THE CHLOROPHYLL-PROTEIN COMPOUND OF THE GREEN LEAF*

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I

INTRODUCTION

Until 1870 it was assumed that chlorophyll extracted from the leaf by alcohol, acetone, or similar solvents, was the same as the green pigment in the leaf. In that year, Hagenbach found that the red absorption band of the leaf was 10 to 20 mμ further towards the red end of the spectrum than the corresponding band in the extracts. He later (1874) noted that the maximum of the weak leaf fluorescence was displaced in the same way with respect to the strong fluorescence of chlorophyll in solution. These observations have been repeatedly confirmed (*e.g.*, Hubert, 1935; Dhéré, 1937), and additional differences between the leaf pigment and chlorophyll solutions have since been observed, particularly with regard to solubility and photostability.

Among the many suggestions that have been offered to explain these differences are that the leaf pigment is dispersed in (Tschirch, 1883) or combined with lipoid (Palladin, 1910); that the pigment is colloidally dispersed (Herlitzka, 1912) and possibly adsorbed as a monomolecular layer on protein (Willstätter and Stoll, 1913; Noack, 1927).

In recent years, under the influence of the progress in the study of the respiratory proteins and enzymes, there has been a steadily growing notion that leaf chlorophyll is combined with protein (Lubimenko, 1927; Osborne, 1928; Mestre, 1930; Hubert, 1935; Stoll, 1936; Smith, 1938). Nevertheless, only little evidence has been forthcoming to prove this viewpoint. It is our intention to show that the properties of the green leaf pigment are best explained in terms of a true stoichiometric combination of chlorophyll with

* Short notes on this work have already appeared (Smith, 1938; 1940).

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protein, and to describe some of the properties of this compound.[1] The studies of French (1938, 1940) have demonstrated that the chlorophyll of photosynthetic bacteria is also bound to protein, showing that this linkage is general in nature.

II

Most of the observations were made with a simple direct extract of the leaves of spinach (*Spinacia oleracia*). Leaves were separated from stems, washed thoroughly, and then ground mechanically in a porcelain mortar with sand and a neutral or slightly alkaline buffer solution. The sand and cell debris were removed by centrifuging at low speeds. Opaque dark green preparations were obtained which show the dull red fluorescence characteristic of the leaf.

Both Osborne and Wakeman (1920), and Noack noted that such crude leaf extracts from spinach show extremely fine particles or globules under the microscope, so that what was actually studied was a suspension of the chloroplast material. The suspended chloroplast material can be separated in a variety of ways. It is sedimented by centrifuging at moderate speeds (3000–4000 R.P.M.), only a yellow or brown supernatant fluid remaining. It can also be separated by filtration through a thick layer of paper pulp or a Seitz bacterial filter, or by filtering through kieselguhr or Celite. All these separations indicate that the chloroplast material is not in a molecularly dispersed solution. The insolubility of the chlorophyll-protein complex appears to be due to the hydrophobic character of the chlorophyll, and the other lipoids associated with it in the chloroplast. This is indicated by the work of Menke (1938), who found that 37 per cent of the dry weight of the chloroplast (including the chlorophyll) is soluble in alcohol and ether.

Some extracts were made from the leaves of *Aspidistra lurida* because it was reported by Lubimenko that this species gives aqueous extracts which are completely water-clear and that the green pigment is in true aqueous solution. We have been unable to confirm this observation. While the *Aspidistra* extracts appeared somewhat clearer than those from the spinach leaf, the extracts were always strongly opalescent. For purposes of comparison, most of the observations in this paper were made with the leaves of both species. Unless specific differences are indicated, observations may be taken to apply to both species.

III

Colloidal Chlorophyll

The characterization of the leaf pigment has depended in large part on the position of its absorption bands. Considerable controversy has attended efforts to explain the position of the red absorption band of the leaf on the basis of Kundt's rule. Mestre has summarized the evidence which

[1] At the moment it seems preferable to leave open the question of a name for this compound. It has been pointed out to us that the term "phyllochlorin" suggested by Mestre which we used in an earlier paper applies to a specific chemical derivative of chlorophyll. Other names which have been suggested are "chlorophylle naturelle" (Lubimenko), "chloroplastin" (Stoll), and "photosynthin" (French).

effectively disposes of this suggestion. However, many insoluble pigments show a shift in their band positions depending on the degree of dispersion. A striking example in a naturally occurring pigment is turacin, the copper-porphyrin compound of turaco feathers (Keilin, 1926). In order to show that such factors were not concerned, a comparison was made between the leaf pigment and colloidal chlorophyll in various states of dispersion. The spectral observations were confined to the region between 520 and 700 mμ since the absorption of other leaf pigments can be neglected in this region.

