THE PHYSICAL STATE OF CHLOROPHYLL IN THE LIVING PLASTID

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Introduction.

In the green parts of plants, the chloroplasts, containing chlorophyll, CO_2 is absorbed. In the presence of light, the CO_2 is reduced, O_2 is liberated and carbohydrates are formed:

$$6\ CO_2 + 6\ H_2O = C_6\ H_{12}\ O_6 + 6\ O_2.$$

The process of photosynthesis might be considered as a transference of hydrogen, as expressed clearly by Muller (48). According to this theory a chlorophyll—CO_2 complex is formed, which accepts, per molecule of CO_2, four hydrogen ions, generated by four molecules of water with the production of two molecules of hydrogen peroxide. The reduction of CO_2 goes parallel with an increase of the free energy of 110.000 to 120.000 cal per mol. The energy needed for this process is absorbed by the coloured complex, present in the plastids. In his theory, the pigment plays therefore a physical as well as a chemical role.

Merely bringing together chlorophyll, CO_2 and water does not start the reduction of CO_2 in the presence of light. In artificial media, containing chlorophyll in some form or another, even in the presence of catalysts, no trace of photosynthesis has ever been found (11) (71).

As, presumably, the pigment plays an active part in the process, it may be that, in the living plastid, the pigment is present in some state still unknown.

Lubimenko (42) calls this hypothetical entity: "la chlorophylle naturelle". but we prefer the name Phyllochlorin, given by Mestre (45).

Statement of problem.

Several theories have been formed to explain the physical state of chlorophyll in the living plastids. These theories are mainly based on spectroscopic or spectrophotometric evidence. Since the spectroscopy of chlorophyll, on the one hand, has been chiefly performed by biologists and chemists, but little attention has often been paid to the spectroscopic technique and accuracy, while the studies carried out by physicists often neglect the known peculiarities (photolability, behaviour towards alcohol, acids and alkalies and allomerisation) of the pigment. We may refer here to the work of Willstätter and Stoll (72) who studied systematically the influence of alcohol, acids and alkalies on the chlorophyll molecule. Stoll (65) has shown that the use of stock solutions of chlorophyll has to be avoided. He found that acetonic solutions of chlorophyll were optically-active, but lost this property rather quickly even if these solutions were kept in absolute darkness

under the exclusion of air. Ursprung (68) has called attention to the spectroscopic technique. For particulars on this subject see Chapter X.

The task we have set ourselves was to investigate and compare the validity of the existing theories concerning the state of the green pigment in the living plastids.

Although in this test we chiefly used the spectrograph, other methods of attack were not neglected. As the colloid chemistry is being more fully investigated by H. A. Bakker (6) and the cytological evidence is being examined by Soeur Christiane Doutreligne, we shall dwell but briefly upon these matters, and only as they are pertinent to the problem at hand.

In the spectroscopical study we have limited ourselves mainly to the study of the position of the first (or main) absorption band in the extreme red end of the spectrum.

The comparative study of the position of the main band in the absorption spectrum of chlorophyll, in different media, yield certain conclusions which enable us to prove certain theories, concerning the state of chlorophyll in the living plastids, to be untenable, while, on the positive side, valuable information may be gathered as to the most probable way in which the green pigment is distributed in the plastids. We have further given attention to the fact that the coloured complex, present in the living plastids, shows a weak be undeniable fluorescence (50) and is highly photostable, two facts which enable us to exclude some theories, which appear to be tenable if only the absorption spectrum of the pigment is taken into account (29).

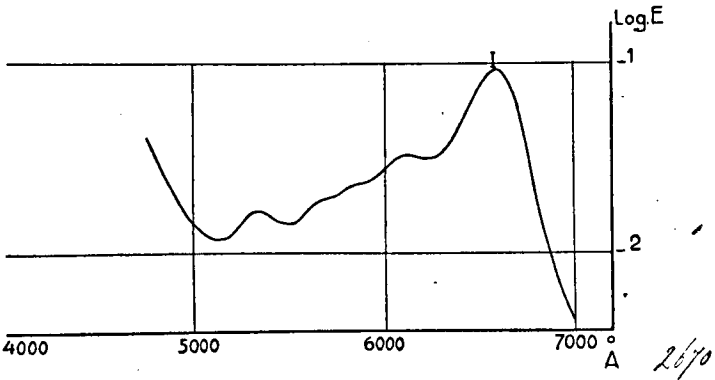

Fig. 1. Extinction curve of an acetone-solution of chlorophyll (a + b).

These points will be elucidated in the following chapter.

Fig. 1 shows the absorption spectrum of the pigment (a + b) in acetone. The curve is obtained with the Keuffel and Esser colour analyser and constructed after the methods described in Chapter IV.

CHAPTER I.

Historical review.

In 1873, Kundt (36) showed that the absorption bands of several pigments have a different position in different media. He came to the conclusion that a rather close connection existed between the band position and the refractive index of the medium. With increasing refractive index of the solvent, the absorption band shifts to the long wavelengths (small frequencies). Formanek (see 18, p. 27) has found that this phenomenon, generally known as Kundt's law, only occurs in 50% of all cases studied by him. S. Hecht (19) has used Kundt's rule to account for the so-called "Purkinje phenomenon" in human vision. It must be remarked here, that Kundt's rule is only used in his paper as an empirical tool and that no theoretical implications are considered. In the original publication of Kundt, six pigments are studied, among which only chlorophyll followed the rule strictly. Further details will be given in Chapter VI.

Reinke (55) in 1883 gives a simple but ingeneous method to estimate the band maximum of the living leaf spectroscopically. To diminish the loss of light by reflection on the cell walls, the leaves are immersed in water. In this way he was able to study the absorption spectrum of a layer of 19 leaves placed before the slit. He made a drawing to this spectrum. By removing the leaves one after another, he obtained nineteen drawings, who, when placed above each other, give an idea of the form of the absorption bands, as the bands narrow gradually with decreasing number of leaves. From his drawing we observe that the band is rather asymmetric. This proves that only very thin leaves should be used to estimate the band maximum spectroscopically. He finds no trace of fluorescence, neither in the living leaves, nor in press juices obtained from those leaves, and states that the majority of workers agree on this point. This statement is very remarkable as Stokes had observed the fluorescence of the living leaf as early as 1852 (see also on this point, Stern (64a). If chlorophyll is imbedded in solid paraffin, all fluorescence disappears on solidification. Reinke comes to the conclusion that the chlorophyll is present, in the living leaf,

in the solid state, and bases this conclusion on the following points;

I. True solutions of chlorophyll are fluorescent.
II. The living plastid is non-fluorescent.
III. Dry chlorophyll is non-fluorescent.

In 1884 he gives an explanation of his failure to detect the fluorescence of the plastids (56). Using a sufficiently high light intensity, he now observes a very weak fluorescence band. Solid paraffin-chlorophyll showed a weak fluorescence too. Starting from the functional side of the problem, he now states that it is highly improbable that chlorophyll should be imbedded in some waxy substance. In analogy with the supposed heamoglobin-protein complex, he mentions the possibility of the existence of a similar chlorophyll-protein complex; this complex being very sensitive towards ether, alcohol, acetone etc.

Iwanowski (29) in 1907 tries to decide between the two leading theories;

I. Chlorophyll is dissolved in a medium of high refractive index.
II. Chlorophyll is present in the solid state.

Fig. 2 may elucidate his results.

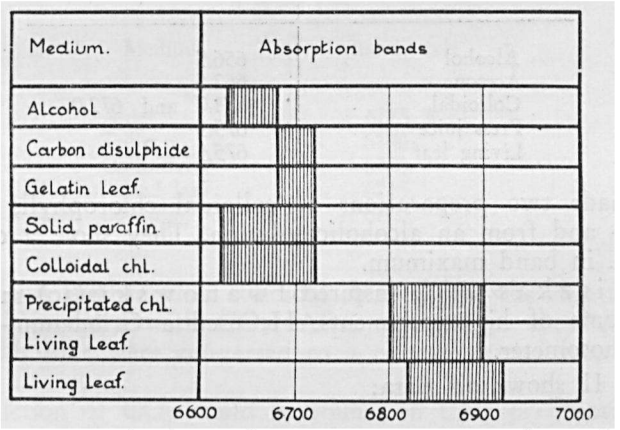

Fig. 2. Band position of chlorophyll in different media according to Iwanowski.

The gelatin leaves of Lommel (41) and the paraffin-chlorophyll of Reinke (55) (56) possess a "solution spectrum". It should be noted that the axis of colloidal chlorophyll lies between the axes of the alcoholic solution and of the CS_2 solution. Iwanowski observed, that, on addition of bivalent ions (Ba, Mg) to colloidal

solutions of chlorophyll, a marked shift of the band maximum towards the long wavelengths could be obtained. The very fine suspensions of chlorophyll, obtained in this way, are non-fluorescent and possess a strong Tyndall effect.

These suspensions possess the exact maximum of the leaves studied by him; in all other states a marked shift to the short wavelengths is observed. As he does not take the fluorescence into account, his final conclusion runs as follows; chlorophyll must be present as a fine suspension of solid chlorophyll particles dispersed homogeneously in the stroma of the living plastids. The measurements were carried out spectrophotometrically.

In 1912, Herlitzka (23) states that the band maxima of colloidal chlorophyll and of the living leaf are identical. A press juice of ground leaves showed an unaltered band maximum, and possessed no fluorescence. His press juices and solutions of colloidal chlorophyll both possessed a negative charge. The measurements were carried out spectroscopically.

Table I shows his data:

TABLE I.

Medium.	Band max. (in m.μ.)
Alcohol	656.5
Acetone	662.5
Colloidal	674.0 and 672.0
Press juice	675.5
Living leaf	675.5

He made two preparations of colloidal chlorophyll, from an acetonic- and from an alcoholic solution. They show a difference of 20 Å. in band maximum.

Herlitzka's article inspired Iwanowski (30) in 1913 to repeat some of his experiments. He used a Grünbaum—Martens spectrophotometer.

Table II shows his data:

TABLE II.

Medium.	Band max.
Mnium (living)	6795 Å.
Colloidal	6695
Alcohol	6645

Willstätter and Stoll (71) in 1918, claim again the identity of the band maximum of colloidal chlorophyll and of the living leaf. From the behaviour of the pigment in the plastids towards several solvents as ether, acetone, benzene etc, they obtained the impression that chlorophyll is present in the living leaf, not in true solution, but in a state very near to that of a colloidal solution. The dry solvents do not extract the pigment from thoroughly dried leaves. On addition of some water the extraction succeeds without difficulty. They have formed the idea that the water, added to the solvents, dissolves some of the salts present in the dried materials, and that these salt-solutions flocculate the colloidal pigments. Later (73) Willstätter decides in favour of an adsorption of the pigment on some polymolecular complex. On boiling the leaves, a shift of the band maximum towards the short wavelengths was observed. A system chlorophyll-lecithin-water had practically the same band maximum and was characterised by a strong fluorescence. He comes to the conclusion that, on boiling the leaves, the pigment passes from the colloidal state into a true solution in some lipoid.

Table III shows W's data:

TABLE III.

Medium.	Band axis.
Alder	6775 Å.
Tulip	6740
Colloidal	6760
Boiled leaf	6715
Lecithin	6700
Phytol	6695

They found further, that colloidal chlorophyll was able to form a chemical complex with CO_2. After some days they observed that the solutions were converted to a certain extend into phaeophytin.

No reduction of CO_2 could be found, in their preparations, in the presence of light.

Stern (64a) in 1921, takes the fluorescence of the living plastid as a starting point. Reinke (56) had already stated that his first idea of the non-fluorescence of the plastids was erroneous. By means of a concentrated bundle of blue light, the fluorescence of *Chlorella* and *Tradescantia spec.* could be measured spectroscopically. The maximum of the fluorescence band was found at 681

m μ. In solutions of colloidal chlorophyll no fluorescence was observed. By shaking colloidal chlorophyll with several solutions and solvents, he obtains a set of fluorescent and non-fluorescent systems.

Table IV shows Stern's data:

TABLE IV.

Colloidal chlorophyll added to;			
Medium.	Fluor.	Medium.	Fluor.
Eggwhite	—	Triolein	+
Peptone	—	Oleic-acid	+
Albumin	—	Lecithin	+
Glycocol	—	Cholesterol	+
Cuccinamid	—	Lanolin	+
Starch	—	Spermaceti	+
Dextrin	—	Soap	+
Sugar	—	Linseed-oil	+
Glycerol	—	Ricinus-oil	+
		Paraffin-oil	+

Chlorophyll imbedded in solid paraffin showed no fluorescence. The observation of Reinke (56) may be explained by the fact, that paraffin has no sharply defined melting point and solidifies after a considerable time. As lecithinoids, in general lipoids, are present in the living cells, these may be the solvents of chlorophyll in the plastids. The band-maximum of a system chlorophyll-lecithin-water was found at 6770 Å. Stern never stated that lecithin-chlorophyll systems showed the same fluorescence-maximum as the living leaf; he only mentions the great similarity of both spectra [1].

Lubimenko (43) finds in 1921 that the position of the absorption band is not altered if leaves are ground and suspended in water. Such suspensions may be precipitated with tannin, ammoniumsulphate and alcohol 50%. If the precipitate, obtained on addition of ammoniumsulphate, is brought in pure water, it dissolves again. From these experiments Lubimenko deduces a chlorophyll-protein complex containing moreover the yellow pigments; "chlorophylle naturelle".

A great number of leaves is studied. He finds several different absorption maxima. The extreme cases are *Ailanthus glandulosa Desf.* 700—680 mμ and *Latania aurea Dunc.* 675—665 mμ. The

[1] Benecke—Jost, 1923. Pflanzenphysiologie. Bd. I, p. 176; „Was die Lage des Fluoreszenzbandes angeht, so liegt es im Spectrum bei 681 mμ und zwar bei Betrachtung von Chlorophylllösungen in Lecithin genau an derselben Stelle, wie bei Betrachtung lebender Blätter". This statement should be modified.

instrument used was a microspectral ocular of Sorby-Browning, which has a small dispersion.

Wurmser (77), in 1921, gives a series of experiments based on the following points:

I. Colloidal chlorophyll has the same band-maximum as the living leaf.
II. It is less stable than the pigments in the plastids.

On addition of several colloids to a solution of colloidal chlorophyll, the photostability is raised considerably. He comes to the conclusion, that chlorophyll is surrounded by a layer of, still unknown, colloids, rendering the system stable against photooxidation.

The protein-chlorophyll complexes obtained by Eisler and Portheim (12) are of little use. We may mention that their fluorescent systems contain a large amount of lipoids. From the work of Stern we know that those solutions are strongly fluorescent. Dolk and v. Veen (11) in 1927 have tested the CO_2 reduction-power of these solutions with negative result.

Noack (50) in 1927, gives a series of experiments to prove the existence of a chlorophyll-protein complex. He comes to the conclusion that it is highly probable that chlorophyll is combined with some protein in the living plastid. Ground plastids showed to be fluorescent. This fluorescence disappeared if the press juice was heated some seconds to 75° C. The disappearence of this fluorescence occurs at the minimum temperature for the denaturation of proteins. If proteolytic enzymes were added to the press juice, a marked decrease of the fluorescence was observed. For further particulars see Chapter X.

Muller (48) in 1930, gives a resumé of the more outstanding theories on photosynthesis. Based partially on the work of Mestre (45) he assumes the existence of a chlorophyll-protein-carotene complex [2]).

Baas Becking and Koning (4) in 1934, for the first time try to verify Kundt's law. They come to the conclusion that the law is obeyed in general, but even in a medium of very high refractive index (1.72) the band maximum shows a marked shift towards the short wavelengths as compared with its position in the living leaf.

[2]) As Mestre's Doctors Thesis was only issued in a few typewritten copies, and the Laboratory copy was loaned and, apparently, lost, several efforts were made to obtain a copy from the Stanford University Library. As no reply was obtained from this library we have to rely on Mestre's short article in the Contrib. to Marine Biol. Stanford Univ. Press.

CHAPTER II.

Purification of the pigment.

