Botany. — *Some chemical properties of the plastid-granum.* By W. F. H. M. MOMMAERTS, (from the Botanical Institute, Government University, Leyden). (Communicated by Prof. L. G. M. BAAS BECKING.)

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§ 1.

In recent years it has become increasingly evident that the chlorophyll in the living leaf is present as a prosthetic group of a proteid. The publications of NOACK, ARNOLD and, in particular, the work done at the Leyden Botanical Laboratory have supplied arguments in favour of this opinion (1). The analogy with the chemistry both of enzymes and of respiratory pigments is apparent. The existence of an "agon-pheron"-unit is believed to be essential to the mechanism of photosynthesis (2). It seems that the chlorophyll-proteid is combined with both carotinoids and lecithinoids into a "symplex" in the sense of WILLSTÄTTER and that, in the granum (3) this symplex is present in a regular pattern (4). The name phyllochlorin, as used by MESTRE (5) to designate this symplex has precedence over STOLL's chloroplastin (6) and will therefore be used in this paper to designate this complex structure.

§ 2.

Attempts were made to isolate this phyllochlorin. According to NOACK and also to LUBIMENKO (7) grinding of leaves in tapwater yields a green suspension containing nuclear and protoplasmic material, cell-wall fragments, intact- and broken plastids, but also green particles of a much smaller dimension than that of the chloroplast. It is believed that these particles constitute the grana (8). By means of fractional centrifugation these grana may be separated from the rest of the cell-fragments; at lower speeds these other fragments are thrown down while the grana are separated from the aqueous phase by high-speed centrifugation. MENCKE (9) who used this method to determine the chemical composition of various cell-constituents, does not recognize the grana as separate entities, as he speaks of "Chloroplastensubstanz".

The reality of the grana, however, seems to us beyond dispute, as will be demonstrated below.

Method of preparation. Washed leaves of sweet pea, clover, spinach, nettle and *Nicotiana macrophylla* are ground in tapwater to which calcium carbonate was added, under continuous cooling with ice. The extract is filtered through cloth, the residue being used again. The dark green suspension is centrifuged for about 30 minutes at 3500—4000 rev./min.

in order to throw down most of the cell fragments. Part of the grana are also sedimented in this way. The remaining liquid is centrifuged for 90 minutes. The sediment is collected and stirred into distilled water, after which it is centrifuged again. This procedure is repeated twice. Impurities appear as a whitish zone in the sediment. This stratum is removed. The effect of each manipulation is controlled microscopically. The green mass obtained in this way consists of grana, which are stored in the ice-chest.

The size of the particles and also the fact that grana become more clearly visible on injury of the plastid gives additional support that the sediment actually consists of structural units. The following observation appeared to us particularly significant. When a small fragment of a leaf is torn with needles, high magnification shows broken as well as intact plastids. The liberated grana seem in all respects identical with those still enclosed within the plastid. They possess the characteristic flat shape. Sometimes a broken chloroplast may be seen with a half-protruding granum. From time to time such a granum separates itself from the plastid by its Brownian movement, it enters the liquid phase and is indistinguishable from other green particles in the liquid.

In all plants examined practically all of the plastids disintegrate during the preparation of the suspensions.

It is hardly likely, however, that the grana remain undisturbed during the treatment, as progressive swelling may often be observed. Therefore hydratation may take place and possibly extraneous material may be adsorbed upon the granum-surface. Both factors may cause uncertainties in the determination of the chemical composition of the granum. For the present investigations they seem of secondary importance.

§ 3.

Suspending the grana in alcohol or acetone (± 40 %), or ammonium sulphate (half-saturated) causes them to lose their structure, and to form an amorphous precipitate, this precipitate being the phyllochlorin in its unorganized form, which is but little soluble, in agreement with the hydrophobic character of most of its constituents [1]).