Herlitzka and later Willstätter and Stoll believed that the leaf pigment was colloidal chlorophyll, mainly on the basis of the similarity in position of the main red absorption band. Ivanovski (1907, 1913) opposed this view on the ground that not only were the band positions slightly different, but that the relative intensities of the various bands were different. Hubert found recently that the main red band of the leaf was at 680–681 mμ while that of colloidal chlorophyll was always further towards the blue but depended on the state of aggregation.

Colloidal chlorophyll was prepared by rapidly diluting crude acetone extracts of the leaf with a slightly alkaline phosphate buffer in order to prevent phaeophytin formation. The colloidal chlorophyll was dialyzed in cellophane membranes in the refrigerator against phosphate buffer in order to remove the acetone completely. The maximum absorption was always found in the region between 671 and 673 mμ as measured with a Hilger-Nutting spectrophotometer. The maximum absorption of aqueous leaf extracts was consistently at 677–678 mμ. Attempts were made to duplicate the appearance of the leaf pigment by preparing colloidal chlorophyll in the presence of proteins such as gelatin and horse serum. In every instance the red absorption maximum was the same as in the ordinary colloidal chlorophyll preparations.

Preparations of colloidal chlorophyll can be clarified, removing the characteristic bluish opalescence by adding a detergent such as digitonin or bile salts. The band position was then found to shift to 674–675 mμ. The shift towards the red can be explained by the removal of the light scattering, since the amount of scattering is proportional to the reciprocal of the fourth power of the wave length according to the Raleigh equation.

Differences in the positions of the absorption band in the red are always apparent; for colloidal chlorophyll the band is at 671–673, for the aqueous leaf extract at 677–678, and for the leaf itself at 681 mμ (Hubert). Ivanovski's observations are confirmed not only on this point, but also on the fact that the relative intensities of the absorption bands are different; the minor absorption bands *circa* 540 and 580 mμ like those of chlorophyll in organic

solvents are always much more prominent in colloidal chlorophyll than in the leaf or its aqueous extracts. This is likewise true for the main red bands of chlorophylls *a* and *b*; the separate *b* band is more prominent in colloidal chlorophyll or in organic solvents than it is in the leaf. These differences are very striking when spectra of the different preparations are observed side by side with a low dispersion spectroscope.

No fluorescence was observed with colloidal chlorophyll preparations confirming the older observations of Noack. Meyer (1939) has claimed that preparations of colloidal chlorophyll do fluoresce. We have made similar observations when relatively large amounts of alcohol or acetone were present; after removal of the organic solvent by dialysis, no fluorescence could be observed.

IV

Some Properties of the Leaf Pigment

As yet no specific catalytic property of the chlorophyll-protein has been observed. In order to characterize the material, it has been studied under various conditions.

Absorption Spectrum.—The absorption spectrum of an aqueous extract of spinach is given in Fig. 1; the data are presented in Table I. The measurements were made with the photoelectric spectrophotometer of Shlaer (1938). Absolute extinction values cannot be given for the unpurified extract because of the presence of various yellow substances (blue-absorbing), and because of the light-scattering produced by the suspended particles. This latter effect is clearly shown by the apparent absorption between 700 and 750 mμ. With an *Aspidistra* extract of comparable concentration (same extinction at 677 mμ) there is nearly the same amount of scattering in this region indicating a similar state of dispersion for the *Aspidistra* and spinach proteins.

The maximum absorption at the red end of the spectrum has always been found at 677 to 678 mμ. Secondary bands are at 625 and 590, with a definite inflection at 650 mμ. The minimum absorption is at 560 mμ. The absorption bands in the short wave region are at 470 and 437. These latter bands are the resultant not only of chlorophylls *a* and *b* but of the carotenoids as well. With *Aspidistra* extracts it is frequently possible, using a low dispersion microspectroscope, to separate two absorption bands, one at 470 and the other at 485–490.

Various substances have been tested for possible effect on the absorption spectrum of leaf extracts either because of their influence on photosynthesis or because they combine with some chromoproteins which are involved in

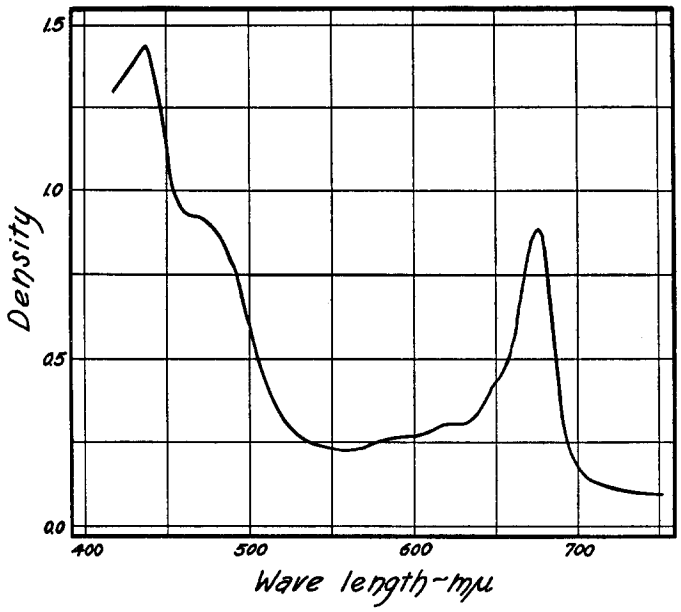

FIG. 1. Absorption spectrum of an aqueous extract from the spinach leaf. The data are given in Table I.