For the purification of the pigment (a + b) we used the method of Willstätter and Stoll (72), with the modification given by Schertz (59). During the numerous experiments several facts were observed which deserve attention as they have been described only partly by other authors, and gave us much trouble.

Materials.

For the extraction of the pigments we used the following plants:

I. Cabbage (*Brassica oleracea var. acephala* (L.)
II. Nettles (*Urtica dioica* (L.) and *U. urens* (L.)
III. *Aquilegia glauca* Lind. and *A. glaucophyllum* Steud.)
IV. *Ginkgo biloba* (L.)

The leaves were dried in a current of air in drying cells at 40° C. No higher temperature could be used due to the thermolability of the pigment (50). The dry meal was ground in a ball-mill to particles of 100 μ in diameter [3]).

Method of extraction.

A portion of the meal is brought on a Buchner funnel and saturated with 80% acetone. After some minutes the acetone is removed by moderate suction. The solution obtained in this way contains the four pigments and many impurities. This process is repeated till the meal is only slightly coloured. Willstätter and Stoll (72) mention that the meal becomes straw yellow; this stage could never be reached by us if dried leaves were extracted; no possible explanation may be given by us.

2. The acetonic solution is shaken with petroleum-ether (b. p. 40—57° C.).

The pigments are taken up by the petroleum-ether layer, and the acetone, containing many impurities, is discarded. During this stage no emulsions are formed.

3. The petroleum-ether solution is washed several times with tapwater, removing in this way the greatest part of the acetone taken up by the petroleum-ether, and many impurities. As the pigments may flocculate during this process, care has to be taken not to remove the acetone quantitatively. During this stage emulsions are formed after vigorous shaking.

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[3]) We are greatly indebted to Prof. Dr. E. v. Slogteren and Prof. Dr. G. J. v. Iterson for hospitality at their laboratories, enabling us to use drying cells and ball mill respectively.

They proved to be very unstable, and were broken in a short time after addition of some NaCl.

4. To remove the xanthophyll, the petroleum-ether is washed with 80% methylalcohol. After washing ten to twenty times, the methylalcohol was still coloured slightly yellow. The use of the methylalcohol of lower concentration (70—80%) gave, in several experiments, a precipitate of xanthophyll in yellow films.

By filtering the petroleum-ether through ordinary filterpaper, the xanthophyll could be removed almost quantitatively, as could be proved by a chromatogram, prepared after the method of Winterstein and Stein (75).

5. The methylalcohol is removed by washing several times with tapwater till the solution loses its fluorescence and the pigments precipitate.

Care has to be taken to avoid emulsions during this stage, as these proved to be very stable. If flocculation did not occur, a small amount of 100% acetone was added to the petroleum-ether solution, and washed again with tapwater. Complete precipitation

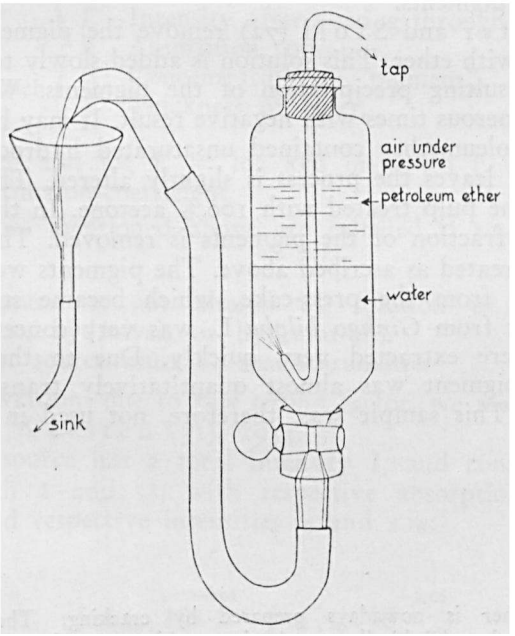

Fig. 3. Apparatus for continuous washing of petroleum-ether solutions of chlorophyll.

was only obtained with *Aquilegia,* be it after intense and continuous washing. Fig. 3 shows an apparatus, constructed by us, to apply continuous washing.

6. The petroleum-ether is shaken with anhydrous sodium sulphate and some talcum, and afterwards filtered over a talcum layer on a Buchner funnel. After washing with petroleum-ether (b. p. 28—40° C.), the talcum layer is dried in a current of air. The filtrate contains the carotene and sometimes a small amount of chlorophyll a.

7. The chlorophyll is removed from the talcum layer with acetone 100% and mixed with petroleum-ether (b. p. 40—57° C.). This solution is washed again with tapwater, till the pigments flocculate.

8. The precipitate is adsorbed on talcum (as ascribed above) and removed by ether. After evaporation of the ether the pigment is dried in a stove at 40° C.

The manipulations described in this chapter were carried out as much as possible in weak artificial light to avoid photodecomposition of the pigments.

Wilstätter and Stoll (72) remove the pigment from the talcum layer with ether. This solution is added slowly to petroleum-ether with resulting precipitation of the pigments. We tried this procedure numerous times with negative result. It may be mentioned that our petroleumether contained unsaturated hydrocarbons [4]).

Using fresh leaves the process is slightly altered. The leaves are ground and the pulp treated with 100% acetone. In this way only a very small fraction of the pigments is removed. The press-cake is dried and treated as ascribed above. The pigments were extracted quantitatively from the press-cake, which became straw yellow.

The extract from *Ginkgo biloba* L. was very concentrated, and the leaves were extracted very quickly. Due to the acidity of cellsap, the pigment was almost quantitatively transformed into phaeophytin. This sample was, therefore, not used in our further experiments.

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[4]) Petroleum-ether is nowadays prepared by cracking. The „Bataafsche Petroleum Maatschappij" kindly provided us with a sample of pure pentane. As this substance did not yield any better results, the lack of succes cannot wholly be attributed to the impurities in the petroleum-ether.

CHAPTER III.

Validity of Beer and Lambert's law.

To our surprise little or no attention has been given to the validity of Beer and Lambert's law for solutions of chlorophyll.

From the data given by Weigert (70) (1916) we may derive that the law is obeyed in acetonic solutions. Wurmser (77), 1921, gives complete curves for an alcoholic and an acetonic solution. He finds a deviation in the lower concentrations. R. Horst (26), 1934, finds that the law is obeyed over a range of concentrations in ether. Baas Bečking and Koning (4), 1934, find that the law is obeyed for a paraffin oil-solution. Colloidal chlorophyll followed the law as far as concentration was concerned, but B. B. & K. found deviations when the thickness of the absorbing layer was studied.

Theoretical.

If a light-absorbing substance is dissolved in a non-coloured medium, the following relation exists:

$$\frac{I}{I^0} = 10^{-kcd}$$

$$\frac{I^0}{I} = 10^{kcd}$$

in wich

I°. Total light intensity.
I. Intensity after passing through the solution.
k. Absorption constant.
c. Concentration of "pigment".
d. Thickness of layer.

$\log \frac{I^0}{I} = kcd$ and $\log E = \log kcd$ in which E is called the extinction-coefficient.

The relations mentioned above are fulfilled if two restrictions are made:

I. The pigment is not altered by solution or by dilution (Ionisation, hydrolysis or hydratation).
II. The light source must be monochromatic.

For the deviation, due to this second factor, we may follow the derivation of Karsten (31), 1934.

The light source has a total intensity I, and consists of light of wavelength λ and λ_1, with respective absorption-coefficients k and k_1, and respective intensities i_0 and $a.i_0$:

$$i_0 = \frac{1}{1+a} . I^0$$

$$I = i_0 . 10^{-kcd} + a . i_0 . 10^{-k_1cd}$$

$$= I^0 . \frac{1}{1+a} \left(10^{-kcd} + a . 10^{-k_1cd}\right)$$

$$= I^0 . 10^{-kcd} . \frac{1}{1+a} \left(1 + a . 10^{(k-k_1)cd}\right)$$

$$= I^0 . 10^{-kcd} \left[1 + \frac{a}{1+a} \left(10^{(k-k_1)cd} - 1\right)\right]$$

The factor $\left[1 + \frac{a}{1+a} \left(10^{(k-k_1)cd} - 1\right)\right]$ gives the deviation from the law.

The magnitude of this error depends on the value of: a, $(k-k_1)$ and the product c.d.

Method.

We used the Leitz "Stufenkolorimeter". Two parallel beams, coming from one light source, pass through the absorption vessels. By means of two plane-parallel dipping rods, the layer of the absorbing fluid may be varied in thickness. The beams are brought together by means of a Hüfner rhombus. The instrument is provided with a set of narrow filters, allowing to estimate the absorption of pigments in different ranges of wavelengths. The extinction-coefficients are estimated with the use of a "greyfilter solution" of known optical properties (10). The light source is mounted on the instrument and may be centered by means of two screws.

Before making measurements the following points must be controlled:

I. If both dipping rods are lowered, the beams must be equal in brightness, and the nonius must stand on zero.
II. For every filter used, the field must be brought into focus.

In one of the absorption vessels is placed a "greyfilter solution". This solution has to be prepared from two stock solutions, supplied by the Firm of Leitz. It follows Beer and Lamberts law, and for a layer of one c.m. $E = kcd = \frac{1}{2}$.

In the other vessel the chlorophyll solution is placed and one of the filters placed before the ocular. Calculations are based on the following equation:

$$\frac{I^0}{I} = 10^{kcd_1} = 10^{E.d_2}$$

$$kcd_1 = E.d_2$$

Calculations are materially simplified by taking a layer of $\frac{1}{2}$ c.m. of chlorophyll solution. In this case E is read off directly from the scale at the greyfilter side:

$$kc. \frac{1}{2} = \frac{1}{2} d_2$$

$$kc = d_2 = E \text{ calculated}$$

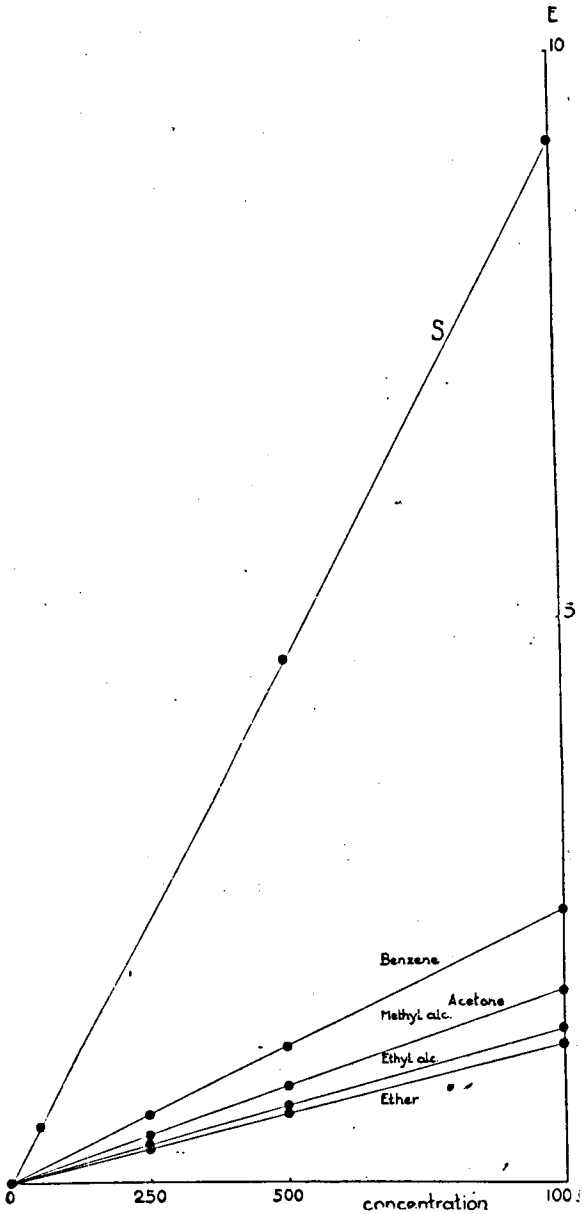

Fig. 4. Relation between E. and concentration for solutions of chlorophyll.

We have calculated E and E/c for a layer of one c.m. of chlorophyll in all our experiments.

Experimental.

The solutions were prepared in weak artificial light to avoid photodecomposition. We never used stock solutions, as chemical alterations of the pigments may take place. We may refer here to the work of Willstätter and Stoll, who found that the pigment underwent some chemical change in alcoholic solutions (allomerisation), and the researches of Stoll (65) who showed that chlorophyll in acetonic solution showed a distinct optical activity. He found that the pigment lost this property rather quickly even if the solution was kept in darkness. From our own experiments we know that traces of acids, present in organic solvents, give rise to the formation of pheaophytin. It is adviseable to use only one filter during a series of experiments, as the eye adapts itself to the wavelength used.

In our first experiment we used a Philips Sodium Lamp. To exclude the light from the neon gas, present in the lamp, we applied the yellow Lifa filter No. 395. A series of concentrations was prepared, starting from 1 mgr per c.c. and ending at 1/640 mgr. per c.c., where the limit of the sensitivity of the instrument was reached.

The third and fourth column of table V give the values of E and E/c respectively, calculated from the experimental data. In fig. 4 we have plotted out E against concentration of the pigment (line S). A straight line is obtained going through the origin.

TABLE V.

Acetone.	Light source: Philips sodiumlamp			
C.	Chlorophyll.	Greyfilter.	E. calculated.	E/c. calc.
1	1.0 m.m.	18.60 m.m.	9.3000	0.01453
$^1/_2$	1.0	9.30	4.6500	0.01453
$^1/_{20}$	5.5	5.15	0.4670	0.01456
$^1/_{80}$	20.5	4.95	0.1200	0.01500
$^1/_{160}$	60.5	7.45	0.0616	0.01540
$^1/_{320}$	80.5	4.65	0.0282	0.01410
$^1/_{640}$	80.5	2.35	0.0146	0.01460

As the points for the lower concentrations fall closely together, they are omitted in this figure. This difficulty is avoided if we plot out log. E against log. c. If Beer and Lambert's law is fulfilled, a straight line must be obtained also in this way.

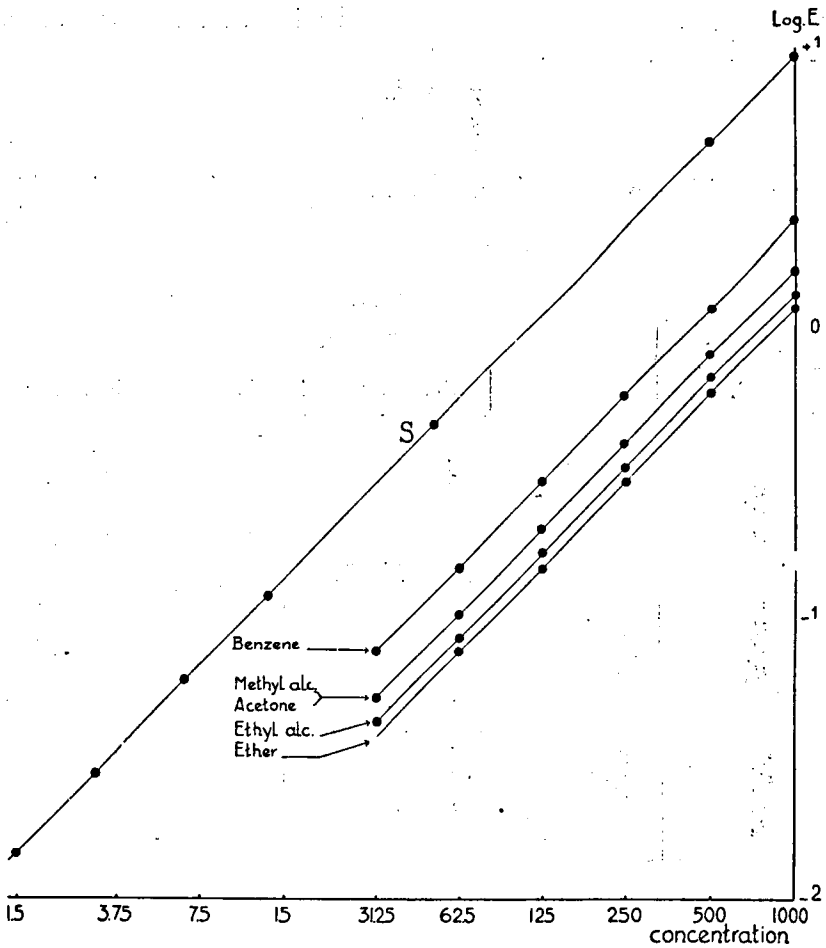

Fig. 5. Relation between log. E. and log. conc. for solutions of chlorophyll.