The sediment is centrifuged, and washed twice with cold distilled water. For chlorophyll- and protein determinations the sediment is suspended in cooled acetone (85 %), centrifuged, and the residue extracted with pure acetone. The pigments are completely removed in this way. The remnant is extracted twice with ether, to dissolve the remaining lipoids out of the protein. The residue shows protein reactions; it is dried over paraffin wax (and, if desired, over phosphoric anhydride) and weighed [2]). The

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[1]) This experiment is inspired by the work of LUBIMENKO (loc. cit.); one cannot say, however, that L. isolated the phyllochlorin because his "chlorophylle naturelle" contained nearly the whole cell.

[2]) Because the N. percentage is not yet known, this procedure gives more reliable results than f.i. micro-Kjeldahl.

chlorophyll-content is estimated by determining the absorbtion coefficient for red light (ruby glass) with a Selenium-cell. For comparison pure chlorophyll ("97 %", according to STOLL) was used; it was kindly put at my disposal by E. A. HANSON, M.A. According to these determinations, the protein-chlorophyll ratio was found to be about 100 : 5.5. *On the assumption, that one protein-unit with a particle weight of 17.000* (SVEDBERG), *carries one chlorophyll molecule (average M.W. 926), one hundred parts of protein should carry 5.45 parts of chlorophyll.* We may conclude, therefore, that the assumption of an agon-pheron compound is correct. It is interesting to note that the molecular ratio protein: prosthetic groups is the same as in other conjugated proteins of biochemical importance, f.i. haemoglobin, cytochrome *c*, and others.

It should be mentioned that during the experiments a serious difficulty presented itself. Particularly in the experiments with nettles, it was observed that grinding of the leaves at a lower pH (SØRENSEN's phosphate buffer pH 6.8) led to irregular results (ratio's of 100 : 10 or more), while extracting with water and calcium carbonate gave results equal to, or close to, the theoretical value. (With nettles exact results are only attainable under special precautions). This may be due to a process of autolysis of the protein, or to different mode of separation of the proteins, caused bij a change in the electric charges. To investigate these possibilities, the following experiment was carried out; leaves were ground with water, calcium carbonate and a trace of cupric chloride, and the grana were purified in the usual way. A second lot of leaves was ground with SØRENSEN's buffer, pH = 6.8; the solid constituents of the suspension were removed and the remaining brownish liquid was mixed with the grana, isolated from the first lot of leaves. The resulting suspension was divided into two portions; of one portion (A), the grana were purified, all the manipulations being carried out as quickly as possible. The other portion (B) was allowed to stand for some hours, and then treated similarly. The results of the determinations of the protein-chlorophyll ratio's were for (A): 100 : 5.5, for (B) 100 : 6.3. This experiment clearly shows that the second supposition (direct "colloid-chemical" influence of the pH) does seem to apply. On the other hand, supposing the protein to be partly autolysed, the difference between (A) and (B) might be expected to be higher; however, it is easily conceivable that the proteolytic agent is mainly removed together with the grana and other cell constituents, and is therefore strongly diminished in fraction (B).

The progressive autolytic action, even at an unfavourable pH, is better demonstrated in another experiment: leaves were ground in SØRENSEN's phosphate buffer (pH = 8.0). From one portion the grana were purified immediately, carrying out all the manipulations at low temperature and as quickly as possible. From the other portion the grana were purified after a few hours standing. The protein-chlorophyll ratio's were found to be 100 : 5.7, resp. 100 : 7.5.

From the foregoing experiments it may be concluded that the assumption of autolysis is justified; the fact that the phenomenon is inhibited by traces of cupric ions, as is autolysis in yeast and in animal tissues, is also in favour of this view.

§ 4.

The question arises whether the quantitative relation between protein and chlorophyll, as demonstrated above, finds support in earlier literature.