TABLE I

Absorption Spectrum of Leaf Extract

Data for an aqueous extract of spinach leaves buffered at pH 7.0 with 0.1 M phosphate buffer.

λ	Density	λ	Density	λ	Density
mμ		mμ		mμ	
750	0.0914	660	0.5224	530	0.2628
740	0.0958	650	0.4282	520	0.3132
730	0.1022	640	0.3382	510	0.4172
720	0.1116	630	0.3014	500	0.5878
710	0.1296	625	0.3026	490	0.7816
700	0.1774	620	0.3002	480	0.8608
695	0.2358	615	0.2908	475	0.9024
690	0.3602	610	0.2800	470	0.9176
685	0.5954	600	0.2672	465	0.9216
680	0.8454	590	0.2622	460	0.9376
678	0.8780	580	0.2516	450	1.0928
677	0.8860	570	0.2362	440	1.3928
676	0.8820	560	0.2246	435	1.4312
675	0.8766	555	0.2260	430	1.3848
670	0.7962	550	0.2304	420	1.3184
665	0.6594	540	0.2428		

tissue respiration. The tests were made by evacuating a control solution in a Thunberg tube and comparing it side by side with the test sample under the microspectroscope. Among the substances which have been tested are: oxygen, carbon dioxide, carbon monoxide, cyanide, hydroxyl amine, sodium azide, hydrogen sulfide, urethane, and mild oxidizing and reducing agents. None of these was found to produce any observable change in the absorption spectrum. The inertness of the chlorophyll-protein compound with respect to these reagents is in contrast to the well known behavior of such iron-porphyrin protein compounds as hemoglobin and catalase.

In contrast to the photolability of chlorophyll in organic solvents, the absorption spectrum of the pigment in the aqueous extracts was found to be stable to high light intensities for long periods. A solution kept at 20° C. was subjected to an intensity of about 200,000 meter candles for 1 hour without measurable effect on the absorption spectrum.

Effect of Organic Solvents.—As mentioned above, the leaf spectrum and chlorophyll dissolved in organic solvents show differences not only in the position but also in the relative intensities of the absorption bands. With an aqueous leaf extract at 20° C., the presence of low concentrations of acetone (10 per cent) does not produce any visible effect. At higher acetone concentrations (20–25 per cent), definite changes take place in the spectrum; the minor bands become more prominent and the main red band shifts slightly towards the blue. With 30 per cent acetone, the protein begins to precipitate and is complete at about 50 per cent acetone but with some color remaining in solution. At higher acetone concentrations the chlorophyll is rapidly extracted from the protein. The effect of the different acetone concentrations is influenced by the pH of the solution, higher concentrations being necessary to produce the same effect for alkaline solutions (pH 8.5 to 9) as compared with neutral ones. Higher temperatures increase the ease of chlorophyll extraction. Ethyl alcohol does not sensibly differ from acetone in its effect.

It is well known that ether will not extract chlorophyll from the leaf and that is equally true for the aqueous leaf extract. However, when the aqueous preparation is vigorously shaken with ether, the preparation is readily emulsified and the spectrum becomes that of free molecular chlorophyll in ether. The fluorescence is also very much brighter. Ether will extract chlorophyll quite readily from dried chloroplast preparations. The failure of ether to dissolve chlorophyll from the leaf or aqueous extracts can be explained by the low solubility of ether in water, just as with moderate acetone concentrations (35 per cent) the spectrum is changed but the chlorophyll not extracted.

Effect of Temperature.—Sorby discovered in 1872 that heating a leaf causes

a shift in the position of the main absorption band in the red. Willstätter and Stoll later showed that the spectrum of the boiled leaf is similar to that of chlorophyll in phytol or lecithin. Noack found that heating a leaf causes the fluorescence first to disappear and on more prolonged heating to reappear. He ascribed the disappearance of the fluorescence to the denaturation of the protein and its subsequent reappearance to the solution of the chlorophyll in some waxy component of the leaf. Mestre found the change in the leaf spectrum to be a function of both time and temperature very similar to those for ordinary protein denaturations.

Heating an aqueous extract of the leaf produces changes in spectrum and fluorescence identical with those directly observed on the leaf. A green protein coagulum is gradually formed on heating a neutral solution above 60° C., with the fluorescence becoming weaker. When the coagulum is evaporated to dryness, the spectrum is identical with that given by Willstätter and Stoll for chlorophyll in phytol; the fluorescence is much more intense than for an unheated control.

