CHLOROPHYLL REACTIONS

Oxidation and Isomerization Reactions of the Chlorophylls in Killed Leaves

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Chlorophyll in killed leaves is altered by chemical reactions such as oxidation, hydrolysis, and isomerization. Oxidation is accelerated by enzymatic reactions. Most of the chlorophyll alteration products are green pigments that are readily separated by chromatographic adsorption in columns of powdered sugar. The course of the reactions and the nature of the products depend upon the plant material and its treatment. In the killed leaves of many plants such as mallow, hydrolytic reactions predominate. In leaves of plants such as barley, oxidative reactions prevail. The most abundant chlorophyll oxidation product obtained from leaves is identical with a green pigment isolated from methanol solutions of chlorophyll a oxidized or allomerized by exposure to air; the second most abundant resembles a pigment isolated from methanol solutions of chlorophyll b exposed to air. The principal allomerization products formed by the action of air upon methanol solutions of chlorophylls a and b are not formed in leaves exposed to methanol and air. Neither these allomerization products nor the enzymatic oxidation products found in leaves exposed to methanol are isomerized by the action of heat upon their solutions. All these compounds exhibit a negative phase test. Ferric chloride in alcohol decolorizes chlorophyll a and the allomerized chlorophylls almost instantly, but addition of water or dimethylaniline regenerates the pigments, indicating that a complex addition product rather than an oxidation product is formed with the ferric chloride. When solutions of methyl and ethyl chlorophyllides a and b are heated, each pigment undergoes isomerization yielding another similar pigment: methyl and ethyl chlorophyllides a′ and b′, analogous to chlorophylls a′ and b′. The isomerization of the chlorophylls does not, therefore, involve cis-trans changes at the double bond in the phytyl group. Isomerization and oxidation products of the chlorophylls were not present in living plants subjected to intense light, darkness, or various oxidation and reduction conditions.

When green leaves are killed with various organic solvents, diverse reactions of chlorophylls a and b often yield a complex mixture of green pigments (*15*). Reversible isomerization, accelerated by heat, produces chlorophylls a′ and b′ (*5*, *14*). Hydrolysis and alcoholysis, catalyzed by the enzyme chlorophyllase, yield the acidic chlorophyllides and their esters (*3*, *8*, *18*); and treatment with acids produces pheophytins (*3*, *8*, *18*). Enzymatic oxidations bleach the chlorophyll (*10*, *11*). Spontaneous oxidation, or allomerization, which occurs rapidly in anhydrous methanol, is usually inhibited by the small amount of water contained in the fresh plant material (*2*, *3*, *8*, *15*, *18*).

Examination of the pigments from various plants has now revealed further enzymatic and isomerization reactions of the chlorophylls and their derivatives. In the presence of organic solvents and leaf material, chlorophylls may undergo only one or two of these reactions, or all the reactions simultaneously. As shown by use of chromatographic adsorption methods, these reactions may yield from two to two dozen or more green pigments.

For brevity, the enzymatic transformations of the chlorophylls are illustrated by observations on mallow leaves, in which the well-known hydrolytic reactions (*3*, *18*) predominate, and by results with barley leaves (*10*, *11*), in which hitherto undescribed oxidation reactions prevail. The enzymatic oxidation products of the chlorophylls are then compared with the allomerization and isomerization products obtained under various conditions. These enzymatic and isomerization reactions of the chlorophylls in killed plants stand in sharp contrast to the unusual stability of the green pigments in living photosynthetic organisms (*9*, *12*).

Extraction and Resolution of Pigments

In most experiments, about 5 grams of freshly chopped leaves (2- to 3-mm. squares) were treated with about 10 ml. of the solvent and kept in the dark. After suitable reaction periods, the pigments were extracted from the leaves by grinding with sand and methanol (100 ml., absolute) or acetone (100 ml., 80 to 90%) to which petroleum ether (50 ml.) had been added. Pigments in the green extracts were transferred to the petroleum ether by the addition of concentrated salt solution (about 500 ml.). The green petroleum ether solution that separated was washed with water, and portions of it were sucked into adsorption columns of powdered sugar (1.2 to 3.2 cm. in diameter by 20 to 26 cm. tall) until a green zone about 1 or 2 cm. deep was formed. The chromatogram was then developed from this zone with a solution of propanol (0.5 to 1% and up to 10%) in petroleum ether. Pigments separated in the columns were compared by their relative adsorbability (Table I). After elution from the sugar, they were identified by chromatographic adsorption with authentic preparations, by spectral absorption properties, and by reaction with alkalies and acids (*2*, *13*, *18*).

