SOME PROPERTIES OF THE CHLOROPHYLL IN RELATION TO ITS BIOLOGICAL FUNCTION

by

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INTRODUCTION.

§ 1. *Protoplasmic Structure.*

In biology water is a ubiquitous compound, in the living cell as well as in the external milieu water is always present. We may certainly say that for the reactions proceeding in the living organism, water is a necessary participant. However, it plays at least a double role, because water is not only present as water of crystallization, in salts, proteins, but also as adsorption-water, more or less strongly bound. In regard to its function, water may be e.g.: reaction-water in oxido-reductions of dissimilation and assimilation. The permeaton of nutritive salts may be explained only with difficulty without the help of water as such and of water as hydrogen- and hydroxylic ions.

Thus we need not be surprised that those compounds in the organism participating intensively in physiological processes show an affinity to water in one way or another.

Inversely, reserve-food materials and skeletal substance, such as calcium carbonate, chitin, suberin, etc., will be kept out of circulation because of their hydrophobic character.

Cellulose is an example of transition between the two extremes. Being skeletal material as well as medium for transport of food and waste products, cellulose adds to the hydrophilic character of the sugars the insolubility of the polysaccharides. At those places of the structure provided with physiologically active groups, we may therefore expect a certain affinity to water. This is only meant as a line of thought and not as a rule.

The proteins are the intermediary par excellence between structure and prosthetic (physiologically important) groups. In a certain sense the proteins are the finest ramifications of the structure, the coarsest manifestation of which is dead skeletal material. Therefore, it is not surprising that the protein molecule is an interesting combination of hydrophobic and hydrophilic properties and the ideal substrate for the prosthetic group. The close relationship between the physiologically active groups and protoplasmic structure is indicated by many enzymes which lose their reactivity if their prosthetic groups are separated from their activating proteinaceous substrate. The reaction proceeding in the structural protoplasmic milieu cannot be realized "in vitro" except if the structural factor

in the process is not too complicated. In such a case a simple addition of the protein is often sufficient to produce a reaction.

As soon as the structural factor becomes too "complicated" such a procedure is ineffectual, and the reaction cannot be realized "in vitro" in consequence of the lack of knowledge of the molecular anatomy of the protoplast, e.g.: the enzymes effecting the reduction of carbon dioxide to sugar (photosynthesis) cannot be extracted with the preservation of their specific activity. Here the supposition arises that, besides the necessary compounds, a suitable grouping of these compounds is needed to create a reaction as improbable as carbon dioxide reduction.

§ 2. *Introductory Remarks on Photosynthesis.*

Photosynthesis is the reduction of carbon dioxide to sugar; the light absorbed being the source of energy of the process.

Carbon dioxide is reduced at the expense of water, and oxygen is set free. This reaction is highly endothermic, the energy for it is won by means of the chlorophyll's capacity of absorbing the visible light.

In nature, another source of the energy of endothermic processes is yielded by simultaneously running oxidations (ultimately sugars are oxidized to carbon dioxide and water) and thus these endothermic processes are run at the expense of reserve materials. Inversely, photosynthesis produces potential energy by recombining the dissimilatory waste products carbon dioxide and water and this in such quantity that the product constitutes practically the only source of energy of living organisms.

It is tempting to hold responsable for this outstanding process not only special compounds, (as there are: chlorophylls a and b, carotinoids, lecithins, proteins.), but also a special "pattern" of these compounds. For a long time chlorophyll was the only comparatively well known substance in this respect, and as it was at the same time the absorbent of the necessary energy, it is comprehensible that early investigators sought for the chemical relation between carbon dioxide, chlorophyll and water.

The mode of contact of these three compounds yields the following points of view. (pt 1, 2, 3) Later on, as the function of chlorophyll became more clear, it was considered either as a specific enzyme (reacting chemically with its reaction products) or simply as a non-specific sensitizer. (pt *a*—*b*)

1. Carbon dioxide is bound to the magnesium centre of the chlorophyll molecule and it can only be reduced in this condition by means of some hydrogen-donor other than chlorophyll itself. (Old

theory Willstätter and Stoll, Assimilation book 1918).

2. Chlorophyll decomposes bound water into hydrogen and hydroperoxide. The hydrogen is used for the reduction of carbon dioxide, the latter not being bound to chlorophyll. (Gaffron 1936)
3. Carbon dioxide as well as water is bound to chlorophyll during the reduction of carbon dioxide. Stoll (1936), Franck (1935), Van Niel (1935).

a. Chlorophyll is present only as a light absorbent. In this non-specific function it might be replaced by other pigments, absorbing light of the same wave-lengths as chlorophyll (Molisch 1903, Gaffron 1936)

b. It is looked upon as an enzyme e.g.: in cases 1 and 2, where the chlorophyll is considered specific to its function.

Ultimately, it has to be remembered that the existence of a chemical bond between chlorophyll and the reaction products is not at all necessary for the transport of the energy (absorbed during the photochemical primary reaction) to the secondary processes. (see Chapter 8)

Notwithstanding a large number of investigations, even these fundamental problems have not been solved unambiguously. However, a process as complicated as respiration was explained with great success as soon as the methods necessary for separating the links of chain-reactions were discovered. The links could be separated not only as a result of their different reaction-velocities; they could also be separated chemically, by purification of the specific enzymes and their substrates.

In regard to carbon dioxide assimilation only a separation in time has been realized by means of the difference in reaction-velocity of the dark and the light reactions. Except for the purification of the chlorophyll (which is the seat of the photochemical primary reaction, see Chapter 8) a separation in place has not yet been realized.

This coherency of the links of the process of photosynthesis is the reason why, in comparison to other subjects of physiological investigation, the knowledge of photosynthesis proceeds only slowly, The usual physiological and biochemical methods failed to simplify the problem. Here other methods of approach might be useful.

§ 3. *Statement of the Problem.*

It is interesting to note the idea of some authors that chlorophyll is adsorbed in the plastid in monomolecular films. The absorption spectrum of living- and of extracted chlorophyll, its fluorescence in

different conditions, and the optical anisotropy of the granum, are indications of the influence of structural factors on photosynthesis. Moreover the chlorophyll molecule itself represents an intricate combination of hydrophilic and hydrophobic properties. In accordance with the above line of thought (§ 1), the hydrophilic groups might bear some relation to the kinetics of photosynthesis, the hydrophobic ones are almost certainly a part of the spatial structural elements involved in this process.

Presumably, carbon dioxide and water (reactionproducts), chlorophyll (sensitizer, eventually enzyme), and protein (carrier and structural factor) possess properties that may provide us with a great deal of information both as to the structure and as to the dynamics of the process, especially if their interaction "in vitro" is studied.

The modern methods of purification of chlorophyll and proteins, of the spreading and piling of monomolecular layers, supported by the X-ray study of these compounds, yield many possibilities. We are now able to build from these different compounds a spatial pattern and thus to imitate what we suppose to be the natural structure of the carbon dioxide assimilation apparatus.

Chlorophyll, water and proteins seem to be the most important. The carotinoids and lecithinoids might be of secondary importance.

In the following pages a description will be given of the purification of chlorophyll, followed by some spreading experiments with this compound. The results will be compared with those obtained by X-ray investigation of amorphous and crystalline chlorophyll and with the results obtained by the spreading and the X-ray analysis of proteins as given in the literature.

CHAPTER I.

PURIFICATION OF CHLOROPHYLL.

§ 1. *General Remarks.*

In 1932, Stoll draws the attention to the fact that Willstätter and his collaborators succeeded in preparing non-allomerised chlorophyll, about twenty years ago.

Although authors like Fischer and Conant founded their chemical investigations on the non-allomerised product, it cannot be denied that the adverse results, obtained by many others, are mainly due to the use of allomerised material as a starting point, an opinion held by Stoll. This is the reason why many workers started investigations to improve the method of purification.

It occurs to us that the original description of Willstätter and Stoll was not followed precisely in many cases. The procedures, given as an improvement upon the original method of Willstätter and Stoll, are either much more laborious or yield a higher percentage of oxidized pigment. In view of the above, it seems important to repeat Willstätter and Stoll's original prescription, which runs as follows: (Chlorophyllbook page 139).

.... "$2^1/_2$ kg of fresh nettle leaves were ground quickly, that is in 20 minutes, to a paste in a ball mill. This paste is dehydratated by shaking it together with 1.5 liters of waterfree acetone and then extracted. By suction and pressure, 2.6 liters of extraction-fluid, containing 90 grams of dry substance, were obtained. Now the press-paste, which is ground anew, is extracted with 1.2 liters of pure acetone, the latter is diluted to 80 volume % by this procedure, while 1 liter of 80 % acetone is added. After sucking off, and washing with 2 liters of the same solvent, we obtained 3.8 liter of extraction-fluid, containing almost the entire chlorophyll content of the leaves, whereas an important part of the yellow pigments is retained by the leaf-paste. By renewed extraction of the leaves with petroleum ether, 0.06 grams of pure carotene was isolated, a further 0.02 grams was obtained by working up the original chlorophyll solution.

Under constant shaking, the extraction-fluid was added to 1.5 liters of petroleum ether, resulting in a clear separation of the layers, the lower layer being only slighty coloured. The petroleum ether was washed once with 0.5 liters of 80 % acetone, and the chlorophyll layer (3.1 liters) is separated from the greater part of the acetone by washing twice with 0.5 liters of water. The volume amounted to 1.7 liters. Chiefly for the separation of the xanthophyll, the washing with 0.5 liters of acetone 80 % was repeated. From this methyl-alcoholic layer 0.15 grams of xantophyll were isolated.

The loss of chlorophyll during these separations was small; finally the solution contained still 4.2 grams of chlorophyll, and this was precipitated quantitatively after being washed five times with 2 liters of water. We gathered the flocculent suspension, by adding 50 grams of talcum, filtered it through a layer of talcum and separated it from the solvent by washing with petroleum ether. After the ether extraction of the chlorophyll from the talcum and slow precipitation from the concentrated solution with petroleum ether, 4.05 grams were obtained, which is more than $^4/_5$ of the chlorophyll present in the leaves. The preparation yielded, after quantitative estimation of the chlorophylls, an a to b ratio of 2.8 and a purity of 97.

The procedure, described above, takes half a day to accomplish. If one renounces from such high yields, the pigment may be isolated much quicker in pure state in small quantities from fresh leaves, that is in 45 minutes. The chlorophyll, thus prepared, shows a close resemblance to the former products, which is shown by the purity of its solution and of its derivatives" ....

The difficulties encountered during the preparation of the chlorophyll, used in the spreading experiments as described in Chapter 3 and 5, were mainly due to the following facts:

1. Chlorophyll is an amorphous compound. Therefore, recrystallization escapes as the ideal method of purification of a highly sensitive compound.
2. In the carbonatoms 9 and 10 (see Figure 6) chlorophyll possesses a hydrogen donating, readily oxidizible, group, the oxidation of which gives rise to the allomerised chlorophyll. Therefore, the purification manipulations causing this allomerisation ought to be omitted.
3. The photodecomposition of the pigment.
4. The crude extract contains the enzyme chlorophyllase. This enzyme splits chlorophyll hydrolytically into the phytol free chlorophyllide and the alcohol phytol.

§ 2. *Discussion of the Willstätter and Stoll Procedure in Regard to the Literature on the Subject.*

sub. 1. Following Willstätter and Stoll the usual recrystallization in purification is replaced by the washing of the amorphous precipitate in petroleum ether. Now petroleum ether is a very good solvent for chlorophyll when the chlorophyll is still mixed with fatty impurities. Only as soon as these impurities are eliminated, the pigment may be flocculated. A certain purity must be achieved to obtain a precipitate sufficiently coarse to be filterable without loss of material. At this degree of purity the chlorophyll is no longer soluble in the washing petroleum ether. This preliminary purification is attained by using acetone as an extraction-fluid and subsequent washing with water and acetone and methanol.

sub 2. As to allomerisation, the mechanism of this process is far from clear. It is surely no simple isomerisation, because oxygen is bound. Furthermore Stoll and Wiedemann (1933) proved this reaction to proceed at the carbon atoms 9 and 10, whereas other parts of the molecule are probably left unchanged. A complete description of this question will be given in another chapter, here only the methods of prevention of allomerisation will be discussed.

Only the method of Conant (1931) gives an exact determination of the degree of allomerisation of the product. One molecule of chlorophyll consumes one molecule of oxygen during allomerisation. This may be measured by means of a Warburg apparatus. However, it is customary to use the phase test instead: a diluted ethereal solution of the pigment (0.010 gram per liter was mostly used) is poured on the surface of a 30 % methyl alcoholic potassium hydroxide solution. The surface of contact shows a yellow, or more or less brown, decolouration (dependent upon the relative amounts of a and b chlorophyll). The colour and especially the time, during which the decolouration persists is a good (though subjective) characterization of the degree of allomerisation. Finally the original green colour is obtained.

The decolouration-time of some mixtures of allomerised and non-allomerised chlorophyll was compared. As was to be expected, the shorter the time of decolouration, the greater the percentage of oxidized product. Our best preparations possessed a decolouration-time of 25 seconds, or more. Only these preparations were used in the experiments. As will be seen later on, this test was sufficient for our purpose.

Allomerisation proceeds in all known solvents. A small quantity of water diminishes the reaction-velocity, though not sufficiently to stop it entirely.

Inversely, adsorption accelerates allomerisation. Very often the chromatographic adsorption analysis is used to separate the chlorophylls from carotinoids and fatty admixtures. At first sight, this chromatographic method seems to be convenient and, therefore, it was tested on its applicability to chlorophyll purification.

The different constituents of a certain mixture of compounds possess a different tendency of adsorption in relation to a certain adsorbent. If the solution of the mixture is sucked through a column of the adsorbent, contained in a glass tube, the stronger adsorbed compound will drive out the less firmly attached one, and therefore the column shows successive layers, each layer containing largely one constituent of the mixture.

This provides a method of purification. We tried its usefulness

in relation to partly purified chlorophyll extracts and found it to be dependent upon two factors. We may ask ourselves:

1. Is the chlorophyll able to withstand adsorption unaltered?
2. How many times the above manipulation has to be repeated to achieve a preparation of sufficient purity?

The most successful method proved to be the following: A glass tube of about 2.5 cm diameter is carefully filled with the highly compressed and air dry adsorbent. (Contrary to other authors, absolutely dry material proved to be unnecessary.) Now petroleum ether is poured in. The contents of the tube expands and thus prevents the chlorophyll solution from escaping between glass wall and adsoption material. (This method of charging the column is better than any other). The bottom of the tube is closed with a rubber stopper and a layer of cotton wool, mounted on a "Buchner funnel", connected to a vacuum pump.

Now a top layer of 5 cm thickness is saturated with petroleum ether (40—60° C, boiling point), and the moment that the upper surface is not yet absolutely liquid free, the vacuum pump is disconnected and 5 cc of a benzene solution of the chlorophyll containing mixture is carefully poured on top. No suction is applied until all liquid has escaped into the adsorption material, otherwise the coloured layer should never attain the equal thickness necessary. Small deviations in the uniform thickness of the original layer will grow during the process and this impedes the final separation of the successive layers. One has to wait until the thickness of the layer grows no longer, whereupon gentle suction is applied.

Now 5 cc of a mixture of $^2/_3$ benzene, $^1/_3$ petroleum ether (40—60°C, boiling point) is added, if possible no-, or little, suction is applied and this process is continued until the separate layers are clearly visible. The result mostly is:

a top layer, greyish green, containing chlorophyll b, with much contamination of compounds adsorbing stronger than chlorophyll b,

a layer of chlorophyll b, olive green,

a layer of chlorophyll a, blueish green, directly followed by a layer of xanthophyll, orange yellow, and last of all:

a layer of non-adsorbing carotene, highly purified by suction.

Very often a brown layer of phaeophytin is obtained between xanthophyll and carotene, or a grey layer, if photodecomposition could not be prevented (Hubert 1935).

The separation of the layers being clearly visible, which state is reached after two or three hours, pure petroleum ether is sucked through and a vacuum of $^3/_4$ atmosphere may be applied now. Finally, 5 cc of light petroleum ether (28—40° C, boiling point) is sucked through to eliminate further impurities and to facilitate the drying of the contents after removal from the glass tube. The contents is pressed out of the slightly conical tube as one mass and divided into the parts: chlorophyll b, chlorophyll a + b, and chlorophyll a. It is dried in the air, until the smell of petroleum ether has gone. Extraction with ether, and vacuum distillation at a temperature not exceeding 25° C, follows.

It should be remembered that a tube containing about 500 grams of adsorbent is charged with a quantity of chlorophyll not exceeding 25 milligrams. At the end, this chlorophyll is extracted with ether

and, therefore, all ether-soluble traces of impurities of the adsorption material may ruin the small quantity of chlorophyll.

On this account, glucose columns cannot be used with success, nor when extraction is performed with benzene or alcohol. Gluconic acids are always present, even in the best glucose preparations, and they are too soluble in ether, benzene and alcohol.

Talcum venetum purissimum of the Ned. Pharm. Groothandel is a very pure adsorbent, but the width of the pores is insufficiently large and, therefore, it takes too much time to achieve a good chromatogram.

Magnesium oxide powder is of no use for the same reason, but a mixture of $^3/_4$ calcium carbonate puriss. powder and $^1/_4$ talcum venetum powder proved to be suitable for the purpose, especially as the calcium carbonate powder may be easily obtained in different degrees of fineness. If such a column is used one is sure to regain the chlorophyll, after vacuum distillation of the ether, as brittle (see page 193) as it was originally.

The only drawback of the method is the decrease in decolouration time of the phase test. This is certainly not the result of chemical activity of the adsorbent, because all adsorption materials showed the same effect. As the oxygen of the air partakes in the allomerisation the chromatogram was made under a nitrogen atmosphere. The result was better. However, rather large quantities of petroleum ether have to be evaporated from the powder and this practically cannot be done in a nitrogen atmosphere. Moreover, one chromatogram delivers a chlorophyll b preparation containing about 20 % of chlorophyll a, therefore, at least three chromatograms are necessary to obtain a purity of more than 95 %. This is the reason why a preparation of such purity is mostly highly oxidized. Last of all, every chromatogram delivers $^1/_3$ of the original quantity as an equal mixture of both the components, therefore, much of the 20 milligrams of a column are lost. In our opinion this method has to be discarded as a means of purification of the crude extract. For the same reason it should neither be used for the separation of the components a and b from the pure product. We prefer the method of Willstätter and Stoll, founded on the difference in solubility in petroleum ether and methanol, (Chlorophyll book, page 161) for the latter separation.

In Chapter 5 the preparation of Zn, Cu, Pb and Hg chlorophyll is described. These compounds are much more resistant to allomerisation and are less damaged by the adsorptive separation. They may be chromatographied three times without any loss of phase, and in this case this method of purification may be applied success-fully.

sub. 3. Photodecomposition is avoided by working in subdued daylight. Artificial light is not necessary.

sub. 4. The enzyme chlorophyllase cannot be extracted from the leaf powder. Therefore, hydrolysis stops as soon as the last leaf powder is filtered off, and the first steps: extraction, filtering of the crude extract, and transfer to the petroleum ether, should be performed as quickly as possible. Once in petroleum ether the preparation is prevented from destruction by chlorophyllase.

§ 3. *Description of the Purification.*

The description will be given in some detail as it was found that slight deviations from the procedure produces allomerised impure preparations.

Some authors (Z s c h e i l e, B a k k e r) redistilled their solvents directly before use. This is unnecessary. The purissimum preparations after the Pharmacopaea Neerlandica may be used. For the final extraction, however, only ether 'pro narcosis' was used.

Nettle leaves are picked the preceding day and stripped from their stems [1]. They are kept wet during the night. The leaves are mixed with calcium carbonate (to neutralize plant acids and thus) to prevent formation of phaeophytin and a quantity not larger than one charge of a common screw press ($^3/_4$ kg) is ground and immediately the water is pressed off. The mass is now mixed thoroughly with 100 cc of pure acetone and then pressed off again. The acetone-water mixture escapes as a dark brown fluid containing many anthocyanins, but no chlorophyll. This extraction is repeated once more and the press fluid is discarded. The following 5 to 8 extractions are carried out with 90 % acetone-water mixture and the different portions of press juice are directly poured on a Buchner funnel on a thin talcum filter (protection against chlorophyllase).

The contents of the funnel is shaken with petroleum ether (40—60° C, boiling point). Water is added and on the mixing the pigments are taken up by the petroleum ether layer. Too much water makes the chlorophyll colloidally soluble in the underlying acetone-water layer. This layer is discarded. Four portions of nettle leaves are treated more or less simultaneously. A larger quantity cannot be finished in a day by one worker.

Up to this stage the treatment should not take more than 40 minutes for every portion (chlorophyllase). This is the reason why the chlorophyll is partly retained by the leaf powder. The concentration of the chlorophyll in the petroleum ether is about 750—1000 mgs/100 cc, in each of the two portions of 100 cc prepared simultaneously as follows.

The portions are washed 4 times alternatively with 100 cc of 80 % acetone and 100 cc of water (B a k k e r 1934).

The xanthophyll is kept in solution by the acetone content of the mixture,

[1] W i l l s t ä t t e r and S t o l l mostly used nettle leaves after fixation in 66 % methanol and made a stock of this material. It is not easy to do so, because the treatment very often damaged our chlorophyll (allomerisation). Therefore fresh nettle leaves were used and a stock of pure chlorophyll was made. This may be kept unchanged for at least two years.

to a smaller degree this is also the case with the chlorophyll. We take advantage of this difference in solubility by precipitating the xanthophyll by washing away the acetone with water (1 to 4 times 100 cc). The precipitate is quickly filtered off, by means of a funnel fitted with a loose plug of cotton wool. If the chlorophyll precipitates also, which happens if the solution is too concentrated, some drops of acetone are added and the manipulation is repeated.

If the precipitate is not produced washing alternatively with 100 cc of 60 % methanol and 100 cc of water will do so and this is repeated until no new precipitate is formed. Great care should be taken in order to separate all xanthophyll from the chlorophyll, because all further purifications are otherwise less effective, as is proved by chromatographic analysis. Finally the petroleum ether is washed with 80 % methanol and water alternatively (Bakker 1934) until the lower layer is only slightly yellow.

Emulsification of the mixture should be prevented carefully, it impedes purification and causes loss of material. If an emulsion is formed it may be broken by adding a small quantity of sodium sulfate, which should be always present in the mixture during this phase of the work.

At this moment the petroleum ether solution of the chlorophyll begins to be slightly colloidal, though the dispersion is still too fine to be filterable. The solution should no longer be fluorescent and ought to be almost black.

In order to make the chlorophyll completely insoluble the solution is washed with water four times or more, until a change in colour is visible, and then it is dried with dry sodium sulfate. Heating not to be feared any more, another amount of salt is added to stimulate precipitation. The preparation is allowed to stand for two hours. In the meantime the second portion is worked up to this state.

Finally, still more sodium sulfate is added until a pasty consistency is reached and the mixture is ground in a mortar.