Aside from the fact that these heating experiments strongly indicate the linkage of chlorophyll to protein, they also provide excellent criteria for determining the native state of the pigment complex. As in experiments with the chlorophyll solvents, the changes which take place are clearly reflected in the character of the spectra and fluorescence.

Effect of Alkali.—At pH 9.0 the leaf extract is quite stable and shows no change in its solubility, precipitation properties, or spectrum. In M/10 NaOH, the band at 678 mμ slowly becomes weaker and a new band at 640 mμ appears; this corresponds to the saponification of the esterified groups which occurs in strongly alkaline solutions with molecular chlorophyll. At the same time, the band at 475 mμ shifts towards the shorter wavelengths, making more prominent the carotenoid band at 485–490 mμ. The rate of saponification seems to be a direct function of the hydroxyl ion concentration. In M/10 NaOH the effect can be detected only after some hours, while with 5 M alkali the reaction is complete in a few minutes. In M/10 alkali, the protein is gradually precipitated. With very strong alkali (5 M), a precipitate of denatured protein forms immediately. Protein denaturation and the change in spectrum appear to be roughly parallel.

Effect of Acid.—Addition of dilute acetic acid causes the complete precipitation of the protein at a pH between 4.5 and 5.0, with no apparent change in the spectrum. Further addition of acid to pH 2 gradually changes the green color to a yellowish green and finally a yellow to brown. The spectrum is that of phaeophytin; the main red band is much weaker, and strong bands appear at 540 and 610 mμ.

The protein precipitated at pH 4.5 is no longer resuspended by neutraliza-

tion; much larger quantities of alkali are required and the protein is suspended only at a pH above 9.0. The precipitation at 4.5 and resuspension at pH 9.0 may be repeated indefinitely and seems to be a useful method of separating the chloroplast material from the cytoplasmic proteins which do not precipitate (*cf.* Menke, 1938). However, the properties of the chloroplast protein aside from its spectrum are definitely changed by this procedure: (1) as already mentioned, it does not resuspend at pH 7.0 but only at much higher pH's; (2) it is readily precipitated by 10 per cent saturation with ammonium sulfate and cannot be resuspended; the original extract requires about 30 per cent saturation for precipitation; (3) boiling the extract for several minutes does not cause any precipitation unless it is brought to an acid pH; it may then be resuspended by adding alkali to bring the solution again to pH 9.0. A control sample at pH 7 not previously treated with acid will form a heavy coagulum by heating to boiling and will not resuspend regardless of the pH.

These changes brought about by treatment with dilute acid are those usually ascribed to protein denaturation. The chloroplast protein is much more sensitive to weakly acid solutions than most proteins.

Effect of Drying.—Leaf extracts were dried by suspending them in cellophane dialyzing tubing in front of an electric fan. In this way, a large volume of extract may be handled, and the extracts kept cool during the entire evaporation process. Small samples of extracts slowly dried *in vacuo* over sulfuric acid gave similar results.

The dried chloroplast material could not be redissolved or suspended in water or neutral buffer solutions, but could be partially suspended in borate buffer at pH 9.0 or with dilute alkali. In these alkaline solvents, the protein appeared to be modified in the same way as with the acid-precipitated material; *e.g.*, the protein could be precipitated by 10 per cent saturation with ammonium sulfate.

Both the chlorophylls and carotenoids are rapidly extracted from the dried material by acetone, alcohol, and ether. Petroleum ether and carbon disulfide extract most of the carotenoids readily and only little chlorophyll even after several hours.

Effect of Detergents.—Because of the insolubility of the chlorophyll-protein complex, the effect of adding various dispersing agents which clarify leaf extracts has been studied. Preparations of the chloroplast material in the detergent were prepared in two different ways. The most direct method was to add the detergent solution directly to the leaf extract. The other method was to add to the leaf extract about 5 per cent Filter-Cel and then filter through a thin layer of Filter-Cel on a Buchner funnel. The

yellow-brown filtrate is discarded, and the filter-cake then washed with water or neutral buffer solution until the filtrates show no yellow color. In this way, all of the water-soluble material extracted from the leaf may be removed. The filter-cake is then extracted with the detergent solution and filtered.

