective chlorophyllide esters. By careful control of the solvent and the availability of oxygen, either the solvolytic or the oxidative reactions could be made to predominate. With acetone, instead of alcohols, as the solvent for the oxidation reaction, the formation of the chlorophyllide esters was not possible, hence the difficult separation of the chlorophyllide esters from the oxidation products was avoided. The unesterified, strongly adsorbed chlorophyllides, formed in the presence of acetone, were readily separable from the oxidized chlorophylls.

If the leaves were dried or if they were heated in boiling water, previous to the treatment with the solvents, the oxidation of the pigments was retarded. If the fresh leaves were treated with the solvents in a nitrogen (oxygen-free) atmosphere, the chlorophylls were not oxidized, but they were hydrolyzed or alcoholyzed, depending upon the solvent. The oxidation of the chlorophylls appeared to take place most rapidly as soon as the leaves were treated with the solvent. This oxidation of the chlorophylls resembles the induced oxidations that take place when unsaturated fats plus pigments are treated with the oxidase system of legume seeds.[17]

Leaves rich in the oxidative enzyme system were not always available. Dandelion leaves were abundant most of the year, except in winter. Hollyhock leaves, of naturalized plants, were used in the fall. For a year-round supply of suitable leaves, barley seedlings and cocklebur (*Xanthium pennsylvanicum*) plants were grown in the greenhouse.

The crystalline oxidized chlorophylls were prepared from hollyhock leaves treated with methanol in air and also from dandelion and cocklebur leaves treated with acetone in oxygen. The procedure with acetone is described here. The deep green leaves were freed of stems and the principal ribs and cut into small sections with scissors. A 250-g portion of this material was placed in a 4-l. Pyrex bottle. The air was displaced with oxygen, and 250 ml of acetone was added. The loosely stoppered bottle was then rotated (9 rpm) for 1–2 hr. At this stage, 500 ml of methanol and 250 ml of petroleum ether (bp 20–40°) were added, and the rolling was continued for 0.5 hr. The green extract was then decanted from the leaf material and filtered through a thin pad of cotton. The leaves were retreated with 500 ml of methanol plus 250 ml of petroleum ether and rotated for 0.5 hr. Again the solvent was decanted and filtered, and this extraction was repeated twice more, making a total of four extractions.

The green extracts, in about 700-ml portions, were treated with an excess of salt solution, which transferred the pigments to the petroleum ether and removed the methanol. After a second washing with water or salt solution, the petroleum ether was divided into three portions, which were filtered into three columns of powdered sugar (8 × 35 cm). The adsorbed pigments were then washed with petroleum ether containing 0.5% 1-propanol. There was very little chlorophyll a, which was adsorbed below the yellow lutein zone. There was much oxidized a, adsorbed in the lutein zone. There was very little chlorophyll b, adsorbed above the lutein zone. There was much oxidized b, adsorbed above the b zone. Much strongly sorbed pigment remained near the top of the column.

The zones of the oxidized a and b were removed from the column, and the respective pigments were eluted with petroleum ether plus ethanol. The pigments were then transferred to petroleum ether with salt solution and purified by readsorption.

The oxidized b was readsorbed in one large column of powdered sugar. It was washed with petroleum ether plus 1.0, 1.5, and 2.0% 1-propanol. The pigment in the principal zone was then eluted from the sugar with petroluem ether plus ethanol. After removal of the alcohol by washing with water, the oxidized b solution was extracted successively with 100-ml portions of 50, 60, and twice with 70% methanol to remove residual xanthophylls and impurities from the sugar. It was washed once with water, and the oxidized b was crystallized by cooling the petroleum ether solution with carbon dioxide. It was collected by centrifugation and dried under vacuum. Yields varied from about 40 to 60 mg.