Talcum is mixed with petroleum ether (40—60° C, boiling point) and a Buchner funnel is charged with a compact layer of it, 1.5 cm thick. On top of this layer, the sodium sulfate chlorophyll paste is pressed. The chlorophyll is washed with petroleum ether (40—60° C, boiling point), until all carotene is carried away, and finally petroleum ether (28—40° C, boiling point) is applied. Not a trace of chlorophyll is lost if precipitation (which runs parallel to the purity of the pigment) was sufficient.

The upper layer of the filter is discarded, because it does not show the right chlorophyll b colouration. A layer rich in component-b follows and then a layer of the a-component. At the bottom of this layer the rest of the xanthophyll is obtained. The separate layers are dried quickly, extracted with ether, the ether is distilled off in vacuo and a brittle residue results.

This chlorophyll is free from carotene and xanthophyll, as may be shown chromatographically. No phytol is lost: 22 % hydrochloric acid extracts no chlorophyllide from the ethereal solution. A purity of 93—95 % is reached [1].

Willstätter and Stoll (Chlorophyll book) obtained the final purity by adding petroleum ether to their concentrated ethereal

[1] After Mg-determinations by P. J. Hubers, Org. Chem. Lab. Amsterdam.

solution of chlorophyll and thus produced a precipitate of the pigment with a purity of 97 %.

Mostly we did not succeed in producing this precipitate and, therefore, altered this part of the procedure: the chlorophyll preparation is dissolved in 30 cc of pure acetone and flocculated with a concentrated solution of sodium chloride, filtered through a talcum layer, extracted with ether and evaporated in vacuo. In this way a purity of 97 % was also reached [1].

Thus we see that after the Willstätter and Stoll-procedure the chlorophyll is first precipitated colloidally, thereupon filtered and washed with the help of talcum. This colloidal chlorophyll is unimpaired by the contact with adsorbents, contrary to the products produced by the chromatographic adsorption method. Here the chlorophyll is adsorbed by the adsorbent from a benzene-solution wherein the chlorophyll is dispersed almost molecularly.

Presumably chlorophyll adsorbed from a molecular solution is less protected against oxidation than chlorophyll mixed in coarse dispersion with the same adsorbent.

§ 4. *Conclusions.*

1. The chromatographic purification of chlorophyll-extracts produces an allomerised product.
2. Therefore, the method of purification of Willstätter and Stoll has the preference over the chromatographic adsorption.
3. If the magnesium is replaced by other metals (as there are: Zn, Pb, Cu, Hg.), these compounds are less damaged by the chromatographic adsorption method in comparison to the magnesium chlorophyll.

CHAPTER II.

THEORETICAL ON MONOMOLECULAR SURFACE FILMS.

§ 1. *General remarks.*

If a drop of a saturated hydrocarbon is deposited on a water-air interface this drop will adopt a lenticular shape. Saturated hydrocarbons lack any affinity towards water, therefore, the interface

water-paraffin tends to become as small as possible, in other words, the drop will contract on the interface until gravity prevents further contraction of the lens. (1)

However, if the affinity of a saturated hydrocarbon towards water is raised by hydrophilic groups the contraction of the lenticular drop will be less pronounced. In this case the lens assumes a larger radius and a smaller thickness. (2)

If a still more effective hydrophilic group is substituted the radius of the lens may become infinitely large, its depth infinitely small. The molecules of the substituted hydrocarbon tend to be situated in the interface, thus forming a monomolecular layer upon the surface. (3)

The above-mentioned possibilities may still be extended. By increased effectivity, or increase of the number, of the hydrophilic groups, or by shortening the hydrocarbon chain, the compound may even become soluble, and in this case the drop will not spread upon the surface, but will dissolve in the water, obeying to the Gibbs-law, the hydrophobic pole of the molecule tending to reach the water-air interface.

The fourth example of the series is given by the inorganic electrolytes, without appreciable preference for the water surface, possessing even a diminished concentration in this surface (4).

We may say: spreading of any substance at an air-water interface only occurs if the molecules of the substance possess separate hydrophilic and hydrophobic groups. In addition, neither the water-soluble, nor the water-insoluble groups of the molecules should be too predominant.

These interfacial films have been discovered in 1891 by A g n e s P o c k e l s. For her experiments she used a flat trough filled to the brim with water. Across the trough a metal strip divided the water surface into two parts. If the water on one side of the strip was covered with a certain "spreadable" substance, the latter could be compressed to an arbitrary surface by moving the strip. Apparently the added substance was enclosed between the strip and the glass-walls of the trough without being able to escape from under the strip.

By moving the strip, the available surface is reduced in size and at a certain point the surface-tension will show a sudden decrease. This moment, when the surface-tension starts to decrease, the film has a depth of one molecule, as defended on theoretical grounds by Lord R a l e i g h (1899). These molecules, therefore, may be considered as floating bodies, each molecule occupying a definite part

of the surface. The molecules repel each other, for a compressing force has to be exerted on the barrier to keep the molecules together in their circumscribed surface. This force is the so-called "surface-pressure" (see below).

In 1917 Langmuir elaborated a technique for measuring these surface-pressures.

Of an oblong, well cleaned, trough, the flat brim is rubbed with pure paraffin. This precaution taken, the trough may be filled to the brim with water. At the same time the paraffin defines sharply the outline of the water surface. The surface is delimited by two barriers. The first one is a paraffined glass barrier and the other is a paraffined metal float. The gaps at the ends of the float are blocked by thin platinum ribbons, which provide sufficient mobility. The float is connected to a torsion balance placed above it.

A known quantity of a "spreadable" compound is dissolved in a volatile solvent and a known volume of it is blown on the surface by means of a pipette. The molecules distribute themselves evenly over the surface between the glass barrier and the float. By straining the torsion spring the surface-pressure may be compensated.

Let us suppose the surface-tension of the water surface behind the balance to be $= \gamma_w$ and in front of the balance $= \gamma'_w$ as a result of the distribution of the floating molecules (see below).

γ_w tending to diminish the surface of the water/air interface behind the float pulls the balance with a force $l \times \gamma_w$, in which l is the length of the balance float.

As $\gamma'_w < \gamma_w$, an apparent surface-pressure of $\gamma_w - \gamma'_w$ will act on the balance float; the force exerted by this pressure will be measured as $K = l(\gamma_w - \gamma'_w)$ dynes.

From another point of view, we may look upon the source of the surface-pressure as a pressure exerted by the floating molecules in the water-air interface, acting as a "two dimensional gas". A change of the area by an amount dA will cost a work of $K.dA = l(\gamma_w - \gamma'_w)$ dA. Thus we see that γ may be defined also as the energy of the surface in erg/cm^2; the numerical value of both the surface-tension $F = \frac{K}{l}$ in dynes . cm^{-1} and the surface energy in erg/cm^2 are identical. The pressure F (force/cm) is given by $F = \frac{K}{l}(\gamma_w - \gamma'_w)$.

In words: "*The outward force F, in dynes per centimeter, exerted upon the balance float, is equal to the drop in surface-tension produced by the molecules spread on one side of the balance strip*".

It is necessary, however, to demonstrate that in the above cases

the monomolecular nature of the layers is beyond dispute. A theory on the subject was proposed by Langmuir (1933). A concise survey of this theory will be given here.

Langmuir proved that the same laws are applicable to oil lenses on water and to monomolecular films. There is a gradual transition between these two conditions. Langmuir proved the reality of this gradual transition and applied the laws, derived from lenses of measurable dimensions, to films of monomolecular depth.

For the experiments as described in the following chapter the

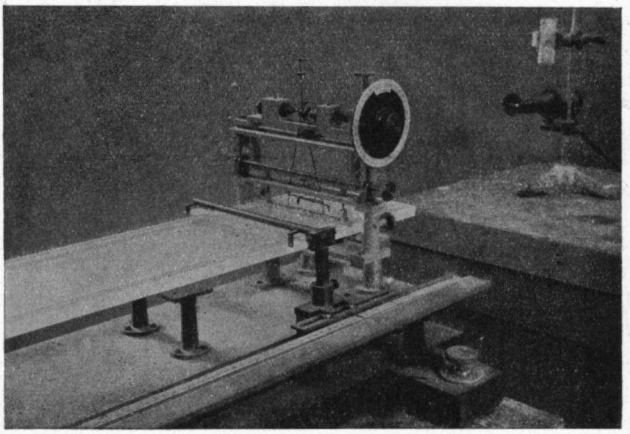

Fig. 1.

same apparatus was used, the balance, however, was not fitted with a torsion thread, but with two watch springs oppositely wound, as described by Gorter and Grendel (figure 1). The advantage of this device is that the pressure exerted upon the monolayer should be directly proportional to the magnitude of the torsion. (Concerning our apparatus up till 50 dynes . cm^{-} this proportionality existed, with a sensitivity of 0.088 $\pm$ 0.003 dyne. cm^{-} per degree of torsion.)

§ 2. *On the Theories of Monolayers* [1].

If an oil lens of appreciable depth is placed upon a water surface the diameter of the lens is certainly influenced by gravity. If the thickness of the oil lens is less than 0.1 mm the gravitational effect

[1] „Monolayer", a customary abbreviation of monomolecular layer.

disappears. If the spreading of such an oil lens is counteracted by a balance float an influence of gravity can no longer be measured. This lens may be called a film.

Notwithstanding the small depth of the film it possesses still a water-oil interface and an air-oil interface distinct from one another.

If this film is deposited upon the water and if forces are in equilibrium, the surface tension γ_1 of the water will balance the sum, γ_2 (oil-air) + $\gamma_{1\cdot2}$ (water-oil), of the film.

$$F_s = \gamma_1 - \gamma_2 - \gamma_{1\cdot2}. \qquad (1)$$

1. If $F_s = 0$ equilibrium.
2. $F_s < 0$ the film contracts until gravity prevents further contraction. After Harkins, F_s is called "spreading-coefficient".
3. $F_s > 0$ the film spreads over the surface.

We now introduce known numbers of stearic acid molecules into the interface between the film and the water. This being done, the excess of hydrophilic material is removed from the free water surface, so that $\gamma_1 = \gamma_{water}$. The molecules introduced into the interface lower the $\gamma_{1\cdot2}$ without changing γ_2 as these hydrophilic molecules (long chained fatty acid molecules) do not dissolve in the hydrocarbon; they succeed in reaching the water-oil interface. Now, the lowering of the surface-tension of pure water may be regarded as due to the spreading force of the adsorbed molecules. Similarly at the interface between water (w) and paraffin oil (o), we may put

$$F_{1\cdot2} = \gamma_{w.o} - \gamma_{1\cdot2}. \qquad (2)$$

where $\gamma_{w.o}$ represents the surface-tension in the interface water-oil if no hydrophilic substances are present. In this way the two-dimensional equation of state of adsorbed molecules at a water-oil interface may be studied in the same way as may be done at the air-water interface.

Let us consider a hydrocarbon film surrounded by an uncontaminated water surface, the interface having a known surface concentration of adsorbed hydrophilic molecules. The spreading coefficient F_s as given by Eq. (1) and Eq. (2) is then

$$F_s = \gamma_w - \gamma_o - \gamma_{ow} + F_{1\cdot2} \qquad (3)$$

Denoting F_0 the spreading coefficient for the lens on water if hydrophilic substances are absent, we have

$$F_0 = \gamma_w - \gamma_o - \gamma_{ow}. \qquad (4)$$

Thus from Eqs. (3) and (4)

$$F_s = F_o + F_{1\cdot 2}. \qquad (5)$$

Langmuir calls these films "duplex films".

Hence, duplex films should be considered as films thin enough not to be influenced by gravity and still thick enough to possess, in view of the small ranges of molecular forces, independent upper and lower surfaces. The duplex films consist largely of a (thin) layer of hydrocarbon placed upon a monomolecular layer of fatty acid molecules. The fatty tails of the latter are dissolved in the hydrocarbon, the carboxyl groups in the underlying water layer. For these films Eq. 5 holds true.

Monomolecular films, consisting only of fatty acid molecules, may be interpreted as duplex films also, as will be explained below.

With ideal gasses the equation

$$PV = R.T \qquad (6)$$

is applicable, where P is the pressure, V the volume of one Mol, R the molal gas constant and T the temperature in degrees Kelvin. If, instead of V, $v = \frac{V}{N}$ is used (v is the volume containing one molecule of gas and N the Avogadro's constant), R must be divided by N as well. In a volume of an ideal gas the molecules exert no attraction or repulsion on one another if the total volume of the molecules is negligible in comparison to the volume of the gas. A monolayer of e.g. a fatty acid may be considered in some cases as a two-dimensional gas. In this monolayer the hydrophilic groups of the molecules tend to reach the water surface. The hydrocarbon chains being in an environment of other hydrocarbon chains have no tendency to do so. They are unimpeded, except that one end of the chain is attached to the hydrophilic group in the interface. Thermal agitation tends to distribute these groups uniformly over the surface. This tendency (in diluted condition) is not counteracted by association- or repulsion forces between the molecules.

These ideal conditions are realized with many fatty acids if surface concentrations are not too high and, therefore, it is not surprising that a modified Eq (6) holds true for two dimensions.

$$F_{1\cdot 2} \times a = K'.T \qquad (K' = \tfrac{2}{3} . K) \qquad (7)$$

v is replaced by a, the spreading surface in $Å^2$. $F_{1\cdot 2}$ is the surface-pressure of the layer. Following the principles of the Van der Waals' law for condensed monolayers, the formula may be changed into

$$F_{1\cdot 2}(a - a_o) = K'.T \qquad (8)$$

a_o, being an empyrical constant, denoting the spreading surface in closely-packed films. In relation to Eq (5), Eq (8) may be changed into

$$(F_s - F_o)(a - a_o) = K'.T \qquad (9)$$

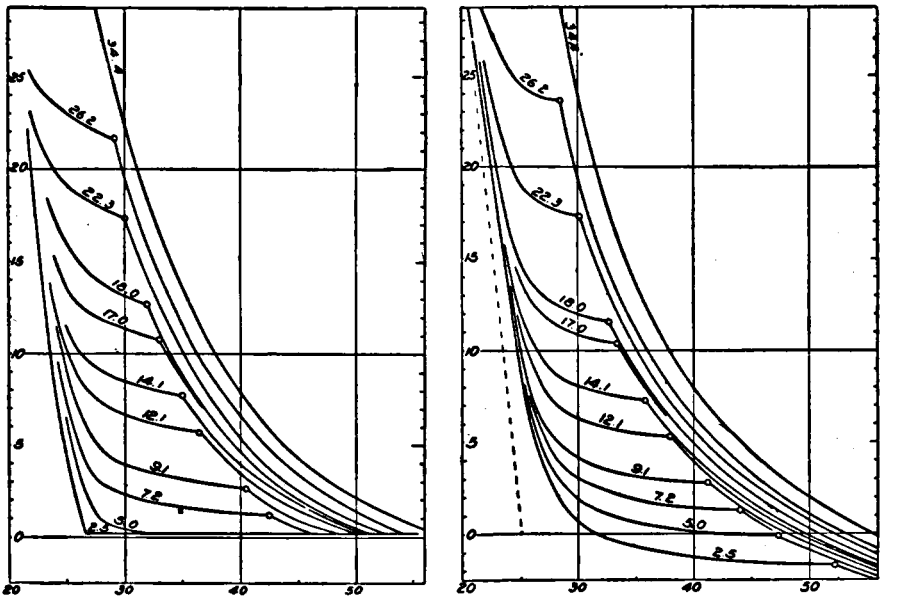

Fig. 2.

It is to be kept in mind that in this formula F_s is the spreading force of the duplex film, as in Eq (5), and F_o the spreading force of that film, if the hydrophilic molecules are absent.

a is the spreading surface of the hydrophilic molecules at an arbitrary state of compression.

a_o is an empyrical constant, being less than a, corresponding to a closely packed film. On other grounds, Langmuir concludes that the van der Waals-factor on molecular attraction is not yet applicable to these cases of low surface concentration.

The last step to be taken is the transition from the duplex film to the monolayer. This is done by proving that Eq (9), derived from lenses and duplex films, is applicable to monolayers. A d a m and J e s s o p (1926) give a family of (F — a) curves for myristic-acid films spread on 0.01 N. H Cl for a series of different temperatures (Figure 2). These curves (only the parts on the right side of the kinks are considered) are almost exactly rectangular hyperbolae. The values of F_o, calculated from these graphs, were practically the same for the whole temperature range from 2.5° C to 34° C, the average value being

$$F_o = -11.2 \text{ dynes. cm}^{-1}$$

The values of a_o are found to vary with the temperature in accordance with the equation

$$a_o = 12 + 0.178\, t$$

If these values are introduced into Eq (9) F and a can be calculated for different temperatures. The agreement between the curves is sufficiently good to justify the assumption that these films of myristic acid are essentially duplex films.

Gaseous films can be recognized by the surface-pressure, this pressure being continuous down to a fraction of a dyne. cm.$^{-1}$. "Ideal gaseous films, however, are met relatively seldom and were not discovered until 1930" (A d a m). Mostly, the film molecules adhere laterally more or less and form monomolecular islands upon the water surface. Many gradual transitions are observed between almost ideal gaseous and condensed films (A d a m (1930).)

§ 3. *Some theoretical Possibilities of the Method.*

A detailed knowledge of the interaction of chlorophyll and water seemed to be necessary for a possible understanding of the process of photosynthesis. In this respect the arrangement and size of the chlorophyll molecules depending on the different degrees of hydratation, as well as on the electric properties of the chlorophyll-water interface, seem to be of principal interest. Spreading alows us to study defined surfaces in which the orientation of the molecules is known.

The change of pH of the spreading substrate enables the study of the behaviour of the chlorophyll molecule in different states of dissociation and hydratation. The potential drop in the interface, as measured by the potential difference between the substrate and

the air (the latter made conductive by ionization by means of a polonium preparation), enables us to draw conclusions on the constitution of the electric double layer.

These experiments will be carried out with natural chlorophyll, as well as with the metal free phaeophytin, and with compounds in which magnesium is substituted by other metals.

Figure 3 shows the relation between F (surface-pressure) and a (spreading surface) for palmitic acid, reproduced from a paper of Adam and Harding (1932). It indicates that if the available surface for each palmitic acid molecule is not diminished below 26 Å^2, the surface-pressure is practically unimpaired by the spread molecules. (Even in these large spreading surfaces palmitic acid does not produce a gaseous layer). Between 26 Å^2 and 21 Å^2 the surface-tension drops as much as 20 dynes. cm^{-1}. This drop may even increase, as follows from the figure. Apparently, the spreading surface depends upon the pressure exerted upon the palmitic acid molecule. This relation becomes linear at high pressures. Extrapolation of this linear part to F = 0 gives the spreading surface a_0 in case the same monolayer should be able to exist without compression.

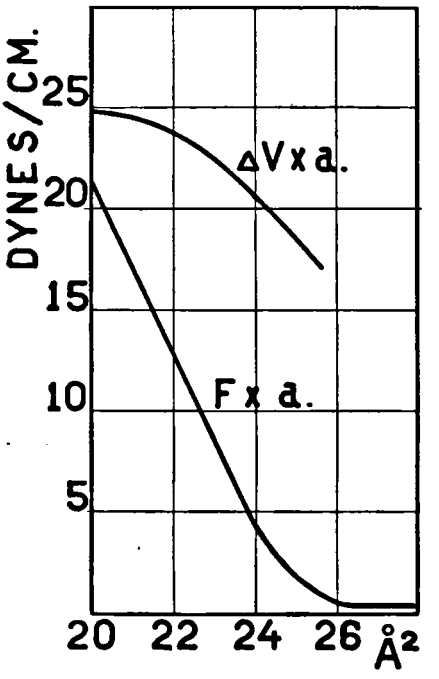

Fig. 3.

a_0 for stearic acid is 20.5 Å^2. This area is identical with the cross sectional area of the molecules in stearic acid crystals in a plane parallel to that occupied by the heads of the molecules. Since the axis of the hydrocarbon chain makes an angle of 63° with this plane, there is a strong evidence that the hydrocarbon chains of the fatty acids on the water are also inclined about 27° from the vertical.

In the following chapters will be explained how analogous results are obtained with monomolecular films and crystals of chlorophyll.

CHAPTER III.

SPREADING OF CHLOROPHYLL.

§ 1. *General remarks.*

In the experiments described in this chapter only pure phase-positive material is used. It is dissolved in acetone.

This solution is blown upon the water by means of a micro-pipette. A concentration of 1—2 milligrams per cc of acetone and a pipette volume of 20—40 mm^3 yields surfaces easily measurable by the instrument. (This concentration is not too high, as 5 mg per cc still spread to a homogeneous monolayer upon a surface of 100—200 cm^2.)

Although acetone is soluble in the buffer solution the above-mentioned quantity of acetone in solution, however, did not alter its surface-tension; at least, readings were not changed in a measurable degree. This conclusion was drawn after comparing acetone- and ether spreadings.

We did not use ether, this substance being too volatile as a stock solvent. Petroleum ether is known as a good spreading solvent but chlorophyll is not dissolved by it.

The pipette is blown out in 30 seconds time, it should not be blown out quicker, as in that case the spreading surface attained is mostly too small. This may be due to the chlorophyll forming colloidal solutions, when large drops of acetone mix with water. Also the solid state of the monolayer, contrary to gaseous and fluid layers (see below), may lead to accumulations of molecules which afterwards are not spread upon the surface.

The mouths of the pipettes were long and thin, assuring a regular outflow of the contents. They were blown out upon the surface with a slight upward inclination.

If talcum powder is blown upon the surface around the mouth of the pipette, it is pushed away by the spreading layer, always yielding a definite boundary. As the final position of this boundary is stationary, a certain number of chlorophyll molecules is capable of covering only a definite area of water. This is a simple demonstration of the fact that the chlorophyll film is condensed. A proof for the solid nature of this film follows from the fact that talcum powder blown on a monolayer of chlorophyll can no longer be displaced even by blowing very hard. In such solid films the molecules possess a strong lateral adhesion and they form islands of monomolecular thickness, from the edges of which molecules very seldom

leave. The gaseous layer of e.g. decane-dicarboxylic-acid leaves to the talcum every freedom of movement (A d a m 1930). These molecules, therefore, do not adhere to one another laterally and travel independently along the surface.

This is an important accessory circumstance during spreading; with solid films a slight leakage of the balance strip does not result immediately in a drop of surface-tension behind the float. In comparison to fatty acids and proteins, leakages are abnormally frequent with chlorophyll, probably because of the affinity of the phytol tail of the compound to the paraffin of the balance, which affinity gives rise to hydrophilic films upon the platinum ribbons. Therefore, a small amount of talcum was powdered on these ribbons.