1. VON EULER, BERGMANN and HELLSTRÖM (10) found that one Elodea-chloroplast of 40 μ^3 contained 2.75—10^{-15} gr. mol. or 16.67×10^8 molecules of chlorophyll. At that time, the granular structure was not yet rediscovered; the Elodea chloroplast is densely filled with small grana. Now, one molecule of a conjugated protein, with a molecular weight of 68.000 (inclusive water and prostethic group) has the size of about $40 \times 40 \times 50$ Å (L. W. JANSSEN, oral communication; in good agreement with the experimental results of ZEILE, KUNITZ and ELFORD). In our case, the protein c.s. would take a volume of:

$$\frac{16.67}{4} \times 10^8 \times 40 \times 40 \times 50 \, \text{Å}^3,$$

or about 30 μ^3, which is of the right order of magnitude.

2. E. A. HANSON (loc. cit.) measured the chlorophyll content of a sample of Hormidium cells and estimated the number of grana in the sample and their approximate dimensions (exact measurement is not possible). By a calculation similar to that employed above, he found a close agreement between the granular volume and the chlorophyll-protein volume.

3. HANSON, MEEUSE, MOMMAERTS and BAAS BECKING (11) determined the chlorophyll-content of the granum indirectly comparing the extinction of red light by the granum (H. D. VERDAM, unpublished), with that of stratified chlorophyll layers, obtained with the Blodgett-technique (12). They found either ± 5 % or 10 %, due to a methodical uncertainty in VERDAM's experiment.

The four different series of measurements, therefore, seem to confirm each other very satisfactorily.

§ 5.

Recently FRENCH (13) prepared a bacteriochlorophyll-carotinoid-protein compound from purple bacteria. It seemed promising to take up the quantitative investigation of this compound.

Bacteria (Rhodospirillum palustre MOLISCH) were centrifuged from the culture liquid. Having no device for high-frequency vibration at my disposal, I desintegrated the bacterial cells by plasmolysis with ice-cold

ammonium-sulphate (half saturated), by which also the chromoproteid and other substances are precipitated. The sediment was washed with CLARK and LUBS' buffer, pH = 4.0. According to FRENCH, the chromoproteid is insoluble at this pH, but is soluble at other degrees of acidity. For pH = 7.2 (CLARK and LUBS' buffer or SØRENSEN's phosphate buffer) I could not confirm this fact; at this pH the degree of dispersity is apparently higher, so that the sedimentation constant in the centrifugal field is lower than at pH 4.0, thus suggesting solubility. However, this difference with FRENCH's result may be due to the different mode of preparation. Making use of this higher dispersity at neutral reaction, I tried a further purification, which seemed to be ineffective.

From this product the protein-bacteriochlorophyll ratio was determined. The protein was estimated gravimetrically, the bacteriochlorophyll on its magnesiumcontent which was determined by means of "Titan-yellow" (KOLTHOFF, 14). Finding 110 mgr. protein, $\frac{24.3}{17.000} \times 110$ mgr. or 157 γ magnesium should be expected. The result was much lower (less than one-half); in agreement with the assumption that the chromoproteid was very impure. Further purification and accurate determinations will be attempted in the near future.

§ 6.

From a study of the action of HCN, WARBURG (15) concluded that "Schwermetallkatalyse" played a part in photosynthesis or more precisely, *an iron-porphyrin compound catalyses the dark reaction.* Although sensitivity to HCN is also conceivable without heavy metal (16), the action of other substances upon the dark reactions supports the idea. Iron determinations in the grana should be, therefore, not without interest.

Grana suspensions were prepared in the usual way; they were placed in SCHLEICHER and SCHÜLL'S dialyzing tubes for two weeks at low temperature. In the dialyzate no iron was detectable with chemical means. A portion was dried, and incinerated in a platinum crucible with some Na_2CO_3 and KNO_3. A second portion was precipitated with acetone 40 %, centrifuged and treated in the same manner. After incineration the residues were extracted with 2n HCl and brought to a volume of 10 cc. Iron determinations were performed by means of ammonium thiocyanate and concentrated by means of ethylacetate, magnesium determinations (for the chlorophyll content) after neutralization, was performed by means of Titan-yellow and 4 N NaOH.