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Effect of Heat, Dehydration, And Freezing

Rapid extraction of chopped mallow or barley leaves yielded unaltered chlorophylls a and b, as indicated by column I in Table I. Chopped leaves that had been heated in boiling water for 1 or 2 minutes yielded chlorophylls a′ and b′ in addition to the unaltered chlorophylls, as shown by column II. Leaves that had been killed by freezing and thawing yielded no additional green pigments. Chopped leaves that had been dried in air (at 20° for about 24 hours) yielded the normal complement of pigments plus chlorophylls a′ and b′ and pheophytins (*5*, *14*).

Reaction of Chlorophylls

Mallow Leaves When treated with methanol (10 ml.), the chopped leaves of mallow (5 grams) turned yellow in from 3 to 6 hours, forming many small, green, microscopic crystals analogous to the so-called "chlorophyll crystals" reported by Borodin (*1*). When these pigments were extracted and adsorbed in columns of powdered sugar, small amounts of the acidic chlorophyllides and large amounts of the less adsorbed methyl chlorophyllides were obtained, as indicated by column III in Table I. The strongly adsorbed, acidic chlorophyllides were not separated from each other until the column had been washed with petroleum ether containing about 5 to 10% propanol. All these green pigments exhibited spectral absorption maxima at the same wave length as the chlorophyll from which they were derived. All gave positive (yellow) phase tests (*18*).

Mallow leaves that had stood with methanol for 20 to 24 hours sometimes yielded a series of pheophytins, some of which were formed in the adsorption column itself. These decomposition reactions, which did not occur with the purified chlorophyllide esters, were retarded by dimethylaniline (5%) added to the methanol during formation of the chlorophyllides and by dimethylaniline (0.5%) added to the petroleum ether–propanol mixture employed for development of the chromatograms.

Mallow leaves that were allowed to stand with methanol or with methanol plus 5% dimethylaniline for 24 hours or more still contained the green crystals, but the chlorophylls and the methyl chlorophyllides had been oxidized to other pigments that no longer gave a positive yellow phase test (*18*). In the sorption columns, one of these oxidation products formed a large yellow-green zone near the neoxanthin zone. Another formed a large green zone between the neoxanthin and the violaxanthin. These oxidation products were not formed in leaves stored with methanol in a vacuum, in heated leaves, or in leaves dried in air and then treated with water and with methanol. Formation of the oxidation products must, therefore, be attributed to labile enzyme or oxidation systems that may catalyze or induce (*10*, *11*) the partial oxidation of the chlorophylls.

Barley Leaves Barley leaves, particularly the first green leaf of seedlings, contained the oxidative enzyme system (*11*) but not the chlorophyllase system. Chopped barley leaves that had been stored with methanol in a vacuum for 1 or 2 days yielded principally unaltered chlorophylls; leaves that had been kept in air yielded most of the green pigments in the form of two nonacidic oxidation products similar to some of those obtained from mallow leaves. One of these products with spectral absorption properties like those of chlorophyll b separated in the adsorption columns as a yellow-green zone above the violaxanthin. The other oxidation product with spectral absorption properties like those of chlorophyll a formed a green zone extending barely below the unoxidized chlorophyll b. With benzene as solvent this second oxidized chlorophyll was more adsorbed than the chlorophyll b, and the two pigments were easily separated by readsorption.

The two chlorophyll oxidation products obtained from barley leaves treated with methanol were identical with the analogous oxidation products obtained in small yields from mallow. These products were also found among the allomerization products of the chlorophylls, one product being derived from chlorophyll b, the other from chlorophyll a (Table II).

As with mallow, the oxidation enzymes or systems of barley resisted freezing and thawing, but did not withstand dehydration in air. This inactivation of the oxidative enzymes by dehydration may account for the better yields of the chlorophyllides usually obtained from dried leaves (*18*).