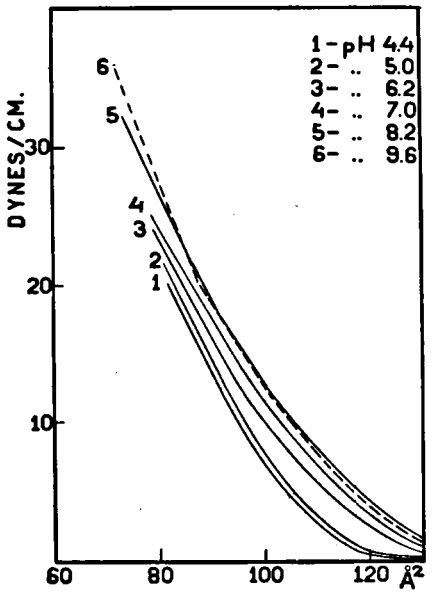

Fig. 4.

§ 2. *Spreading of Chlorophyll a + b.*

Figure 4 shows the relation between the area, a, and the surface-pressure, F, for a preparation of chlorophyll a + b at different hydrogen ion concentrations.

If, at pH 4.4, the spreading surface per molecule exceeds 120 Å², the pressure exerted on the float is only very small and highly variable (as it ought to be with condensed films, see Chapter 2, § 2). However, from 2 dyne. cm^{-1} upwards, F is accurately reproducible and at first rises but slowly. At last F increases quickly and directly proportional to the decrease in a. This point is reached at about 90 Å².

If the pressure is raised above a certain value (20 dynes. cm^{-1} at pH 4.4) the force on the balance suddenly drops in a spurious way, denoting the collapse of the layer; different parts of the layer probably transgress.

The pressure at which the layer collapses we shall call the m a x i m u m p r e s s u r e. We thus find for this mixture of chlorophyll

a + b an $F_{max.}$ of 20 dynes. cm^{-1} at pH 4.4, with a minimum spreading surface of 83 $Å^2$.

a_0, the spreading surface extrapolated to a pressure F = 0, is 108 $Å^2$ ± 3.

The same figure shows the F-a curve at pH 7.0, and contrary to the first curve an accurately reproducible pressure of 3.5 dynes. cm^{-1} is already attained at 120 $Å^2$. Obviously the chlorophyll molecules exert the same lateral pressure on each other at a much larger distance than at pH 4.4. Another difference is that the linear part of the curve is less inclined to the abscissa. This means that the lower the hydrogen ion concentration the more dynes. cm^{-1} are necessary to bring about the same reduction of spreading area.

In other words: *compressibility increases with decreasing hydrogen ion concentration.*

If compressibility (c) is expressed (following Philippi, 1935) as the relative change in area when the force exerted on the layer is increased by 1 dyne. cm^{-1}, and if the area at 20- and the extrapolated value at 0 dyne. cm^{-1} are used for the calculation, we may say

$$c = \frac{1}{\bar{a}} \cdot \frac{\triangle a}{\triangle F} = \frac{a_0 - a_{20}}{20} \cdot \frac{2}{a_0 + a_{20}}.$$

a_0 and a_{20} are the spreading surfaces at 0 and 20 dynes. cm^{-1}.

TABLE 1.

substance	compressibility in cm/dyne. 10^3	author
Palmitic acid (C_{16})	9.1	calculated from Adam's results, shown in fig. 4.
Behenic acid (C_{22})	7.1	——
Ovalbumin	16.5	Philippi
Lactoglobulin	18.7	——
Serumalbumin	24.2	——
Chlorophyll a+b (pH 4.4)	12.2	This investigation
Chlorophyll a+b (pH 7.6)	15.0	——
Phaeophytin	13.1	——

This table shows compressibilities of monolayers of chlorophyll and some other compounds. The compressibilities of monolayers of straight-chained fatty acids are smaller than those of proteins. The layers of fatty acids possess a simple mode of packing and are not or only little influenced by hydratation. The protein layers, however, are of more complicated architecture and their hydratation influences packing very much. Chlorophyll shows an intermediate position.

In table 1 the compressibilities at pH 4.4 to 7.6 are given . c increases gradually, concomitant with pH. At the same time, a_0 and $F_{max.}$ show a synbate change of magnitude.

What may be the reason of the change of a_0?

In general, except by pressure, a change in area may be caused either by reorientation or by hydratation. As will be discussed in detail later on, a reorientation of the molecules is unlikely in this case for three reasons.

1. In the following experiments with chlorophyll derivatives, the changes of spreading surface, caused by reorientation due to pH differences, occurred in a much shorter pH range (less than one unit). The increase described above, however, is very gradual.
2. The changes in spreading surface of chlorophyll derivatives caused by reorientation were always much larger (about 30 % of a_0 at pH 4.1).
3. a_0 changes antibate to the pH in all cases of reorientation observed with the chlorophyll derivatives.

Therefore, the idea of reorientation may be discarded in this case and a_0 must be a function of hydratation.

It has been explained in Chapter II that, after L a n g m u i r's theory, in monolayers of fatty acids the hydrophilic carboxyl groups tend to reach the water surface, while the hydrophobous saturated hydrocarbon tails try to surround themselves by other hydrocarbon tails. These carboxyl groups become hydratated; they surround themselves with a certain number of water molecules. In conformity with these simple compounds the chlorophyll molecule will also turn its hydrophilic groups towards the water. These polar groups will change the distribution of negative and positive ions in the substrate, and thus will give rise to an electric double layer. The groups will surround themselves with hydratation spheres, a number of water molecules also penetrating into the layer. The hydratation is shown by the experimental increase of a_0 with increasing pH. The concurrence of the following facts points also into this direction.

1. The compressibility increases synbate with a_0. The layer comparatively dry at pH 4.1 swells and therefore softens if the pH is raised.
2. $F_{max.}$ increases synbate with the change of a_0 and c.

$F_{max.}$ may be influenced by orientation and packing of the spread molecules, the "anchoring" of the molecules into the water surface is surely an important contributor to the magnitude of $F_{max.}$, especially in our example of persisting orientation. It is difficult to imagine other functions than hydratation responsible for this anchoring (see for an explanation of the character of $F_{max.}$ Chapter 5, § 10).

It follows from figure 4 that notwithstanding an increase in hydratation the minimum spreading area diminishes from 83 Å^2 to 78 Å^2 with increasing pH (4.4 to 7.0). This is easily explained as $F_{max.}$ is able to attain larger values, as a result of stronger "anchoring", by stronger hydratation.

Considering these arguments, the conclusion may be drawn that experimenting with a compound containing a constant hydrophobic pole, $F_{max.}$ might be a measure for the hydratation of the hydrophilic pole.

Something may be said about the results of spreading at low- and at high hydrogen ion concentrations.

At a pH higher than 8, kinks are observed in the F—a curves. One example is given in figure 4 for pH 9.6. A kink occurs at 20 dynes. cm^{-1}, corresponding to two different values of a_0 ($a_0 = 108$ resp. 121 Å^2). At pH 8.2 a kink is sometimes observed, mostly it is smoothed out. At a pH larger than 9.6 more kinks, up to three in the same curve, are observed. The smallest a_0 observed was 104 Å^2.

L a n g m u i r explains the kink in the F—a curve of fatty acids as the result of the initial strong hydration of the carboxyl group ($a_0 = 26$ versus 20.5 Å^2). If the hydrogen atom is replaced by a methyl group, the kink disappears and the usual a for fatty acids, 20.5 Å^2, is recovered.

As in case of chlorophyll, a_0 up to pH 11 is never smaller than the a_0 for a comparatively dry compound at pH 4.1, the molecules in the monolayer are probably also dehydratated by pressure and not reorientated, conditions directly comparable with those of the fatty acids given above.

Chlorophyll cannot be spread at a pH lower than 4.1. If so, it loses its magnesium and phaeophytin is formed. In regard to spreading this compound should not be mistaken for chlorophyll (see Chapter 5).

§ 3. *The electric double Layer.*

As it was made acceptable that the charge and, hence, the hydratation of the chlorophyll molecules is increased at higher pH, we may conclude that the sign of this charge is negative. At a higher pH more positive ions (hydrogen ions) must have dissociated from the layer, thus giving rise to an outer positive- and an inner negative layer.

In the chlorophyll-water interface (which in this case of a monolayer is of measurable dimensions) a separation of charges takes

place. At pH 4.2 some initial separation of charges certainly exists already, it is, however, changed in the sense described above if the hydrogen ion concentration be reduced.

The chemical cause of the affinity of the chlorophyll to water ought to be discussed first, in order to achieve a better understanding of the processes involved.

CHAPTER IV.

CHEMICAL DESCRIPTION OF THE CHLOROPHYLL MOLECULE.

§ 1. *The Phytol.*

Fischer (1936) proposed a formula for the chlorophyll molecule given in figure 5. Although this formula is the result of more than 25 years continuous research at the laboratories of Fischer, Willstätter, Stoll, Conant, Noack and others, it still seems unconvincing in certain minor aspects.

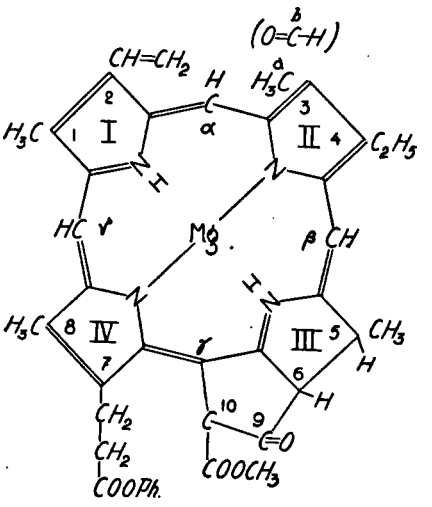

Fig. 5.

The chlorophyll molecule is a compound of two widely different parts: the phytyl ester group and the coloured tetrapyrrole group (pigment nucleus).

The phytol (figure 6), a saponification-product of the chlorophyll, is an unsaturated primary alcohol, a viscous fluid with high boiling point.

G. Fischer and K. Löwenberg (1929) gave a synthetic proof of the structural formula. The starting point for the synthesis was pseudoionone, which shows the relationship between phytol and

the carotinoids. However, the conjugated double bonds of the carotinoids are absent in phytol, which is the reason why phytol does not absorb visible light, as the carotinoids do.

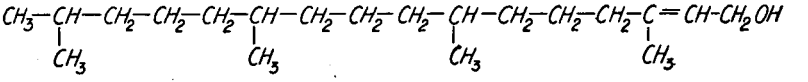

Fig. 6.

§ 2. *The Pigment Nucleus.*

In regard to the structural formula of the pigment nucleus, mainly the line of investigation of H. Fischer is followed here.

A starting point for the research after the general structure was given in the phyllo-, pyrro- and rhodoporphyrins, characteristical degradation products of chlorophyll, having the 2-vinyl-group of the natural chlorophyll saturated to ethyl, the cyclopentanon broken and shortened, and possessing an unimpaired propionic acid group (Fischer 1929, 1933).

The structure of e.g.: pyrroporphyrin was known in so far as it should be a tetramethyl-diethyl-propionic acid of porphin. (Porphins possess a so called "chlorin" ring which is a tetrapyrrole ring without a cyclopentanon closure. If the cyclopentanon closure is present, the ring is called "phorbin" ring. Fischer 1935). The structural formula of this compound was proved by synthesis. The 3-methyl group of chlorophyll-a appeared to be a formyl group in chlorophyll-b (Fischer 1936), which is the only difference between a and b chlorophyll.

Amongst the tetrapyrrole pigments (as there are: heme, chlorophyll, katalase, Warburg ferments, cytochromes) the chlorophyll is characterized by its cyclopentanon. Two carbon atoms (9 and 10) (figure 5) are added to the chlorin ring between the γ-carbon and the 6-carbon atom. The 9-carbon is part of a keto-group, the 10th is a quaternary carbon atom. Synthetical proof of this structure is as follows:

Chloroporphyrin-e_4 (figure 7) (4 denoting the number of oxygen atoms) (1 . 3 . 5 . 8 . tetramethyl-2 . 4 . diethyl-, methyl-carbonic acid-porphin 7 propionic acid) was synthesized, the methyl group of which proved to be very reactive. In chloroporphyrin-e_4 this reactivity is caused by the presence of an active group (COOH) at carbon atom 6. With the help of sodium ethylate chloroporphyrin e_4 may be condensed into phylloerythrin (see figure 8), a well known product of biological degradation of chlorophyll (Fischer 1932, 1936). There are several closure reactions.

Closure-reactions may be followed spectroscopically as the phorbin-rings

possess an absorption spectrum differing characteristically from the chlorin rings.

More arguments will not be given, being primarily of chemical importance. The closed chain of conjugated double bonds will be in

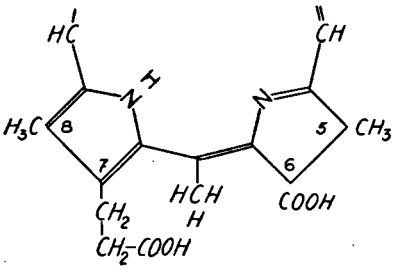

Fig. 7.

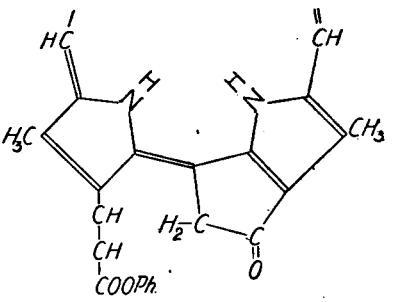

Fig. 8.
(Here the phytyl-compound is given).

discussion many times and therefore some experiments to be found in the literature are gathered here and some ideas on the subject will be given.

§ 3. *The closed Chain of conjugated double Bonds.*

Many pigments in nature owe their colour to conjugated double bonds. The carotinoids are an ideal example of this fact. The same holds true for chlorophyll, as will be explained in the following manner.

An important question in this respect is the number of conjugated double bonds. This number, to be derived from the number of hydrogen atoms, is not so easily settled, the high molecular weights of the compounds concerned making the determination uncertain. Hence, exact information on these conjugated double bonds must be derived from other considerations.

This information may be obtained from what is known about the position of the (most probably) 28 hydrogen atoms (chlorophyll-a) belonging to the pigment nucleus (figure 5).

The carbon atoms connecting the pyrrole groups (methin-bridges) surely belong to the conjugated ring and, therefore, they all possess only one hydrogen atom, except the γ-carbon atom being a member of the cyclopentanon. Moreover, the optical activity of the chlorophyll (F i s c h e r 1935) is not produced, as we know at present, by the double bonded γ carbon atom, but, among others, by the

carbon atom 10, a fact substantiating the position of only one hydrogen atom at carbon atom 10.

The remaining hydrogen atoms may be placed at the peripheral groups, except two of them. The following facts furnish the key to the elucidation of the position of these two hydrogen atoms.

Stoll (1932) found on hydrogenation that only two hydrogen atoms may be introduced into the natural chlorophyll without flattening out the absorption spectrum. This hydrogenation, therefore, does not proceed in the conjugated ring, as Kuhn (1932) found that saturation of already one double bond in the ring is sufficient to weaken the colour. Hence, Stoll concludes that this hydrogenation saturated the vinyl group.

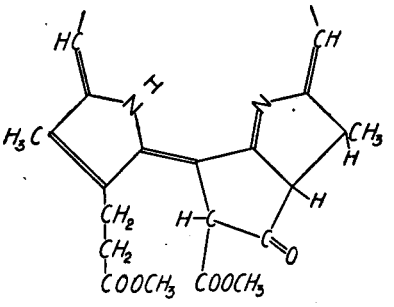

Fig. 9.

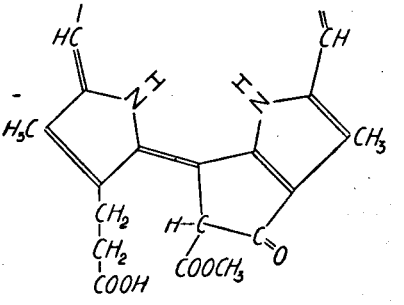

Fig. 10.

We shall now combine the above data with those on phaeophorbid-a (figure 9) and phaeporphyrin-a_5 (figure 10).

Phaeophorbid-a and phaeoporphyrin-a_5 are isomers (Fischer 1935, 1936), differing only in the place of two hydrogen atoms. Two hydrogen atoms coming from some place of the phaeophorbid-a molecule are able to saturate the vinyl group and thus produce the phaeoporphyrin-a_5, without changing the colour. Obviously, these two hydrogen atoms have nothing to do with the chromophoric ring and, hence, may be placed at carbon atoms 5 and 6.

The position of these two hydrogen atoms at the 3-pyrrole group gives at the same time a suitable explanation of the higher reactivity of the cyclopentanon in phaeophorbid-a compared to this reactivity in phaeoporphyrin-a_5. In phaeophorbid-a the cyclopentanon is activated by the proximity of these two hydrogen atoms (Fischer 1933).

The rest of the hydrogen atoms may be placed at the peripheral hydrocarbon groups.

In this way, every hydrogen atom present, after the best elementary analyses, has an explained- and explainable place, and at the same time the continuous closed chain of conjugated double bonds is obtained.

§ 4. *The Absorption Spectrum of the Chlorophyll.*

The chlorophyll possesses a much stronger light absorption than the carotinoids. In the chlorophyll molecule special chromophoric groups are absent. The spectra of the more simple tetrapyrrole compounds and the spectra of the metal free porphins possess the same selective character; these spectra are all composed of many distinct, often very narrow, bands. Peripheral groups of the molecule do not change this remarkable character of the spectra. Peripheral groups may have a shifting influence upon the spectrum.

As the hydrogenation of already one double bond of the conjugated system (K u h n 1932), or the change of one methin-bridge, into a keto-bridge (C o n a n t 1931) weakens the colour considerably, we may be conclude that the conjugated double bonds

1. are the seat of the selective absorption and
2. that they are arranged in one system.

The indication that this system should be a c l o s e d system of conjugated double bonds may be attained along two different ways.

1. M ü l l e r and E n g e l (1931) gave the absorption spectra of bilirubin solutions. The only difference between bilirubin and porphins is the break in the ring between two of the four neighbouring pyrrole groups. In this compound the closed chain of conjugated double bonds is broken and has changed into a straight chain.

Comparing the spectrum of this straight-chained tetrapyrrole pigment with the spectrum of chlorophyll and its ring-like chained derivatives, we were struck by the perfect selectivity of the latter, and thus consider this a good support of the view given above. The same holds true for the carotinoids being straight-chained pigments as well.

2. It is known from experimental- and theoretical evidence that the resonance phenomena (P a u l i n g 1933, W h e l a n d 1933) in a system of conjugated double bonds are much more marked if these bonds are arranged in a ring-like structure. In addition, the symmetry of this structure plays an important role. Quantum theory proves that the energy-levels of electrons in a field of high symmetry are in general degenerate, which means that different states of the total system, as described by different states of the individual electrons, possess the same energy. In a field of lower symmetry these levels are split, thus giving rise to a more complicated system

of energy-levels. As the absorption bands, corresponding to the transition between different energy-levels, are always fairly broad in molecules, we may expect a spectrum composed of distinct bands only when a few energy-levels are present. In other cases (low symmetry) the result will be a broad absorption region.

Hence, figures 5 and 11 do not seem to be the best expression of the optical properties of the chlorophyll molecule: the chromophoric ring possesses a low symmetry.

Figure 12 is in better agreement with the optical character of the chlorophyll and as the changes in the carbon skeleton do not influence the number, the place, or the mode of binding of the different groups bound to the tetrapyrrole skeleton (except magnesium, which

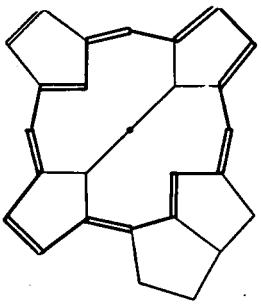

Fig. 11.

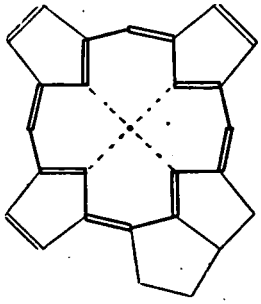

Fig. 12.

The chromophoric ring is given in heavier lines.

is another question, see below), figure 12 is suggested here as another structural formula for chlorophyll.

One objection may be made, as there is an important difference between the two formulas in the mode of binding of the metal atom.

In the analogous molecule of phthalocyanine Robertson and Woodward (1937) established, by means of complete X-ray analysis, the equal distances between the central metal (Ni) and each of the four pyrrole nitrogen atoms, indicating the absence of a preferential attraction between the metal and two of the hydrogen atoms (figure 32).

The porphins as well as the phthalocyanines possess the same spectral characteristics. Considering the analogy between the molecules of phthalocyanines and the porphins, we may apply the above data on the mode of binding of the central metal to chlorophyll also, which removes an important objection against the proposed formula.

§ 5. *Hydratation of the Chlorophyll Molecule.*

Applying the above knowledge on the structure of the chlorophyll molecule, we will try to explain the pH-sensitive hydratation of this pigment. The principal hydratation centra of the molecule are the formyl group of chlorophyll-b, the central metal atom and the cyclopentanon.

1. The formyl group is not present in the a-component and as chlorophyll-a still possesses the same pH-sensitive hydratation, this sensitivity cannot be caused by the formyl group. (see Chapter 5, § 1)

2. After Conant (1932, 1933), the central metal in porphins possesses the property of binding one or two molecules, e.g.: two molecules of pyridin. The pigment nuclei should be bound to their proteinaceous substrates by the same forces (Haurowitz 1935). Therefore a part of the field, not used in binding the magnesium to the pigment nucleus, may be a means of attracting hydratation water molecules.

From the X-ray experiments, described in Chapter VII, may be concluded that in crystalline state these forces of the magnesium are used in attracting the neighbouring two chlorophyll molecules. And as the packing of the molecules in the monolayer is exactly the same as in the crystal, the residual field of the magnesium will be saturated in the monolayer and it can no longer be applied for hydratation (see Chapter VII, § 3).

3. During the early investigations of Willstätter and Stoll, it was observed that chlorophyll binds ½ aq., even in vacuo. It was not until 1932 that the water free formula could be given. This strong affinity to water is a "highly unexpected property of a waxy substance" (Stoll 1936), soluble in the usual lipoid solvents.

Fischer (1938) gives examples of magnesium containing porphins without cyclopentanon. A particular hygroscopicity is not mentioned as a property of these compounds.

The location of this hygroscopic function is to be sought most probably in the cyclopentanon, which is fundamentally a tautomeric acetyl-acetic-ester group. Enolisation of this group has been discovered by Stoll (1932, 1936). It could be shown by benzoylation that the hydrogen atom of carbon atom 10 is able to change its place to carbon atom 9, forming a hydroxyl group.

As enol-isomers are stable in alkaline milieu, keto isomers in acid milieu, it is tempting to suppose this acetyl-acetic-ester group to be the cause of the pH-sensitive hydratation. If this group is oxidized (as it is in allomerised chlorophyll) the pH-sensitivity of the chlorophyll changes its character fundamentally, furnishing another indication of the correctness of the above view, as the reactions of

allomerisation proceed only in the cyclopentanon and not in other parts of the molecule.

The degree of hydratation is also influenced by the presence and by the character of the central metal, as will be described below.