The oxidized a removed from the three columns was readsorbed in one column of powdered sugar and washed with petroleum ether alone, thus separating yellow pigments below the strongly adsorbed, oxidized a. The principal oxidized a zone was removed. The pigment was eluted with petroleum ether plus ethanol and transferred to the petroleum ether by washing the elutriate with water. This petroleum ether solution was readsorbed in a single, large column of powdered sugar, and the adsorbed pigment was washed with petroleum ether containing 0.5 and then 1.0% 1-propanol. The principal green zone was removed, and the oxidized a was eluted with ethanol plus petroleum ether. This green solution was washed with water, to remove the ethanol, and then extracted with 100-ml portions of 50, 60, and twice with 70% methanol to remove impurities. It was then washed with water and evaporated in the rotary evaporator. The residue was dissolved in only about 10 ml of the low-boiling petroleum ether and cooled with solid carbon dioxide. Crystals that separated were collected by centrifugation and dried under vacuum. A second crop was often obtained by cooling the mother liquors. Yields varied from 60 to 90 mg. If the oxidized a failed to crystallize under these conditions, the solvent was evaporated; the residue was dissolved in about 10 ml of acetone, and the pigment was crystallized by the careful addition of water.

Anal.[18] Calcd for $C_{55}H_{72}N_4O_6Mg$: C, 72.63; H, 7.98; N, 6.16; OCH_3, 3.41. Found: C, 72.85; H, 8.20; N, 6.29; OCH_3, 3.96.

Allomerization. All allomerization experiments were carried out at a chlorophyll concentration of about 0.0067 *M*. Chlorophyll a (166 mg) was dissolved with stirring in 20 ml of spectrograde methanol. After the solution had been allowed to stand at room temperature in the dark for 3 days, the solvent was evaporated away in a stream of nitrogen. The solid residue was chromatographed on powdered sugar using petroleum ether with 0.5–1.0% propanol as a developing solution. Two major fractions were obtained, plus a minor third fraction, which moved most rapidly on the column, and traces of other minor products, which were sorbed near the top of the column. Both major fractions were cut from the columns and the pigments eluted with anhydrous diethyl ether. The ether was washed with water, 50% methanol, 70% methanol, and then water. The ether solution was dried over anhydrous sodium sulfate and the ether removed under vacuum. Of these two major products, the one obtained in the smallest yield (about 40 mg) was identical with the enzymatically produced product. Nmr spectra (see Table I) and infrared spectra (Table II) indicate that this fraction is 10-hydroxychlorophyll a.

The second major product (about 60 mg) had an nmr spectrum (see Table I) consistent with the 10-methoxy lactone structure.

(17) M. Holden, *J. Sci. Food Agr.*, **16**, 312 (1965).

(18) By Crobaugh Laboratories, Cleveland, Ohio 44114.

Allomerization of deuteriochlorophyll a (120 mg) was carried out in essentially the same way, using 18 ml of methanol. The product corresponding chromatographically to 10-hydroxydeuteriochlorophyll a was isolated (51 mg) and found to have the resonances associated with the 10-hydroxyl and the δ protons (see Table I).

Chlorophyll b (105 mg) was dissolved in methanol (17.3 ml). Dissolution of the chlorophyll b was much slower than for a. After 3 days, the solution was processed as described above for the chlorophyll a allomerization. Again, there were two major fractions, but they did not separate as well as the products did in the a series. Nmr spectra clearly indicated that the product obtained in lowest yield (34 mg) was the same as the enzymatically produced 10-hydroxychlorophyll b. The nmr spectrum of the second major product (54 mg) was consistent with the 10-methoxy lactone formulation (see Table II).

Allomerization of deuteriochlorophyll b was analogous to that of chlorophyll b.

Spectra and Molecular Weights. Nmr spectra were recorded on a Varian HA-100 spectrometer by techniques previously described.[5] All nmr spectra were recorded on tetrahydrofuran-d_8 solutions. Chemical shifts are given in δ, ppm, downfield from hexamethylsiloxane (HMS). Infrared spectra were recorded on a Beckman IR-7; approximately 10% w/w solutions were used in an Irtran cell.[4] Absorption spectra in the visible and ultraviolet were recorded on a Cary 14 spectrophotometer. Molecular weights were measured on a Mechrolab vapor phase osmometer.[4]

Studies on Polynucleotides. LXXII.[1] Deoxyribooligonucleotide Synthesis on a Polymer Support[2]