In the presence of methanol, the relative activity of the chlorophyllase and of the oxidative enzymes varied greatly in different plants. Chlorophyllase activity was most pronounced in the leaves of an arum (*Arum italicum*), Catalina cherry (*Prunus*), *Coreopsis lanceolata*, hollyhock (*Althaea*), iris, Swiss chard (*Beta*), water cress (*Rorippa*), and chloroplasts of Swiss chard. Oxidation reactions of the chlorophylls predominated in potato leaves as well as in barley. There was little enzymatic alteration of chlorophylls in the leaves of *Bryophyllum*, California poppy (*Eschscholtzia*), incense cedar (*Libocedrus*), ivy (*Hedera*), madroño (*Arbutus*), and tobacco (*Nicotiana*). Geranium (*Pelargonium*) and *Bryophyllum* yielded pheophytins. With the exception of *Bryophyllum*, leaves of all these plants yielded green crystals, yet a variety of products had been formed in many leaves, as shown by the chromatographic analysis.

Table I. Pigments Obtained from Mallow Leaves

The color and the names of pigments are listed in the order in which they occur in the adsorption columns of powdered sugar. The xanthophylls and carotenes, which vary little with the different conditions, are indicated by Y. Relative proportions of the several green pigments are indicated by the + and − signs. The solvent was petroleum ether plus propanol (0.5 to 10%).

Color	Name, and Adsorption Sequence of Pigments in Columns of Powdered Sugar	Extraction I Rapid methanol or acetone	Extraction II Heated	III Reaction with Methanol, 3 to 6 Hours	IV Reaction with Acetone or Methyl Acetate, 10 to 20 Hours
YG	Chlorophyllide b	−	−	+	+++
G	Chlorophyllide a	−	−	+	+++
	Neoxanthin	Y	Y	Y	Y
YG	Methyl chlorophyllide b	−	−	+++	−
	Violaxanthin	Y	Y	Y	Y
YG	Chlorophyll b	++++	+++	+	±
YG	Chlorophyll b′	−	+	−	−
G	Methyl chlorophyllide a	−	−	+++	−
	Lutein + zeaxanthin	Y	Y	Y	Y
G	Chlorophyll a	++++	+++	+	±
G	Chlorophyll a′	−	+	−	−
	Carotenes	Y	Y	Y	Y

Reaction of Chlorophylls with Ethanol, Acetone, and Methyl Acetate

Ethanol reacted with the chlorophylls in chopped leaves of mallow and of barley, yielding products analogous to those obtained with methanol. Relative to the xanthophylls, the ethyl chlorophyllides obtained from mallow were slightly less adsorbed in columns of sugar than the corresponding methyl compounds. In separate tests, mixtures of ethyl and methyl chlorophyllide a or of ethyl and methyl chlorophyllide b were resolvable in columns of sugar, provided the initial zones of the adsorbed pigments were only a few millimeters deep in columns about 25 cm. tall.

When treated with acetone, or with methyl acetate, for 10 to 20 hours, freshly chopped leaves of mallow formed large

amounts of the strongly adsorbed chlorophyllides (*3*, *18*) with concomitant loss of the chlorophylls, as illustrated by column IV in Table I. Chopped mallow leaves that were permitted to stand in acetone in the presence of air for more than 24 hours formed several green oxidation products that were more adsorbed than the unoxidized acidic chlorophyllides. These substances gave a negative (green) phase test, and they were extractable from their solution in ether with 0.01*N* aqueous potassium hydroxide.

Barley leaves treated with acetone, or with methyl acetate, in the presence of air yielded primarily the chlorophyll oxidation products that were observed when these leaves were treated with methanol or ethanol and that have now been found among the allomerization products of the chlorophylls.

Allomerization of Chlorophyll a

For comparison of the enzymatic oxidation products with the allomerization products, chlorophyll a, isolated from 15 grams of fresh barley leaves, was dissolved in absolute methanol (50 ml.), and this solution was allowed to stand in a loosely stoppered flask in the dark for 1 to 2 days (*3*, *18*). When transferred to petroleum ether and adsorbed in a column of powdered sugar (6.7 by 27 cm.), this allomerized chlorophyll yielded in largest proportion, a "blue-green" pigment that was slightly more adsorbed than chlorophyll a, and that exhibited absorption maxima at wave lengths about 10 mμ shorter than those of chlorophyll a. It yielded smaller amounts of a more adsorbed green pigment with absorption maxima near those of chlorophyll a, and still smaller amounts of several other more adsorbed and less adsorbed green pigments (*15*).