Finally, a consideration of the two carboxyl groups remains. The two carboxyl groups are esterified; the effect is a strong decrease in hydratation. Replacing the hydrogen atom of a carboxyl group of an organic acid by an alkyl group yields a compound of strongly reduced solubility. In addition, the comparatively unimportant hydratation of the esterified carboxyl group is, most probably, not influenced by the hydrogen ion concentrations applied, and cannot be the cause of the pH-sensitive hydratation.

CHAPTER V.

SPREADING OF CHLOROPHYLL DERIVATIVES.

§ 1. *Spreading of Chlorophyll a and Chlorophyll b.*

Large quantities of the pure separated components were not prepared, because of the time involved. Therefore, an exact surface determination was not possible. Within the limits of the error (3 % in this case) no difference in spreading surface between the two components was observed at pH 4.1. There was, however, a large difference in $F_{max.}$, which is shown in figure 13.

From this figure it may be inferred that this difference in $F_{max.}$ is comparatively small at pH 4.1. $F_{max.}$ for the b-component is 21 dynes. cm^{-1}, and for the a-component 19 dynes. cm^{-1}. $F_{max.}$ grows steadily with diminishing hydrogen ion concentration. At pH 9 the $F_{max.}$ for b is more than 50 dynes. cm^{-1}, and for the a-component we obtain 34 dynes. cm^{-1}. Only at pH 10.5 the a-component reaches a value comparable to chlorophyll-b.

A good idea of the strong hydratation is obtained observing the time necessary for a balance reading. In regard to chlorophyll-b it asks only some seconds at pH 6. At pH 8.5, however, 20 minutes are needed before the increase in pressure applied has made its influence felt in a layer of 40 cm of length. With chlorophyll-a a comparable time is reached at a much higher pH (10).

In other words, the layer becomes viscous in consequence of the

uptake of water molecules, and the greatest change in viscosity is found at a pH larger than 6.

This is a striking proof of the fact that chlorophyll is hydratated by increasing pH.

We may conclude that the formyl group of chlorophyll-b increases the hydrophilic properties of the molecule. The substitution of the 3-methyl group by a 3-formyl group does not result in a qualitative change of the pH-sensitive hydratation the differences bear only a quantitative character.

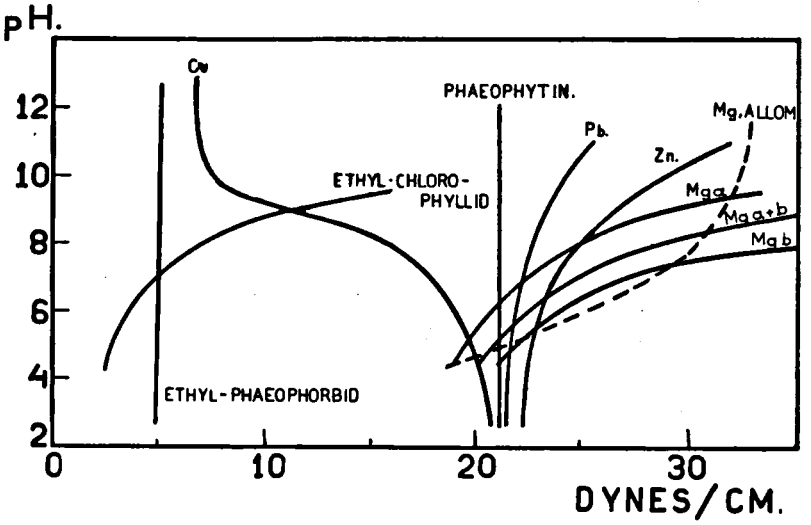

Fig. 13.

Therefore, in the other experiments the mixture was used, instead of the separate components, the former being more easily prepared.

As is explained in the introduction, water is a necessary constituent of carbon dioxide assimilation. Consequently, if water will have anything to do with chlorophyll during this reaction, the pH of the living plastid, being the milieu for the chlorophyll, should be in accordance with the sensitivity of the hydratation of this compound to hydrogen ion concentration. Weber discovered that the pH of the plastid-milieu, in the living cell, ranges from about 7.0 to 9.5. In fact a strong hydratation of the monomolecularly spread chlorophyll is found in the pH-range of the living plastid. (Still it

remains questionable whether, in these small structural dimensions, "a pH of the plastid" may be applied to the milieu directly surrounding the cyclopentanon).

This coincidence might be explained as a support for the assimilation theories of Stoll and of Van Niel, suggesting a chemical bond between the chlorophyll and the water during photosynthesis. Our experiments need not be explained as such an intimate interaction, most probably it is a hydratation, influenced by pH.

Another point, worth considering, is the obvious sensitivity of hydratation to a change in pH in the vital region of hydrogen ion concentration. This suggests a sensitivity of the whole process of carbon dioxide assimilation to this factor. The plastid and, within the plastid, especially the granum, being the pigment-containing assimilatory agent, seems to be perfectly isolated osmotically and therefore hitherto experimentally inaccessible for induced changes in pH.

§ 2. *Spreading of ethyl-Chlorophyllide a + b.*

Furthermore, the influence of the phytyl-chain on spreading is investigated by spreading of ethyl-chlorophyllide a + b. It was prepared after the procedure of Willstätter and Stoll (1911, 1913), see Chapter VII. Here, at pH 4.1 an $F_{max.}$ was obtained of only 2.5 dynes. cm^{-1}. This was enlarged to as much as 16 dynes. cm^{-1} at pH 9.6. The agreement with the curves found for the natural chlorophyll is striking (figure 13).

With ethyl chlorophyllide it is difficult to obtain exact spreading surfaces for two reasons.

At high hydrogen ion concentrations the F—a curves do not end recti-linearly and consequently no a_0 is yielded, in other words, the curves are too short. In the second place, spreading surfaces tend to be too small with ethyl-chlorophyllide. Obviously, it is the increase of solubility, obtained by the loss of the long saturated phytol-tail which is the cause of this phenomenon.

Thus, at pH 5.4 a_0 was found to be 70 $Å^2$, this value increasing to 94 $Å^2$ at pH 9.5. As explained above, a "dry' spreading surface at pH 4.1 could not be obtained.

The conclusion may be drawn that the loss of phytol did not result in a characteristic change of the pH-sensitive group. Hydratation is diminished only quantitatively.

The loss of the most hydrophobous part of the molecule resulted in a strong decrease of the $F_{max.}$. It is an astonishing fact that, notwithstanding the relative preponderance of the hydrophilic character of ethyl-chlorophyllide, hydratation still increases the $F_{max.}$.

§ 3. *Spreading of the bicarbonic Acid.*

By means of saponification with methylalcoholic potash and driving back into ether directly by careful acidulation, the bicarbonic acid of chlorophyll was prepared. It was tried to spread this compound from ether, but we did not succeed, not even at pH 4.1 (at a lower pH Mg was replaced by hydrogen), when the carboxyl groups are least dissociated. Decreasing the pH to a value smaller than 4.1, we did not succeed either.

In other words, the pigment nucleus is too water-soluble to spread, if both the carboxyl groups are free. If at a low pH the carboxyl groups are practically non-dissociated the substance is insoluble enough to be extracted by ether from an aqueous medium, it is however still "unspreadable". Therefore, a good chance exists that the pigment nucleus will be spreadable if deprived of its carboxyl groups.

§ 4. *Spreading of the Magnesium-free Compound.*

The magnesium atom may be easily replaced by two hydrogen atoms. By addition of hydrochloric acid to the acetonic or alcoholic solution we obtain the phaeophytin. Such a solution is not stable. It is better to apply the following method:

A bulb-burette of a volume of 35 cc, the lower part of which is a semi-micro-burette of 10 cc, is charged with about 15 mg of chlorophyll, to which are added a few drops of ether. 8 to 10 cc of 90 % ethanol are added. In a solution of this concentration both the colour and the fluorescence are best observed.

Now hydrochloric acid is added until the solution becomes "black" as an indication of the simultaneous existence of the absorption spectra of chlorophyll and phaeophytin. Some more hydrochloric acid is carefully added until the solution becomes olive-green, a slight excess of acid does not harm at first.

The mixture is shaken well, after which the phaeophytin is separated from the acid by extraction with ether. To this purpose water and ether are added and the water layer is discarded. This procedure is repeated two or three times. The ether is evaporated and the residue is dissolved in acetone, the volume of the solution is read on the calibrated stem of the bulb-burette.

10 % of water should be added to the ethanol solution, as with absolute ethanol blue degradation-products arise. This is also the reason why methanol proved to be inadequate.

If the above prescription is followed a very good phaeophytin spectrum is obtained. The a_0 of this compound, on spreading, shows only an error of $\pm$ 3 %, which is in conformity with the original chlorophyll and a good proof of the adequacy of the method to prepare the phaeophytin quantitatively from 2 to 10 mg of chlorophyll. The phase-test is positive and yields the gold-coloured intermediary product.

The F—a curve curve for pH 2.6 is of about the same shape as the "dry" chlorophyll curve at a low pH. The a_0 (118 Å^2) is decidedly larger than the one of dry chlorophyll, being 108 Å^2. The compressibility is 13.1 . 10^3 cm/dynes and the $F_{max.}$ 21 dynes. cm^{-1}.

A striking fact is that these three variables do not change from pH 2.6 to 9.6. Above pH 9.6 the veronal buffer of Michaelis cannot be used and the glycocol buffer of Sörensen was used instead. It was diluted 3.33 times, thus giving the same $F_{max.}$ as the four times diluted veronal buffer. The compressibility and the a_0 seem to be slightly larger, but the probable error is enlarged too at these high pH's. We may therefore say that the hydratation of the phaeophytin is absolutely independent of the hydrogen ion concentration, from a pH 2.6 to pH 12.5.

It has been explained above why the central magnesium cannot influence hydratation directly. It looks as if the magnesium made its presence felt in different parts of the molecule by the intermediary of the carbon-nitrogen skeleton of the chlorophyll molecule.

In spreading, phaeophytin is a "dead" compound which has lost its pH-sensitivity by substitution of the magnesium by two hydrogen atoms. This situation substantiates our supposition that it is the central magnesium atom which possesses the physiologically important function of activating the cyclopentanon's activity towards water.

§ 5. *Spreading of ethyl-Phaeophorbid a + b.*

Ethyl-phaeophorbid (the magnesium free component with one ethyl- and one methyl-ester group) was prepared from ethyl-chlorophyllide in the same way as phaeophytin from chlorophyll. It follows from figure 13 that spreading of ethyl-phaeophorid is also independent of the pH.

§ 6. *Spreading of allomerised Chlorophyll a + b.*

Allomerised chlorophyll can be distinguished from the natural compound by the lack of the yellow phase (Molisch 1903). It can be distinguished also by special degradation products, (Willstätter 1911) and by the fact that allomerised ethyl-chlorophyllide has lost its property of crystallization. There are many investigations on the nature of allomerisation, conclusive results, however, have not been obtained.

Conant (1931) proved that in ethanol solution 1 Mol oxygen reacts with 1 Mol chlorophyll without producing acetaldehyd in the alcohol. It is also known that allomerisation is a complex process. (Stoll 1932). After Conant and Fischer chlorophyll is

dehydrogenated, is accepting oxygen and a transitory opening of the cyclopentanon, or some addition of KOH (or similar compounds), in the conjugated double bond system, proceeds (F i s c h e r 1933). These last two processes should be responsable for the yellow colour of the intermediary product (F i s c h e r 1932). A stated fact is that in the oxido-reductive changes of allomerisation the carbon atoms of the cyclopentanon are involved. From the literature on this subject it might be concluded that the hydrogen atom of carbon atom 5 might be participating also. Be it as it may, other parts of the molecule are unimpaired.

The allomerised chlorophyll is obtained by free exposition to the air of a solution of the phase-positive material. In ethanol solution the phase disappears sometimes within the time of half an hour. The yellow phase might be slightly visible in ether even after eight hours.

At pH 4.1 we obtain $a_0 = 110$ Å$^2 \pm 3$, a magnitude directly comparable to the spreading surface of the natural product. This surface, however, diminishes, when the pH is raised from 7 to 9, to $a_0 = 88 \pm 3$. This decrease is too large to be explained as a simple result of dehydratation. Reorientation should be its cause. Moreover, $F_{max.}$ increases distinctly which makes dehydratation improbable. This increase comes to a standstill at high alcalinity, contrary to the course of the $F_{max.}$-pH curve of the natural product.

Therefore, the hydratation of chlorophyll changes its character radically when oxidized. The changes in the cyclopentanon do not deprive the compound of its pH-sensitivity as substitution of the magnesium by two hydrogen atoms did. Only the character of the sensitivity is changed, which is the first indication of the direct relation the cyclopentanon bears to the hydratation of chlorophyll.

§ 7. *Spreading of allomerised Phaeophytin.*

Spreading allomerised phaeophytin, a monolayer results with a behaviour independent of hydrogen ion concentration. Its spreading surface can not be distinguished from the non-allomerised product. Its $F_{max.}$ is about 1 dyne. cm^{-1} larger, which may be expected of a more highly oxidized substance.

Thus we see that the natural- as well as the oxidized cyclopentanon is activated towards water by the central magnesium atom.

§ 8. *Substitution of Magnesium by other Metals.*

In their "Chlorophyll book" W i l l s t ä t t e r and S t o l l state that withdrawing magnesium from derivatives of chlorophyll, as there are: phaeophorbid, phytochlorin, phytorhodin and different

TABLE III.

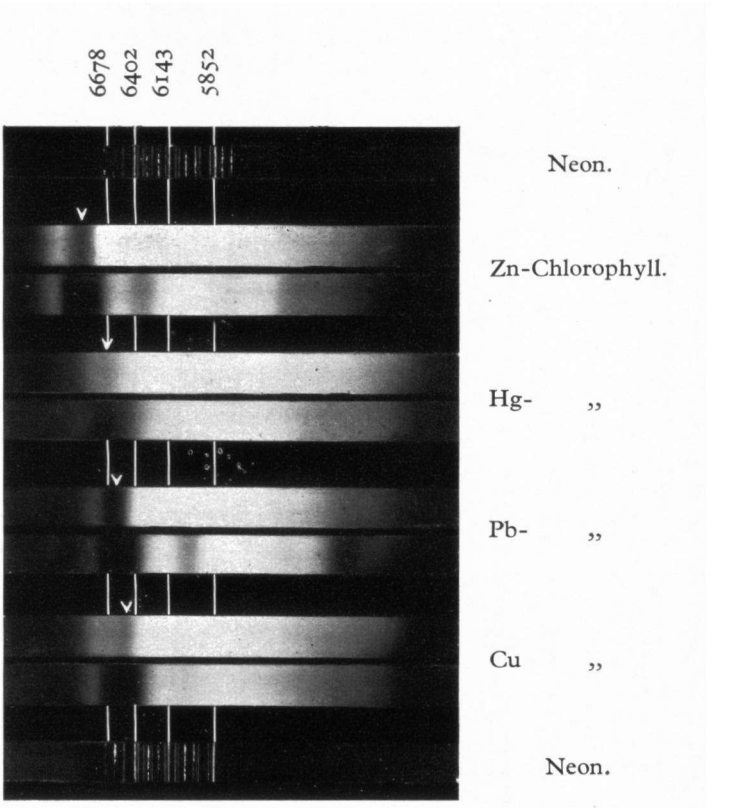

Fig. 14. Absorption spectra of Zn-, Hg-, Pb- and Cu chlorophyll in acetonic solution, each at two different concentrations. The figure shows the shift of the maximum absorption band (left) to shorter wavelengths in the direction Zn Cu.

porphyrins, the obtained substances may be combined with zinc and copper. These compounds are very stable to acids.

As it would be interesting in view of the theory of photosynthesis, to study the behaviour of the compounds of the natural product with foreign metals, we tried to prepare these compounds. Happily chlorophyll proved to fulfil the above conditions without allomerisation.

The acetates of the bivalent metals: zinc, lead, copper and mercury lend themselves to the purpose. Of the chlorophyll derivatives only the magnesium-free substances are able to add a new metal atom. The same proved to be true for the chlorophyll itself.

For the quantitative preparation, the same calibrated bulb-burette as for the preparation of phaeophytin is used. The superfluous hydrochloric acid is not washed away now, as it facilitates further reactions.

Shaking a 90 % ethanolsolution of phaeophytin with an excess of finely powdered metal acetate the substitution proceeds spontaneously with copper salts. The lead reacts very slowly (heating to 40° C is advantageous) and the zinc compound cannot be obtained without heating. All steps in the process were checked for allomerisation; as allomerisation occurred only in the ethanol solution fresh ethanol solutions should be used within 5 minutes time.

The compounds of the foreign metals proved to be much more stable to allomerisation. Especially mercury chlorophyll produced a convincing phase test, even after eleven days.

The colour of the four compounds is green, zinc greyish green, copper blue green, lead and mercury yellow green. It is directly observed visually that the power of colouration is surely strongest in the magnesium compound, zinc possessing decidedly less colour, while the mercury compound is only little coloured.

The absorption spectra of the foreign metal compounds are given in figure 14. As may be observed, they all possess e.g.: the characteristic strong absorption band in the red. This band shifts to the shorter wave-lengths when passing from Zn- to Hg-, to Pb-, to Cu-chlorophyll.

Iron is an important constituent of heme, it has a bivalent place in the centre of this molecule. It was tried, therefore, to substitute the magnesium by iron. We did not succeed with ferro acetate, nor with ferro lactate, which is a comparatively stable ferrous compound. Probably because of the oxidizability of the iron, even the addition of zinc and diluted acetic acid under a layer of petroleum was not successful. However the possibility exists, as T r e i b s (1932) succeeded in preparing ferrous compounds of heme derivatives. This looks as if only bivalent metals may be bound.

Stoll proved that the zinc-compounds of chlorophyll derivatives are decomposed by concentrated strong acids, contrary to the copper compounds.

In spreading natural chlorophyll lost its magnesium at pH 4.1, contrary to the other compounds which were spread upon 0.1 N HCl without being decomposed.

In this chapter the same sequence is observed in reaction-velocities, e.g. the zinc compound may be only obtained by heating lead substitutes very slowly, and copper substitutes spontaneously. At present magnesium cannot be substituted in chlorophyll and only with difficulty in chlorophyll derivatives by means of the Grignard method.

In Chapter VI, the E.M.F. of the chlorophyll-water interfaces for different substituted metals is measured; it is observed that magnesium shows the largest E.M.F., followed by Zn, Hg, Pb and finally Cu.

The sequence of the stability of the metal compounds $Cu > Pb > Hg > Zn > Mg$ may be understood referring to the ionic sizes and the electronic configurations. The stronger polarizing ions of the 18-electron configuration (Zn, Hg) yield more stable compounds than the Mg-ion with noble gas configuration. In addition, the ions of bivalent Cu and Pb, which have no closed configuration, give the most stable compounds. The same sequence is observed in ordinary complex compounds, such as ammoniakates.

The chlorophyll-iron compound could not be obtained, probably due to technical difficulties (see above). From the above sequence, however, the following in regard to the place of the iron may be derived. Bivalent iron as well as bivalent copper and lead possesses no closed electron configuration. The place of the iron in the succession will probably be in the neighbourhood of copper and lead, distant from the magnesium.

§ 9. *Spreading of the Metal Compounds.*

The zinc compound is much more stable to acids than the natural chlorophyll, it may even be spread on N 0.1 hydrochloric acid, figure 15 shows the dependence of $F_{max.}$ and a_0 of hydrogen ion concentration.

At pH 2.6 a_0 is $= 116\ Å^2 \pm 3$. This value is kept to pH 7 and then drops to 82 $Å^2$ between pH 7 and 8. Above pH 8 a_0 increases again, slowly but definitely.

This sudden decrease of spreading surface can only be caused by reorientation. Surfaces change gradually by hydratation. In addition, a diminishing spreading area should mean dehydratation and

dehydratation is not to be expected in this case, as with all chlorophyll compounds studied we observe a parallel increase in $F_{max.}$ and hydratation. Upon the whole $F_{max.}$ increases, although it should be admitted that an irregularity exists between pH 7 and 8. This, however, is the pH range of reorientation and as soon as stable packing is reacquired hydratation becomes visible again as an increase of a_0 and $F_{max.}$, their values growing in orders of magnitude usual for hydratation.

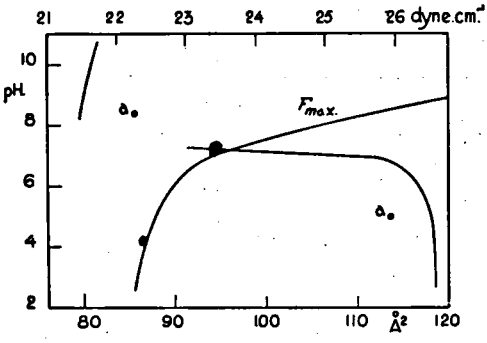

Fig. 15.

The course of the compressibility may be easily accounted for by the same reasoning. The non-vertically orientated chlorophyll molecules yield a less compressible layer.

The lead, copper and mercury compounds show the same reorientation in the same pH region, a_0 dropping from about 110 Å² to about 80 Å².

§ 10. *Conclusions.*

The conclusions to be drawn from this chapter are the following:

1. The cyclopentanon is the seat of the hydratation of the chlorophyll molecule.
2. There exists an interrelation between the central metal (Mg, Zn, Pb, Cu and Hg) and the cyclopentanon. The metal sensitizes the hydratation capacity of the cyclopentanon to the hydrogen ion concentration.

   In case of the natural magnesium chlorophyll, magnesium sensitizes the cyclopentanon to take up water, concurrent with the pH. This hydratation is remarkably strong. The dry monolayer (pH 4.1) possesses a compressibility comparable to the crystalline films of fatty acids, while at pH 7.6 the consistency of the layer resembles very much a hydratated protein monolayer (tabel 1). From crystalline, the layer becomes viscous (Chapter V, § 1).
3. In figure 13 $F_{max.}$ tends to achieve a certain value (20—25 dynes. cm^{-1}) with all phytol-containing derivatives. Hydratation seems to reach a comparable magnitude in all these compounds; in other words, hydratation tends to reach a basal low magnitude,

which is steadied by the magnesium-free compound at a pH ranging from 2.6 to 11.6.

4. Bound magnesium increases this basal hydratation very much, this hydratation increasing also concurrent with the pH. Apart from reorientation, zinc, though less pronounced, behaves very much like magnesium. The other metals seem to stimulate in a still smaller degree.
   As reorientation complicates the course of $F_{max.}$, a_0 and c, another method will be applied in Chapter VI to discover the sequence of sensitizing power of the metals.
5. The inactive phaeophytin and phaeophorbid are resensitized by the addition of a metal.
6. Hydratation of chlorophyll is already very strong in the vital range of pH. If not, a collaboration between water and chlorophyll should be unlikely in photosynthesis, actually facts point to an interrelation between the two in this process.
7. $F_{max.}$, a magnitude hitherto not used in the literature on spreading, was a valuable help for the interpretation of conditions in the monolayers.