Extraction with a 1 to 5 per cent digitonin[2] solution after filtration yields a clear dark green solution which shows no trace of particles under an oil

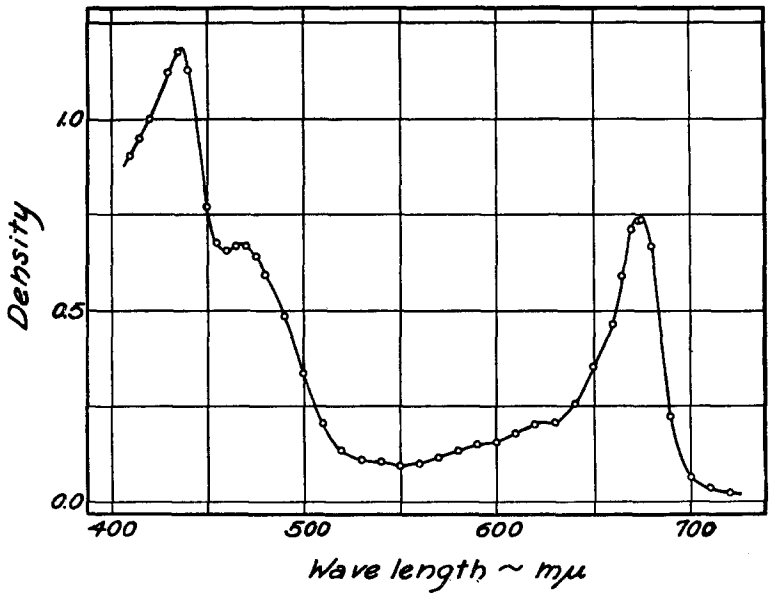

FIG. 2. The absorption spectrum of an extract from the spinach leaf prepared in 2 per cent digitonin and diluted 1 to 10 with distilled water. The data are given in Table II.

immersion lens. The absorption spectrum of a diluted solution is shown in Fig. 2 and the data are given in Table II. When this curve is compared with that of a direct aqueous leaf extract (Fig. 1), several differences are apparent. The absorption drops very rapidly on the long wave side of 700 mμ; it has already been pointed out that the apparent absorption in this region shown by an aqueous extract is due to light scattering. The removal of scattering is probably also responsible for the decrease in the

[2] The digitonin was obtained from Eimer and Amend, New York, as crystalline digitalin. This digitonin was dissolved by heating to a gentle boil when the solution becomes water clear. On cooling, the solution will remain clear for some weeks at room temperature. Over longer periods, some precipitation occurs.

relative height of the middle region of the spectrum. In addition, the pigment in digitonin shows a shift of the main red band from 677–678 mμ to 675 mμ, and of the minimum region of absorption from 560 mμ to 550 mμ. The sharper character of the band at 470 mμ in the digitonin solution is undoubtedly due to the removal of the yellowish impurities.

Solutions clarified by the addition of digitonin show a somewhat increased fluorescence when compared visually with a direct leaf extract. It is likely that the apparent increase in fluorescence may be due to the decrease in light scattering caused by the presence of the detergent.

TABLE II

Absorption Spectrum of Spinach Leaf Extract in Digitonin

Data of Fig. 2. Extract prepared in 2 per cent digitonin and diluted 1 to 10 with distilled water.

λ	Density	λ	Density	λ	Density
mμ		mμ		mμ	
720	0.0254	610	0.1794	480	0.5930
710	0.0346	600	0.1602	475	0.6414
700	0.0678	590	0.1506	470	0.6694
690	0.2218	580	0.1374	465	0.6670
680	0.6658	570	0.1182	460	0.6562
675	0.7370	560	0.0998	455	0.6734
673	0.7346	550	0.0974	450	0.7712
670	0.7086	540	0.1018	440	1.1294
665	0.5914	530	0.1090	435	1.1754
660	0.4654	520	0.1350	430	1.1202
650	0.3518	510	0.2054	420	1.0090
640	0.2590	500	0.3356	415	0.9522
630	0.2090	490	0.4854	410	0.9046
620	0.2034				

The precipitation properties of the chloroplast protein are distinctly modified by the presence of the digitonin. Even saturation with ammonium sulfate is quite ineffective. Most of the digitonin can be removed by ultrafiltration through a 3 per cent Bechold collodion membrane without loss of pigment, but prolonged dialysis is necessary for complete removal of the detergent. The absence of the digitonin in the dialysate can be readily tested by shaking vigorously since all of the detergents produce a persistent foaming. After dialysis, the pigment is readily precipitated by a tenth saturation with ammonium sulfate, and can be redissolved in digitonin solution. The pigment can also be precipitated by acidification to pH 4.5 and redissolved by buffer solution at pH 9.0. This process can be repeated indefinitely. In this respect, the properties of the pigment are similar to those produced by direct acid precipitation.

Bile salts (a purified mixture of sodium glycocholate and taurocholate) and sodium desoxycholate have also been used for dispersing the chloroplast pigment. The properties of the pigment in these detergents closely resemble those in digitonin solutions. For equivalent concentrations, the desoxycholate is somewhat more effective than either bile salts or digitonin. However, desoxycholate has the disadvantage of being insoluble at acid pH's and it tends to precipitate or gel even at slightly alkaline ones.

The absorption spectrum of the pigment in these detergents is almost identical with that found in digitonin. The only difference is that in both, the position of the main red band is shifted further towards the blue, and is found at 671 to 672 mμ.