The green pigment isolated from the allomerized chlorophyll a is identical with the principal enzymatic oxidation product obtained from barley leaves (see Table II). A mixture of these two preparations formed a single green zone in columns of powdered sugar when either benzene or petroleum ether plus propanol was used as the solvent. This mixture was slightly less adsorbed than chlorophyll b when petroleum ether plus 0.5% propanol was employed as solvent; it was more adsorbed than chlorophyll b when benzene was the solvent. This green oxidation product formed by allomerization or by enzymatic oxidation exhibited a pronounced spectral absorption maximum at the following wave lengths: in petroleum ether (blue-green solution), 662 mμ; in methanol (green solution), 667 mμ; in methanol plus 10% potassium hydroxide for 1 to 2 minutes, 667 mμ, for 15 minutes, 647 mμ; in ethyl ether after treatment with methanol plus potassium hydroxide for 15 minutes, followed by transference to the ether with water and acetic acid, 652 mμ; in ethyl ether after treatment with methanol plus potassium hydroxide for 15 minutes, followed by transference to ether with water plus acetic acid and then treated with concentrated hydrochloric acid and retransferred to ether with water, 667 mμ (*14*). This green pigment, obtained by allomerization or by enzymatic oxidation, gave a negative (green) phase test, and it formed a pheophytin with absorption maxima at 655 mμ in methanol plus hydrochloric acid and at 667 mμ in methanol plus dimethylaniline. With ferric chloride in methanol it formed yellow solutions from which the unaltered pigment could be regenerated with dimethylaniline. In so far as the formation of this green pigment is concerned, the enzymatic oxidation of chlorophyll a in barley leaves and the allomerization of chlorophyll a in absolute methanol are analogous reactions.

The blue-green pigment from allomerized chlorophyll a exhibited pronounced spectral absorption maxima at the following wave lengths: in petroleum ether (blue solution) 658 mμ and below 440 mμ; in methanol (blue solution) 656 mμ and below 440 mμ; in methanol plus 10% potassium hydroxide for 2 to 15 minutes, 675 mμ; in ethyl ether after treatment with methanol plus potassium hydroxide, followed by neutralization with acetic acid, 650 mμ; and in ethyl ether after treatment with methanol plus potassium hydroxide, transference to ether by acetic acid, and reaction with hydrochloric acid, followed by retransference to ether, 670 mμ (*2*). The blue-green allomerization product itself gave a negative phase test, and it formed a pheophytin with absorption maxima at 650 mμ in methanol plus hydrochloric acid and 760 mμ in methanol plus dimethylaniline. With ferric chloride in methanol, it also yielded a yellow solution from which unchanged pigment could be recovered after treatment with water or with dimethylaniline.

When mixed with an extract of leaves and adsorbed in columns of powdered sugar (petroleum ether plus 0.5% propanol as solvent), the blue-green allomerized chlorophyll formed a blue-green zone just below the lutein and well above the chlorophyll a, so that small quantities of this pigment were easily detectable when added to the leaf extracts. Even with this sensitive analytical procedure, the blue-green allomerization product was not found among the green enzymatic oxidation products extracted from barley leaves that had stood with methanol in air. With respect to the formation of this blue-green pigment, allomerization of chlorophyll in methanol differs from the enzymatic oxidation of chlorophyll in barley leaves.

Each of the allomerization products yielded only very small amounts of more adsorbed pigments when permitted to stand in solution in anhydrous methanol. When solutions of the allomerization products in propanol were heated to 100° for several minutes, other pigments were not formed.

Most of the minor allomerization products exhibited spectral absorption properties similar to those of chlorophyll a, but one weakly adsorbed product exhibited maximum absorption at 680 and 640 mμ in methanol.

An allomerization of chlorophyll a′ in methanol yielded the same products obtained from chlorophyll a. Sealed in a vacuum, a methanol solution of the chlorophylls remained unchanged for weeks. In solution in acetone and exposed to air, chlorophylls a and a′ were allomerized very slowly, yielding small amounts of the more adsorbed green pigments (presumably the hydroxy derivative) but none of the blue-green pigment (presumably the methoxy derivative) (*3*, *8*).

Table II. Principal Pigments Formed by Oxidation of Chlorophylls a and b Under Various Conditions

Relative sorbability was determined in columns of powdered sugar with petroleum ether plus 0.5% propanol as solvent.