In a fluid-gas interface spreading depends largely upon two factors: insolubility and hydratation. Only insoluble hydratating compounds can spread. Natural chlorophyll fulfils both conditions. Therefore, substitution of phytyl by ethyl, thus decreasing the hydrophobic character of the molecule, results in a much smaller $F_{max.}$, $F_{max.}$ decreasing from 50 to 17 dynes. cm^{-1}.

At first sight, in case of ethyl-chlorophyllid, a further increase of the power of the hydrophilic pole, brought about by rising the pH and thus resulting in a comparative decrease of the hydrophobic pole, ought to diminish $F_{max.}$ anew.

This does not happen, $F_{max.}$ i n c r e a s e s with pH. Presumably, two different factors act simultaneously.

It is comprehensible that increasing insolubility, caused by an increased hydrophobic character, results in a firmer monolayer, able to withstand a stronger compression. Thermal agitation tends to diminish the stability. Evidently this $F_{max.}$-decreasing influence of thermal agitation may be counteracted by a stronger hydratation, a stronger "anchoring" of the molecules.

On this account, increasing hydratation may still increase the resistance against collapse of a layer built by molecules possessing a comparatively weak hydrophobous pole.

The above might be an explanation of the character of the magnitude $F_{max.}$. Experimentally it is more easily achieved than a_0 and c.

CHAPTER VI.

ON THE ELECTRIC DOUBLE LAYER IN THE INTERFACE CHLOROPHYLL-WATER.

§ 1. *General Remarks.*

In the bulk of a liquid every molecule is attracted by the surrounding molecules, it is subject to the same attraction from all sides during periods of time long compared with molecular vibrations. At the liquid-air interface, however, there is hardly any outward attraction to compensate the inward pull, thus giving rise to the surface-tension.

/ Some parts of the molecules at the surface are attracted more han other parts, e.g. as a result of polarity of the molecules. It follows that the molecules at the surface are definetely orientated, which gives rise to an electric double layer, the potential difference of which is a characteristic for any liquid, e.g. 400 millivolts for pure water.

As the constellation of the molecules giving rise to this double layer is of molecular dimension, the spreading of a new monolayer of strange molecules on top of the liquid will necessarily change the original surface potential. If the magnitude and the direction of the original and the induced surface potentials are determined, the "surface potential difference" ($\triangle V$) of the monolayer, which is a characteristic for any monolayer on a certain liquid, may be calculated by subtraction of the E.M.F. of a "clean" surface from the E.M.F. of a surface covered with a monolayer.

The determination of surface potentials may be tackled along two different ways.

After the first method, a horizontal metal plate is fixed directly above the surface. Plate and liquid together form a condensor and vibrations of the plate induce an alternating current in the condensor circuit, which may be rectified and determined. Surface potential differences measured by this method agree well with those found by the ionization method. This confirms the reliability of both methods.

After the ionization method, the one used in these experiments, an air-electrode, consisting of a small piece of gold wire covered with a radio-active polonium film, is fixed above the surface layer to be investigated. This air-electrode is the connection between the upper

surface of the chlorophyll film and the potentiometer, making the air conductive between the top of the layer and the electrode. The lower side of the monolayer = the water of the tray is connected to the instrument by means of a calomel-electrode.

If care is taken to leave the whole circuit unchanged except the conditions in the surface layer, the influence of spread monolayers and of the pH of the underlying solution may be investigated.

Helmholtz was the first to consider the surface potentila from the point of view of a condensor.

If n is the number of molecules per cm^2 of the surface,
e the charge of the dipoles,
d the distance of the two opposite poles,
$\triangle V$ the potential difference of the double layer,
$\triangle V = 4\pi \times d \cdot e \times n = 4\pi \mu n$,
μ representing the electric moment of the dipoles. In case of a dielectric constant K, $\triangle V$ should be divided by K.

§ 2. *The Apparatus.*

The apparatus used for the $\triangle$ V measurements is essentially the same as the one of Schulman and Rideal (1931), it was built by G. Th. Philippi (1935). It consists of a spreading trough filled with the aqueous solution, the air interface potential difference of which is measured. All precautions are taken to prevent short-circuiting of the connections of the upper and the lower electric layer against earth. Therefore, the tray has to be dried carefully when filled with the solution and it is placed upon ebonite adjusting screws, fitted upon a paraffin block. For these experiments the ordinary balance was used, the same one as is described above. The balance is carefully insulated by paraffin blocks.

The saturated calomel-electrode after Michaelis is equipped with a special basin connecting the agar bridge with the tray-fluid. The air-electrode is mounted upon a carriage. This carriage is moved parallel to the longer side of the trough by means of a dial. On top of this carriage a second pair of rails is fixed, whereupon a second carriage, bearing the polonium-electrode, can be moved in a direction perpendicular to the first. This device permits the investigation of the entire surface of the trough and thus questions regarding the homogenenity of the surface layer can be answered.

The spreading tray and the electrodes are mounted in a Faraday cage with a small hole in it to handle the torsion balance. This is not only necessary as a prevention against dust, which annihilates the electric insulations of the apparatus, but at the same time makes all hand-effect impossible, which is very troublesome when eventu-

ally the laboratory air is conductive.

Figure 16 gives a scheme of the measuring circuit. The only non-conductor in it is the air between the air-electrode and the upper side of the layer. This small volume of air is ionized by the polonium and, hence, behaves similar to a solution of an electrolyte. The electrode was kindly prepared by the Laboratoire Curie and had a power of 400 unités, which permits a difference in distance of 0.5 to 1.5 cm from the surface without changes in $\triangle V$.

After filling the tray with the buffer solution the electrode should

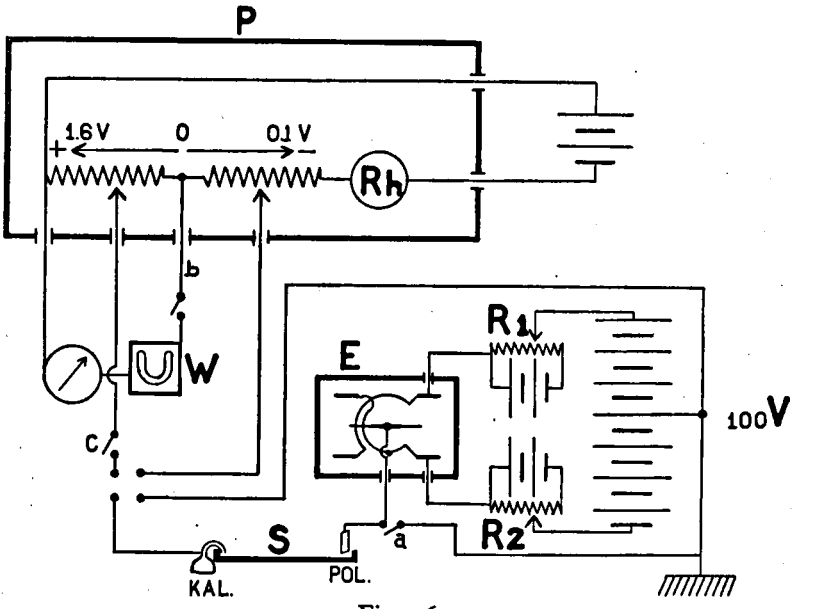

Fig. 16.

stand above it for an hour or longer to obtain a constant surface potential. As soon as the E.M.F. is constant the chlorophyll is pipetted upon the surface and $\triangle V$ may be measured.

In starting an experiment the potentiometer P is adjusted by means of the rheostat, the switch c is opened, b closed and after adjustment b is reopened. E is an electrometer, the quadrants of which are charged by a 100 Volts battery earthed at about 50 Volts. By means of the two resistances R_1 and R_2, in contact with two 4 Volts batteries, the movable part of the electrometer may be adjusted to a certain position when a is closed. Now both the contacts p_1

and p_2 are moved to zero, c is closed and a is carefully opened. The result is mostly a strong deflection of the electrometer needle, which is directly stopped by means of the earth contact a. If the calomel-electrode was positive to the polonium-electrode, this deflection is readily compensated by moving p_1 and p_2 respectively to the left and to the right and thus the E.M.F. of the surface layer is measured. If the charges of the electrodes are the reverse, the commutator e is used and the sign of the potential drop is determined.

Two different polonium-electrodes were used, the comparison of which yielded a potential difference of no more than 0.001 Volts. In some occasions, however, differences up to 10 milli-volts were detected. They were either caused by faults in the insulations of the apparatus, by impurities of the solutions, or of the surfaces.

The determination of some fifteen surface potentials, belonging to corresponding places of a F—a curve, takes no more time than half an hour. A second control of some points directly after the measurements yields the same results, denoting that the layer of chlorophyll is not injured by the radiation of the electrode.

§ 3. *Surface Potentials and Hydratation.*

The potential drop across a monolayer is dependent upon the number (n) and the electric moment (μ) of the molecules, measured in a direction perpendicular to the surface.

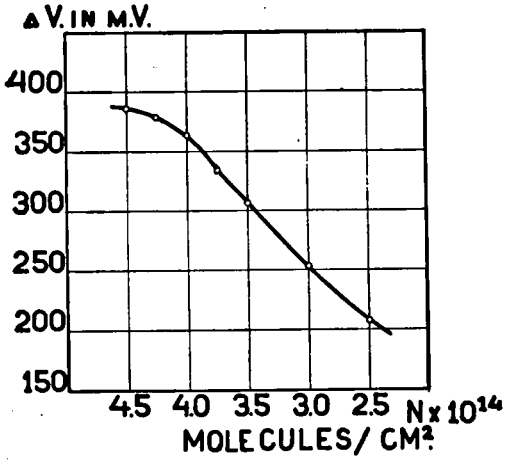

Fig. 17.

The proof was given by Adam and Harding (1932), in a paper on the electric double layers of films of fatty acids. They give F—a and $\triangle$V—a curves of myristic-, palmitic-, behenic- and stearic acids, and especially the results for myristic acid (figure 17), are the most convincing. With this substance definite pressures are already observed at comparatively large surfaces. Therefore, long ranges of surfaces may be studied, which is necessary for the investigation of the interrelation of surface concentration and $\triangle$V.

From a surface concentration of 2.10^{14} up to 4.10^{14} molecules per cm^2 the $\triangle V$—n curve is practically rectilinear indeed. According to Langmuirs theory, in this range of areas no hydratation water is squeezed out of the films. This happens at surface concentrations higher than 4.10^{14} molecules per cm^2, until the layer breaks down at very high pressures. Here the surface potential difference-surface concentration curves deviate from the linear form, indicating a decrease in the share each molecule contributes to the total $\triangle V$. Probably the decrease of this share is a result of dehydratation (Philippi).

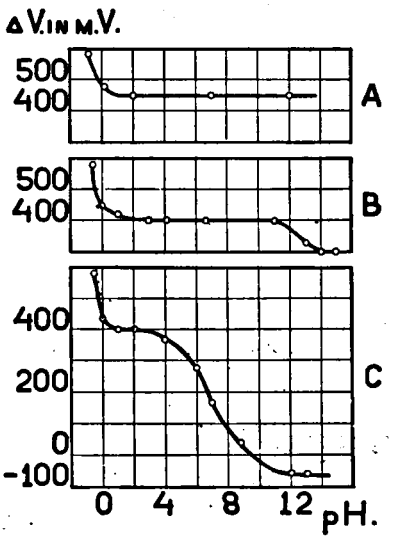

Fig. 18.

Apart from dehydratation by pressure, the hydratation of the carboxyl-"head" groups may be influenced by the hydrogen ion concentration of the underlying liquid. Shulman and Hughes (1932) examined this influence of hydratating group and pH by determining $\triangle V$ for three long chain hydrocarbons: octadecyl methyl ether, tetradecyl alcohol and myristic acid. The spreading surfaces of these compounds are pretty well comparable. In figure 18 the $\triangle V$'s, corresponding to an area of approximately 20 $Å^2$, are given.

Except for a $pH < 1$, $\triangle V$ changes only when the compound possesses an ionizable group. When these groups become ionized $\triangle V$ changes very much. With carboxyl groups this sudden change proceeds at a much higher hydrogen ion concentration than with the less dissociating hydroxyl groups.

These examples show that value and sign of $\triangle V$ are highly dependent upon the degree of hydratation. This hydratation may be influenced by pressure. It is influenced by pH only if the group is ionizable.

§ 4. *Experimental on $\triangle V$ and Hydratation of Chlorophyll a + b.*

Figure 19 shows the F—a curve of chlorophyll a + b at pH 4.5. Above it the $\triangle V$—a curve is given, $\triangle V$ counted in millivolts. Starting with a pressure of 4 dynes. cm^{-1}, $\triangle V$ is rather good re-

producible, rising almost rectilinear to about 420 m-Volts, the curve then bends to the left until a surface of 82 Å² is reached. Here the layer breaks down and $\triangle V$ directly decreases and is no longer reproducible.

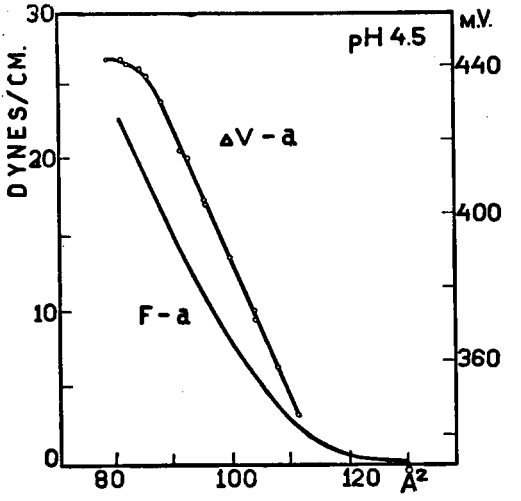

Fig. 19.

If μ is the dipole moment of a chlorophyll molecule, counted perpendicular to the surface, and n the number of molecules per cm² inelectrostatic units:

$$\frac{\triangle V}{300} = 4\pi n\mu.$$

If the surface per molecule is expressed in Å², we may say

$$n = \frac{10^{16}}{a},$$

a, representing the spreading surface in Å² per molecule. Hence,

$$\mu = \frac{\triangle V \times a}{12\pi} \cdot 10^{-18}.$$

μ is given in figure 20 for pH 4.5 and 8.2, and it is observed that μ is practically constant at pH 4.5 up to 90 Å². At pH 8.2 μ is decreasing regularly from the beginning of the curve to the end of it.

If μ of a monolayer is looked upon as originating from an electric double layer, the form of the curves may be explained by hydratation concurrent with

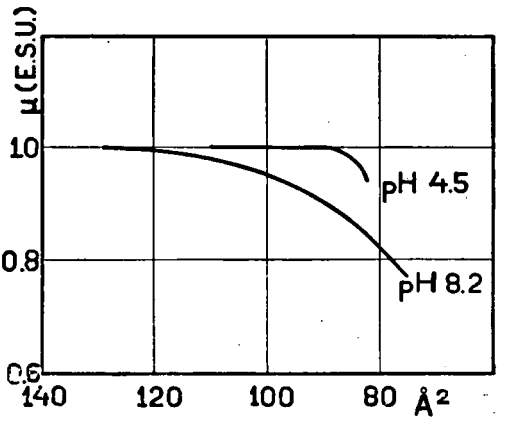

Fig. 20.

pH. With diminishing hydrogen ion concentration the hydratating chlorophyll dipole is able to attract more water molecules. These water molecules possess permanent and induced electric dipoles and thus are directed in one or a few double layers enlarging the electric moment of the originating dipole.

At a low pH (4.5) hydratating water molecules are present only in small quantities; their number is not diminished by compression of the monolayer. At a higher pH (8.2) a large hydratation sphere is present and the water molecules in it might be squeezed out of it (or reorientated), thus diminishing μ already at large spreading surfaces.

We cannot explain why μ, at large spreading surfaces (e.g. 120 Å^2), does not grow synbate with pH, the absolute value of μ depending upon many factors.

§ 5. *Changes in $\triangle V$ by Substitution of other Metals.*

In Chapter V, figure 13 was given. It shows the hydratating activity of the metal compounds of chlorophyll contrary to phaeophytin, which is absolutely inactive. This figure might be at the same time the expression of a decrease of charges in the direction from magnesium to lead, charges becoming zero at phaeophytin (hydrogen instead of metal) and rising again from phaeophytin to the left side of the picture possessing the opposite sign now. Hence, it is interesting to determine the relation between pH and E.M.F. for these metals. The results are given in figure 21. The curves represent the E.M.F.'s at a pressure of 4 dynes. cm^{-1}. At higher pressures the same sequence and model of curves is obtained, only a parallel displacement is observed.

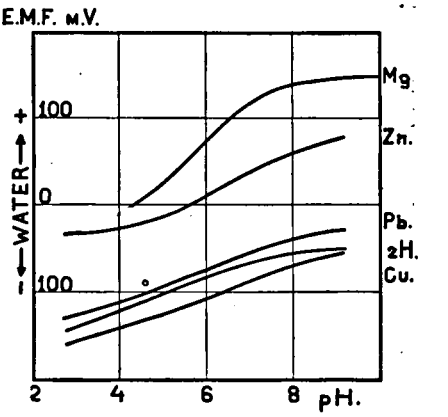

Fig. 21.

Contemplating these figures, it should be kept in mind that E.M.F. and $\triangle V$ depend upon the orientation of the dipoles to the surface. If reorientation occurs μ should change its magnitude. The situation of the hydratating dipole is not known and, therefore, the influence of the pigment nucleus changing from a tilting position

to a more or less vertical position cannot be predicted. In case of the chlorophyll molecule many other dipoles might join in, complicating the effect.

Regarding μ at large spreading surfaces (when μ is not changed by dehydratation), μ decreases from

1.12, at pH 5.3, to 0.82, at pH 8.5,

in case of zinc chlorophyll.

At the same time a decreases from 119 Å^2 to 82 Å^2 and n (surface concentration) increases in the same degree. This non-expected coincidence of a comparable decrease in μ and increase in n, during reorientation, makes it possible to compare the $\triangle$V-pH curves of reorientating zinc (copper, lead and mercury) monolayers with the steady layers of magnesium chlorophyll.

We may consider the $\triangle$V of a monolayer bearing interface to depend upon:

1. the relative position and the magnitudes of the charges within the molecules of the film. This potential gradient across the molecules is unknown.
2. the magnitude of the hydrophilic dipole.
3. the dielectric character of the substances surrounding these hydrophilic groups, e.g. water molecules and neighbouring film molecules.
4. the number and orientation of the adsorbed water molecules and the distribution of counter ions of the buffer solution. In combination with the E.M.F. of the original clean buffer surface these instances determine the final $\triangle$V of the layer.

The composition of $\triangle$V or E.M.F., is rather complicated, as was demonstrated in the above lines. Hence, a comparison of different monolayer forming molecules is scarcely permitted. However, the difference between these five molecules is comparatively small and, therefore, it is likely that this magnesium-copper sequence is the same as the one found in hydratation, reaction-velocity and so on.

In our monolayer the hydratating dipole (which might be the lowest dipole) is directly in contact with the underlying solution and may be detected by comparing the courses of the E.M.F. curves. It is immediately observed that the magnesium chlorophyll changes its E.M.F., in relation to the inactive phaeophytin, much more than the zinc compound does. The magnesium compound re-establishes the original positive charge (of the clean buffer surface) of the water side of the interface to a higher degree than zinc does. Lead and copper do not show a clear pH-sensitivity. This is not in

conflict with figure 13 as the $F_{max.}$—pH curves for lead and copper run rather vertically in compasrison to the curve of the magnesium compound. At high pH's, however, $F_{max.}$ increases. This fact, combined with reorientation and so on, shows the pH-sensitivity of hydratation for the lead and the copper compounds, and this obviously much better than E.M.F. measurements may do; on the contrary E.M.F. measurements are a suitable means to discover the sequence of the metal compounds.

This sequence is constant for the whole range of pH's investigated: Mg - Zn - Hg - Pb - (2H) [1] - Cu.

A more detailed understanding of this sequence is obtained if the phaeophytin curve is considered as a curve of a compound lacking the pH-sensitive dipole of the metal compounds. Phaeophytin may be considered as such, as was explained in the preceding chapters, and consequently the E.M.F.—pH curve of phaeophytin should run parallel to the E.M.F.—pH curve of the clean buffer surface. The general trend of the curves was found to be identical, although no exact parallelism exists, which is most probably a "concentration effect" of the buffer solution. If a more diluted solution were applied (we used a four times diluted veronal-buffer solution of Michaelis 1931), a more exact parallelism might have been expected. More diluted solutions, however, are too unstable at higher pH's to be used successfully on the air.

There is still enough evidence to the insensitivity of the phaeophytin to use its E.M.F.—pH curve as a zero curve. This being done, the following conclusions are drawn.

The magnesium compound re-establishes the positive charge of the water side of the interface if the pH is raised. The zinc compound acts in the same way, although in a smaller degree. The lead compound behaves like zinc- and magnesium chlorophyll.

As the metal free compound does not possess any influence upon the pH-sensitive separation of charges, the copper compound will exert the opposite effect, charging the water negatively. Mercury is to be found between lead and zinc.

§ 6. *On the Interrelation of Groups in the Chlorophyll Molecule.* A characteristic property of chlorophyll is the interaction of groups very often widely apart in the molecule. It will be of interest to give some results of experiments to be found in the literature

[1] 2H means phaeophytin, the metal being substituted by two hydrogen atoms.

on the interaction of groups within the molecules of different compounds.

S m y t h e and S c h m i d t (1930) find colorimetrically that, at a suitable and constant pH, the amount of ferric iron bound by carboxylic acids is nihil with normal carboxylic acids. With the α hydroxylic carbonic acids much of the iron is bound, with the β acid very little iron is bound, and with the γ acid no effect could be observed.

From determinations of the dissociation constant of normal, mono-, di-, and trichloracetic-, propionic- and butyric acids, L a n g m u i r (1929) concludes "that the electric polarization, produced by the presence of the chlorin, decreases in a ratio 2.7 : 1, when transmitted from one carbon atom of the chain to another". After two or three of these transmissions this influence is practically extinguished.

The above mentioned examples suffice to show that in aliphatic carbon chains an interaction of groups further apart than two carbon atoms is almost imperceptible.

In aromatic compounds matters are different. For instance the ortho- and para-hydrogen atoms in phenol are more reactive than those in benzene.

The aromatic character (conjugated double bonds) will be responsible for the remarkable interaction of groups in the chlorophyll molecule. Facts like the ones cited above will be of outstanding interest in physiology. They are, however, still in a too chemical degree of development.

CHAPTER VII.

X-RAY INVESTIGATION OF NATURAL CHLOROPHYLL AND OF ETHYL-CHLOROPHYLLIDE.

§ 1. *The X-Ray Diagram of natural Chlorophyll.*

The diagram of the natural product, the preparation of which is described in Chapter I, is given in figure 22. One ring is clearly visible, another larger ring is faintly observed. These two rings denote the first and second order of a period of 4.2 Å. This period of 4.2 Å is of the order of magnitude of the thicknesses of the flat carbon skeletons in crystals of benzene derivatives. In table 2 some data are given.