The grana, prepared in this way, contain iron, apparently in an organic state. There seems to be one iron atom to several tens of chlorophyll molecules. The nature of the iron compound will be investigated further [1].

[1]) Some provisional experiments showed that the grana suspensions possess a weak catalase action. Many iron-porphyrin compounds have this property which I believe to be of no interest to the mechanism of photosynthesis, because the inequality of the catalase-theory has been definitely shown (20).

§ 7.

From the foregoing it is seen that it is possible to isolate a compound of protein, chlorophyll, carotinoids and probably lipoids. The nature of the mutual attraction of the components will be briefly discussed.

Concerning the chlorophyll, a protein-bound state is very probable because of the exact stoechiometric relations and the spectral properties (HUBERT, loc. cit.).

As to the carotinoids, the answer is more difficult. Without giving a definite opinion at this place, I will mention some facts which seem to oppose the assumption of a protein-bound state of the carotinoids:

1. If the carotinoids were bound to the same protein as the chlorophyll, there should be a definite relation between the number of chlorophyll molecules and the number of carotinoid molecules. This is not the case. For a protein carrier, apart from the carotinoids, there is "no room" (§ 4); moreover such a protein was not detected during the present investigation.
2. If the carotinoids were bound to a protein, very considerable alteration of their spectral properties should be detectable (compare the data about the astacin (17) and the visual pigments (18)). A systematic study of the leaf carotinoids on this point, comparable with HUBERT's work on the chlorophyll, does not exist. From the data available, however, it appears that such a band-shift does not exist.
3. Photochemical function correlates with protein-bound state: chlorophyll, phycoerythrin and phycocyanin. Also the photoreceptoric carotinoids in the retina are protein-bound. The fact that the plastid carotinoids have no photochemical function in photosynthesis, fits therefore well with the assumption that they are not bound to a protein.

The most probable conclusion is, therefore, that the chlorophyll and the protein form a stoichiometrical compound and that the carotinoids, and probably also the other lipoids are kept together in the system by (homoiopolar) cohesion-forces. The lipophilic nature of the symplex explains its insolubility. It may be expected that a higher dispersity of the system may be obtained with the aid of suitable chemicals. This gives the explanation of the fact that, according to a recent publication, SMITH (19) obtained "solutions" of the phyllochlorin with digitalin or bile-salts which solutions show no visible particles. It will be clear that such systems are wholly artificial and that the "molecular weights" of its components have neither chemical nor physiological significance, except when the reality of these compounds is proved by other means [1]).

As an argument for the combined state of the pigments, it is often stated that for the extraction of the pigments from the leaf, a certain amount of water is necessary which should "hydrolyse" the compound. From carefully dried grana, the pigments are easily extracted with dry acetone,

[1]) Moreover, SMITH's phyllochlorin is certainly not pure. The material which is extracted also contains nuclei, cell-wall fragments, and so on (see § 2).

ether, benzene, petroleum-ether (low-boiling), CS_2 and CCl_4. The phenomenon is therefore not real and seems to be caused by the protective action of the surrounding cell structures.

SUMMARY.

1. The significance of the protein-bound state of chlorophyll is briefly mentioned.

2. A method is described for the preparation of grana-suspensions, with additional evidence about the identity of the isolated material.

3. From such a grana-suspension, the phyllochlorin may be obtained in an unorganized form.

4. In the phyllochlorin the ratio protein-units to porphin-nuclei is 1 : 1, as is the case in other conjugated proteins of high biochemical importance.

5. A comparison is made between these results and other data about the chlorophyll content of the photosynthetic system. These data show satisfactory agreement.

6. In the case of the purple-bacteria, the preparation of the symplex did not yet succeed.

7. The grana-preparations contain iron in a bound state which is of great interest in relation to the so-called "dark-reaction". They show a weak catalase-action which may be due to this compound.

8. It is probable that chlorophyll is chemically bound to the protein but that the carotinoids are only loosely attached by cohesion-forces. The protein-bound state seems to be essential for photochemical action.

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