Line S in fig. 5 shows our data plotted out in this way. The experiments show that, in contrast with the observations of W u r m s e r (77), acetonic solutions of chlorophyll follow the law for the wavelengths 5895.9—5890.0 Å. over a considerable range of concentrations.

The next point to investigate was the validity of the law for partly monochromatic light (glass filters). From the work of K a r s t e n we know, that deviations from the law may arise if

the light source is not purely monochromatic. We used the Leitz "Stufenkolorimeter", which is provided with a set of glass filters. The following filters were used:

7000 Å.
5690
4510

Table VI shows our data. Obviously the law is fulfilled within the experimental error. The results of this set of experiments will be used in the following chapter.

TABLE VI.

Acetone.	Filter 7000 Å.			
C.	Chlorophyll.	Greyfilter.	E. calculated.	E/c. calc.
$^1/_1$	1.50 m.m.	5 m.m.	1.6650	0.1041
$^1/_2$	3.05	5	0.8200	0.1050
$^1/_4$	6.05	5	0.4130	0.1033
$^1/_8$	12.00	5	0.2080	0.1040
$^1/_{16}$	23.95	5	0.1045	0.1045
		Filter 5690 Å.		
$^1/_1$	4.50	5	0.555000	0.03469
$^1/_2$	9.05	5	0.276200	0.03452
$^1/_4$	27.90	7.5	0.136200	0.03405
$^1/_8$	36.05	5	0.069350	0.03462
$^1/_{16}$	36.00	2.5	0.034725	0.03472
		Filter 4510 Å.		
$^1/_1$	0.9	5	2.77500	0.1736
$^1/_2$	1.85	5	1.35000	0.1687
$^1/_4$	3.75	5	0.66650	0.1666
$^1/_8$	7.50	5	0.33333	0.1666
$^1/_{16}$	15.00	5	0.16666	0.1666

In table VII the results are given for the following set of solvents:

Methyl alcohol.
Ether.
Acetone.
Ethylalcohol.
Benzene.

Filter λ 7000 was used, and the concentration varied from 100 mgr. per liter to 6.25 mgr. per liter.

Fig. No. 4 and No. 5 show that, for this set of solvents, the law is fulfilled. The validity of the law for colloidal solutions will be considered in chapter VII.

TABLE VII.

Filter 7000.	Solvent: Methylalcohol.			
C.	Chlorophyll.	Greyfilter.	E. calc.	E/c. calc.
$^1/_1$	1.5 m.m.	5 m.m.	1.66500	0.0523
$^1/_2$	3.0	5	0.83000	0.0519
$^1/_4$	6.0	5	0.41650	0.0526
$^1/_8$	12.0	5	0.21300	0.0532
$^1/_{16}$	24.8	5	0.10500	0.0525
$^1/_{32}$	48.4	5	0.05165	0.0516
	Solvent: Ether.			
$^1/_1$	2.05	5	1.2200	0.0762
$^1/_2$	4.10	5	0.6100	0.0762
$^1/_4$	8.20	5	0.3005	0.0751
$^1/_8$	16.00	5	0.1560	0.0785
$^1/_{16}$	32.00	5	0.0780	0.0785
	Solvent: Acetone.			
$^1/_1$	1.50	5	1.66500	0.0523
$^1/_2$	3.00	5	0.83000	0.0519
$^1/_4$	6.00	5	0.41650	0.0526
$^1/_8$	12.05	5	0.21300	0.0532
$^1/_{16}$	24.05	5	0.10620	0.0531
$^1/_{32}$	48.15	5	0.05165	0.0516
	Solvent: Ethylalcohol.			
$^1/_1$	1.85	5	1.3550	0.0423
$^1/_2$	3.60	5	0.6940	0.0433
$^1/_4$	7.15	5	0.3500	0.0437
$^1/_8$	14.50	5	0.1744	0.0436
$^1/_{16}$	28.90	5	0.0865	0.0432
$^1/_{32}$	56.90	5	0.0431	0.0431
	Solvent: Benzene.			
$^1/_1$	1.05	5	2.38000	0.0743
$^1/_2$	2.05	5	1.21500	0.0759
$^1/_4$	4.10	5	0.60950	0.0762
$^1/_8$	7.90	5	0.31645	0.0791
$^1/_{16}$	15.90	5	0.15720	0.0786
$^1/_{32}$	31.95	5	0.07950	0.0795

CHAPTER IV.

Photodecomposition of chlorophyll [5]).

If we plot out log. kcd = log. E against wavelength, a curve is obtained, the shape of which is independent upon the concentration of the pigment. By reduction of the concentration to 1/n

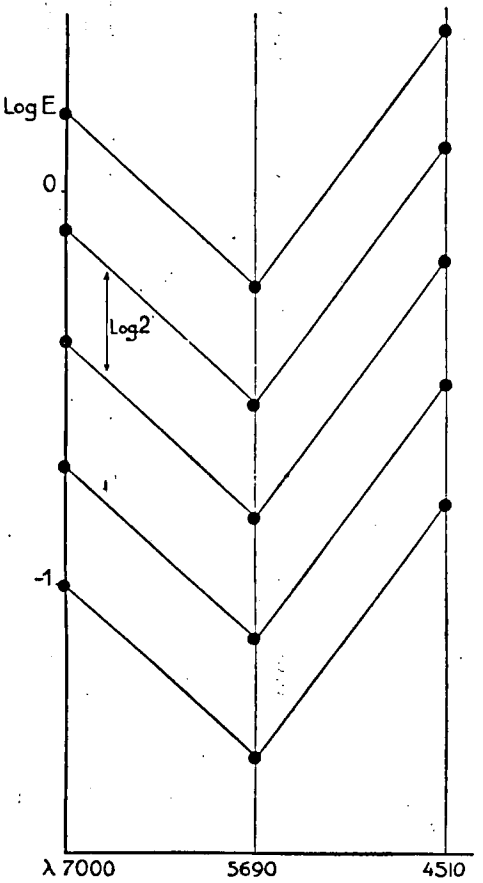

Fig. 6. Explanation see text.

of its value log. E = log. kcd—log. n, every point of the curve is displaced along the ordinate over a distance equal to log. n. The

[5]) See also our preliminary communication in Proc. Kon. Akad. Amsterdam, 37, p. 684, 1934.

truth of this statement depends upon the applicability of Beer and Lambert's law. In fig. 6 we have plotted out the data from table II of the previous chapter. Here log. E. is plotted out against wavelength for a set of five concentrations (n = 2). A set of equidistant curves is obtained, each displaced along the ordinate over a distance equal to log.2.

As in our experiments the typical "colour curve" of chlorophyll, as given by Weigert (70) in 1916, could not be reproduced, neither with the Keuffel and Esser colour analyser, kindly put at our disposal by Dr. H. P. Wolvekamp, nor with the König—Martens spectrophotometer, it seemed worth while to investigate the reason of this discrepancy.

v. d. Honert (25), using the Keuffel and Esser instrument too, was unable to duplicate the curve of Weigert. The curve of Wurmser (77), obtained with the König—Martens instrument, shows a similar deviation. It is our belief that this deviation may be explained by the photolability of the pigments. For both instruments a light source of great intensity has to be used.

The leaf-extract was prepared as described by Weigert; ten grammes of fresh spinach were extracted with 100 c.c. of 85% acetone and the extinction measured with the König—Martens instrument.

Quick measurements were made every 15 minutes at the following wavelengths:

7000
6800
6440
6250
5900
5500
5000
4800 Å.

Photodecomposition should go parallel with a change of log. E, and if d is kept constant, the only variable remaining is c. Plotting out log. E against observation-time shows (fig. 7), that log. E decreases between λ 5900 and λ 6800, while between λ 5000 and λ 5900 log. E seems to increase.

This decrease in log. E should be due to a disappearence of chlorophyll, while the increase in log. E at the shorter wavelengths could be brought about by the appearence of the photooxidised product. We may observe moreover that, in the range of 6250—6800 Å., the decrease is homogeneous, and, therefore, strongly

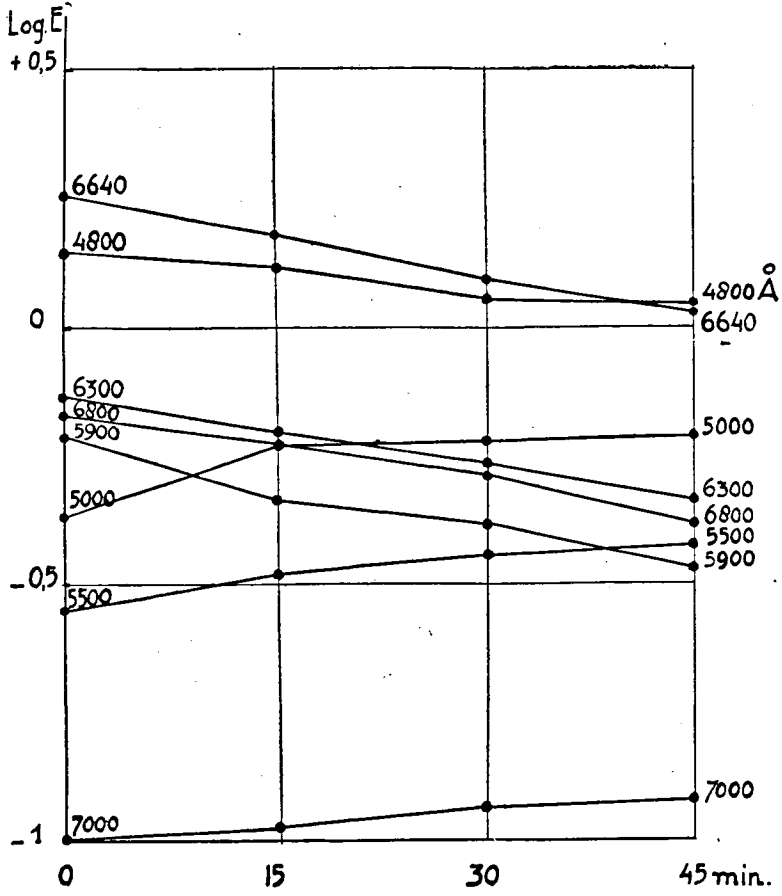

Fig. 7. Influence of observation-time on log. E.

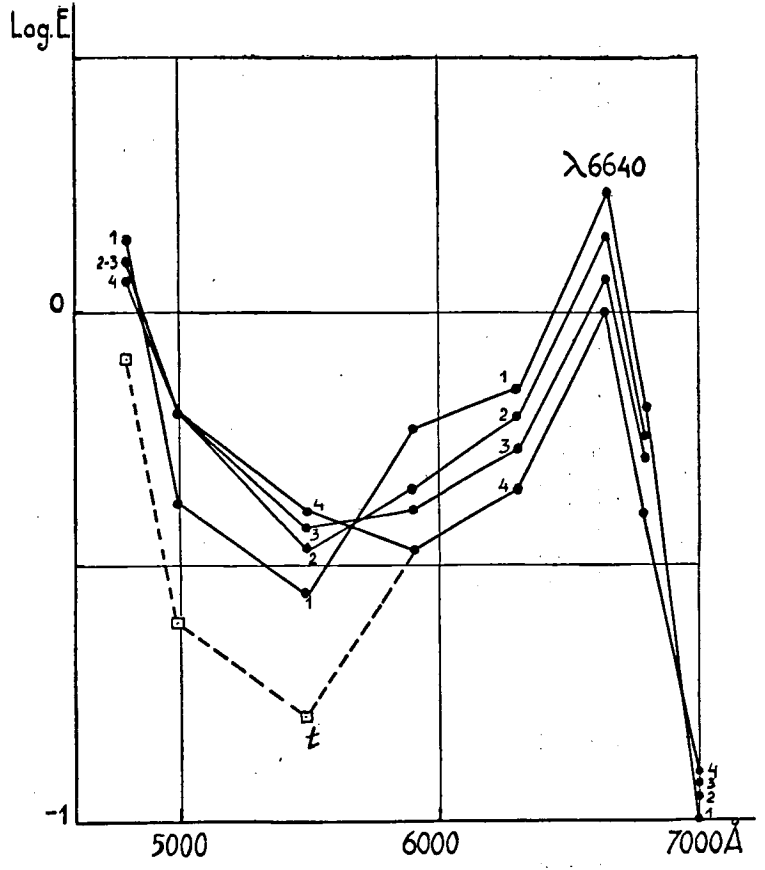

Fig. 8. Influence of observation-time on the typical „colourcurve" of chlorophyll (a + b).

suggests a diminution in concentration, while, in the range of the shorter wavelengths no such law seems to hold. At 7000 Å. an increase in concentration of some substance seems to be indicated. Now if we follow curve 4 (fig. 8) from the red to the blue we may extrapolate (along the dotted line) a theoretical curve, which curve represents a reduction of chlorophyll corresponding to that found for λ 6640. Obviously the ordinate-distance point for point between this dotted line and the observed curve represents a spectrum-characteristic of the photooxidised product.

When we call the extinction E and the corresponding (extrapolated) extinction Et, the relation between λ and (log. E—log. Et) indicates in a rough way the absorption spectrum of the photodecomposed product. The possibility exists that a series of photodecomposed products are formed in this way. (log. E—log. Et) is represented in figure 9.

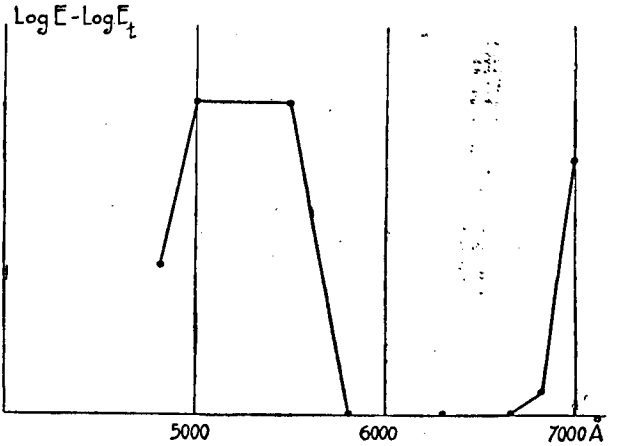

Fig. 9. Relation between (log. E—log. Et) and wavelength.

If the photooxidised product absorbed radiations in the range between λ 6000 and λ 6800, the chromatographic analysis of Tswett (67) might show more than two green coloured zones. We used the modificated method as given by Winterstein and Stein (75) in 1933 and Zscheile (79) in 1934.

A glucose-talcum column was prepared with petroleum-ether, containing some acetone. A chromatogram was prepared of a petroleumether solution of chlorophyll, which solution had been exposed to full sunlight during 30 minutes. Two green zones appea-

red, but also a grey zone, appearing below the green zones.

The following conclusion may be made:

If a visual spectrophotometric method is used to measure the extinction of chlorophyll solutions, a great number of observations have to be made to obtain reliable results. During this time the pigment is exposed to the radiations of powerful light sources, with resulting destruction of the pigment. From the considerations given above it follows that, if measurements are taken throughout the whole spectrum, the curve obtained is a mixture of the chlorophyll curve and the curve of the photodecomposed product. If we start measurements from the long wavelengths, the values obtained for the short wavelengths must be altogether wrong, as the pigment is partly decomposed during the rather long observation-time nessessary to obtain a complete curve. The reverse is true if we start at the short wavelengths. Willstätter and Stoll (71) avoided this effect by using a cuvette in which the solution was continuously renewed and Zscheile (79) changed the solutions after a short time.