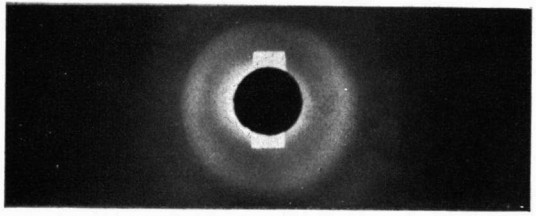

Fig. 22.

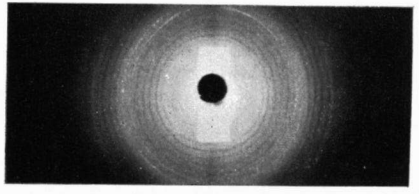

Fig. 23.

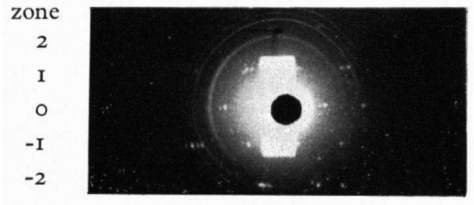

Fig. 26.

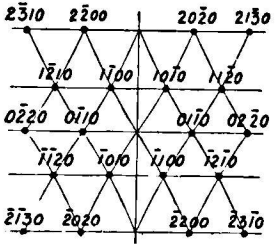

Fig. 28.

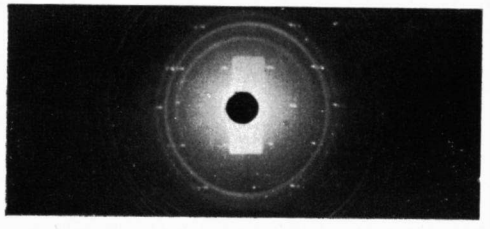

Fig. 27.

Figure 28 shows some indexed reflections of figure 27.

TABLE IV.

TABLE 2.

compound	thickness of the flat molecule in Å.
hexa methyl benzene	3.69
hexa ethyl benzene	5.16
phthalocyanine	3.38
ethyl-chlorophyllide	3.87
natural chlorophyll	4.2

In the paragraph on ethyl-chlorophyllide will be shown that the pigment nucleus of chlorophyll is a flat plate.

The period of 4.2 Å in "amorphous" chlorophyll points to a certain, although imperfect, orientation of the flat pigment nuclei in this solid substance. The imperfect parallel orientation only extends over a few molecular diameters (the rings are very broad), denoting the existence of faintly defined swarms of chlorophyll molecules, which have reached a certain parallelism in the orientation of their pigment nuclei.

§ 2. *The X-Ray Investigation of Ethyl-Chlorophyllide.*

The so-called "crystallized chlorophyll" was prepared after the procedure of Willstätter and Stoll (1913). This crystallized product is ethyl-chlorophyllide, the phytyl chain being substitued by an ethyl group. The ground leaf-substance, possessing a natural content of the saponifying enzyme, "chlorophyllase", is shaken in ethanol for many hours. The enzyme cannot be extracted from the leaf-substance and, therefore, the preparation of ethyl-chlorophyllide can only be achieved with the help of chlorophyllase-containing, ground, plant material (Chlorophyll book).

The ground leaves of Heracleum Burmannianum Bunge contain comparatively more of this enzyme than those of Datura. As no other species of high chlorophyllase content were present in sufficient amount, the ethyl-chlorophyllide was prepared from Heracleum leaves.

The loss of the fatty phytol chain changes "amorphous" chlorophyll into a crystallizable product. The crystals were not larger than 10 μ, which is too small to obtain a single crystal diagram.

In order to obtain larger crystals, the product of the Willstätter-Stoll treatment was recrystallized several times in ether. An addition of a few per cents of methanol increased the weight of the crystals to about 2 γ. This sufficed to orientate them microscopically in the X-ray spectrograph and to obtain a single crystal diagram.

The general habitus of the crystals is shown in figure 24. The crystal form is, as was already stated by Willstätter and Stoll, trigonal hemiedric (symmetry class C_3). The hkil integers are given for the peripheral crystal faces. The I integer, could not be obtained, the crystals being too small.

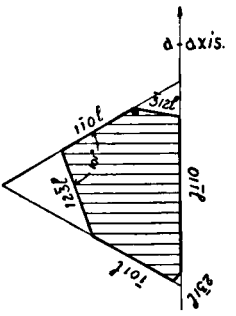

Fig. 24.

The large face, 0001, originally turned to the glass wall of the crystallization vessel, was perfectly flat and highly reflecting. This quality was of significant help in the orientation of the crystal on the spectrograph needle.

With Cu Kα rays X-ray diagrams were taken with the crystal rotating through a definite restricted angle around the side of the triangle as an axis, and around an axis in the basal plane at right angles to the former axis. From the distance between the different zones on the diagrams the lattice period in the direction of the rotation axes was calculated.

It was found that this period in the direction of the side of the triangle is 8.95 $\pm$ 0.02 Å, and at right angles with this direction 15.48 $\pm$ 0.03 Å. This shows that the side of the triangle is parallel to the a-axis of 8.95 Å, the lattice period at right angles to this direction being about $\sqrt{3}$ larger.

With the aid of a graphic method, based upon the reciprocal lattice, the indices of the reflections (550 different reflections were observed) could be determined (figure 25), which allowed to calculate the dimensions of the elementary period of the c-axis. This was found to be 38.4 $\pm$ 0.2 Å.

Pycnometrically a density of 1.28 was found for ethyl-chlorophyllide. The number of ethyl-chlorophyllide molecules contained in the cell was found to be 3.1, or approximately 3. The exact value of the density is, therefore, 1.23.

X-ray diagrams confirmed the presence of a threefold axis with no symmetry plane through the c-axis, or at right angles to this axis. Therefore, only the symmetry classes C_3 and C_{3i} are possible, the latter being improbable according to the external form (hemiedric) of the crystals (figure 24). As only those reflections 0001 for which $l = 3n$ are found and as many of the reflections are inconsistent with a rhombohedral lattice, the space group will be one of the pair of enantiomorphic groups, C_3^2 and C_3^3. In these space groups the molecules will be arranged in spirals around the c-axis.

Examples of X-ray diagrams are given in figures 26 and 27. A complete description of the arrangement of all the atoms cannot yet be given because of the high complexity of the molecule. However,

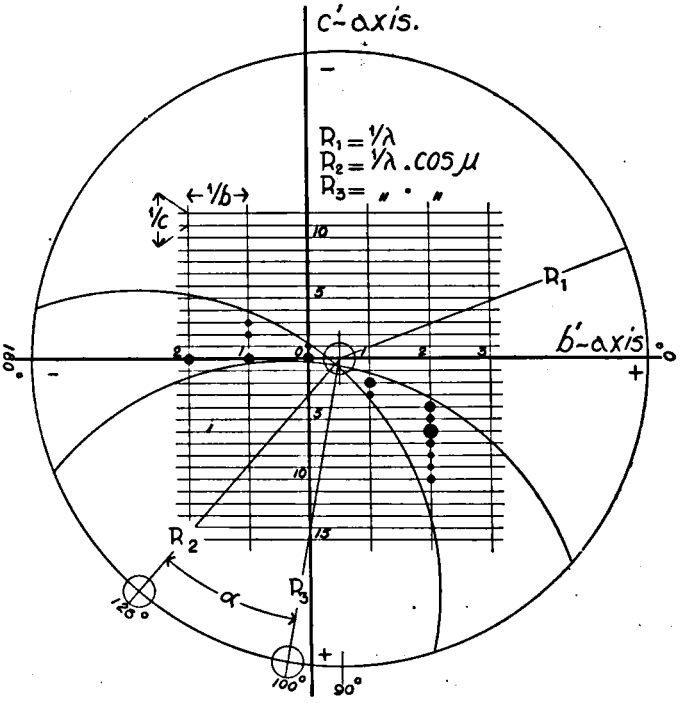

Fig. 25 shows the reciprocal plane belonging to the —1 zone of the X-ray oscillation diagram given in fig. 26. The crystal was oscillated around the a-axis in such a way that the beam of X-ray light incided in directions enclosing an angle from 10° to 36° in regard to the direction of the c-axis. The planes, reflecting when the crystal is irradiated at these angles, are found between the circles, circumscribed around R_2 and R_3 (α is the angle of oscillation). For example, the sequence of the seven reflections on the —1 zone of the photograph is represented in fig. 25 by the vertical series of dots: $\bar{1}\,2\,\bar{1}\,4$ to $\bar{1}\,2\,\bar{1}\,10$, the larger dots denoting stronger reflections. On the —1 zone on the left side of figure 26 the three left points (—8, —9 and —10 on the —c'-axis) of the series of seven were too weak on the original photograph to be reproduced in press. Only the stronger ones, —4, —5, —6 and —7, are represented in figure 26.

conclusions can be drawn on the arrangement of the molecules in the elementary cell and on the dimensions of the molecules.

As the reflection of the $2\,\bar{1}\,\bar{1}\,6$ (and $\bar{1}\,2\,\bar{1}\,6$, $\bar{1}\,1\,2\,6$) plane is

the strongest reflection observed, we concluded that this plane will be occupied by the majority of the atoms, which means that the presumably flat molecules are arranged parallel to these planes. This arrangement is shown in figure 29. ABCD is the basal face of the elementary cell, AE is $^1/_3$ of its height. A set of 2 $\bar{1}$ $\bar{1}$ 6 planes (e.g. plane PQRS) is drawn. The distance between these planes yields the thickness of the molecules (3.87 Å), and also DB and CE

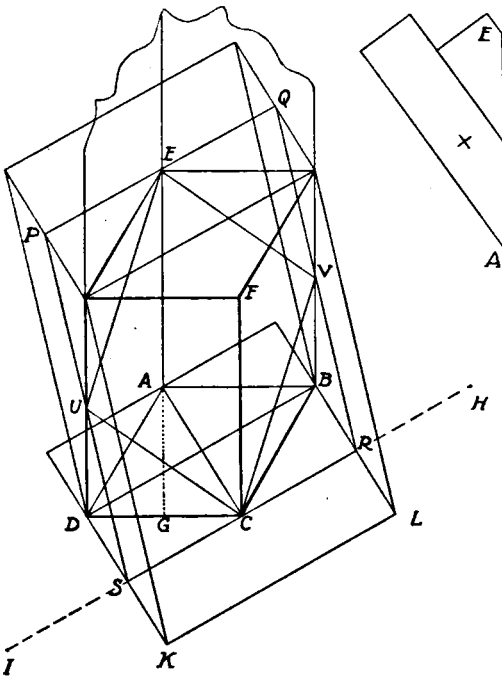

Fig. 29.

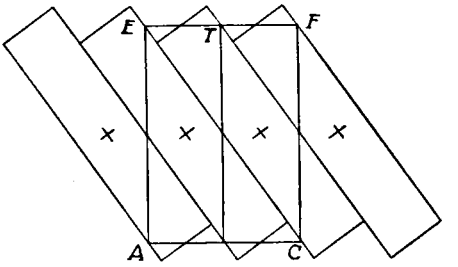

Fig. 30.

the dimensions within the plane of the carbon skeleton (15.48 and 15.62 Å).

A side view on plane EFAC is given in figure 30, showing the circumferences of the molecules sideways. The plane of the Mg-atoms should be near the crosses in the middle of the rectangles.

Above the plane ABCD the molecules are situated in a monolayer, each parallel to the plane EVCU, enclosing an angle of 55° with plane ABCD.

As pointed out above, the systematic extinction along the c-axis pointed to a threefold screw-axis. This means, stereometrically,

that at $^1/_3$ of the height (AE) of the c-axis a second plane is found parallel to ABCD, covered by a monolayer of molecules each enclosing an angle of 55° with it, but now the molecules parallel to a plane "EUVC" turned 120° around the c-axis. The same change of position is found above this second floor of the elementary cell, but with another rotation of 120° in the same sense. The fourth floor belongs to the following cell to be found along the c-axis.

The monolayers of molecules are packed in a special pattern. If the carbon skeleton plane of the first molecule sections plane ABCD along a horizontal line of the length AB, the neighbouring two

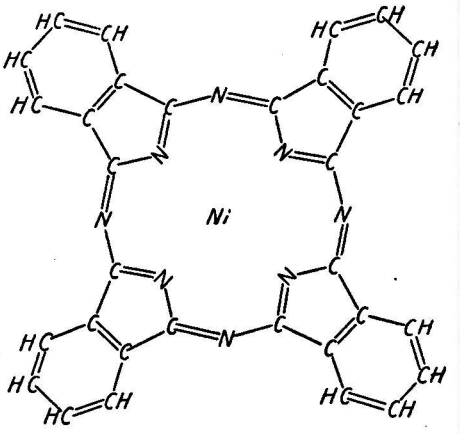

Fig. 31.

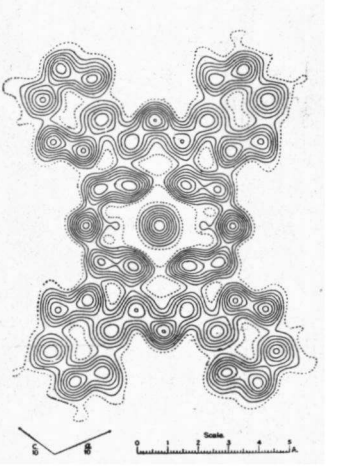

Fig. 32. (J. M. Robertson and I. Woodward. J. Chem. Soc. 1937:220).

molecules in front of it cut ABCD along CH and CI, and the following again along KL, etc.

The molecules show a parallel shift of 2.56 Å (figure 30 and 33). Their upper surface is 242 Å.

Robertson and Woodward (1937) elaborated a complete X-ray analysis for different phthalocyanines. The phthalocyanine molecule is also a tetrapyrrole compound. The pyrrole groups are connected by nitrogen-bridges and every pyrrole group is peripherally followed by a benzene ring (figure 31). Different metals may be substituted in the centre of the tetrapyrrole ring (e.g. Ni). The result of their analysis, the electronic densities in the plane of the carbon-nitrogen skeleton of the phthalocyanine molecule

projected on a plane enclosing an angle of 45.8° with the first, is reproduced in figure 32.

The surface of a phthalocyanine molecule is (13.45^2 Å^2) 181 Å^2. This is smaller than the surface of ethyl-chlorophyllide. The side chains of the chlorophyllide molecule, and especially the formyl- and acetyl groups, take more room. The thickness of the phthalocyanine molecule is 3.38 Å, of the ethyl-chlorophyllide molecule 3.87 Å. This greater thickness is caused by the fact that the carbon atoms of the benzene rings are placed in the plane of the pyrrole groups, whereas the peripheral side chains of ethyl-chlorophyllide, replacing the benzene rings of phthalocyanine, are irregularly placed, probably partly above, partly below this plane. See also table 2.

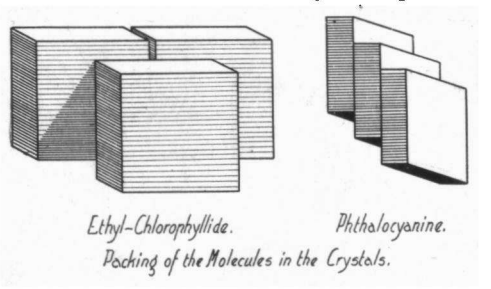

Fig. 33.

Robertson and Woodward's calculations show that every central metal atom is covered by (and placed upon) one of the four imin-bridge nitrogen atoms of its neighbouring molecule. These molecules are situated, with a slight parallel shift, in continuous piles.

In ethyl-chlorophyllide, the molecules are placed alternately, see figure 33.

§ 3. *The Results applied to the Chlorophyll Monolayer.*

Figures 29 and 30 show the structure and the dimensions of a minute part of the ethyl-chlorophyllide crystal. If these figures were extended parallel to the plane ABCD they should have reproduced an ethyl-chlorophyllide film of a depth of one molecule, cut from the crystal. The depth of this film is 12.80 Å and the "spreading surface" per molecule on the plane ABCD is 69.2 Å^2. (69.2 Å^2 is the surface of the ABCD plane divided by the number of covering molecules.)

In Chapter V, § 2, ethyl-chlorophyllide has been spread upon a buffer solution. At pH 4.1 (the lowest possible pH to spread the magnesium-containing compound) an $F_{max.}$ of only 2.5 dynes. cm^{-1} was found. This $F_{max.}$ is too small to obtain reliable a_0 values. At pH 5,4 an exactly reproducible a_0 is obtained of 70 Å^2.

It was shown with chlorophyll that at a pH 4.1 the a_0 of the monolayer of this compound is only little influenced by hydratation.

The increase of a_0 from pH 4.1 to 5.4 amounts: 106 ± 3 Å² to 110 ± 3 Å². Therefore, a_0 of dry ethyl-chlorophyllide should be 66 ± 3 Å². Regarding the difference of the methods employed, this value is in good agreement with 69 Å², observed by X-ray data, the more so, as this a_0 of ethyl-chlorophyllide should be looked upon as a minimum magnitude, because of the comparative solubility of the compound (Chapter 5, § 2).

The depth of the ethyl-chlorophyllide monolayer was found to be 12.2 Å (pycnometrical density 1.28), X-ray data yielded 12.8 Å. If the X-ray spec. weight is used (1.23), the depth of the monolayer is found to be 12.7 Å. In this way, however, no independent value is obtained. From the above follows that the ethyl-chlorophyllide monolayer may be looked upon as a two-dimensional crystal in the basal plane, which is in accordance with the solid condition of the monolayer.

The X-ray diagram of amorphous chlorophyll shows a periodicity of 4.2 Å, which has been explained as the result of a certain, although imperfect, parallel arrangement in solid condition, the phytyl preventing a perfect crystalline packing. Therefore, it is supposed here that a parallel association at an inclination of 35° to the vertical exists also in the monolayer of the phytol containing chlorophyll.

In regard to reorientation, this cannot be the work of the hydrophobic phytyl, it is the result of a changed cooperation of hydratation and association of the pigment nucleus. A stronger hydratation presumably prevents parallel association at higher pH's. Therefore, in a reorientated phytol-containing layer, the phytol covers 82 Å²—60 Å² = 22 Å². (60 Å² is the spreading surface of the vertical nucleus).

In a "layer of 35° of inclination" the phytol area amounts to 106 Å²—69 Å² = 37 Å², which means that the phytyl surface has been reduced also by reorientation. After reorientation the spreading surface of the phytyl rest is about 20 Å² (about as much as the a_0 of straight chained fatty acids). Hence, after reorientation the chlorophyll monolayers should be covered on top by parts of the phytyl chains, which is in accordance with the depth of this layer, 16.1 Å (Zn-chlorophyll), being larger than the diameter of 15.6 Å of the pigment nucleus. A somewhat larger difference would have been more satisfactory.

CHAPTER VIII.

PHOTOSYNTHESIS.

> ...., "it would seem appropriate to limit the use of the word "chlorophyll" strictly to these two chemical substances (chlorophyll a and chlorophyll b of Willstätter and Stoll 1913), and to refer, without prejudice as to their exact nature, to the green pigments of the plastid as "leaf green" or "phyllochlorin", at least until such time as their precise relationship to chlorophyll is made clear. There is more than a possibility that phyllochlorin may prove to be a compound of chlorophyll with protein, or with some other substance, or even to be of different chemical nature".
>
> Harold Mestre (1929).

§ 1. *The Composition of the Chloroplast.*

It is a well known fact that photosynthesis proceeds within the chloroplast, the only chlorophyll bearing organ of the cell. The presence of chlorophyll is specific to photosynthesis. An up to date review of the literature on the structure of the chloroplasts has been given by Frey-Wyssling (1937). It is sufficient to state here that the chloroplast consists of a stroma containing a large number of coloured particles.

In regard to the localisation of the green pigment the first decisive proof was given by Doutreligne (1935): the chloroplast contains more or less isodiametrical accumulations of chlorophyll, called "grana". After Heitz (1936), these grana are variable in size, ranging from 0.5 to 2 μ in diameter, their general form being mostly rotatory elliptic. Scarth (1924), Menke (1934) have shown that the grana possess an optical anisotropy. Ambronn and Frey (1926) conclude from glycerol imbibition experiments to the existence of flat parallel layers within the granum.

Up to 1938, nothing was known about the chemical constitution of the chloroplasts. The presence of proteins was deduced from the curves showing the shift of the maximum absorption band of chlorophyll to the shorter wave-lengths, in dependence of time, for different temperatures (Mestre, 1929). This set of curves resembles closely the coagulation curves of egg-albumin (Heilbrunn, 1928). Arnold (1933) found that on irradiation of suspensions of Chlorella, with visible and ultraviolet light, the respiration remained fairly constant, although photosynthetic activity diminished rapidly. It is the idea of Hubert (1935) that ultraviolet light

exerts a coagulative effect upon the ligatory group, probably a protein, as proteins are liable to coagulate when irradiated by ultraviolet light. Chlorophyll itself was not affected. Noack, Mestre and Arnold show that most probably chlorophyll is combined with protein.

On the other hand, myelin-desintegration of chloroplasts and grana points to the presence of comparatively large quantities of lipoids. In chloroplasts, myelin figures arise only if some hydrolising agent has been added. Hence, the lipoids were esterified in the intact condition (Frey-Wyssling 1937). The literature on this subject is found in Weber (1933), Menke (1934), Hubert (1935), Wakkie (1935). This year, Menke (1938) published an important paper on the chemical constitution of cytoplasm and chloroplasts. For the chloroplast he finds 47.7—57.6 % of proteins and 29.7—32.7 % of lipoids. He separates the chloroplast substance from the cytoplasm by centrifuging paste obtained by grinding spinach leaves. It is not certain that Menke separated the grana from the matrix by this process. Therefore, it is uncertain that these data really refer to the granum. However, we think that the error made, by inclusion of the stroma into his calculation, will not be very important, because the spinach chloroplasts are well filled with grana. The conclusion to be drawn from these experiments is that the chloroplast (spinach) contains more than 50 % of proteins, 30 % of lipoids including the pigments, 5—11 % of ashes and an unknown rest of about 4 %. Most probably, an analysis of the granum should yield comparable results.

Taking the hydrophilic polarity of the chlorophyll molecule as a starting point for their reasoning, Bungenberg de Jong and Hubert (1935) adopted the existence of hydrophobic and hydrophilic (lecithin-protein) layers within the granum, in the interfaces of which the chlorophyll molecules should be adsorbed.

§ 2. *The Granum as a photosynthetic Unit.*

In 1894, Engelmann demonstrated that a very narrow beam of light, hitting a minute part of the chloroplast of a Spirogyra cell, induces photosynthesis in the illuminated part of the chloroplast. This illuminated part was almost immediately surrounded by large numbers of Bacterium vulgare, which forms are positively chemotactic to oxygen. Up to these days, the smallest photosynthetic unit in physiological experiments has been the plastid. A specific character of the photosynthetically active plastid is the possession of chlorophyll. These facts lead to the idea that chlorophyll itself is a specific implement of photosynthesis.