Chlorophyll a	In absolute methanol	Blue-green pigment λ max., methanol, 656 mμ. Sorbed below lutein and above chlorophyll a
	In barley plus methanol	Green pigment λ max., methanol, 667 mμ. Sorbed above lutein and barely below chlorophyll b
Chlorophyll b	In absolute methanol	Light green pigment λ max., methanol, 636 mμ. Sorbed above chlorophyll b and below violaxanthin
	In barley plus methanol	Green pigment λ max., methanol, ca. 653 mμ. Sorbed just above violaxanthin and below neoxanthin

Oxidation of Chlorophyll a With Ferric Chloride

Chlorophyll a reacts instantly with ferric chloride in absolute methanol, forming a yellow solution from which chlorophyll a can be regenerated with reducing agents, a phenomenon frequently attributed to oxidation and reduction of the green pigment (*6*, *17*). Attempts to isolate chlorophyll oxidation products from the yellow solution have now revealed, however, that unaltered

chlorophyll a may be regenerated from the yellow solution containing ferric chloride without the addition of reducing agents. Upon addition of petroleum ether and water to the yellow solution, unchanged chlorophyll a was transferred to the petroleum ether. Addition of dimethylaniline or of dimethylaniline plus an excess of ferric chloride to the yellow solution also regenerated the chlorophyll a. Addition of dimethylaniline either to the ferric chloride solution or to the chlorophyll solution prevented the formation of the yellow product when the solutions were mixed. With reaction periods of about 24 hours, several pheophytins and green oxidation products were formed from chlorophyll a that had been permitted to stand with ferric chloride and dimethylaniline in 90% methanol.

Allomerization of Chlorophyll b

Allomerization of chlorophyll b in methanol, as described with chlorophyll a, produced a variety of green pigments that were separable in the adsorption columns. As with chlorophyll a, the principal allomerization product was lighter green than the parent chlorophyll. It exhibited spectral absorption maxima at wave lengths shorter than those of chlorophyll b. A more adsorbed product, formed in much smaller proportions, exhibited spectral absorption properties like those of chlorophyll b. Both pigments gave negative phase tests, and both decomposed very rapidly when preserved in alcohol-free petroleum ether, in which they were slightly soluble (see Table II).

The principal light green allomerization product exhibited the following absorption maxima: in petroleum ether, 631 and about 442 mμ; in methanol, 636 and about 458 mμ; in methanol plus 10% potassium hydroxide, 635 and about 455 mμ; in ethyl ether after treatment with potassium hydroxide and acetic acid, 631 and about 446 mμ; and in ether after treatment with potassium hydroxide and hydrochloric acid, 656 and less than 431 mμ. The light green allomerization product formed a pheophytin that exhibited absorption maxima at 643 and less than 425 mμ in methanol plus hydrochloric acid and at 661 and less than 425 mμ in methanol plus dimethylaniline. In columns of powdered sugar, the allomerized product was adsorbed below violaxanthin and above chlorophyll b. It was not found among the enzymatic oxidation products of chlorophyll b in barley leaves.

Chlorophyll b itself showed the following properties: absorption maxima in petroleum ether, 643 mμ; in methanol, 652 mμ; in methanol plus 10% potassium hydroxide, 621 mμ; in ether after treatment with potassium hydroxide and acetic acid, 626 mμ; and in ether after treatment with potassium hydroxide and hydrochloric acid, 654 mμ. Pheophytin b exhibited absorption maxima at the following wave lengths: in methanol plus hydrochloric acid, 647 mμ; in methanol plus dimethylaniline, 659 mμ.

Isolation and Isomerization of Methyl and Ethyl Chlorophyllides

Chopped leaves of mallow (5 grams) were permitted to stand with methanol or ethanol (10 ml.) plus dimethylaniline (0.5 ml.) in a stoppered flask for about 5 hours. The pigments were then extracted, transferred to petroleum ether, and adsorbed in two columns of powdered sugar (3.2 by 26 cm.). The chlorophyllide esters (cf. column III in Table I) were eluted from the sugar with methanol and transferred to about 30 ml. of petroleum ether plus a little ether, and each ester was readsorbed in short columns of powdered sugar (3.2 by 10 cm.). After the columns had been washed with petroleum ether plus benzene (10 to 20%) in order to carry residual carotenoids below the green bands, the chlorophyll esters were eluted and crystallized from ethyl ether by the addition of petroleum ether (*3*, *18*).