Concentrated urea solutions (50 per cent) also clarify aqueous solutions of the chloroplast pigment. The absorption spectrum is identical with that of the pigment in digitonin with the main red absorption band at 675 mμ.

V

Relationship of Chlorophyll to Protein

If a true combination exists between chlorophyll and protein, there should be a definite quantitative relationship between them. This point has been investigated by purifying the chloroplast material in different ways, and then evaluating chlorophyll in relation to the dry weight, and in a few cases, to the chloroplast nitrogen as well.

Estimation of Chlorophyll Concentration.—The usual method of estimating chlorophyll colorimetrically by matching against a standard solution of chlorophyll is subject to the difficulty of obtaining chlorophyll solutions of known purity. Moreover, the absolute extinction coefficients of chlorophylls *a* and *b* are still subject to some revision although it does not appear likely that they will change very much. We have preferred to estimate chlorophyll by measuring the extinction at the maximum absorption at the red end of the spectrum where there is no interference by the yellow pigments of the leaf. Using the best absolute extinction values, it is then possible to compute the chlorophyll concentration.

Although the position of the absorption band in the aqueous preparations is different from that of chlorophyll in organic solvents, the same preparation has an identical extinction value in the aqueous extract clarified by digitonin, or in ether or petroleum ether.[3] This comparison was made by diluting an aliquot portion of the concentrated aqueous extract until the extract had several times the chlorophyll concentration suitable for spectrophotometric estimation. The extract was then diluted with a 5 per cent solution of digitonin until the final digitonin concentration was 1 or 2 per cent.

[3] In a preliminary communication (Smith, 1940) it was inadvertently stated that the "extinction value in water as protein compound, or in ether or petroleum ether" is the same. The statement should read "aqueous digitonin" in place of "water."

The chlorophyll from another sample of the extract was transferred to ether by adding ten volumes of acetone to precipitate the protein, washing with more acetone, and finally washing the chlorophyll into the ether by adding water. After several additional washings of the ether with water, the ether extract was brought to a definite volume and the chlorophyll estimated spectrophotometrically. The data of four separate experiments are given in Table III. Single determinations with ethanol and acetone as solvents are in agreement with the data for ether and petroleum ether.

The aqueous extract cannot be directly compared with the organic solvents since the former shows a large and variable loss of light caused by scattering, giving an appreciably higher extinction value. While digitonin has been used to eliminate this scattering, it is likely that other clarifying agents, such as bile salts, would also serve the same purpose. The absorption of the pigment in digitonin was found to follow the Lambert and Beer law over a tested concentration range of one to ten.

TABLE III

Comparison of Chlorophyll Absorption in Different Solvents

Experiment	Solvent	λ maximum	Density	Averages
		mμ		
1	Ether	660	1.14	
		661	1.16	
		660	1.17	1.16
	Digitonin	675	1.18	
		675	1.17	1.18
2	Petroleum ether	661	1.25	
		660	1.22	1.24
	Digitonin	675	1.25	1.25
3	Ether	660.5	1.67	
		660	1.81	
		660	1.80	1.76
	Digitonin	674.5	1.80	1.80
4	Ethanol	665	1.36	
	Acetone	663	1.32	
	Digitonin	675	1.40	

Accepting the findings of Willstätter and Stoll, it has been assumed that the leaf pigment contains chlorophylls *a* and *b* in a ratio of three to one. On this basis, values for the mixed pigments have been computed from the best available data. Using the molecular extinction coefficient ϵ where

$$\epsilon cd = \log_{10} I_0/I = D$$

the data of Zscheile (1934) give 5.4×10^4. When the data of Winterstein and Stein (1933) are converted from $\log_e$ to $\log_{10}$, the same value is obtained. MacKinney's (1940) recent data on chlorophylls *a* and *b* yield the value 5.6×10^4. Since the higher value indicates purer components, the absolute chlorophyll concentrations are calculated

in terms of this value. The most convenient method of expressing the relative values is as optical density (D) per mg. of dry weight per ml. of solution per cm. absorption. The percentage of chlorophyll is $D(900)(100)/5.6 \cdot 10^4$ or 1.61 D.

Purification.—Most of the preparations were purified by repeated salt precipitation and resuspension in slightly alkaline phosphate buffer. The spinach protein could be precipitated by 0.3 saturation with ammonium sulfate or by saturation with sodium chloride. *Aspidistra* protein could not be precipitated with sodium chloride but was precipitated by half saturation with ammonium sulfate. The ammonium sulfate was always added from a saturated solution made slightly alkaline (phenol red) with 0.1 N sodium hydroxide. It was necessary to carry out all of the manipulations in slightly alkaline solutions in the cold; otherwise denatured protein was obtained which could not be resuspended.