If a simple spectroscopic method is used, no displacement of the first band has to be dreaded. The only effect observed will be a gradual narrowing of the band, which is even favorable, as Reinke (55) in 1883, has already shown that the band is rather asymmetrical. The influence of the asymmetry of the band on the estimation of the real maximum deminishes in diluted solutions or with decreasing thickness of the layer.

CHAPTER V.

Methods and Instruments.

a. The instrument [6]).

We used a spectrograph of great luminosity, designed by Dr. A. C. S. v. Heel (21). An instrument of this type with one prism is put on the market by the Firm of Kipp and sons. The camera lens is a "Cooke" anastigmat of Taylor and Hobson (f/2) and the

[6]) The apparatus was constructed under the direct supervision of Dr. A. C. S. v. Heel by the Master Mechanic of the Botanical Laboratory, Mr. A. J. Stuivenberg, who spared no efforts to obtain the best possible results. At this place I want to thank him for unfailing aid in this work.

Mr. H. Vylbrief, assistant-mechanic at the Botanical Laboratory, materially assisted in the construction of the instrument and the Firm of Kipp and sons were kind enough to place the original working drawings at our disposal.

Due to this combined effort the apparatus was constructed with comparatively small cost, without impairing its precision.

collimator (E) a common achromat (f/10). The diameter of both lenses is 6.5 c.m. The camera lens has a focal distance of 13 c.m. and the collimator a focal distance of 65 c.m. The spectrum may be brought into focus by means of a focussing drum (F).

A small lens (I), behind the plate, allows to control the spectrum visually.

The instrument is provided with a slit (A) (2 c.m. high) with one jaw moveable. To obtain sufficient dispersion two prisms (D_1, D_2) were used. The prisms were filled with ethyl cinnamate, a liquid which has the advantage of giving a greater dispersion, with the same lose of light, as glass prisms.

As the liquid has a relative small N_D (1.56), a large angle could be chosen (65°), giving a long spectrum.

The height of the prisms was 7 c.m. and the baselength 14 c.m.

A glass prism of the same dimensions is very costly. The liquid, used in the prisms, has to be filtered before the instrument is used, as polymerisation products are formed after some time. We filtered the liquid on a Buchner funnel through ordinary filter paper.

v. Heel (21) states, that a change in temperature of 0.3° C. is not at all harmful. To avoid great changes in temperature of the liquid in the prisms during the experiments, (which changes might give rise to convection-currents in the ethyl-cinnamate), we inclosed

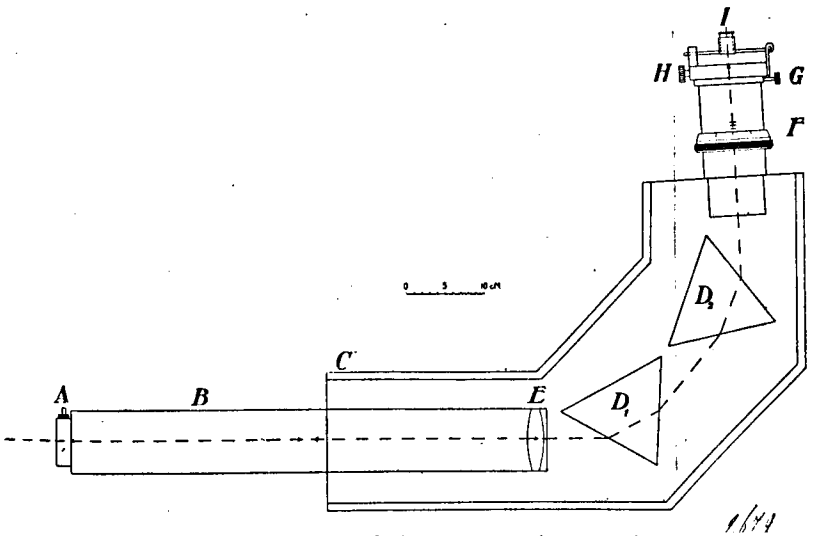

Fig. 10. Diagram of the spectograph v. Heel.

the whole apparatus in a box of heavy oak (C). The inside of this box was covered with velvet paper, to diminish the false light, due to reflections on the walls of the instrument. After these precautions mentioned above, it was still necessary to place a red filter before the slit in all our experiments, as a certain amount of false light was still present in the range used by us.

Figure 10 shows the construction and dimensions of the instrument.

Before the apparatus we mounted an optical rail, carrying the red filter, the light sources and the cuvette holder.

The lamp was placed in a metal box, provided with a photographic shutter. In the side of the lamp box we made a small opening, in front of which a thermocouple was mounted to control the light intensity of the lamp. The slit width was kept constant at 0.19 m.m. during our experiments. The dispersion curve is given in fig. 11.

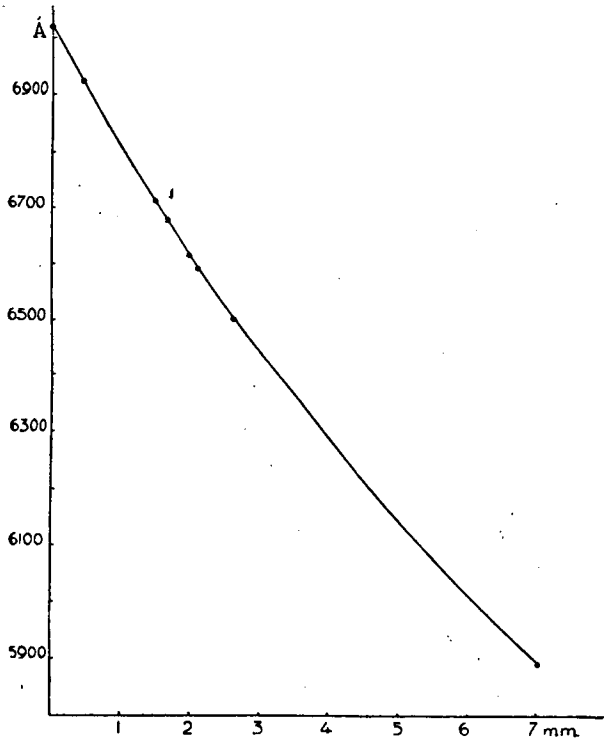

Fig. 11. Dispersion curve of the spectograph.

b. Estimation of band maximum.

1. Method.

We photographed the spectra on Agfa "Superpan" plates. As a simultaneous exposure at several intensities was nessessary, we used a socalled "Stufenabschwächer" constructed with small pieces of smoked glass of different transmitting power. I want to thank Dr. v. Heel for his kind advice in this matter.

The "Stufenabschwächer" consisted of three pieces of smoked glass, deviding the slit in five equal parts. Fig. 12 shows its construction.

The transmitted intensities were 65, 39 and 23% in the range used by us. The upper and lower part of the slit was left uncovered, these parts representing 100% intensity. The height of the spectrum on the negative was 4 m.m. and every step was 0.8 m.m. high.

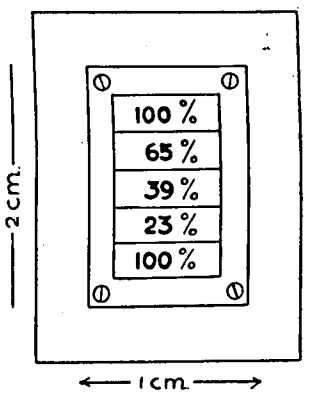

Fig. 12. Explanation see text.

2. Line spectra.

For the construction of the dispersion curves the following light sources were used:

I. Philips neon-nightlamp, running on 127 Volts. A. C.
II. Philips sodium lamp, running on 127 Volts. A. C.

TABLE VIII.

Neon.	Helium.	Sodium.
7032 Å.	7065 Å.	5895.9 A.
6929	6678	5890.0
6717		
6678		
6598		
6532		
6506		

III. A Helium discharge tube, connected with a Ford interruptor, running on a six volt storage battery. This instrument has the advantage of being very cheap and reliable.

Table VIII gives the lines used in our experiments:

3. For the absorption of solutions we used plane-parallel cuvettes (diameter 1 c.m.).

4. To obtain the continuous spectrum an Osram "Nitra" lamp was used. This lamp has a short linear filament, which was placed vertically. The lamp ran on a 6 volts storage battery. By means of a variable resistance the voltage was kept constant at 3.8. Changes in the intensity of the lamp could be controlled on a voltmeter, enclosed in the circuit, and on a mirror galvanometer connected with a thermocouple.

The lamp was placed at a distance of 50 c.m. from the slit and by means of an Amalux Sperrschicht cell an illumination of 50 lux was found at the level of the slit. We chose a weak light source to avoid the destruction of the pigment (see chapter IV).

To obtain homogeneous illumination of the slit it is nessessary to center the lamp as carefully as possible. By means of a lens an image of the light source was produced in the collimator lens. When this image is found in the middle of this lens, the lamp is centered. When measuring the band in solutions no lenses were used before the slit. If the spectrum of a living leaf was photographed, an unsharp image of the lamp was produced on the leaf, and the comparison spectrum obtained by placing a greyfilter solution of Leitz before the slit. In this way the spectral energy-distribution remains unchanged.

5. Preparation of negatives etc.

The exposure times for the solutions varied between 30 and 180 seconds. If living leaves were photographed an exposure time of 20 minutes was nessessary to get sufficient density of the plate.

The plates were developed during 5 minutes with methol-hydroquinone in absolute darkness. A few drops of a 10% potassium bromide solution were added to prevent fog. After fixation during 15 minutes, the plates were brought in running water during one hour, and afterwards allowed to dry in a dust-free place at room temperature.

6. Registration.

For the registration of our negatives we used the Moll microphotometer. We have to thank Prof. Dr. H. Dorgelo for his permission to use this instrument at the institute of Technical Physics, Technical Institute, Delft.

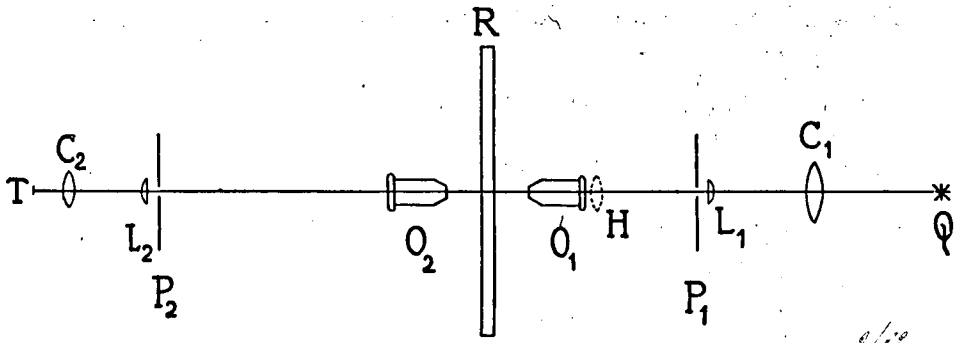

Fig. 13. Explanation see text.

As this instrument is described in detail by Ornstein, Moll and Burger (51), a short description may be given here. (fig. 13).

The image of an Osram "Nitra" lamp is projected on slit P_1 by collimator C_1. Objective O_1 throws an image of this slit on the photographic plate R. The spectrum on the plate is placed horizontally. The illuminated part of the plate is projected magnified on the slit of the thermocouple T by means of objective O_2. This thermocouple is connected with a mirror-galvanometer of Moll. Driven by an electromotor the plate moves horizontally, while the galvanometer deflections are recorded on a brome silver paper strip wrapped on a drum, which moves synchronously with the plate, but with an acceleration of × 40. These brome silver papers are developed and fixed in the normal way. As the continuous spectrum has no "fixed wavelength", it was nessessary to

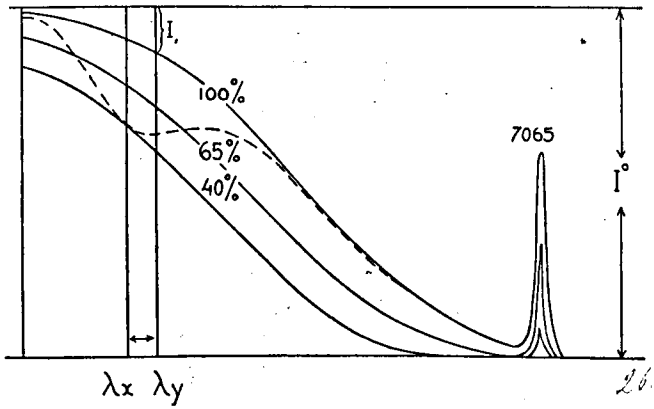

Fig. 14. Explanation see text.

throw a line spectrum over it. Care has to be taken that this spectrum has no lines at the place where we expect the band maximum. For this purpose we used the Helium or Sodium spectrum.

As the "Stufenabschwächer" had four steps we obtained 8 densitograms and one line spectrum from one plate. Fig. 14 shows a set of partly schematical densitograms. As "fixed wavelength" in the continuous spectrum, we find the Helium line 7065. The three densitograms represent the continuous spectrum taken at 100, 65 and 40% intensity.

The dotted line is the densitogram of the continuous spectrum, with some absorbing substance before the slit. The zero line is obtained be screening off the light, and the fog line by recording the galvanometer deflections at an unexposed part of the plate.

It may easily be seen that the real maximum must be found at λx, while the visual maximum lies at λx (method Baas Becking and Koning).

λy-λx is dependent upon many factors, among which we may mention:

I. The plate used.
II. Energy distribution of the light source.
III. Wavelength.
IV. Exposure time.

On our negatives λy-λx was 30 Å. The maxima of Baas Becking and Koning (4) are generally shifted to the longer wavelengths.

7. Estimation of band maximum.

The density is defined by $S = \log \frac{I_0}{I}$, in which formula I_0 is the galvanometer deflection at an unexposed part of the plate, and I the deflection at an exposed part of the plate.

If S is plotted out against the logarithm of the intensity of the light source, we obtain a so-called density-curve. A change in the unit of the light intensity does not alter the shape of the curve, but only a parallel displacement along the abcissa.

At a certain wavelength, calculated from the dispersion curve and the „fixed wavelength", we calculate $\frac{I_0}{I}$ for the four intensities used.

The data are plotted out on double logarithmic paper (Schleicher and Schüll No. 365½). In this way two parallel curves are obtained. The distance of these curves is a measure of the absorption of the pigment at that wavelength. At the maximum distance of the two curves we find the maximum of the absorption band. An example may elucidate this process.

Table IX shows the data obtained by measuring the galvanometer deflections at a certain wavelength.

TABLE IX.

Continuous spectrum.			
Intensity.	I°	I	I°/I
100	60	12	5
65	60	20	3
39	60	30	2
23%	60	43	1.4
	Continuous spectrum + object.		
100	60	27.3	2.2
65	60	37.5	1.6
39	60	50	1.2
23	60	57	1.05

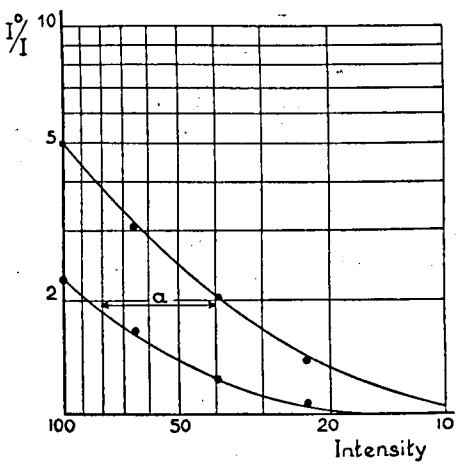

Fig. 15. Density curves from densitometer-records.

Fig. 15 shows the density curves constructed from these data. This process is repeated over a certain range of wavelengths to the left and to the right of the place, where we expect the band. The band maximum is found at the maximum value of a. Density curves were constructed every 20 Å. and, very near to the maximum, every 10 Å.

A calculation of the possible error is very difficult as the method is rather complicated. The following sources of errors are present:

I. Bleaching. This factor is practically excluded due to the short exposure times and the low intensity of the light source.