When heated in propanol solution at 100° for a few minutes, each methyl and ethyl chlorophyllide yielded a mixture of two pigments that were separable in the adsorption columns. As shown by chromatographic experiments, one of these two pigments was identical with the unheated chlorophyllide ester. Each pigment of each pair was converted into the other when the solutions were heated. This same interconversion occurred almost instantly in the presence of traces of potassium hydroxide. Because these reactions are analogous to the isomerization of the chlorophylls, which is also catalyzed by alkalies, the new isomers of the chlorophyllides should be called methyl and ethyl chlorophyllides a′ and b′.

Reactions of Chlorophylls In Living Plants

Once plants have become green, the chlorophylls in the living tissue are remarkably constant in kind, regardless of the conditions to which the plants are exposed (*4*, *7*, *9*, *12*, *16*). For example, leaves of *Byrophyllum* exposed to light from a north window and kept in oxygen, in hydrogen, or in air plus 12% carbon dioxide for a month or two yielded the normal complement of chlorophylls a and b with not more than traces (less than 1%) of other green pigments. Similar leaves kept in the dark in air for a month also yielded primarily chlorophylls a and b.

Siphonaceous green algae exposed to full sunlight for several hours or kept in darkness for several days yielded chlorophylls a and b and little if any other green pigment. Under similar conditions, another alga, *Polyedriella helvetica*, yielded the normal chlorophyll a and no other green pigment. Green leaves of barley seedlings kept for several days at 18° in a stream of hydrogen and exposed to light from a north window or preserved in darkness yielded the normal amounts of chlorophylls a and b.

Discussion

The enzymatic hydrolysis, alcoholysis, and oxidation of the chlorophylls in leaves occur most rapidly in the presence of solvents that are miscible with water, dissolve the pigments, and do not completely dehydrate or harden the tissues and enzyme systems. These results and the stability of the pigments in frozen and thawed leaves suggest that, in the chloroplasts, the chlorophylls are isolated or protected from the oxidative and hydrolytic enzymes.

Because the methyl and ethyl chlorophyllides lack the phytyl group yet undergo isomerization analogous to that of the chlorophylls, the reversible isomerization of the chlorophylls must involve a modification of the phorbin nucleus rather than a cis-trans isomerization of the unsaturated phytyl group. Formation of identical allomerization products from chlorophylls a and a′ and the failure of the allomerization products to yield isomers when heated in propanol indicate that the molecular structure involved in the a $\leftrightharpoons$ a′ equilibrium is stabilized or destroyed by the oxidation. As allomerization affects the chlorophyll molecule through introduction of a hydroxyl or methoxyl group at carbon atom 10 (*3*, *8*), and as this change prevents the formation of interconvertible isomers, spatial changes of the hydrogen atom and the carboxyl group normally attached to this carbon atom might account for the reversible isomerization of the chlorophylls and the chlorophyllides.

The absence of chlorophyll alteration products in leaves exposed to various conditions supports the view that chlorophylls do not undergo extensive chemical change during photosynthesis (*12*).

Literature Cited

(1) Borodin, J., *Botan. Ztg.*, **40,** 608 (1882).

(2) Conant, J. B., Dietz, E. M., Bailey, C. F., and Kamerling, S. E., *J. Am. Chem. Soc.*, **53,** 2382 (1931).

(3) Fischer, H., and Stern, A., "Die Chemie des Pyrrols," II Band, 2. Hälfte, Leipzig, Akademische Verlagsgesellschaft, 1940.

(4) Frank, S. R., *J. Gen. Physiol.*, **29,** 157 (1946).

(5) Manning, W. M., and Strain, H. H., *J. Biol. Chem.*, **151,** 1 (1943).