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Abstract: The concept of carrying out stepwise deoxyribooligonucleotide synthesis on a polystyrene support, which permits condensation reactions in completely homogeneous organic medium such as pyridine, has been successfully developed. A portion of the total phenyl groups in polystyrene was derivatized to yield monomethoxytrityl chloride groups. Condensation reactions with deoxyribonucleosides afforded polystyrene-supported 5′-monomethoxytrityl deoxyribonucleosides. Previously developed methods involving condensations with protected deoxyribonucleoside 5′-phosphates were now used for internucleotide bond synthesis. After condensations, polymer-supported deoxyribooligonucleotides were separated from the excess of reagents and mononucleotide by precipitation from an aqueous medium. Methods for the removal of protecting groups were adapted for work with polymer-supported compounds. The compounds prepared by the new method include: thymidylyl-(3′→5′)-thymidine, thymidylyl-(3′→5′)-deoxycytidine, thymidylyl-(3′→5′)-deoxyadenosine, thymidylyl-(3′→5′)-deoxyguanosine, and a trinucleotide, thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine. The yield at each internucleotide bond synthesis ranged between 88 and 96%.

Chemical synthesis of polynucleotides containing defined nucleotide sequences has formed the subject of extended investigations in recent years and the methods developed have been used successfully in the synthesis of a large variety of ribo- and deoxyribopolynucleotides.[3] In all of the synthetic work hitherto reported, the products obtained after each condensation step to form internucleotide bonds are separated by time-consuming procedures involving mainly column chromatography. Marked rapidity in synthetic work would result if after the condensation reactions the product is present in a form readily separable from the remainder of the reaction components. This concept, while having been expressed in literature from time to time,[4] has recently been developed with striking success by Merrifield for the synthesis of polypeptides.[5] In the Merrifield procedure, a polypeptide chain is built up in a stepwise manner from one end while it is linked by a covalent bond at the other end to an insoluble polymeric support. Shemyakin and co-workers have more recently reported an alternative approach to polypeptide synthesis in which the polymer carrying the growing peptide chain is soluble in the medium of reaction, and therefore the repetitive condensations are performed in completely homogeneous phase.[6]

Clearly, it would be desirable to develop the use of similar concepts for work in the polynucleotide field and in this paper we report on an approach to deoxyribooligonucleotide synthesis by the use of a soluble polymer as a support. In concept, therefore, the approach is similar to that used previously by Shemyakin and co-workers in the peptide field. Preliminary reports of this work have already appeared.[7] A number of other laboratories have also reported activity in this area. Thus, Letsinger and Mahadevan[8] reported an

(1) Paper LXXI in this series is by R. D. Wells and J. Blair, *J. Mol. Biol.*, in press.

(2) This work has been supported by grants from the Life Insurance Medical Research Fund (Grant No. G-62-54), the National Science Foundation (Grant No. GB-3342), and the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service (Grant No. CA-05178).

(3) Selected references which give literature citations are: H. G. Khorana, T. M. Jacob, M. W. Moon, S. A. Narang, and E. Ohtsuka, *J. Am. Chem. Soc.*, **87**, 2954 (1965); R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka, and H. G. Khorana, *ibid.*, 88, 819 (1966); and H. G. Khorana, H. Büchi, T. M. Jacob, H. Kössel, S. A. Narang, and E. Ohtsuka, *ibid.*, **89**, 2154 (1967).

(4) I. H. Silman and E. Katchalski, *Ann. Rev. Biochem.*, **35**, 873 (1966).

(5) For a review see R. B. Merrifield, *Science*, **150**, 178 (1965).

(6) M. M. Shemyakin, Y. A. Ovchinnikov, A. A. Kinyushkin, and I. V. Kozhevnikova, *Tetrahedron Letters*, 2323 (1965).

(7) H. Hayatsu and H. G. Khorana, *J. Am. Chem. Soc.*, **88**, 3182 (1966); Abstracts, 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 1966, p 59C.

(8) R. L. Letsinger and V. Mahadevan, *J. Am. Chem. Soc.*, **87**, 3526 (1965); **88**, 5319 (1966).