Determinations of the dry weight were made after the solutions were thoroughly dialyzed in cellophane tubing first against slightly alkaline phosphate buffer, and finally against distilled water. Dry weights were determined by evaporating an aliquot portion of the solution over a steam bath and finally by drying in an evacuated dry chamber over sulfuric acid.

Initial extracts of the leaves had a D/mg./ml./cm. between 1.2 and 1.4 both for spinach and *Aspidistra*. This is roughly one-fourth of the average value obtained for the purified material after three or four precipitations. There was generally only a small change in the D value after the second precipitation. The final values are given in Table IV.

In one experiment purification was effected in a different manner. The leaf extract was sedimented in an air-turbine concentration centrifuge at 8000 R.P.M. The preparation was sedimented twice, resuspending in M/10 Na_2HPO_4, and finally three times more, resuspending each time in distilled water. The precipitates were collected in a little distilled water. To a carefully measured volume, an equal volume of 5 per cent digitonin was added. This was centrifuged at 2500 R.P.M. for 15 minutes, and aliquot portions of the clear solution were taken for chlorophyll, dry weight, and nitrogen estimation. A sample of the digitonin solution was also taken for dry weight. Estimation showed that the digitonin preparation was nitrogen-free. The results of this experiment also given in Table IV are in keeping with the others.[4]

Table IV summarizes the purification data. Using the actual chlorophyll determinations, the total nitrogen values (micro Kjeldahl) were corrected for the 6.2 per cent nitrogen present in chlorophyll assessed on the

[4] Thanks are gratefully acknowledged to Dr. E. A. Kabat of Cornell University Medical School for the use of the air-turbine centrifuge and for his aid with this experiment.

basis of three parts of *a* to one of *b*. The remaining nitrogen was assumed to be protein nitrogen, using the customary factor 6.25. The average chlorophyll content of the isolated chloroplast material was 7.86 per cent. For the three experiments where nitrogen was determined, the protein content of the chloroplasts was 46.5 per cent in good agreement with the average value of 47.7 per cent found by Menke (1938) for spinach leaves.

The average chlorophyll content was 16.1 parts of chlorophyll per 100 parts of protein.[5] This is in contrast to the results of Granick (1938) and Mommaerts (1938). Granick found 27 parts of chlorophyll per 100 parts of protein calculated from his statement of 30 molecules of chlorophyll

TABLE IV

Relationship of Chlorophyll to Protein in Chloroplast

Species	Nitrogen	Protein N	Protein (protein N·6.25)	Density	Chlorophyll	Chlorophyll per 100 parts of protein	Method of purification
	per cent	*per cent*	*per cent*		*per cent*		
Spinacia.....	8.3	7.8	48.8	4.93	7.94	16.3	High speed centrifuging
				5.26	8.47		$(NH_4)_2SO_4$ precipitation
				4.88	7.86		" " "
				5.06	8.15		Sodium chloride precipitation
				5.40	8.69		" " "
Aspidistra.....	7.4	6.9	43.1	4.43	7.13	16.5	$(NH_4)_2SO_4$ precipitation
	8.1	7.6	47.5	4.56	7.34	15.5	" " "
				4.33	6.97		" " "
				5.07	8.16		" " "
				4.92	7.92		" " "
Averages.....		7.4	46.5	4.88	7.86	16.1	

per 100,000 molecular weight of protein. Mommaerts found about 5.5 parts of chlorophyll per 100 parts of protein. The decided discrepancy between the results of these two investigators and the data given here may be at least partly explained by the fact that Mommaerts removed the chlorophyll from the chloroplasts with ether and determined the dry weight of the ether-insoluble residue, assuming that it was entirely protein. Granick determined chlorophyll colorimetrically but did not specify his standard of comparison. If his standard was of lower purity than MacKinney's it would aid in explaining the difference.

[5] After this work had appeared in preliminary form, the paper of Menke (1940) became available in which he reported an average value of 17.2 parts of chlorophyll per 100 parts of protein. This is in excellent agreement with the value of 16.1 reported here when one considers the different methods used.

The value of 16.1 per cent chlorophyll may have to be lowered somewhat if the absolute extinction coefficients for pure chlorophylls *a* and *b* are found to be higher. This does not appear likely since the results of Zscheile, Winterstein and Stein, and MacKinney agree within 5 per cent. On the other hand, further purification of the chloroplast protein may necessitate some revision of this figure. Some of the chloroplast nitrogen may not belong to the chlorophyll protein. Our evidence is negative in that other purification methods were unsuccessful in changing the chlorophyll to dry weight ratio. The pigment was readily adsorbed at pH 6.6–6.8 by alumina cγ, and gelatinous calcium triphosphate but elution at pH 8 to 9.5 was unsuccessful. When partial adsorption was carried out by using an amount of adsorbent insufficient to remove all of the pigment, the remaining pigment did not differ from the starting material already purified by salt precipitations. Other adsorbents such as copper hydroxide and calcium hydroxide behaved similarly. At pH 6.6 the green pigment was not adsorbed by bone charcoal or kaolin, nor did these adsorbents remove enough impurities to change the chlorophyll to dry weight ratio.