II. Irregularities of the plate.
III. Construction of the dispersion curve.
IV. Registration and calculation.

Another factor is the shape of the absorption band of chlorophyll solutions. The top of this band is rather flat, and this property is a source of errors. From the values found for the living leaves we may calculate, that an error of 10 Å. may be possible. Small irregularities of the plate may be eliminated graphically on the densitograms. If the density curves did not run parallel, caused by inhomogeneous illumination of the slit, we averaged over a certain distance, which is allowable inasmuch as we are not measuring quantitative absorption. As the living leaf is very inhomogeneous, no second density curve could be constructed. So we had to rely on the distance of one point to the comparison curve.

CHAPTER VI.

Chlorophyll in molecular solutions.

Kundt (36) in 1878, showed that the absorption bands of dissolved pigments shift to the longer wavelengths with increasing refractive index of the solvents. He used an alcoholic extract of chlorophyll as stock solution. 2 c.c. of this solution were added to 10 c.c. of the solvent, in which the maximum had to be studied.

Unhappily he does give his maxima expressed in nonius readings.

Calling the band axis in ether O, the other shifts are given in relative units.

The following table shows his data.

TABLE X.

Solvent.	Nonius.	Shift in Å.	Refract. ind. (Kundt)
Ether	0.0	0.0	1.3594
Acetone	1.2	5.3	1.3617
Alcohol	3.2	14.2	1.3633
Amylalc.	4.9	21.8	1.4033
Chloroform	6.8	30.2	1.4492
Benzene	7.3	32.5	1.5002
Cassia-oil	13.6	60.5	1.5780
Carbon-disulph.	18.6	82.8	1.6248

From his other data we see, that we may take his dispersion curve practically as linear between the Fraunhofer lines B and C.

Starting from the band in ether we may calculate the shifts in Angström units.

Column 3 shows the shifts in Å. calculated from Kundt's data.

Kundt states that the pigment in methylalcohol underwent some change.

It is our belief that this solvent may have contained some formic acid.

Several authors give a more or less complete set of media showing similar shifts. Baas Becking and Koning (4) in 1934 (for the first time) tried to verify the observations of Kundt. A set of 14 media was used to test Kundt's law. From their curve we see that a marked tendency exists, but even in a medium of very high refractive index the band-position of the living leaf is not reached.

The authors state that their data cannot be regarded as quantitative due to the imperfect methods used.

We have repeated their experiments. Table XI shows our maxima:

TABLE XI.

Solvent.	Band max.	(N_D) Refr. index.	Solubility.
Methylalcohol	6625	1.3292	Rather diff.
Ether	6640	1.3525	Very quickly
Acetone	6665	1.3585	Quickly
Ethylalc.	6680	1.3617	Rather diff.
Benzene	6720	1.5014	Good
α-Mono-brome naphtalene	6710	1.6588	Good
Carbon-disulph.	6725	1.6279	Good
Methylene-iodide.	6740	1.7400	Almost insoluble

The band maximum in carbon tetra chloride was found at 6640. Estimating the band the following morning, spectroscopically (in CCl_4) a marked difference was found with the maximum found in the spectrographical way. As in general the values obtained in both ways differed only 10—20 Å, we measured the maximum in the morning and 10 hours later (6710). The band shifted to the longer wavelengths. We can give no explanation for this phenomenon.

In fig. 16 we have plotted out the band maximum against the refractive index of the medium. With the exception of the place of methylalcohol there is a striking similarity with the curve obtained by Baas Becking and Koning (4). Between N_D 1.2 and N_D 1.4 a rapid shift of the band maximum may be observed,

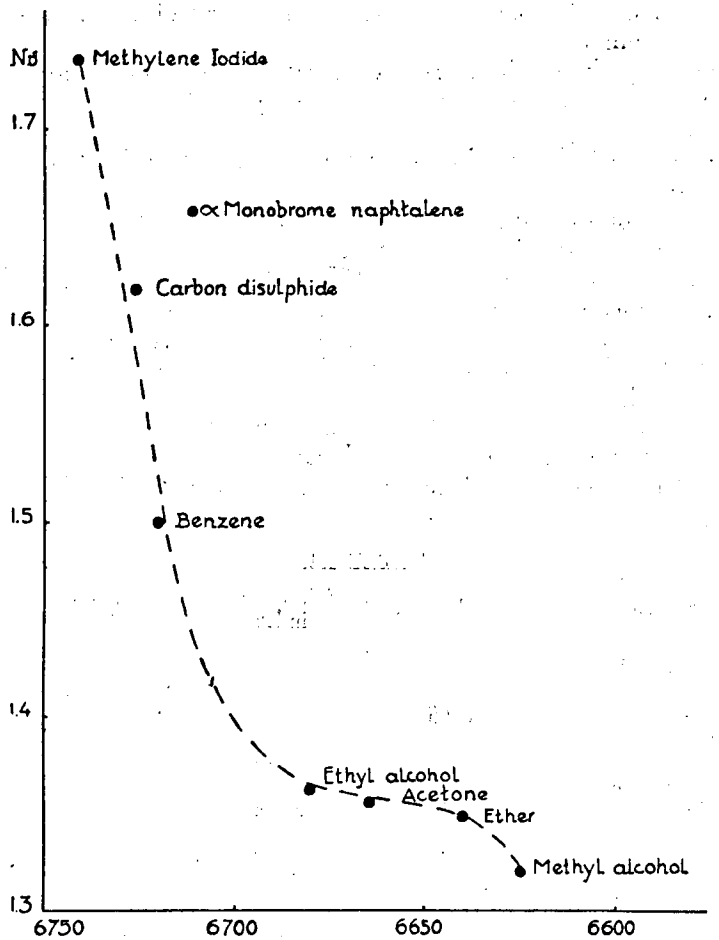

Fig. 16. Band maximum against refractive index.

while between N_D 1.4 and N_D 1.74 only a shift of 40 Å. is obtained. The curve is drawn in analogy with the older litterature, and may not be used for interpolation, as is shown by the maximum found for the solution in α-monobrome-naphtalene.

With the exception of methylene-iodide we may conclude that, in general, the band shifts to the short wavelengths in polar media.

Scheibe (58) has studied the place of the band maximum of acetone in several alcohols. In spite of the fact that the dipole moment remains fairly constant in the set of solvents used by him,

a marked shift is obtained. He comes to the conclusion that steric influences play an important role in this effect.

The pigment is strongly fluorescent in all the media studied by us and is highly photolabile.

Specific extinction.

If a pigment is dissolved in some indifferent medium, two effects may be observed (66):

I. The absorption maximum shifts.
II. The specific extinction is altered.

These effects may occur in all their possible combinations. As a marked shift of the band has been observed in solutions of chlorophyll, we might as well expect a change in the specific extinction.

Experimental. A set of six solutions was prepared in the following way:

1 mgr. chlorophyll was dissolved in ten c.c. solvent.

1 c.c. of this solution was added to ten c.c. of pure solvent. The absorption was measured on the Keuffel and Esser colour analyser. A description of this instrument is given by Wolvekamp (76). The light intensity is varied by means of a set of rotating discs. The absorption is measured in per cents. Measurements were made every 25 Å. The readings from the wavelength drum are given uncorrected. A correction of 20 Å. has to be made. The corrected maxima still show a marked difference with those obtained with our spectrograph.

It may be observed that the maxima shift in the same way as those obtained spectrographically. As the absolute wavelengths do not interest us here, the instrument proved to be very useful, as a great amount of observations may be made in a very short time. A correction has been made for the loss of light due to the reflection and absorption of the cuvette and the solvent. Within the experimental error, this correction proved to be 8% for all the solvents used. Table XII shows our corrected data. In table XIII the solvents are given with corresponding refractive index and maximum absorption. It is clear that the specific extinction is practically the same in all the solvents used by us. Perhaps a faint maximum is present.

TABLE XII.

Conc. 9 m.gr. chlorophyll per litre						
Wavelength.	Methylalc.	Ether	Acetone.	Ethylalc.	Benzene	Carbon-disulp.
6500 Å	46.0 %	50.5	52.0	44.0	43.0	34.0
6525	47.5	54.5	58.5	48.0	49.0	38.0
6550	50.5	57.0	60.0	54.0	51.5	43.0
6575	54.5	59.0	63.0	57.0	53.0	43.5
6600	58.0	63.0	63.5	59.5	58.0	48.0
6625	59.5	64.5	65.5	62.0	60.5	51.0
6650	58.5	57.5	65.5	63.0	61.0	55.0
6675	57.0	54.0	60.0	61.0	62.0	58.0
6700	52.0	47.5	51.0	55.5	54.0	57.0
6725	51.0	40.0	39.5	48.0	49.0	56.0
6750	44.5	30.0	32.0	45.5	48.0	54.0

TABLE XIII.

Conc. 9 m.gr. chl. per litre.		
Solvent.	N_D.	Max. absorption.
Methylalcohol	1.329	59.5%
Ether	1.352	64.5
Acetone	1.358	65.5
Ethylalc.	1.361	63.0
Benzene	1.501	62.0
Carb. disulph.	1.627	58.0

CHAPTER VII.

Colloidal systems and dry chlorophyll.

a. Colloidal chlorophyll. [7])

In 1907, Iwanowski (29) showed that colloidal chlorophyll did not possess the band maximum of the living plastid.

Herlitzka (23) in 1912 stated, that the band maximum of the living leaf and of solutions of colloidal chlorophyll were identical.

Iwanowski (30) in 1913, repeated some of his experiments, confirming his older data. Willstätter and Stoll (71) in 1918 find that colloidal chlorophyll shows the same band maximum as the living leaf.

[7]) Part of this work was done in collaboration with Mr. H. A. Bakker (6) at the Laboratory of Medical Chemistry, Leyden. We want to thank here Prof. Dr. H. G. Bungenberg de Jong for hospitality at his laboratory and for helpful advice.

In table No. XIV. are collected the data of several authors with corresponding maxima of living leaves.

TABLE XIV.

Date.	Author.	Colloidal chl.	Living leaf.
1907	Iwanowski.	6620—6720 Å	6800—6900 Å
1912	Herlitzka.	674. 672.	6755
1913	Iwanowski.	6695	6795
1918	Willstätter and Stoll.	6760	6775
1934	B. Becking and Koning.	6760	6810

We want to call attention to the very low value found by Iwanowski (30). In Chapter I. we have called attention to the fact that the band maximum of colloidal chlorophyll was found by this author between the band maximum of a solution of chlorophyll in alcohol and of the band maximum of a solution in carbon-disulphide.

In this chapter we hope to give an explanation of this remarkable fact, which seems to be in contradiction with the results of many other authors.

Preparation of colloidal chlorophyll.

If water is added quickly to an acetonic solution of pure chlorophyll. we obtain a green liquid having the following characteristics:

I. It is non-fluorescent.
II. It is rather photostable.
III. It may be flocculated with positive bivalent ions, which proves that it is negatively charged (6).
IV. Shaking the solution with ether or benzene does not extract the pigment.

If the water is added slowly a rough flocculation occurs. This is the method used by Willstätter and Stoll (72) in 1913 to prepare fairly pure preparations of chlorophyll (a + b) directly from an acetonic solution, still containing many impurities.

Experimental.

For the preparation of our solutions we used a sample of pure chlorophyll (a + b) kindly put at our disposal by Prof. A. Stoll and a sample of pure chlorophyll obtained by us from *Aquilegia spec.* leaves, purified after the methods of Willstätter and Stoll. A special method was used to disperse the chlorophyll in water.

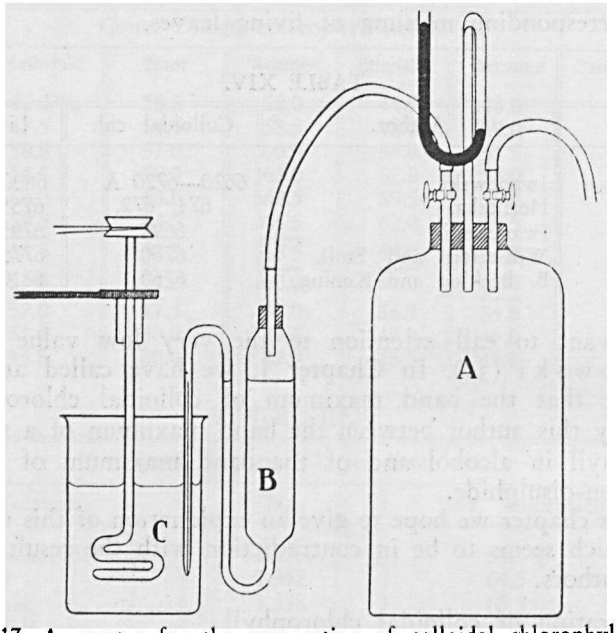

Fig. 17. Apparatus for the preparation of colloidal chlorophyll.

Fig. 17 shows the apparatus. The water in vessel C. is stirred by a glass stirrer, driven by an electromotor. By means of slightly compressed air from reservoir A., carrying an open manometer, the acetonic solution is driven from B. to C. Due to the great difference in size of A and B, a constant pressure is obtained, giving a constant outflow in C, where the pigment is instanteneously and thoroughly mixed with water (final concentration of acetone 20%).

Under reduced pressure (4 c.m. Hg) and between 30—40° C. the acetone was distilled over in two hours. Care had been taken to keep the solution in darkness during this procedure to avoid photodecomposition as much as possible.

In some preliminary experiments it proved to be impossible to dialyse the solution due to the formation of phaeophytin.

Our solution showed the following characteristics:

I. It is non-fluorescent.
II. It is rather photostable.
III. It flocculates with bivalent ions (20—100 m.aeq. $MgCl_2$).

IV. It possesses scarcely any opalescence.
V. The pigment is not extracted by ether, benzene or paraffin-oil.
VI. On addition of some bivalent ions the pigment is quantitatively extracted by ether and benzene.

In contradiction with Baas Becking and Koning (4), we found that our solutions followed Beer and Lambert's law, which proves that our solutions are more finely dispersed. The solutions of these authors showed a distinct Tyndall effect, which could hardly be observed in our preparations.

Table XV shows our data:

TABLE XV.

Greyfilter.	Chlorophyll.	E. calc.	
2.5 m.m.	1 m.m.	1.25	
5.0	2.0	1.25	
10.0	3.9	1.28	
15.0	6.0	1.25	
20.0	7.9	1.26	
25.0	9.9	1.26	
Greyfilter.	**Chlorophyll.**	**E. calc.**	**Conc.**
5.0	2.0	1.25	1
5.0	4.0	0.625	$^1/_2$
5.0	8.1	0.308	$^1/_4$
5.0	15.9	0.157	$^1/_8$
5.0	32.0	0.078	$^1/_{16}$

The band maximum of our solutions was found at λ 6680. As we did not expect this maximum, we tried to use the helium lines as "fixed wavelengths". To our surprise the Helium line 6678 Å. was found practically in the middle of the band.

We repeated the experiment with the sample of Prof. A. Stoll with the same result. In our final experiment we used the sodium line.

It is our idea that the position of the maximum is dependent upon the degree of dispersion of the pigment. It should be remarked moreover, that the band of colloidal chlorophyll is very asymmetrical. Working with a spectroscope it many easily be observed, that, in concentrated solutions, the band axis is found more to the red side than in very diluted solutions.

I am indebted to Mr. H. A. Bakker for examining one of our

solutions spectroscopically. The band was found at 6700 Å and of a living leaf at 6800 Å.

b. Influence of bivalent ions on colloidal chlorophyll.

As Iwanowski (29) had already observed a marked shift of the bandmaximum if electrolytes were added to colloidal solutions of chlorophyll, it seemed worth while to investigate this point, which has obviously failed to attract attention in the literature.

Iwanowski used totally flocculated solutions suspended by means of a diluted gelatin solution. From the curves of Bakker (6) we see, that an increasing flocculation occurs between 10—100 m.aeq. $MgCl_2$.

We chose an intermediate concentration (33 m.aeq. $MgCl_2$). The absorption maximum of the suspension, obtained by us, in this way, was found at 6760 Å.

Addition of salts to a colloidal solution of chlorophyll gives an increasing Tyndall effect, which means that larger aggregates of the pigment are formed; this phenomenon going parallel with a decrease in hydratation of the micelles.

These experiments confirm our idea, that the maximum is dependent upon the method of preparation of the solutions.