(6) Rabinowitch, E. I., "Photosyn-

thesis," Vol. I, p. 464, New York, Interscience Publishers, 1945.
(7) Shiau, Y. G., and Franck, J., *Arch. Biochem.*, **14,** 253 (1947).
(8) Stoll, A., and Wiedemann, E., *Fortschr. Chem. org. Naturstoffe*, **1,** 159 (1938).
(9) Strain, H. H., in Loomis, W. E., and Franck, J., "Photosynthesis in Plants," Chap. 6, Ames, Iowa State College Press, 1949.
(10) Strain, H. H., *J. Am. Chem. Soc.*, **63,** 3542 (1941).
(11) Strain, H. H., "Leaf Xanthophylls," Washington, Carnegie Institution of Washington, 1938.
(12) Strain, H. H., *Science*, **112,** 161 (1950).
(13) *Ibid.*, **116,** 174 (1952).
(14) Strain, H. H., and Manning, W. M., *J. Biol. Chem.*, **146,** 275 (1942).
(15) Strain, H. H., Manning, W. M., and Hardin, G., *Ibid.*, **148,** 655 (1943).
(16) Wassink, E. C., *Antonie van Leeuwenhoek*, **12,** 281 (1947).
(17) Watson, W. F., *J. Am. Chem. Soc.*, **75,** 2522 (1953).
(18) Willstätter, R., and Stoll, A., "Untersuchungen über Chlorophyll," Berlin, Julius Springer, 1913.

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INSECTICIDE RESIDUE ANALYSIS

Sodium Reduction Technique for Microdetermination Of Chlorine in Organic Insecticides

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The sodium digestion technique has been modified for application to the determination of residues of organic chloride materials on food products. This technique offers the simplicity of the conventional sodium reduction method, with accuracy and precision comparable to those obtained with the combustion technique. This procedure could be useful in the study of residues where the spray history is known and in the quality control laboratory where such information may not be complete. The technique is particularly well suited for use as a screening test in the food packer's quality control laboratory.

ROUTINE CONTROL DETERMINATION of residual amounts of organic chlorides is a major problem faced by food processors as a result of the development and use of modern pesticides. There are specific methods for determining most of the compounds that fall in this category, but at best these methods are difficult to employ routinely in a quality control laboratory. It is not practical for the average control laboratory to perform a wide variety of specific tests on all fresh foodstuffs to be packaged; nevertheless, there is a definite need for specific methods. The practical approach is to employ a rapid, sensitive screening test that will indicate the presence or absence of chlorinated hydrocarbons, followed by colorimetric analysis when further identification is necessary.

The available methods for determining total organic chlorine were investigated, in order to select the one that would be most suitable for routine control analyses, and would permit participation in a collaborative study of the effects of canning procedures on dieldrin residues on peaches.

The most widely used methods of determining residues of organic chlorides are the sodium reduction (*3–5*) and combustion (*1, 2, 6*) techniques. The sodium reduction method, although readily adapted to large volume analyses, was not found satisfactory for estimating dieldrin, and its sensitivity, approximately 100γ of chlorine (*3*), was not adequate. The combustion technique described by Agazzi *et al.* (*2*) exhibits good precision, accuracy, and sensitivity, but would be difficult to employ as a routine procedure in the small food control laboratory.

The sodium digestion method was modified to improve its sensitivity and effectiveness. In brief, the procedure consists of six steps and the modifications described herein are concerned with steps 3, 5, and 6.

1. Removal of the organic residue from the surface of the raw material.
2. "Cleanup" of the strip solution and evaporation of the solvent.
3. Refluxing the residue with metallic sodium in isobutyl alcohol.
4. Elimination of excess sodium.
5. Preparation of the reflux mixture for amperometric titration with silver nitrate solution.
6. Titration and final calculation of results.

Special Apparatus

Amperometric Titration Assembly. The apparatus employed consisted of a sensitive current-measuring instrument (Fisher Elecdropode), a rotating platinum electrode, and a saturated calomel electrode. This equipment has been described and discussed (*1, 2, 6, 8*).

Büchner funnel, M porosity, 65 mm. in diameter.

Burets, 2-ml., graduated in 0.01-ml. units.

Flasks, 50-ml., round-bottomed, semi-ball joint 28/15.

Condensers, West type, length 16 inches, ball and socket joint No. 28/15.

Heating mantles, 50-ml. size.

Reagents

Acetone, C.P.

Acetone solution, equal parts by volume of acetone and distilled water.

Calcium carbonate, low in alkalies.

Celite No. 545 (Johns Manville Co.).

Filter-Cel (Johns Manville Co.).

Gelatin Solution, 1%. Dissolve 1 gram of C.P. gelatin in 100 ml. of hot distilled water and add 1 ml. of C.P. chloroform.

Isobutyl alcohol, boiling point 106–108° C.

Isopropyl Alcohol Solution. Mix equal parts by volume of 99% isopropyl alcohol and distilled water.

Mineral oil, white, chlorine-free.

Nitric Acid Solution. Mix 1 volume of concentrated acid with 1 volume of distilled water.