A few attempts were made to obtain an independent estimate of the chlorophyll concentration by measuring the magnesium content of leaf extracts or purified material by the Titan yellow method after digestion with sulfuric acid or with nitric acid and H_2O_2. The values obtained, especially with the unpurified extracts, always gave chlorophyll estimations much higher than those found by the spectrophotometric method, indicating the presence of magnesium not bound in the chlorophyll molecule.

VI

DISCUSSION

From the evidence of the spectral and chemical properties of the chloroplast pigment, it seems certain the chlorophyll exists in the leaf as the prosthetic group of a definite protein. The constant proportionality of chlorophyll to protein must be regarded as one of the more important facts indicating this linkage in spite of the fact that some uncertainty remains attached to the absolute ratio.

It is still undetermined whether the large quantity of non-protein material associated with the chloroplast protein represents a molecular combination or only an association complex. If the entire complex is in molecular combination, then the average chlorophyll content of 7.86 per cent would indicate a minimum molecular weight of 11,500 for the complex. Using the chlorophyll-protein ratio of 16.1 to 100, the minimum molecular weight is

5600, or a little over three chlorophyll molecules for the Svedberg protein unit of 17,500. Because of the much smaller light absorption at the standard wave length of chlorophyll *b* compared to chlorophyll *a*, the three and a fraction may represent three molecules of chlorophyll *a* and one of *b*. In their analyses of the leaves of many green plants, Willstätter and Stoll found that the ratio of chlorophyll *a* to *b* seldom deviated from three to one. Using a different method of chlorophyll estimation, Winterstein and Stein found the same ratio. This suggests a definite combining ratio of three molecules of *a* and one of *b* in the same protein unit. Although many hypotheses have been advanced ascribing different functions to chlorophylls *a* and *b*, this is, we believe, the first suggestion to explain the constant ratio.

There is some doubt whether the carotenoids are also bound to protein. None of the purification methods which have been attempted has served to separate any of the chlorophyll or carotenoid components of the chloroplast. The fact that petroleum ether readily extracts the carotenoids but not chlorophyll from dried chloroplast material indicates that the carotenoids may be only loosely associated rather than bound by true chemical linkage. On the other hand, sedimentation studies in the ultracentrifuge (Smith and Pickels, unpublished) in the presence of sodium dodecyl sulfate reveal no separation of chlorophylls and carotenoids even though the protein is split into smaller units. The existence of carotenoid-protein compounds in nature such as the astacene compounds of Crustacea, and visual purple, shows that such combination is not unlikely.

Whether the close association of all the pigment components of the chloroplast is a loose one or is in the form of a giant molecule as postulated by Lubimenko, this association must be of importance in the photosynthetic mechanism. In any case, the combination of chlorophyll with protein must be taken into consideration in dealing with the problem of photosynthesis.

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SUMMARY

1. Aqueous extracts of spinach and *Aspidistra* leaves yield highly opalescent preparations which are not in true solution. Such extracts differ markedly from colloidal chlorophyll in their spectrum and fluorescence. The differences between the green leaf pigment and chlorophyll in organic

solvents are shown to be due to combination of chlorophyll with protein in the leaf.

2. The effect of some agents on extracts of the chlorophyll-protein compound has been investigated. Both strong acid and alkali modify the absorption spectrum, acid converting the compound to the phaeophytin derivative and alkali saponifying the esterified groups of chlorophyll. Even weakly acid solutions (pH 4.5) denature the protein. Heating denatures the protein and modifies the absorption spectrum and fluorescence as earlier described for the intact leaf. The protein is denatured by drying. Low concentrations of alcohol or acetone precipitate and denature the protein; higher concentrations cause dissociation liberating the pigments.

3. Detergents such as digitonin, bile salts, and sodium desoxycholate clarify the leaf extracts but denature the protein changing the spectrum and other properties.

4. Inhibiting agents of photosynthesis are without effect on the absorption spectrum of the chlorophyll-protein compound.

5. The red absorption band of chlorophyll possesses the same extinction value in organic solvents such as ether or petroleum ether, and in aqueous leaf extracts clarified by digitonin although the band positions are different. Using previously determined values of the extinction coefficients of purified chlorophylls *a* and *b*, the chlorophyll content of the leaf extracts may be estimated spectrophotometrically.

6. It was found that the average chlorophyll content of the purified chloroplasts was 7.86 per cent. The protein content was 46.5 per cent yielding an average value of 16.1 parts per 100 parts of protein. This corresponds to a chlorophyll content of three molecules of chlorophyll *a* and one of chlorophyll *b* for the Svedberg unit of 17,500. It is suggested that this may represent a definite combining ratio of *a* and *b* in the protein molecule.

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