Willstätter and Stoll (71) in 1918, state on page 259:

"an Stelle der dunkelroten Fluorescenz der molecularen Lösung tritt blaugrüne Opalescenz".

on page 260:

"Drei solche separate Darstellungen werden von vereinzelt vorkommenden Flöckchen abfiltriert".

on page 258:

"Die Darstellung des kolloiden Chlorophylls erfolgt durch Kondensation aus molekularen Lösungen, in indifferenten organischen Solventien, bei der fällung mit Wasser, die so rasch und vorsichtig vorzunehmen ist, dasz dabei eine grobdisperse Abscheidung vermieden wird".

As we never observed any flocs in our samples, and hardly any opalescence could be observed, we get the impression that our solutions possessed a higher degree of dispersion.

c. Dry chlorophyll.

Several authors (for the older literature, see Iwanowski) held the view that the pigment is present in the solid state, dispersed homogeneously in the stroma of the plastids.

For the estimation of the band maximum of dry chlorophyll we

prepared a highly concentrated solution of chlorophyll in ether. A drop of this solution was brought on a thin glass plate and quickly smeared out by means of a glass rod. In this way a rather homogeneous film of the pigment was obtained. To diminish the scattering of the light, the film was covered with a layer of 30% glycerol and the whole covered with a second glass plate. The dry pigment is absolutely insoluble in dilute glycerol. The band maximum was found at 6805 Å.

Dry chlorophyll is non-fluorescent (50).

d. The system chlorophyll-lecithin-water.

Stern (64) found in 1920, that the position of the fluorescence band of the living plastid *(Chlorella* and *Tradescantia spec.)* and of chlorophyll in lecithin were practically identical. His data show, however, that there existed still a difference of 40 Å. From our own experience it is more difficult to find, spectroscopically, the maximum of the fluorescence band, than the maximum of the absorption band of chlorophyll. Moreover, Stern worked with a very wide slit.

We prepared a system chlorophyll-lecithin-water in the following way:

½ gr. of lecithin (planticin, Riedel de Haen) and 8 m.gr. chlorophyll were dissolved in 20 c.c. ethyl alcohol. This solution was squirted into 500 c.c. water after the method described for colloidal chlorophyll.

The alcohol was removed by destillation under reduced pressure. The solution obtained in this way showed a weak Tyndall effect and a marked fluorescence in blue light.

By vigorous shaking, during a long time, a small amount of the pigment may be extracted with ether, a fact already observed by Willstätter and Stoll (71).

If a colloidal solution of chlorophyll is added to a lecithin sol, no fluorescence was observed by us, which confirms the observations of Bakker (6).

The maximum of the absorption band of our solution was found at 6700 Å. This value corresponds strikingly with the band axis, calculated from the data of Willstätter and Stoll (682—658 mμ).

A solution prepared in this way was studied spectroscopically by Bakker (oral communication). The band maximum was found at 668—669 mμ.

CHAPTER VIII.

Leaves and Plastids.

a. Living leaves.

As a great amount of light is scattered, by reflection on the cellwalls, the leaves were immersed in water and the air in the intercellulary spaces replaced by water. This method was already used by Reinke (55).

In this way this author was able to study a spectrum of a layer of 19 leaves.

Seybold (61—62) in 1932—33 has studied these effects quantitatively.

He uses a photo-electric method to estimate the amount of light lost by reflection on the epidermis, and the amount of light scattered on the cellwalls in the spongy parenchyma. The influence of injecting the leaves with water is illustrated in several graphs.

In spite of the precaution juist mentioned it proved to be necessary, in our experiments, to concentrate the light on the leaf by means of a lens. In this way we were able to photograph the absorption spectrum of the living leaf in twenty minutes.

The following table represents the band maxima found by us:

TABLE XVI.

Plant.	Band maximum.
Aquilegia glauca Lindl.	6810 Å
Sapium spec.	6810
Desmodium girans D. C.	6810
Iatropha multifida L.	6800
Wistaria multijuga Van Houtte	6795
Ulva lactuca L.	6800
Potamogeton spec.	6810
Trichomanes spec.	6810

Comparison with the data of other authors shows, that the band maxima of Iwanowski (30) and of Baas Becking and Koning (4) agree very well with ours. Wurmser (77), using the spectrophotometer of König—Martens, finds the band maximum of *Ulva* at 6800 Å.

The maxima of Willstätter and Stoll (71) and of Herlitzka (23) show a marked deviation towards the short wavelengths. It may be observed that these authors estimated their maxima spectroscopically, moreover Willstätter and Stoll use very thick leaves (alder and tulip), which is unfavorable, as we know

already from the work of Reinke (55), that the band is very asymmetrical, so the band axis, estimated spectroscopically, and the real maximum must show a marked difference if a thick leaf is used.

The following table shows the data of Baas Becking and Koning, which are only partly published elsewhere (27):

TABLE XVII.

Plant	Band max.
Aspidistra spec.	6810 Å.
Impatiens spec.	6810
Hookeria spec.	6810
Selaginella spec.	6810
Hymenophyllum spec.	6810
Laminaria spec.	6810

Lubimenko (42, 42a) states, that the band maximum of the living plastid is not at all constant. The very great deviations (see Chapter I), found by this author, may be traced to experimental errors, as he uses a microspectral ocular, which has a very small dispersion. Moreover the influence of the slit plays an important role.

Ursprung (68) found the maximum in an alcoholic solution of chlorophyll at λ 6610, widening the slit displaced the maximum to λ 6370.

b. Press juices.

As Noack (50) had found that press juices, obtained from living green leaves, proved to be still fluorescent, it seemed worth while to investigate this point further. If the band maximum of these juices show the same position as the living leaves, conclusions may be drawn from the behaviour of these liquids towards several agents, in respect to the state of the green coloured complex in the living plastids.

Preparation.

Living leaves were ground in a mortar with quartz-sand and tap water.

The green coloured sap was centrifuged a short time at 500 revolutions per minute to remove the cell walls and other rough particles. The supernatant fluid was centrifuged during one hour at 2400 revolutions per minute. A green coloured sediment settled to the bottom of the vessel, consisting of broken and intact plastids and small crystals.

The remaining fluid still showed a strong green colour and showed a marked Tyndall effect.

A microscopical examination showed that the fluid contained many tiny green coloured particles, of much smaller dimensions than the intact plastids.

These fluids have the following characteristics:

I. On heating to 100° C. the green coloured substance coagulates.

Under the microscope, the coagulate proved to consist of large aggregates of very small droplets.

II. Placed in full sunlight the fluid bleaches very slowly; no coagulation occurs.

III. If the fluid is shaken with ether, we observed a rapid coagulation followed by a quantitative solution of the chlorophyll in the ether; the coagulate became colourless. This confirms the observations of Noack (50).

IV. The fluorescence of these solutions could not be investigated by lack of suitable instruments, but it has been demonstrated very convincingly by Noack (50).

Noack, working with several saps, states that the press juices of some plants show a different behaviour. After centrifuging the sap of *Aspidistra spec.* the sediment proved to be fluorescent, but the supernatant fluid, which was still coloured strongly-green, missed this property.

Colloidal chlorophyll shows the following chracteristics:

I. It is non-fluorescent. Noack has observed that colloidal solutions of chlorophyll became fluorescent after boiling the solution during ten minutes. This may be explained by the saponification of the pigment and subsequent solution of the unchanged pigment in phytol, set free in this reaction.

II. It bleaches slowly in full sunlight.

III. On shaking the solution with ether the pigment is not extracted.

IV. On addition of some $MgCl_2$ the pigment flocculates and dissolves readily in ether.

Sap of the fruits of *Solanum melongena* L. proved to coagulate on boiling; it contained no chlorophyll. If this sap was added to a solution of colloidal chlorophyll nothing occurred. If this mixture was heated to 100° C., it coagulated and the pigment was taken up, quantitatively, by the coagulum. In contrast to boiled press juices, this coagulate did not give off the pigment, if shaken with ether.

The following table shows the band-maxima of the press juices:

TABLE XVIII.

Plant.	Band maximum.
Aquilegia glauca Lind.	6795 Å.
Polygonatum officinale All.	6795

Practically no shift of the band maximum has taken place by grinding the plastids, which proves that the green coloured complex, present in the living plastid, is only slightly affected by this process.

Herlitzka (23) and Lubimenko (42, 42a) came also to the conclusion that the maximum did not shift if the leaves were ground. We have investigated this point, as Noack (50) has tried to prove the existance of a chlorophyll-protein complex, and bases this theory on the behaviour of press juices.

As Noack has shown that the saps are fluorescent, and we have found that the band maximum has not shifted, we come to the conclusion, that his conclusions are justified. A further discussion of the experiments of Noack will follow in Chapter X.

c. Boiled plastids.

Willstätter and Stoll (71) observed that, after heating the leaves to 100° C., the band maximum had shifted towards the shorter wavelengths.

Lloyd (40) and Noack (50) showed, that, after this process, the chlorophyll is present again in some fluorescent state.

As the band maxima of a system chlorophyll-lecithin-water and of the boiled plastid are found to be practically identical, Willstätter and Stoll came to the conclusion that, after heating, the chlorophyll is dissolved in lecithin. As lecithinoids are present in the living cell and the system chlorophyll-lecithin-water shows a brilliant red fluorescence this supposition is not very speculative.

The same shift has been found bij Baas Becking and Koning (4).

The following table shows the data of these authors compared with the maximum found by us for *Ulva lactuca* L. The alga was heated during three minutes to 100° C. and photographed in the same way as the living leaf.

TABLE XIX.

Author.	Band maximum.
Willstätter and Stoll.	6715 Å.
B. Becking and Koning.	6730
B. Hubert.	6710

CHAPTER IX.

Structure of the plastids.

Several authors have tried to explain the structure and chemical composition of the chloroplasts.

Molisch (47), in 1916, comes to the conculsion, that the greatest part of the proteins of the leaves is present in the chloroplasts.

Biedermann (9), in 1918, finds that the chloroplasts consist of proteins, lipoids and the four pigments: chlorophyll a, chlorophyll b, carotene and xanthophyll.

Of the structure of the chloroplasts we know next to nothing.

The question of a plasmatic layer surrounding the chloroplast (see Schürhoff (60) for the older literature) is not yet settled. This question, however, is of great importance as Ewart (14) in 1897, Kny (34) in 1898 and Molisch (46) in 1904 have found, that isolated plastids are able to reduce CO_2. The plastids seem to lose this capacity if they are totally deprived of the surrounding cytoplasmic layer.

Haberlandt (17) in 1905, studying the chloroplasts of *Selaginella martensii Spring.*, finds, that a differentiated part of the cytoplasm surrounding the chloroplasts belongs functionally to the plastid.

Irving and Priestley (53) from a microscopical observation of the chloroplasts of *Selaginella. martensii* and of *Chlorophytum elatum*, arrive at the conclusion that the chloroplast is inhomogeneous; chlorophyll containing parts are imbedded in a colourless stroma. A cross-section showed that a big central vacuole was present only the outer layer is coloured.

Ponomarew (52) in 1914 was unable to detect any inhomogeneity. According to him the chloroplast should be a droplet of gel structure.

Zirkle (78) in 1926 finds a great central vacuole in the chloroplasts of several higher plants. This vacuole is connected with the plasma outside of the chloroplasts by several very fine pores.

Soeur Chr. Doutreligne has photographed chloroplasts in monochromatic light at the Botanical Laboratory, Leyden.

This method to detect the localisation of pigments within the living cells had already been tried, with succes by Baas Becking. His experiments are described by Hof and Frémy (24).

The chromoplast of a strain of *Aphanothece halophytica* Frémy. showed a rather curious, variegated, appearence.

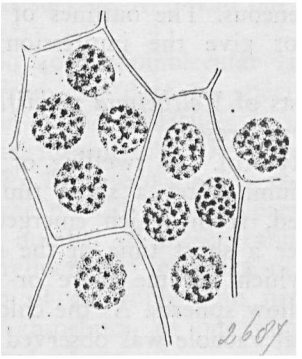

Fig. 18. Plastids of a moss, after a photograph in red light.

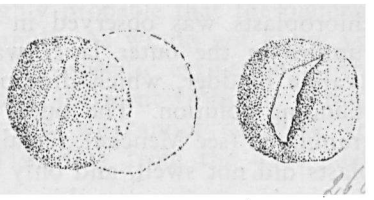

Fig. 19. Plastids of Veltheimia viridiflora Jacq. in dilute glycerol.

As two pigments, chlorophyll and phycocyanin, are present in these plastids, it was supposed that the pigments were localised in different parts of the chromoplast. Camera drawings were made in light of varying wavelengths. The instrument used was a small Leitz monochromator.

Several parts of the chromoplast never showed dark but other parts were observed as dark parts between 6200—6300 Å., due to the absorption of the phycocyanin and still others became visible between 6700—6800 Å., due to the absorption of the chlorophyll.

Without any attempt to interfere with the work of Soeur Chr. Doutreligue, which is nearing completion, it proved to be necessary to make at least one photo.

The photographs of Soeur Chr. Doutreligne show a pronounced inhomogeneity of the chloroplasts. As the chlorophyll absorbs the red radiation, the chlorophyll-containing parts are seen as dark patches on a colourless background. M e n c k e (44) comes to the conclusion that the granulation of the chloroplasts is pathological and often occurs after placing a piece of a leaf in water, a fact often observed by us.

To avoid this effect we chose moss-leaves *(Mnium sp.)*, who have the advantage og being very thin, so that the plastids may be photographed without disturbing the cells.

We used the Metaphot microphotographic apparatus. To obtain more or less monochromatic light a filter of ruby glass was used.

The light source was a 25 Watt Osram "Nitra" lamp. The exposure time was 20 seconds.

A drawing (Fig. 18), constructed after our negative, shows that

the chloroplasts are distinctly inhomogeneous. The outlines of the granula are rather diffuse and do not give the impression of droplets.

A casual observation of the chloroplasts of *Veltheimia viridiflora* Jacq. showed that a central vacuole was present.

The plastids were placed in dilute glycerol. No swelling of the chloroplasts was observed in this medium. After a short time a rupture in the outer layer was observed, from which emerged a hyaline bladder, which disappeared after a short time in the surrounding solution. The outer layers, which became more or less granulated (see Mencke), remained as hollow spheres. As the chloroplasts did not swell, and only one central vacuole was observed we get the impression that this vacuole is not an artefact. (see Fig. 19).

Theoretical considerations.

As we have at our disposal a certain amount of data from other authors, concerning the amount of chlorophyll in the chloroplasts, the dimensions of those plastids and the demensions of the chlorophyll molecule, some calculations are possible.

v. d. Honert (25) finds that 45.7 m. *Hormidium* cells contain 27.1×10^{-6} gr. chl.

One cell and so one plastid contains $\frac{27.1 \times 10^{-6}}{45.7 \times 10^{5}} = 0.5 \times 10^{-11}$ gr. chl.

900 gr. chlorophyll $= 6 \times 10^{23}$ molec, so one plastid of *Hormidium* contains: $\frac{5}{9} \times 10^{21} \times 0.5 \times 10^{-11} = 3\,3 \times 10^{9}$ molec. chl.

v. Euler, Bergman and Hellström (13) found that one plastid of *Elodea densa* contained 2.75×10^{-15} gr. mol. chlorophyll $= \pm 1.7 \times 10^{9}$ molec. chlorophyll. The volume of the plastids of this plant was 40 μ^3.

Prof. Dr. E. Gorter has estimated the area covered be one molecule of chlorophyll at the interface water-air (oral communication). The results are not published yet but are nearing completion. He uses the method of Langmuir. The appartus used is described by Grendel (16). Prof. Gorter has found that one molecule of chlorophyll covers an area of 100 Å.^{2} if spread out in a monomolecular layer at the interface water-air. As the chlorophyll molecules consist of a hydrophilic group (porphine) and a lipophilic group (phytol), Prof. Gorter has the idea that, if the chlorophyll molecules are spread in a monomolecular layer, the porphine groups are turned towards the water.