We do not consider the chloroplast as the smallest photosynthetic unit any longer, as it consists of grana and matrix. The classical proof of Engelmann is, therefore, no longer exactly applicable and other evidence should be looked for in order to define the smallest photosynthetic unit in photosynthesis. Is it still the plastid, or is it the granum?

This is really an important question as never did anyone succeed in proving photosynthesis of chloroplast fragments (quickly prepared from living cells) to proceed for a sufficiently long time. This speaks for an interrelation of granum and matrix.

Moreover, Emerson (1932) proved that from 1000 to 2000 molecules of chlorophyll cooperate to reduce one molecule of carbon dioxide, and as chlorophyll is certainly not the only agent in photosynthesis, large areas of molecules, contained in some ten millions of $Å^3$, are operating dependently. These groups might extend from the inner part of the granum into the stroma. Photosynthesis may not be compared to a simple chemical substitution reaction resulting from the very local collision of the two reacting groups.

It is to be deplored that arguments as simple and direct as Engelmann's cannot be given anew. They can only be derived by deduction and by comparison with experiments on models.

Conditions are as follows: photic energy has to be absorbed and there is a direct conformity between the spectrum of the chlorophyll and the amount of photic energy transformed into chemical energy for very different parts of the absorption spectrum investigated. (Except in the blue, where the carotinoids might play a subordinate role. Warburg, 1923). Combining the morphological knowledge of the chloroplasts with these experiments on absorption of light and photosynthetic activity, we may only say that the chlorophyll is specific to photosynthesis and that it is present in the grana. For the presence of the whole photosynthetic apparatus within the granum other evidence should be supplied.

To solve this problem a definition must be given of what will be called "the photosynthetic apparatus". It is the smallest combination of some definite molecules, e.g. chlorophyll-, carotinoid-, protein-, lipoid-, water molecules, the direct cooperation of which may reduce CO_2 molecules to the reduction-level of formaldehyde.

We regard the question as to the seat of this photosynthetic apparatus settled as soon as one of the two following answers is given:

This apparatus (or a number of them) is to be found

1. entirely within the granum,

2. partly within the granum, and partly within the matrix (e.g. the chlorophyll in the granum, the other species of contributing molecules in the matrix).

Since 1913 the working-hypothesis of Willstätter and Stoll has been of enormous help to the study of photosynthesis. This hypothesis was the following. The affinity of carbon dioxide to chlorophyll in an aqueous medium was taken as an indication that a similar reaction (yielding the magnesium-chlorophyll carbonate) should take place in photosynthesis. As the reduction of the H_2CO_3 to H_2CO (formaldehyde) may proceed only in separate steps, and as these intermediary products must have all an acid character and, therefore, possess an affinity to the magnesium of the chlorophyll, only the non-acid reduction stage, H_2CO, is able to dissociate from the pigment molecule.

Recently the great hygroscopicity of the chlorophyll was added to the theory: The water attracted by the chlorophyll should be assimilation water, the hydrogen of which is transmitted to the carbon dioxide by means of the photic energy absorbed by the same carbon dioxide-chlorophyll-water complex. In 1935, Stoll completed this theory by adding an activating proteinaceous substrate, on the analogy of enzymes in nature. Thus we see that this hypothesis makes it unnecessary to place the photosynthetic apparatus partly within partly outside the granum.

However, difficulties arose from several sides, e.g.

a. the bond chlorophyll-carbon dioxide is easily broken (assimilation book, Willstätter-Stoll) and yet carbon dioxide is very strongly bound by the leaf. This implies the removal of carbon dioxide from a certain unknown substance in the leaf, binding carbon dioxide very strongly, to chlorophyll, which shows less affinity to CO_2.

b. The connection between primary and secondary reactions is still a difricult problem. This is caused by the fact that a chemical bond between the sensitizing and the reacting molecules has never been demonstrated in vitro, e.g. not in the well studied case of chlorophyll having allyl-thio-ureum as an acceptor (Gaffron 1927). Only an energetical contact exists between chlorophyll and allyl-thio-ureum, a definite compound, in equilibrium with the free reaction constituents, is not formed.

Water, the other reaction-partner, seems to play an entirely different role.

Van Niel demonstrated the uptake of H_2S by sulphur bac-

teria. No oxygen is evolved (as in green plants), but sulphur is deposited intracellularly, in equivalent amounts, during photosynthesis.

Muller (1933) found that several photosynthetic bacteria are able to use different organic acids in photosynthesis. "Just as H_2S and H_2 are dehydrogenated (oxidized) completely, the hydrogen being transferred to CO_2, so the organic substances are completely dehydrogenated (to CO_2 and H_2O) with CO_2 as an acceptor" (Van Niel, 1935).

Czurda (1936) proved the intermediary evolution of oxygen by the oxidation of methylene-blue and Nakamura (1937) showed that evolution of a gas takes place, as soon as the H_2S-bacteria begin to suffer a lack of H_2S. The gas evolved is, most probably, oxygen.

Now, it seems unlikely that substances as different as H_2O, H_2S, H_2 and organic substances should act with the same substance (e.g. chlorophyll), actions, which should yield the same results. It is more reasonable to assume that water plays the same role in these different processes and that the function of the other compounds is to act as oxygen acceptors, coming into action after the liberation of oxygen (Baas Becking and Hanson 1937, Nakamura 1937.)

On this line of thought it will be endavoured to accomplish a uniform explanation of photosynthesis in its strictest sense, separated from subsequent processes of different nature.

The above arguments lead us to the idea

1. that the formation of a compound of carbon dioxide and chlorophyll is not at all necessary:
2. that nothing may be said against an intimate and essential contact between water and chlorophyll during photosynthesis. In addition, a chlorophyll-water cooperation in photosynthesis furnishes the possiblity of a uniform photosynthetic process (and apparatus) in nature.

But herewith another question arises: The carbon dioxide must be bound somewhere in the neighbourhood of the active chlorophyll-water complex. Very often a carbamino-bond with some proteinaceous substance has been regarded as a possible means of fixing carbon dioxide (Willstätter-Stoll 1913, Warburg 1920, Gaffron 1935). In human physiology this reaction was unknown until 1928 (Henriques). It was proved by Meldrum and Roughton (1932) to occur in the hemoglobin molecule. They found that about 5—20 % of the total carbon dioxide content of the blood

is taken up in this "carbhemoglobin" form. That this fraction of the CO_2 content of the blood is actually bound by hemoglobin is shown by the effect of oxygenation and reduction upon the CO_2 content of the blood: reduced blood takes up more CO_2, as carbhemoglobin carbon dioxide, than oxygenated blood, under otherwise analogous conditions. The compound to which this carbon dioxide is bound must, therefore, be one which is reversibly affected by oxygenation. "There is no compound except hemoglobin in the blood, which is known to be reversibly affected by oxygenation, and it must therefore be hemoglobin in which carbon dioxide is bound in the carbamino-form" (Roughton, 1935).

Roughton was able to adduce further evidence for the existence of this carbomino-bond.

"CO_2 is bound carbaminically only with substances containing an $—NH_2$ group, but not with substances containing an $—NH_3+$ group, e.g.: CO_2 combines with NH_3, CH_3NH_2 and with $CH_2NH_2COO—$, but not with NH_4+, CH_3NH_3+, or $CH_2NH_3+COO—$".

"The binding of CO_2 as carbhemoglobin proceeds in a time much shorter than the $CO_2 + H_2O = H_2CO_3$ reaction requires. The carbamino reactions in vitro proceed much faster than the hydratation of CO_2 to H_2CO_3, which is promoted in blood by the enzyme: carbonic anhydrase. A rise of temperature reduces the amount of carbhemoglobin-bound CO_2, which is the behaviour of carbamino equilibrium in vitro also. Carbamino compounds decompose according to

$$CO_2 + \text{Protein-}NH_2 \rightleftarrows \text{Protein-}NHCOOH \rightleftarrows \text{Protein-}NHCOO— + H+$$

The rate of decomposition in CO_2 and protein is extremely slow in alkaline solution of pH 12, presumably because the equilibrium

$$\text{Protein-}NHCOOH \rightleftarrows \text{Protein-}NHCOO— + H+$$

is so much displaced to the right. Hence, it may be expected that in the alkaline range of pH the compound hemoglobin dissociates only slowly, which is consistent with experimental results in blood. Inversely, acidulation to pH 6 or 5 splits off the CO_2 readily".

One interesting fact should not be omitted: Henderson (1920) and Van Slyke (1922) have suggested that there is one particular hydrogen ion in hemoglobin which dissociates off from the neighbourhood of each hematin nucleus and does so more readily, if oxygen is attached to the latter. For this reason Henderson and Van Slyke called this the oxylabile hydrogen ion, in order to distinguish it from other hydrogen ions given off at various hydrogen ion concentrations.

Ferguson and Roughton (1934) point out that oxygenated blood absorbs less CO_2 in the form of carbhemoglobin than reduced blood does and that the oxylabile group, at which the special $H+$ ion becomes attached, is almost certainly an $—NH_2$ group.

Ferguson and Roughton's picture may be summarized as follows (Roughton 1935): It is believed that there is an oxylabile $—NH_2$ group in the neighbourhood of each hematin nucleus of the hemoglobin molecule, that CO_2 and H^+ compete with one another for combination with this group, that the affinity for the group, both of CO_2 and of H^+, is decreased by oxygenation of the hemoglobin molecule, and that finally the attachment of either CO_2 or H^+ ions to the oxylabile NH_2 group decreases the affinity of hemoglobin for oxygen. In regard to the last point, Margaria and Green (1933) found that at pH 7.4 the dissociation curve of oxyhemoglobin in the presence of CO_2 is displaced considerably in a solution of NaCl of the same ionic strength but containing no CO_2.

What use may be made of this for the elucidation of the process of photosynthesis, in which process both the roles of oxygen and carbon dioxide differ from their roles in the processes related to above? In macroscopical anatomy little analogy is apparent between objects as far apart as animals and plants. In microscopical anatomy, however, directly comparable phenomena are observed, and in molecular anatomy most certainly the resemblance is of the same or even of a higher degree. Especially in prosthetic groups and their protein substrates we may expect analogous appearances in plants and in animals. As biochemistry of hemoglobin and chemical knowledge of blood proteins are comparatively advanced one might make use of the results obtained in these fields in photosynthesis.

The principal suggestions to be gathered from the above data on hemoglobin are the following.

1. In photosynthesis carbon dioxide may be bound carbaminically to a protein in the neighbourhood of the chlorophyll.
2. There is a perfect interrelation between the carbamino-reaction on the globin and the oxygenation of the heme-nucleus: bound oxygen makes the CO_2 bond labile, bound CO_2 results in a labile oxygen bond. In case of CO_2assimilation, the CO_2 bound to protein, therefore, need not be "out of reach" of the energy absorbing chlorophyll molecule in case this chlorophyll molecule were bound to its protein in the same way as the heme-nucleus is bound to its globin.

After Roughton, no enzyme (carbo-anhydrase) stimulating the velocity of the reaction $CO_2 + H_2O = H_2CO_3$ is present in the chloroplasts of green plants. If CO_2 is present in carbamino form in photosynthesis, and not as a hydrate (as it is e.g. in the theory of Willstätter and Stoll and in the theory given by Baas

Becking and Hanson, 1937), the enzyme carbo-anhydrase may be absent without interfering with the views suggested in this chapter where advantage is taken of the strong hydratation capacity of the chlorophyll.

Before attempting to utilize these facts in photosynthesis we should keep in mind certain data on the molecular weights of animal respiratory pigments.

One of the most remarkable facts, discovered by Svedberg, is the uniformity of molecular weight observed in the hemoglobins of all Vertebrates, in the erythrocruorins of Molluscs, Insects, Crustaceans, Polychaetes and in some other groups. These chromoproteids, containing a tetrapyrrole compound as a chromogen, occur in molecular weights of 34000 or 68000. Roche (1937) attributed a probable physiological meaning to the Svedberg units and especially to the unit of 17000, being one half of 34000 and a quarter of 68000. Such units exist in nature (intraglobular respiratory pigments, e.g.: hemoglobin, chlorocruorin, erythrocruorin) in degradation-products of natural proteins and in solutions of a hydrogen ion concentration larger or smaller than in the stable region of pH. The physico-chemical unit would coincide with the physiological unit, containing one protein molecule of 17000 and one tetrapyrrole nucleus (iron- and colorimetrical determinations) and possessing the power of binding one Mol O_2 (or CO). In plasmatic medium this M. W. of 68000 may rise to as much as 2.10^6, the ratio porphin: globin remains steadily 1 : 1.

Besides hemoglobin, erythocruorin and chlorocruorin, respiratory chromoproteids, derived from the porphin compounds are: catalase, Warburg's assimilatory pigments and the cytochromes. The yellow assimilatory pigments are altogether different compounds, among others possessing no tetrapyrrole nucleus and a protein carrier of M.W. 80000 for every non-tetrapyrrole prosthetic group (Keckwick 1936).

The molecular weight of catalase is 68000, as of hemoglobin, and it contains four porphin nuclei just like hemoglobin does. (K. Stern 1933).

The properties of cytochrome-c point to a globin. The molecular weight differs but little from the globin unit of M.W. 17000, viz. 16500 (Theorell 1935, 1936).

The hemoglobins of different Mammals, and even of one species, yield a different ultraviolet absorption spectrum, which means differences in the amino acid combination of the globin moleculel however, molecular weights do not differ within the experimenta,

error (Roche 1937).

As is communicated above, many authors suggest a proteinaceous carrier for the "living" chlorophyll molecule. Nobody succeeded in the preparation of this chlorophyll-protein compound. Presumably the bond between the two compounds is much weaker than in hemoglobin, catalase, cytochromes, etc.

Haurowitz and Conant accept the assistance of heme-iron in the bond to globin. Conform to their suggestion we suppose the magnesium to act also as a link between the protein and the chlorophyll nucleus. Most likely these metals will be bound to protein-nitrogen or protein-sulphur. In such a case, the weaker polarizing influence of the magnesium upon the polarizable nitrogen or sulphur will result in a chlorophyll-protein bond weaker than a heme-protein bond. This might explain why the chlorophyll-protein is not yet known. Unpublished experiments of this laboratory (Mommaerts 1938) point to the existence of such a substance, which renders the following reasoning more acceptable.

Summarizing we may say: chlorophyll is a porphin compound. Free porphin compounds occur in nature only as decomposition-products of the physiologically active chromoproteids. Many authors suggest a chlorophyll-protein in photosynthesis. This protein carrier ought to be capable of binding carbon dioxide. Especially globins are bound to porphins and it appears that these globins are able to bind one CO_2 molecule carbaminically in the direct neighbourhood of each heme nucleus (van Slyke, 1922). The globins occur in nature by preference in molecular weights of 68000, containing four physiological units of 17000, every one of these units bears one porphin nucleus [1].

We will now answer the question: "Is there enough room in the granum to contain such large numbers of protein molecules, and does the calculated protein content of the granum agree with the protein percentage found by Menke? (1937)

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[1] In the literature, the ratio chlorophyll a to b is always given as 2.9 : 1. The original numbers of Willstätter and Stoll, however, range from 2.3—3.0 : 1.0, differing even rather much in the same species. In case the data smaller than 3 : 1 were caused by secondary changes the ontogenetically older ratio 3 : 1 might be another indication of the tetrad condition of "living" chlorophyll, one tetrad containing three molecules of the a and one of the b component.

This idea is not sustained by conditions in purple bacteria. In purple bacteria the a component is present only in very small amounts compared to the ratio in green plants (Schneider 1934).

§ 3. *Chlorophyll Content and Protein Content of the Granum.*

In the preceding paragraph, the probability is discussed of the simultaneous existence of one chlorophyll molecule with one protein molecule of a molecular weight of 17000. From experiments on sedimentation-velocity on pore width of collodion membranes, and on X-ray and spreading data, is concluded to a diameter of 50 Å for a protein molecule of M.W. 68000. This is a maximum value: the specific weight of this molecule of $50^3 Å^3$ will be 0.9, which is too low for a protein, hence a value of 46—47 Å will not be far from right.

Let us assume the volume belonging to four molecules of chlorophyll, therefore, to be 125000 $Å^3$ ($50^3 Å^3$). By means of these data it may be endeavoured to determine the number of chlorophyll molecules per granum.

To this purpose, 712.10^6 Hormidium flaccidum cells of a fresh culture were extracted quantitatively with acetone. The chlorophyll content was determined photometrically: it amounted to 0.597 mg. This means:

$$\frac{597 \times 10^{-6}}{915 \times 712 \times 10^6} \times 6 \times 10^{23} = 0.55 \times 10^9$$

molecules per cell, if the M.W. of chlorophyll ½aq. is taken as 915 (6.10^{23} being Avogadro's number).

It was first tried to determine the dimensions of one granum. This, however, proved to be too difficult. Particles of the order of magnitude of 1 μ may be measured if a number of them are amassed along a straight line. However, the grana are scattered throughout the matrix. The only thing to be done was to count their number. The mean number of grana of 50 cells was taken, it amounted to 26 ± 2. The volume occupied by the protein in one cell will be

$$\frac{0.55 \times 10^9}{4} \times 50^3 \times 10^3 = 17 \times 10^{12} Å^3.$$

If M e n k e's protein content is taken, we shall say 60 % of the granum, the total granum volume of the cell is $29.10^{12} Å^3$, and one granum has a volume of $1.1 . 10^{12} Å^3$. Therefore, the radius of the granum is 0.6 μ, which is entirely within the range of magnitudes given in the literature (0.5 — 2 μ). 0.6 μ is a maximum radius, as $50 Å^3$ is too large a diameter for a globin molecule.

A protein carrier molecule of M.W. 80000, for every prosthetic group, (as is the case in the yellow dissimilation ferments, see above)

yields a granum much too large for Hormidium flaccidum containing grana of a diameter of about 1 μ.

If Ambronn and Frey (1926) are right in their supposition that the granum is built of a pile of submicroscopical layers [1] of alternating optical density and if the protein content of the granum is about 60 % (Menke and see chapter 8, § 1), the existence of the granum as a structural entity will probably be largely dependent upon this protein content. As furthermore the chlorophyll will be most likely bound by proteins, of an M.W. of about 68000 for every four chlorophyll molecules, and as the diameter of these protein molecules is maximally 50 Å, the simplest manner of devising a pattern for the granum is one of the patterns given in figure 34. The granum might be built of alternating protein-lipoid layers; when crossing the granum the number of protein layers

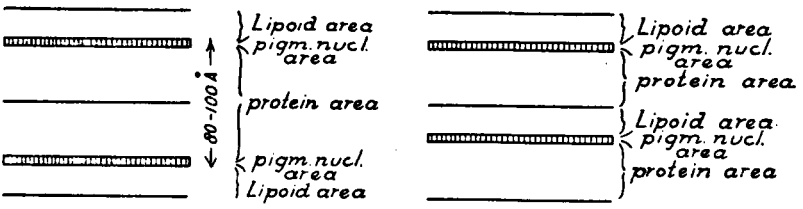


Fig. 34.

encountered should be, therefore, of the order of magnitude of 150.

Assuming the diameter of a protein molecule (M.W. 68000) to be 45—50 Å, its upper surface will be 45^2—50^2 $Å^2$. This surface contains four chlorophyll nuclei of 16 × 16 $Å^2$ (Chapter VII). This means that there should be enough room even for flat lying chlorophyll nuclei. The chlorophyll nuclei of the adjacent chlorophyll-protein molecules, therefore, must needs come into the direct neighbourhood of each other [1].

§ 4. "*The photosynthetic Apparatus*".

Experimental facts described in Chapters III, IV, V, VI and VII, contributing to a better understanding of the photosynthetic apparatus (as defined in Chapter VIII, § 2), are the following.

[1] Frey-Wyssling suggests flat layers of protein. As CO_2 and assimilation products have respectively to penetrate and to leave the grana in large quantities, we think that this idea is in better agreement with the function of the granum than the assumption of concentric protein-lipoid layers. Also the discoidal form points to the existence of flat layers.

1. The nucleus of the chlorophyll molecule is a flat plate, contrary to earlier ideas (Pfeilsticker).
2. The cyclopentanon, therefore, occupies a peripheral place in the molecule.
3. The cyclopentanon is the principal hydratation centre of the chlorophyll molecule. This hydratation centre is only weakly hydratated at high hydrogen ion concentrations. In physiological ranges of actual acidity hydratation is very marked.
4. Hydratation proceeds concomitant with the rise of a negative charge at the cyclopentanon and a compensating positive charge of the water. Obviously, water is attracted and an ionic double layer is formed by adsorbed OH^- ions on the chlorophyll side, H^+ ions on the side of the water.
5. This 'activity" of the cyclopentanon is only observable if a central metal atom is present, magnesium yielding the most active chlorophyll, zinc and lead, respectively, yielding less active chlorophyll's.
6. Total absence of the central metal results in a non- or weakly hydratated chlorophyll. In this case hydratation is not pH-sensitive, it is constant from pH 2.6 to 12 and does not differ much from the hydratation of ordinary chlorophyll at pH 4.1.
7. The formyl group at carbon atom 3 of chlorophyll b has an activating influence on hydratation.
8. A calculation of the volume of a presumable chlorophyll-globin compound, founded on an estimation of the number of chlorophyll molecules per granum, indicates the volumetrical possibility of the existence of such a compound.
9. A globin monolayer built of the usual isodiametrical "fourfold" globin molecules of M.W. 68000, the latter possessing a diameter of 46-47 Å, offers a surface large enough to bear a monolayer of chlorophyll molecules (surface in plane of carbon skeleton 242 $Å^2$) of rather high surface concentration. These chlorophyll molecules may even adopt a horizontal position without touching or covering one another.

Before attempting to design a more definite model for the photosynthetic apparatus, a description of the thermodynamical conditions to be fulfilled will be given.

In thermodynamics it seems to be difficult to combine the very

[1] An analysis meanwhile carried out in this laboratory by Mommaerts (1938) suggests that the granum substance freed from lipoids contains about 5 % of chlorophyll. The theoretical 1 : 1 ratio, protein (M.W. 17000): chlorophyll, (M.W. 915) should require 5.4 % chlorophyll.

efficient way in which the absorbed energy is used in photosynthesis with the general accepted idea that four consecutive reactions are needed to reduce one CO_2 molecule.

Gaffron (1936) computes that four quanta of visible light absorbed for the reduction of one carbon dioxide molecule are insufficient to cover the want of energy for this reduction. According to the accepted theory, several intermediary products have to be activated again before reacting anew. In addition, these intermediary products have to be rather stable in order not to decompose. Stable products, however, mostly need a large activation-energy. Finally H_2O_2 arises, a very expensive waste product from the point of view of energetics.

Warburg (1922) shows that the process of photosynthesis in green algae is a most economical process, the quantum yield may be even 0.92, which indicates that it is improbable that four consecutive reactions proceed during the process of reduction of one CO_2 molecule.

To cover the above mentioned incongruity, Gaffron tries to use two consecutive hydrogenations instead of the usual number of four.