As the radius of the plastid of *Elodea densa* Casp. is 2 μ we get a surface of $4 \eta r^2 = 0.5 \times 10^{-6}$ c.m.^{2}

In one plastid we find 2×10^{9} molecules chlorophyll with a

total surface, if spread out in a monomolecular layer, of 2×10^{-5} c.m.^{2}

So 40 monomolecular layers are possible.

From the data on specific extinction we may calculate that a train of 60 molecules in an organic solvent absorbs 60% of the light, measured in the maximum of the absorbtion band.

We have seen already that it is highly probable that the pigment in the plastids may be present in granula. As we do not know yet the number of these granula and as the specific extinction of the "phyllochorin" complex may be widely different from the extinction of chlorophyll in organic solvents, we cannot calculate yet the absorbtion of one single plastid starting from the data mentioned above.

CHAPTER X.

Discussion and Summary.

We have mentioned that the reduction of CO_2 takes place in the chloroplastids. The energy needed for this reaction is taken up by a green coloured complex in the form of light. From fig. I we see that a strong absorbtion exists in the red part of the spectrum.

With Mestre (45) we want to call this hypothetical complex „phyllochlorin".

When leaves are extracted with 80% acetone, the complex is apparently destroyed and we obtain a series of four pigments: chlorophyll a, chlorophyll b, carotene and xanthophyll. These pigments „per se" lack the CO_2 reduction-power.

From the many data available we may form some idea of the properties of the complex in the living plastid.

Willstätter and Stoll (71) have shown that neither the amount of the pigment nor the ratio of the two pigments a and b undergo any appreciable change during photosynthesis.

On every 75 molecules of chlorophyll a we find 25 molecules chlorophyll b, 20 molecules xanthophyll and 10 molecules carotene, calculated from the data of Willstätter and Stoll.

The phyllochlorin complex has the following characteristics:

I. It is fluorescent.
II. It is photostable.
III. It is stable against weak acids.
IV. It possesses an absorption band in the extreme red part of the spectrum.

The fluorescence of the chloroplastids, disregarded by many authors is not very strong but may be easily detected by the simple but ingeneous method of Lloyd (37) (38) (39) (40).

Kautsky and Hirsch (32) in 1934 have measured this fluorescence quantitatively. Fresh leaves, which had been kept in absolute darkness, during 30 minutes, were irradiated with ultraviolet light. During the first second they observed that the fluorescence increased rapidly, but after reaching a maximum the fluorescence decreased slowly. At a certain low intensity the fluorescence became constant.

The rapidity of the increase of the fluorescent light is independent upon temperature, while the decrease is highly dependent upon temperature. This proves, that in photosynthesis we have two processes; a photochemical process being independent of temperature and an enzymatic process (dark reaction) dependent on temperature.

If the phyllochlorin complex reacts with the CO_2, the energy absorbed by the complex must be used to reduce the CO_2 and so cannot be reradiated as fluorescence light, which explains the decrease of the intensity of the fluorescent radiation.

Several theories have been formed to explain the physical state of the pigments in the living chloroplastids:

I. The pigment is dissolved in some waxy medium of high refractive index.
II. Chlorophyll is present in the solid state, dispersed in the stroma of the chloroplasts.
III. Chlorophyll is present in the colloidal state.
IV. Chlorophyll forms some colloidal system with lecithinoids.
V. Chlorophyll is absorbed on- or combined with some protein.

These theories are based for the greatest part on the position of the absorption maxima of these artificial systems.

For the estimation of the band maxima several methods have been used:

I. Spectroscopy.
II. Spectrophotometry:
    a. Visual.
    b. Bolometric. etc.
    c. Photo-electric.

We have pointed out in Chapter IV that the influence of

bleaching may be a source of serious errors if the visual spectrophotometric method is used.

Ursprung (69) has shown that the place of the band maximum is dependent upon the slit width. Working with a Hüfner spectrophotometer, he found the maximum in alcohol at λ 6610, widening the slit displaced the maximum to λ 6370.

With the spectroscopic method very good results may be obtained if a narrow slit is used, and the solution is very diluted. As the band is rather asymmetric, we find that, in concentrated solutions, the band axis has shifted to the shorter wavelengths.

Bleaching does not affect the position of the band, the only effect observed is a narrowing of the band. We have used this rather complicated method for the estimation of the band maximum, as we got the impression, when we started our experiments, that the many contradictions found in the literature were brought about by the use of instruments of low dispersion, and powerful light sources.

From the data of Mr. Bakker, who was so kind to estimate some band maxima with a Hoffman spectroscope, we see that the maxima estimated spectroscopically do not differ widely from the maxima estimated by us.

We have no experience with the photo-electric method, but as a very weak light source may be used, we get the impression that with this method the effect of bleaching should be practically excluded.

Our method has the advantages that the effect of bleaching is practically excluded and as we used an instrument of great luminosity a great purity of spectrum could be reached.

With an Amalux "Sperrschicht" cell a light intensity of 50 Lux was found at the level of the slit. This cell was kindly calibrated at the University Physics Laboratory, Utrecht by Dr. M. Wouda.

The theories concerning the state of the pigment in the plastids may be conveniently tested by spectroscopic means.

I. Chlorophyll in molecular solutions.

Inasmuch as we have found that solutions of chlorophyll follow Beer and Lambert's law, we may conclude that the position of the band maximum is not influenced by the concentration of the pigment.

Kundt (36) showed that the absorption maximum of a dissolved pigment shifts with the refractive index of the solvent. Unhappily the maxima of chlorophyll in several media, studied by Kundt, are given in arbitrary units, moreover Kundt used a mixture of the solvent and alcohol.

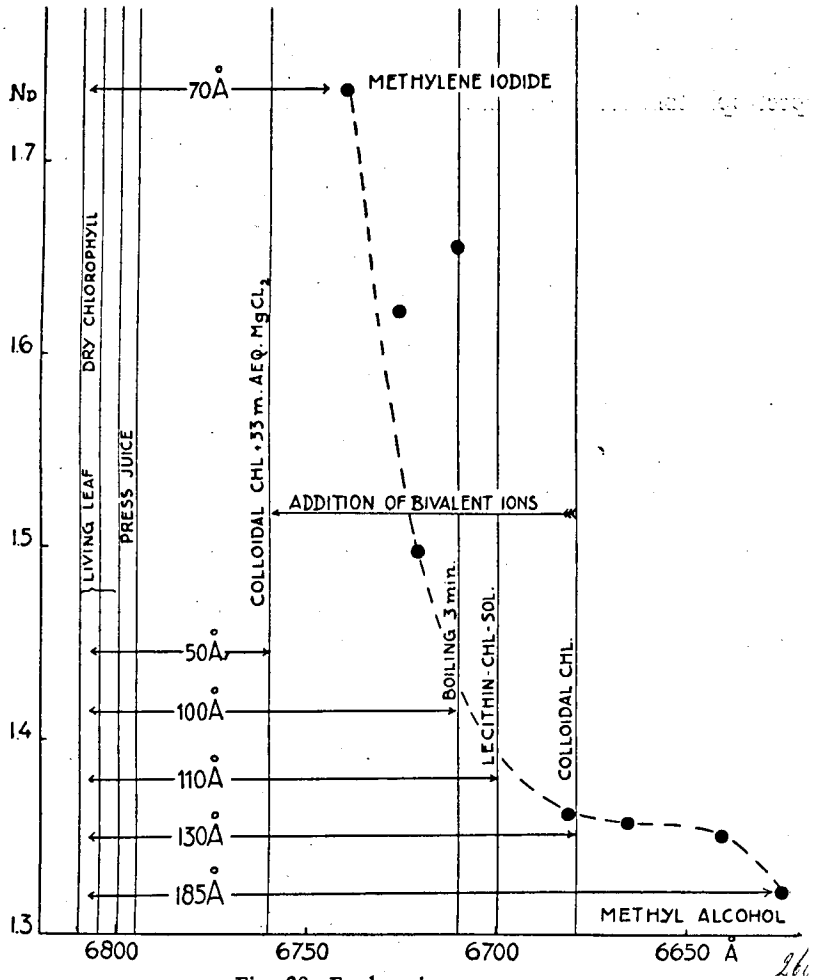

Fig. 20. Explanation see text.

We have repeated these experiments for a set of solvents. We may conclude that, in general, the "law" is obeyed.

Fig. 20 shows all the band-maxima obtained by us.

Even in a medium of very high refractive index we see that there exists still a shift of 70 Å. towards the shorter wavelengths as compared with the living plastid. The greatest shift was obtained by us in methyl alcohol (185 Å.).

To our surprise no attention has been given in the literature to the total possible shift of the absorption band-maximum.

Speculations on the identity of two systems containing chlorophyll have to be based on this factor.

From the work of Stern (64, 64a) we know that between the fluorescence band maximum of the lecithin system and the maximum of the living leaf still a difference of 40Å. exists. Compared with the total possible shift this is not at all negligible.

Way may observe, moreover, that the greatest shift towards the shorter wavelengths is obtained in polar media. Methylene iodide is an exception to this rule.

It may be observed that the pigment is almost insoluble in this medium.

The data of Baas Becking and Koning are shifted to the longer wavelengths as compared with our data. An explanation of this fact has been given in Chapter V.

In all the solvents used the pigment is strongly fluorescent and highly photolable.

II. Colloidal chlorophyll.

Willstätter and Stoll (71) held the view that chlorophyll was present in the colloidal state as the band maximum was identical with the maximum of the living plastid. They observed that the pigment, if brought in this state, was capable of giving a chemical union with CO_2. No reduction of CO_2 was found, however, in the presence of light.

These authors have disregarded the fact that the pigment in this state is non-fluorescent.

From the behaviour of the leaves, dried or fresh, towards acetone and other solvents, they come to the conclusion that the pigment cannot be dissolved in some organic medium. The pigment, which dissolves readily in 100% acetone, can only be extracted from the leaves if a certain amount of water is added to the acetone. This is indeed very remarkable as the solubility of the pigment diminishes if the acetone is diluted.

In a later publication Willstätter states that the pigment may be adsorbed on some polymolecular substance.

We have used a special method to prepare the colloidal solutions.

Willstätter and Stoll (71) observed that if water is added slowly to an acetonic solution of chlorophyll a coarse flocculation occurs. To avoid this phase the pigment has to be brought in water as quickly as possible.

As we never observed any flocculation and as our preparations

showed only a weak Tyndall effect and followed Beer and Lambert's law, we have the impression that our solutions were highly dispersed.

The band maximum of our solution was shifted to the shorter wavelengths as compared with the data of Willstätter and Stoll and of Baas Becking and Koning (4).

We were able confirm the observations of Iwanowski (29) who found that the band-maxima of solutions of colloidal chlorophyll are situated between the bands of carbon-disulphide and alcohol. It is our impression that the band-maximum of solutions of colloidal chlorophyll shifts with increasing dispersion towards the shorter wavelengths.

Iwanowski observed that the maximum of solutions of colloidal chlorophyll shifted towards the longer wavelengths on addition of electrolytes.

These "totally" flocculated solutions possessed the identical band maximum of the living leaves studied by him.

We have found, that on addition of bivalent ions, this shift may indeed be obtained. Addition of salts diminishes the dispersion of the pigment and coarser suspensions are formed.

III. Dry chlorophyll.

The final stage in the process, just mentioned, must be the flocculation of the "dry" pigment.

We have estimated the band maximum of a film of the pigment on a thin glass plate. The maximum was found at 6805 Å. and is practically identical with the maximum of the living plastids, studied by us.

Several authors have shown already that the dry pigment is non-fluorescent. In this state the pigment is highly photostable.

IV. The system chlorophyll-lecithin-water.

Stern held the view that the pigment was dissolved in a lipoid medium. He observed, that the plastids were fluorescent in a concentrated beam of blue light. When a lecithin sol was shaken with a colloidal solution of chlorophyll, he obtained a fluorescent system. The band maximum of this system showed a shift of 40 Å. to the shorter wavelengths. The accuracy of these measurements cannot be very great as we know from our own experience that it is far more difficult to find the maximum of the fluorescence band than the maximum of the absorbtion band. Moreover Stern worked with a very wide slit, which "diffused" the band still more.

Willstätter and Stoll have shown already before the experiments of Stern, that the maximum of the absorption band in lecithin was

practically identical with the maximum of a phytol solution and the boiled leaf.

It is remarkable that our colloidal solutions of chlorophyll did not give fluorescent systems if shaken with lecithin sols, a fact observed also by Bakker (6).

Willstätter and Stoll (71) mention that on boiling a mixture of a colloidal solution of chlorophyll and a lecithin sol the position of the absorption band remains unaltered and showed the maximum of a colloidal solution of chlorophyll.

The system chlorophyll-lecithin-water prepared by us showed a weak Tyndall effect and a strong fluorescence in blue light. The maximum of our system was found at 6700 Å.

V. Leaves and plastids.

a. Living leaves.

The maxima found by several authors for different leaves show differences of 200 Å. The main sources of error must be the influence of the slit width and the dispersion of the prism used. Another source of error may be found in the shape of the band. We may refer here to the work of Reinke (55). An examination of the fig. on page 399 of his article shows that the axis of the absorption band in thick leaves must show a shift of the band towards the shorter wavelengths as compared with the axis of the band of a very thin leaf.

The greatest deviation found by us is 15 Å., and the maximum was found practically at 6800 A. The same maximum has been found for a series of plants by Baas Becking and Koning (4).

b. Press juices.

We have ground living leaves with quartz-sand and tap water. The sap was centrifuged to remove the rougher particles. The remaining fluid was still strongly coloured and proved to contain tiny green particles of much smaller dimensions than the chloroplasts. These saps show the same maximum as the living leaves, which proves that the hypothetical "phyllochlorin" complex is not, or slightly, altered by this process.

As Noack has shown that these saps were still fluorescent, they should be an excellent starting point for further experiments as we have here the practically unaltered complex "in vitro". As Molisch (46) has already shown that such suspensions liberate O_2 if placed in sunlight, but lose this property within a few hours, it might be interesting to repeat this experiments manometrically. The liberation of the O_2 was studied by means of the luminosity

of *Micrococcus phosphoreus* Cohn. The presence of a plasmatic layer (peristromium) around the plastids, seems to be nessessary to obtain positive results.

VI. Experiments of Noack.

Noack (50) in 1927 has started a series of interesting experiments with press juices. We want to consider his observations more closely as they yield very valuable data for the study of the "phyllochlorin" complex and may be completed with our observations.

As the living plastid shows red fluorescence and as Willstätter (71) has shown that, from the behaviour of the pigments, in the plastid, we may derive that a linkage of the chlorophyll molecule with some polymolecular substance is highly probable, Noack tries to obtain fluorescent adsorbates of chlorophyll.

The "dry" pigment proved to be absolutely non-fluorescent.

The following table gives the result of his experiments:

TABLE XX.

Adsorbens	Medium	Fluor.
Moist Al $(OH)_3$	Colloid. (a + b)	—
" " "	Petrol-ether	—
Dry " "	" "	+
Globin.	" "	+
Albumin.	" "	—
Casein.	" "	—
Legumin.	" "	—
Hordenin.	" "	?
Clupein-sulph.	" "	—
Globin.	Colloid. (a + b)	—

In these series of experiments care has been taken to remove the lipoids as much as possible. The chlorophyll had been prepared after the method of Willstätter and Stoll. The experiments show, that in principle, the fluorescence is not dependent upon the presence of lipoids.

The following table shows the results of some experiments with leaf extracts, still containing lipoids and other impurities.

TABLE XXI.

Adsorbens	Medium	Fluor.
Al $(OH)_3$	Colloid. (a + b)	+
Al $(OH)_3$ with lecithin film	" "	+
Kaolin	" "	+

We see, that in the presence of lipoids it is far more easy to obtain fluorescent adsorbates. If the absorbing layer of $Al(OH)_3$ is covered with a film of lecithin, the fluorescence becomes very strong.

We have seen already that chlorophyll may form monomolecular layers at the interface water-air. We have the impression that the adsorbates of colloidal chlorophyll on $Al(OH)_3$ are built up in an analogous way. Both components of the adsorbate are attracted due to the opposite electric charges of the constituting elements. From the work of Herlitzka (23) and Bakker (6) we know that colloidal chlorophyll has a negative charge. It is our impression that, in the case of colloidal chlorophyll adsorbed on $Al(OH)_3$, a monomolecular layer of chlorophyll is formed around the particles of the adsorbens and that the hydrated porphine groups are turned towards the adsorbens while the phytol chains are turned towards the water. If the $Al(OH)_3$ is treated first with lecithin, the surface of the adsorbens becomes covered with a monomolecular layer of lecithin. The lecithin molecule contains two fatty chains and the phosphoric acid-cholin group. In this case the cholin group must be turned towards the adsorbens and the fatty chains are turned towards the water.

This system now forms the adsorbens for the colloidal chlorophyll.

As the surface of the $Al(OH)_3$ is totally covered with lecithin, the chlorophyll must be adsorbed in another way as decribed in

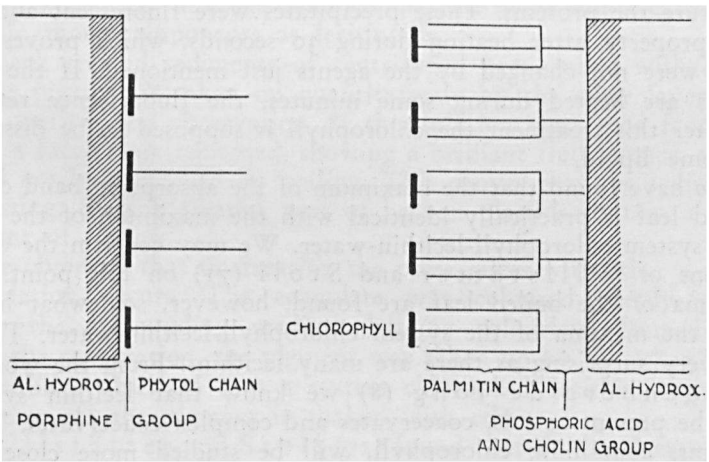

Fig. 21. A tentative interpretation of Noack's results.

the previous case. We hold the view that in this case the phytol chains are attracted by the fatty chains of the lecithin molecules as both groups are lipophilic. The phytol chains are pushed between the two fatty chains of the lecithin molecule and the porphine group is turned towards the water. We mention here that this last system is strongly fluorescent. I am indebted to Prof. Dr. H. G. Bungenberg de Jong for many valuable suggestions on these points. Fig. 21 may elucidate our idea.

Now we may discuss the results of Noack, obtained with press juices.

If leaves were ground with water and the resulting sap was centrifuged, a green sediment was formed consisting of very small particles.

These sediments could easily be dissolved in water and showed fluorescence in ultraviolet light. If these sediments were dried in a vacuum dissicator, the fluorescence did not disappear. An analysis of the chlorophyll proved that the pigment had not changed during these treatments.

If the fresh sediments are heated 30 seconds at 75° C. the fluorescence dissappears. Noack states that this is the minimum temperature for the denaturation of proteins.

He comes to the conclusion that the supposed chlorophyll-protein complex is broken down and the chlorophyll is then present in the colloidal state or adsorbed on the denatured protein.

The fresh saps may be flocculated with lead acetate or ammonium sulphate. It is a well-known fact that these agents do not denature the proteins. These precipitates were fluorescent and lost this property after heating during 30 seconds, which proves that they were not changed by the agents just mentioned. If the sediments are heated during some minutes, the fluorescence returns.

After this treatment the chlorophyll is supposed to be dissolved in some lipoid.

We have found that the maximum of the absorption band of the boiled leaf is practically identical with the maximum of the band of a system chlorophyll-lecithin-water. We may confirm the observations of Willstätter and Stoll (71) on this point. The maxima of the boiled leaf are found, however, somewhat higher than the maxima of the system chlorophyll-lecithin-water. This is not very surprising as there are many lecithins. From the work of Bungenberg de Jong (8) we know that lecithin systems may be present as sols, coacervates and complex coacervates. These systems, containing chlorophyll, will be studied more closely at the Laboratory for Medical Chemistry, Leyden.

Noack observed further that the fluorescence of the fresh sap could be destroyed by enzymes. As he assumed the existence of a chlorophyll-protein complex, he expected a destruction of this complex on addition of proteolytic enzymes. Good results were obtained with trypsin, the fluorescence deminishing rapidly. If the saps, rendered non-fluorescent by the enzyme, were heated to 100° C., the fluorescence returned as the chlorophyll now dissolved in the lipoids. The pigment proved to be unchanged after these treatments.

VII. Experiments of other authors.

Mestre (45) observed that, if the velocity, with which the maximum of the band shifts to the shorter wavelengths, is plotted out against temperature, a curve is obtained resembling closely the coagulationcurve of egg albumin obtained by Chick and Martin (see Heilbrunn (22).

Arnold (1) has found that on irradiation of suspensions of *Chlorella* spec. with ultraviolet light, the respiration remains fairly constant but the photosynthetic activity diminishes rapidly. When the photosynthetic activity had sunk to 10% of its normal value, the pigment was extracted and examined chemically. It proved to be neither photo-oxidised nor changed in any other detectible way. It is our idea that here the ligatory group is affected as it is well known that ultraviolet light has a coagulative effect on proteins (22).

The experiments of Noack, Mestre and Arnold show that it is probable that chlorophyll is combined with some protein, but we know too that the complex may be more complicated and contain more components as lecitinoids.

Noack treated sediments of centrifuged press juices with ether. The pigment is taken up quantitatively by the ether layer. We may confirm this observation. If this solution was freed from the ether a fatty mass remained, showing a brilliant fluorescence, which could not be destroyed by boiling. This proves that the sediments, consisting, for the greatest part of broken plastids contain a large amount of lipoids.

We observed that on treating the press juices with ether a rapid coagulation occurs. The coagulate was coloured deeply green, while the surrounding medium became colourless. Immediately after the coagulation, the pigment was taken up by the ether layer.

We have observed that a system chlorophyll-lecithin-water did not give off the pigment if shaken with ether.

Willstätter and Stoll (71) found that the band maximum of a mixture of colloidal chlorophyll and colloidal lecithin did

not shift on boiling. This proves that the pigment in the complex, present in the living plastid, must be already in close contact with the lecithin molecules.

If sediments of the press juices are heated, the coagulate too gives off the pigment to ether.

If a colloidal solution of chlorophyll is added to plant sap containing no chlorophyll nothing occurs. On heating this mixture a rapid coagulation takes place, the coagulate does not give off the pigment to ether.

It is our idea that in this case the chlorophyll is adsorbed on the denatured protein and does not dissolve in the lipoids. This strengthens our idea that the pigment must be in close contact with the lipoids.

Scarth (57), Mencke (44) and Küster (35) have shown that the plastids are anisotropic, which proves that the constituting elements of the complex in the living plastid must be arranged in some regular pattern.

Küster mentions that the plastids lose this property after a treatment with alcohol, which is not at all surprising inasmuch as after this process the whole structure of the complex is disturbed. Dead cells of *Closterium* spec., however, still showed this phenomenon, which is apparently in agreement with our idea that the complex is not broken down immediately after killing the cells, a view also held by Noack.

We may mention here that Prof. Dr. H. G. Bungenberg de Jong has observed that relatively "dry" coacervates of lecithin proved to be anisotropic (oral comm.).

Mencke (44) showed moreover that myelin figures could be obtained from chloroplastids after treatment with a solution of sodium-oleate. Bungenberg de Jong (oral comm.) has found that the myelin figures may be obtained on treating lecithin coacervates with several agents.

Baas Becking (oral communication) has found that the unicellular flagellate *Dunalliella viridis* Teod, when treated with a 0.1% solution of methylviolet, often shows a myelin-desintegration of the plastid. Similar effects could be obtained in *Asteromonas gracilis* Artari.

Muller (48) holds the view that the carotinoids play an active role in the photosynthetic process, and bases his conclusions on the fact that the yellow pigments are found in those organisms who use H_2O or another less active hydrogen donor, than H_2S.

VIII. Structure of the chloroplastid.

We may now form some idea of the structure of the chloroplastid. We are well aware of the highly speculative character of these considerations, but they may be at least a stimulans for other workers on the subject to probe the weak points in our scheme.

From the work of Schürhoff (60) we know that the form of the chloroplastid is not at all constant. Where the form of the plastids of the higher plants is rather uniform, varying from spherical to ellipsoidal, we may find almost every imaginable form in the lower plants. It is possible that the green coloured substance is surrounded by a differentiated plasmatic layer, peristromium, belonging cytologically and functionally to the chloroplastid, but this question is not yet settled.

From the work of Irving and Priestley (53), Zirkle (78) and from our own observations we may assume the presence of a big central vacuole in the chloroplastid. The outer layer of this sphere contains the green coloured light-absorbing complex.

Soeur Chr. Doutreligne has found that the light-absorbing substance is distributed inhomogeneously in the outer layer.

As Mencke held the view that the granulation of the chloroplastid was pathological, we repeated some of the experiments, taking care not to wound the cells in any way.

Photographed in red light a large number of dark patches could be observed, distributed regularly over the surface of the plastid.

From the data of v. d. Honert (25) and v. Euler, Bergman and Hellström (13) we have calculated that the pigment cannot be spread over the total surface of the chloroplastid in one monomolecular layer.

We have seen that the chloroplastids are anisotropic, so the constituting elements must be arranged in a regular pattern.

Combining the facts related in this chapter we come to the conclusion that the complex in the chloroplastids consists of the following elements:

I. A. coloured light-absorbing pigment or series of pigments.
II. A protein complex.
III. Lecithinoids.

Fig. 22 shows a possible construction of the complex present in the granula.

It is our idea that the outer layer consists of lecithin molecules linked together in the way shown in Fig. 23. The porphine groups of the chlorophyll molecules are combined in some way or another

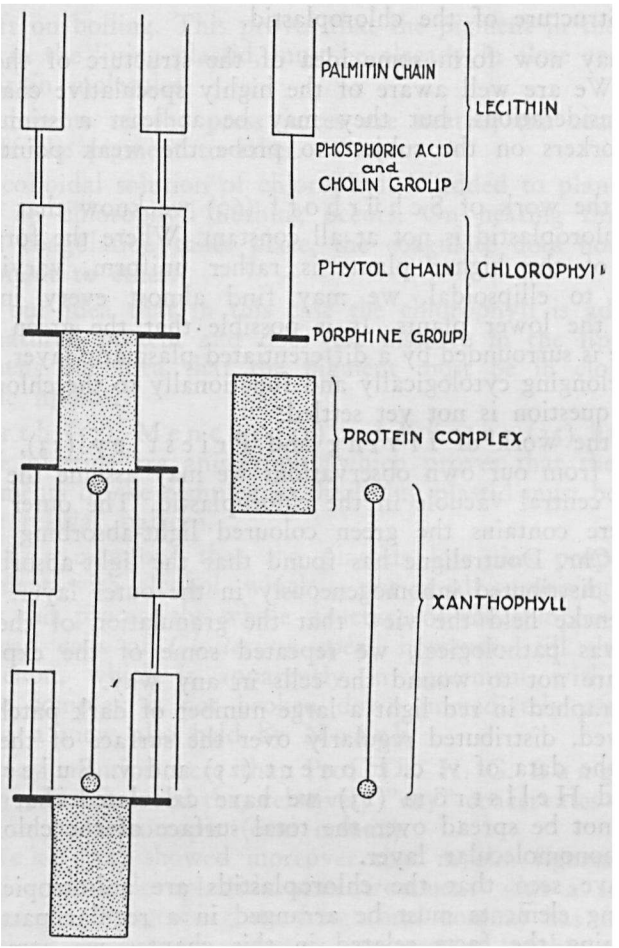

Fig. 22. Possible relation of chlorophyll, protein, lecithin and xanthophyll in the living plastid.

with a protein complex and the phytol chains pushed between the fatty chains of the lecithin molecules.

The xanthophyll molecules may be connected loosely with the protein complex. This last supposition is based on the fact only the carotene molecules are extracted by petroleum-ether or benzene from carefully dried leaves. The xanthophyll may be in a state analogous to that of the chlorophyll molecules.

Finally we may mention that Arnold and Kohn (2) in

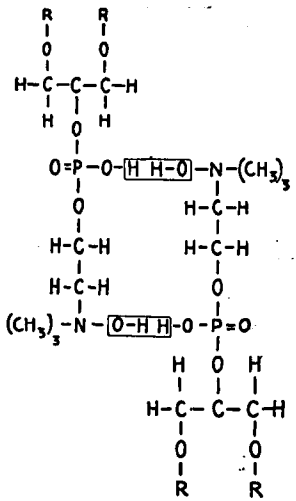

Fig. 23. Lecithin-linkage, according to Bungenberg de Jong.

1934 accept (in accordance with the idea of Emerson) the possibility of the existence of a *chlorophyll unit* in the living chloroplastid. They have found that, when suspensions of *Chlorella pyrenoidosa* were illuminated with intermittant ultraviolet light, the ratio: Mols of chlorophyll/Mols CO_2 reduced per flash was fairly constant in six species of plants. When light-and CO_2 saturation existed this ratio proved to be 2500—5000. They state that this hypothetical unit consists of 2000—3000 chlorophyll molecules. These experiments are in agreement with our idea which states that a regular pattern must exist in the chloroplastid. From the work of v. d. Honert (25) and v. Euler, Bergman and Hellström (13) we may calculate that the plastid may contain one million of these units.

This investigation was carried out at the Botanical Laboratory, Leyden, Director Prof. Dr. L. G. M. Baas Becking.

Part of the expenses were defrayed by monthly grants from the „Stichting tot Werkverruiming voor Academisch Gevormden" and a donation from „Het Bataafsch Genootschap".

Both organisations we tender hereby our thanks.

At this place I want to express my thanks to Prof. Dr. L. G. M. Baas Becking, who enabled me to work at this subject and whose critical remarks were a constant source of inspiration to me.

SUMMARY.

I. A photographic spectrophotometric method has been used to estimate the position of the band-maximum of chlorophyll (a + b) in different media and in the living leaf.

A description of the methods and instruments is given.

II. The pigment was prepared after the method of Willstätter and Stoll.

III. The pigment follows Beer and Lambert's law over a considerable range of concentrations in all the media studied.

IV. The effect of photooxidation is studied. Spectroscopic measurements of the position of the first (main) absorption band are not influenced by photooxidation. An approximate absorption spectrum of the photooxidised product is given.

V. The shifting of the band maxima in organic media follows, in general, Kundt's "law". The specific extinction is not altered in these media. The band moves "bodily" through the spectrum, excepted in methyl alcohol. In this medium the absorption band shows a kind of wing to the longer wavelengths.

VI. A special method is described to prepare colloidal solutions of chlorophyll. The position of the band-maximum of the pigment in the colloidal state is dependent upon the degree of dispersion of the pigment. Addition of bivalent ions to solutions of colloidal chlorophyll decreases the dispersion. With decreasing dispersion of the pigment, the band maximum shifts towards the longer wavelengths.

VII. Dry chlorophyll has the same absorption maximum as the living plastid, but the pigment in this state is non-fluorescent.

VIII. The system chlorophyll-lecithin-water is strongly fluorescent but shows a shift of 100 Å to the shorter wavelengths.

IX. The band maxima of several leaves are found to be practically identical. The maximum is found between 6800—6810 Å.

When leaves are ground the maximum shows no appreciable shift.

On boiling the leaves, the maximum shifts to the shorter wavelengths. The maximum of the band of boiled leaves is found practically on the same place as the maximum of a system chlorophyll-lecithin-water.

X. Plastids photographed in red light show that the absorbing complex is distributed inhomogeneously in the stroma of the chloroplastid.

Some calculations have been made on the amount of chlorophyll in one single chloroplastid. The pigment cannot be present in one monomolecular layer around the surface of the plastid.

XI. An attempt has been made to explain the structure of the plastid. Based on our experiments and the work of Noack (50) and Mestre (45) the tentative conclusion is reached that the "phyllochlorin" complex consists of a protein complex, lecithinoids and the four well known pigments.

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