In the following we shall try to devise a scheme, less subject to the above objections, at the same time applicable to all known forms of photosynthesis.

Emerson proved by means of intermittent light that from 1000—2000 chlorophyll molecules cooperate to reduce 1 CO_2 molecule. We regard this as another indication for the absence of a bond between a particular chlorophyll- and a particular CO_2 molecule, which is in accordance with the conclusions in § 2 of this chapter.

Assuming that the CO_2 molecule is not bound to chlorophyll it should be bound to the protein carrier of the chlorophyll molecule.

The other reaction constituent, water, is fixed by the cyclopentanon. Porphin-protein compounds preferably occur as tetrads of M.W. 68000, suggesting the existence of chlorophyll tetrads in the photosynthetic apparatus also.

In these tetrads the peripheral hydratated cyclopentanons should be turned to the inside, surrounding a carbon dioxide molecule which is fixed carbaminically upon the protein.

For photosynthetic reduction the situation becomes thus: in every tetrad "reduction water" molecules are always ready in a special position at the cyclopentanon groups. They are indeed almost always in this situation, as obviously water is almost never the

limiting factor in photosynthesis and for their adsorption no light-energy is needed (spreading experiments were carried out in the dark room).

Four quanta are ready at hand, as Emerson proved the existence of energy conduction between 1000—2000 chlorophyll molecules. If enough light is distributed we shall say 1000 chlorophyll molecules, corresponding to 250 tetrads, wait for the binding of a CO_2 molecule in one of them. As soon as the CO_2 molecule is bound 4 quanta are at hand and 4 activated water molecules are always ready (even if each chlorophyll molecule is able to activate only one H_2O molecule) to react in the immediate proximity. These water molecules are split into H and OH.

Three different methods of splitting may be distinguished.

1. Splitting of water into its free radicals H and OH.
2. Splitting of free water into H^+- and OH^- ions. The chlorophyll molecule arranges H^+- and OH^- ions in such a way as to form a double layer. Therefore, the possibility of the following mechanism arises.
   4 chlorophyll molecules push away 4 H^+ ions. 4 OH^- ions are attracted by their cyclopentanon groups, discharged, and turned into H_2O_2. The four electrons taken up by the CO_2-protein-chlorophyll molecule, cooperating with the 4 H ions, reduce the CO_2 to $H_2CO + H_2O$.
3. No free radicals or ions are formed; water is present as OH bound to another molecule. CO_2 is also a part of this molecule: the reaction proceeds intramolecularly.

Sub 1. The splitting of water into its free radicals is impossible: the four visible quanta are insufficient to cover the want of energy necessary for the splitting of four molecules into their free radicals.

Sub. 2. The property of the chlorophyll molecule to bring about a double layer of H^+- and OH^- ions (already present in the surrounding water), which ions might be used in photosynthesis, yields only little advantage. The reason is that the ions are strongly hydratated and have to be dehydratated before participating in photosynthesis. In regard to the knowledge of the hydratation energies of these ions, the advantage of working with H^+ and OH^- ions is annihilated by their hydratation energies.

Sub 3. Hence "camouflaged" water, OH bound to other molecules functioning in photosynthesis, is the only possibility left (see below).

The fact,

1. that especially the four different chlorophylls, as there are:

chlorophyll a and b, and bacteriochlorophyll a and b, possess a cyclopentanon group, contrary to the other known tetrapyrrole compounds,

2. that this tetrapyrrole group possesses a strong hygroscopicity (which is not mentioned for the magnesium-porphins), as discovered in solid condition (S t o l l), and a characteristic pH-sensitive hydratation when surrounded by water,
3. that this pH-sensitivity is activated by the presence of metals in the centre of the pigment nucleus and that magnesium is a metal of particularly strong activating influence upon this hydratation of the cyclopentanon, whereas the metals Cu and Fe, present in dissimilatory tetrapyrrole pigments, possess hardly any activating influence upon the cyclopentanon, in other words, the very fact that especially Mg takes an outstanding position among the metals as an activating agent in hydratation of the chlorophyll molecule,
4. that water must be looked upon as a substance always present in and of central importance in photosynthesis,

substantiates the view, that *chlorophyll possesses the function of binding water in photosynthesis* also. As pointed out above, this assimilation water cannot exist as free H_2O or H^+ and OH^- ions. It will be part of the molecules of the reactants: chlorophyll, protein-bound carbon dioxide and its intermediary products.

In order to arrive at a more definite scheme, it should be remembered that photosynthesis always needs 4 visible quanta, under very different conditions (W a r b u r g 1923, R o e l o f s e n 1935 F r e n c h 1937). As a means of comparison the following reaction formulas may be given:

green plants	$H_2O + CO_2 = \overline{CH_2O} + O_2$	— 144.000 cals
purple bacteria	$2H_2 + CO_2 = \overline{CH_2O} + H_2O$	± 0 „
green sulphur bacteria	$2H_2S + CO_2 = \overline{CH_2O} + H_2O + S_2$	— 35.300 „

The conclusion to be drawn from these facts is that photosynthesis should possess a comparable structure in these different cases, for if the number of 4 quanta were only a question of energy balance bacteria should be content with a smaller number of visible quanta per CO_2 molecule.

Combining the above facts with the existence of chlorophyll-protein tetrads and a protein-bound CO_2 molecule, the scheme of

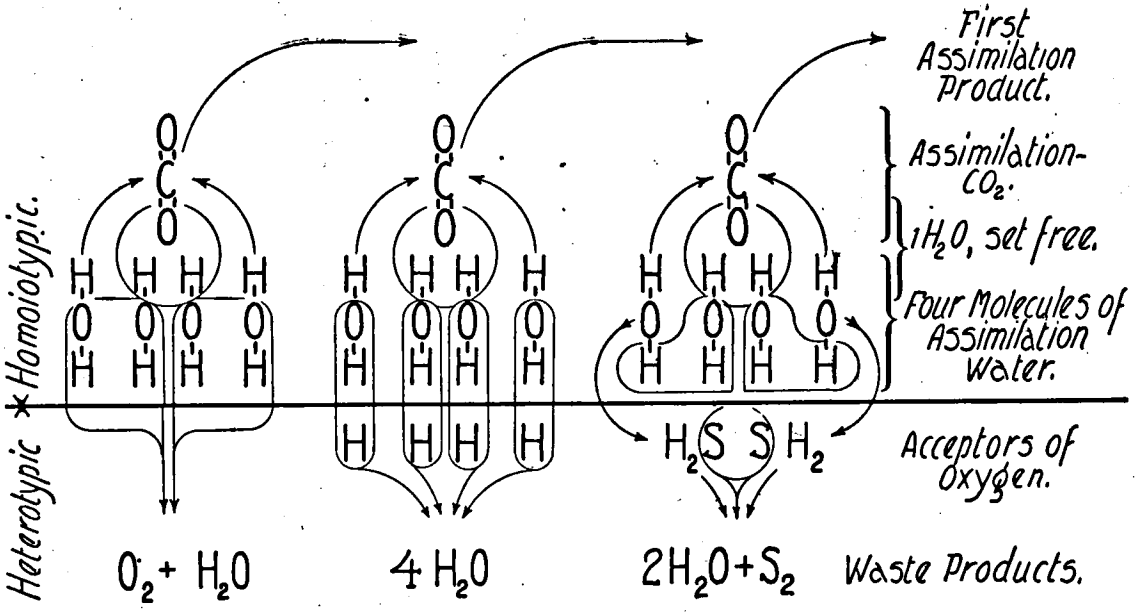

First Assimilation Product.
Assimilation-CO_2.
$1H_2O$, set free.
Four Molecules of Assimilation Water.
Acceptors of Oxygen.
Waste Products.
Homoiotypic.
Heterotypic
$O_2 + H_2O$
$4H_2O$
$2H_2O + S_2$
H_2S S H_2
Green Plants.
Streptococcus varians.
Thiorhodaceae.

Fig. 35.

figure 35 may be given, showing photosynthesis for green plants and bacteria.

This scheme may be explained as follows:

At the beginning of a photosynthetic act, by which one CO_2 molecule is reduced, all molecules standing "above the horizontal line" are bound by the tetrad. The reactions proceeding above the horizontal line belong to the photosynthetic process in its strictest sense. It is observed from the figure that this part of photosynthesis is "homoiotypic" for green plants and bacteria.

What happens "below the horizontal line" might be a special adaptation of the organism to make use of photosynthesis under its (the organisms) special intracellular- and milieu conditions.

In this scheme, H_2, H_2S and organic substances are oxidized in bacteria, they act as oxygen-acceptors. These reactions do not proceed in the tetrad mechanism, the latter being too specific to photosynthesis to allow these very different substances to play a role in it.

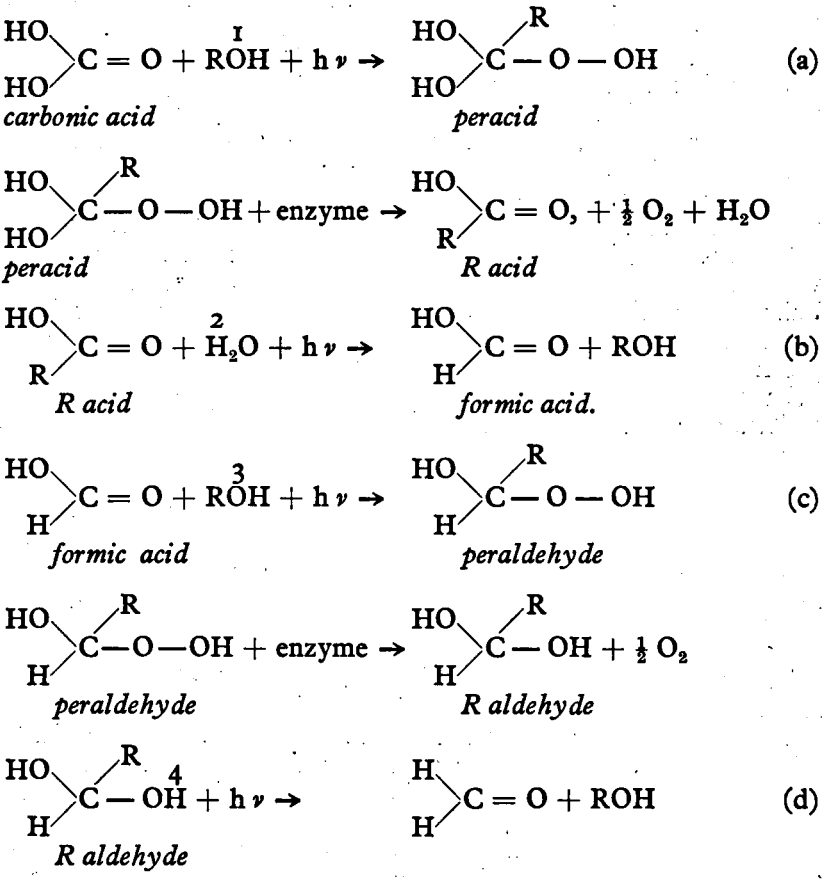
In regard to the destiny of the hypothetical primary assimilation-product, which should be something like formaldehyde, the chlorophyll-protein-tetrad does not necessarily exert an influence upon it. Hexoses recombine from molecules and radicals of less than 6-carbon atoms under very different conditions in the living cell. Hence, the function of formaldehyde-condensation is not necessarily a function of the tetrad In addition, the condensation of formaldehyde to d-glucose is an exothermal process and may run, therefore, independently of the photosynthetic mechanism.

The advantages of the scheme are the following.

1. The scheme is a uniform expression of photosynthesis in very different organisms, running at the expense of very different substrates.
2. The presence of the required number of molecules participating in the process of photosynthesis at one and the same point of the structure. The stability necessary for a sufficient long life time of the intermediary products ought to be large when these products have to diffuse first from those parts of the structure where they were formed originally. The scheme allows the use of products of low stability, which requires smaller activation-energies.
3. As the production of free radicals (or other free highly activated, products) is impossible by lack of energy, photosynthesis most probably exists of rearrangements of chemical bonds present as one continuous entity. The chlorophyll-protein-tetrad molecule, and especially the active centre of it, may be looked upon as an entity of this kind.

At first sight, there seems to exist a relation between the number of four quanta and the number of four water molecules acting in the tetrad. Although it is possible that such a relation actually exists, it is mere speculation at present: nothing is known about the intermediary products.

Our scheme (figure 31) does not specify the intermediary reactions. It summarizes in a sense all possible schemes and likewise the scheme of Franck and Herzfeld (1937), founded on energy-calculations. The chemical expression of it is given here.



It may be observed that also in the scheme of Franck and Herzfeld four water molecules are applied, three of them are

directly visible (1, 2, 3), the fourth is bound in R-aldehyde (4). This may be regarded merely as a matter of orthography, as in the analogous second (b) reaction this water molecule is given as H_2O.

§ 5. *The Stroma.*

Practically nothing is known about the chemical constitution of the grana bearing stroma. Functionally, however, a definite distinction between granum and stroma may be made. Carbon dioxide is reduced by the granum to a product of the reduction-level of formaldehyde. This substance is a hypothetical first product. The first visible product of photosynthesis is starch, which is often deposited in granules within the chloroplast. Apparently, an important link between the two substances mentioned is d-glucose, the presence of which has been indicated by the authors: Weevers, Brown and Morris. Many different hexoses are found in nature; d-glucose seems to be a specific product of photosynthesis in higher plants. We do not consider this indicative of its origin within the granum as it may be formed katabolically as well.

The starch grain, however, is a typical product of the stroma, which may be deduced from the fact that starch grains are also built by colourless plastids from sugar in parts of the plant lacking chlorophyll. The sugar was originally produced by the assimilating green leaf cells.

In regard to the function of the stroma the following may be said.

1. The stroma has a structural function being the matrix of the grana.
2. It is the physiological milieu for the grana, possessing properties which have to be known in order to be able to perform photosynthesis in vitro by means of grana or chloroplast suspensions.
3. It might play a role in the phototaxic properties of the chloroplast.
4. It is the seat of the amylum apposition.

The manner of starch grain apposition yields another suggestion for the independence of this function of photosynthesis.

The structure of the starch grain shows cubic starch particles of 1 μ^3 volume. Hanson and Katz (1934) found that these particles are deposited in concentric "monolayers" of 1 μ thickness, excentric grains showing a number of non-closed layers on one side besides a certain number of totally enclosing layers. Crystallographically these cubes consist of great numbers of parallel trichites [1]

[1] It is possible that these trichites show a dichotomous contact after Meyer and Nägeli (Frey-Wyssling, 1936).

The long axis of these crystalline trichites should be situated parallel to the radius of the starch grain.

On the other hand, it is found by Farr and Eckerson (1934) that cellulose walls may be macerated into fibrilla of 1 μ thickness, these fibrilla may be further macerated into cubic bodies also of 1 μ^3 volume.

The cellulose fibrilla are placed more or less parallel to the long axis of the fibre, resulting in a strong tensile strength of the fibre. This strength can only be explained (Meyer and Lotmar 1935) by the fact that the valence chains run continuously along the total length of the fibrilla, which is realized by the exact correspondence

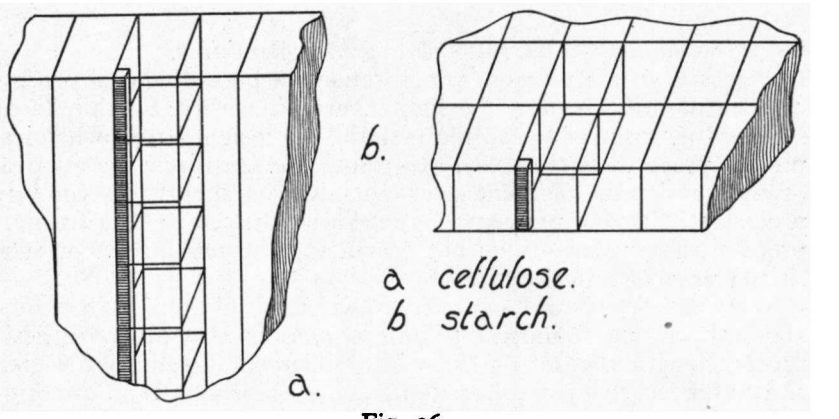


Fig. 36.

of the apical faces of the cubes. (Apical faces are formed by the ends of the valence chains). It should not be astonishing if these fibrilla combined sideways to a cylindrical elementary cellulose layer surrounding the cell. Figure 36 explains the conditions, showing the relation between mode of apposition and technical properties of starch and of cellulose.

In the starch grain, however, an elementary layer is deposited perpendicular to the direction of the valence chain resulting in an inhomogeneity (discontinuity) at the apical ends of every cube deposited by the chloroplast. This explains why in starch grains the elementary layers are easily broken from one another during maceration.

Notwithstanding these differences and the only slight chemical difference between cellulose and amylum, the analogy in the elementary building stones in both cases is apparent enough to

assume the existence of a similar protoplasmic apposition apparatus. This is a further support to the idea that the starch building functions need not bear any relation to photosynthesis.

There is one important missing link in the chain of processes within the chloroplast, starting with carbon dioxide and water and producing starch grains as an end product. We do not know where, within the plastid, sugar is formed, i.o.w.: what happens to the carbon dioxide as soon as it is reduced to the "first assimilation product", containing still one carbon atom per molecule. Physiological experiments with granum suspensions might solve this problem.

§ 6. *Is Chlorophyll an Enzyme or is it a Sensitizer?*

A sensitizer is a pigment which renders a photochemical process capable of utilizing also the light energy absorbed by this "sensitizing" pigment added to the system. The original photochemical process proceeds with as well as without the sensitizer; the removal of the sensitizing pigment does not stop or alter the secondary processes. Different pigments may sensitize the same photochemical process; these pigments do not bear a specific relation to the sensitized photochemical process.

Some authors discard a specific function of chlorophyll in photosynthesis, a view founded e.g. on observations that photosynthesis proceeds with the aid of H_2O, H_2S, different organic acids etc., and on the fact that two different pigments: chlorophyll and bacteriochlorophyll run photosynthetic processes apparently closely related to one another. In addition, in vitro chlorophyll and many other pigments do act as sensitizers (Gaffron 1936).

In the preceding paragraphs, we defended a theory of photosynthesis founded on a chlorophyll-water cooperation. In photosynthesis H_2O is just as important as CO_2 and hence we may say: in regard to photosynthesis chlorophyll acts specific towards H_2O.

In addition, it was suggested that CO_2 is not bound to chlorophyll. Therefore, in regard to CO_2 specificity should not be attributed to chlorophyll. However, chlorophyll, as known in chemistry, is no biological unit; chlorophyll and related derivatives exist only in nature as decomposition products of vegetable food. Chlorophyll in function only appears as the pigment-protein complex: phyllochlorin, and this complex is at the same time photosynthetically specific to CO_2 as well as to H_2O.

Hence, the question as to the sensitizing versus enzymatic function of chlorophyll has no biological sense. This question may only

be put forward in case of in vitro experiments with chlorophyll solutions; phyllochlorin should be taken as a starting point in biology.

As Emerson proved, by means of photosynthesis in intermittent light, that 1000—2000 chlorophyll molecules act dependently in photosynthesis, it may be defended that not even the chemical entity: phyllochlorin is a suitable base for theories on photosynthesis. About 1000 to 2000 phyllochlorin molecules, in natural structural condition, should be used as such. In regard to its perfect specificity under different natural conditions this phyllochlorin structure might be called an enzyme, but it is difficult to apply the term enzyme in its strict biochemical sense to this phenomenon.

This work was carried out at the Botanical Institute of the Government University, Leyden, Holland.

At this place I want to thank Professor Dr L. G. M. Baas Becking sincerely for his hospitality and for his constructive criticism. The years I spent in his laboratory will be a stimulating remembrance to me.

I am much indebted to Professor E. Gorter for his hospitality and for the inspiration I gathered from his researches on spreading.

Also to Dr J. A. A. Ketelaar I want to express my thanks for his constant interest and help in the chemical part of this work.

SUMMARY.

1. The principles of the usual methods of investigation of photosynthesis have been exposed. It was explained why at the same time an approach of the problem from a structural point of view might be useful.
2. From the latter point of view, compounds as proteins, chlorophyll, carbon dioxide and water might be of first importance, probably followed by lipoids, carotinoids and ashes.
3. Chlorophyll was prepared. The choice of the method of purification of this compound was explained. Chlorophyll a+ b, of a purity of 97 %, both the components, phaeophytin, Zn-, Pb-, Cu- and Hg-chlorophyll, ethyl-chlorophyllide, ethyl-phaeophorbid and allomerised chlorophyll were prepared quantitatively. The absorption spectra of the foreign metal compounds are given. The iron-compound could not be obtained, probably in consequence of technical difficulties. The sequence Mg-, Zn-, Hg-, Pb-chlorophyll, phaeophytin, Cu-chlorophyll was discovered in different properties of the compounds. Deductively, the place of iron in the sequence will be found in the neighbour-

hood of Pb and Cu. This shows a certain contrast between the assimilatory Mg and the dissimilatory Cu and Fe.

4. These compounds were spread monomolecularly on buffer solutions from pH 2.6 to 12.
In addition, the potential differences of the monolayer-bearing interfaces water-air were measured at different pH's. In this way a very sensitive means of investigation of the interaction of chlorophyll and water was obtained.
Chlorophyll attracts + ions at its cyclopentanon group and it repels — ions, the natural Mg compound is most active in this respect, Zn less active and the other metals the least active (Cu might possess an inverse activity). Phaeophytin is absolutely inactive. At high pH the charge of the double layer as well as hydratation increases.
5. A magnitude "$F_{max.}$" which might be of use when measuring hydratation in spreading experiments was applied and a possible explanation of its character was given.
6. To achieve a better understanding of the structure of the above monolayers X-ray diagrams were taken of the crystallizable compound ethyl-chlorophyllide. The dimensions of the chlorophyll molecule are 3.87, 15.48 and 15.62 Å. The surface of the molecule in the plane of the carbon skeleton is 242 $Å^2$. The molecule is a flat plate. The measurements yielded a similar structure for the crystal and for the monolayer, the molecules of the monolayer enclosing angles of 55° with the water surface.
7. It was explained, from data in the literature on other porphin compounds (hemoglobin, catalase, Warburg ferments, erythrocruorins, cytochromes, etc.), that chlorophyll occurs most likely in plants in tetrads: 4 molecules together on a protein carrier of globin dimensions (M.W. 68000). It was endavoured to prove the volumetrical possibility of this supposition by measuring the number of chlorophyll molecules per granum. The probability was discussed of a carbamino-CO_2 bond at this protein molecule in photosynthesis.
8. It was tried to plan a structural model for the photosynthetic mechanism, covering the demands of the present knowledge of the process.
A model is described consisting of a chlorophyll-protein tetrad,

a. suggesting a uniform mechanism for photosynthesis in green plants as well as in bacteria,

b. allowing of photosynthesis running entirely within an area of small dimensions, within one molecule,

c. able to prevent much of the loss of energy, otherwise needed for the activation of intermediary products.

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