THE EFFECT OF DETERGENTS ON THE CHLOROPHYLL-PROTEIN COMPOUND OF SPINACH AS STUDIED IN THE ULTRACENTRIFUGE*

By EMIL L. SMITH** and EDWARD G. PICKELS

(From the Laboratory of Biophysics, Columbia University, and the Laboratories of the International Health Division of The Rockefeller Foundation, New York)

(Received for publication, March 13, 1941)

I

INTRODUCTION

It has been demonstrated that in the green leaf chlorophyll is combined with protein by true chemical linkage. The chlorophyll-protein compound of the spinach leaf is insoluble in water and buffer solutions, but can be dispersed by detergents yielding brilliantly clear solutions. In addition to clarifying these solutions, the detergents denature the protein and change the absorption spectrum and other properties (Smith, 1941 *a*, *b*). Observations of the action of various detergents on the chlorophyll-protein compound have now been made using the ultracentrifuge.

II

Materials and Methods

All of the observations were made with fresh extracts of spinach leaves obtained by grinding mechanically in a porcelain mortar with sand and buffer solutions. Most of the sand and debris were removed by squeezing through muslin, and the extract was then centrifuged in a cold room at 1800–2000 R.P.M. The extracts were used within 1 or 2 days, and from the time of preparation until use were kept cold.

For most of the work the preparation was not further purified. In one instance, an extract was concentrated by centrifuging at 8000 R.P.M. in an air-turbine concentration centrifuge. The supernatant fluid containing the soluble proteins of the leaf extract was discarded, and the green pellets rubbed up in 0.2 M Na_2HPO_4. This preparation was used for three runs.

Solutions of the detergents were added immediately before beginning the measurements. Four detergents were used: digitonin obtained as crystalline digitalin from Eimer and Amend, New York; sodium desoxycholate obtained from Riedel de Haen,

* A preliminary report of this work was presented to the American Physiological Society in March, 1940 (Smith, 1940).

** John Simon Guggenheim Memorial Fellow (1938–1940).

New York; bile salts, a colorless crystalline preparation prepared from commercial sodium glycocholate in the Laboratory of Biophysics; and sodium dodecyl sulfate, a crystalline preparation obtained from Imperial Chemical Industries by Professor Keilin of the Molteno Institute, Cambridge, whom we wish to thank for a supply of this substance.

The air-driven vacuum ultracentrifuge described by Bauer and Pickels (1937) was used in these studies. The solutions were observed with the optical absorption method of Svedberg, and simultaneously with an automatic refractive index system designed especially for the ultracentrifuge and utilizing a scanning system similar to that described by Longsworth (1939) for electrophoresis measurements. Sedimenting solutions were photographed alternately with red and with violet light. For red light the Wratten "monochromat" filter No. 70 was used, and for the violet, a 1.5 mm. No. 511 Corning filter together with a 3 mm. No. 038 Corning filter. A 100 watt projection lamp was the source. The runs were made at a speed of 46,800 R.P.M. at temperatures near 25°C.

III

EXPERIMENTAL

Absorption measurements were carried out with both red and violet light in order to ascertain whether the carotenoid pigments sedimented differently from the chlorophyll. One difficulty that was encountered with all of the measurements was that the protein concentration could not be made very high. The relatively high concentration of chlorophyll present in the chloroplast complex, 7.9 per cent or 16 per cent in relation to the protein alone (Smith, 1941 *a*), together with the high extinction coefficient of the chlorophyll necessitated keeping the total concentration of protein low in order not to make excessively long exposures both for the absorption and refractive index readings. For this reason, the refractive index diagrams usually showed only protein boundaries of low concentration. Nevertheless, in order to obtain refractive index diagrams simultaneously, the pigment concentration had to be kept too high to give ideal conditions for absorption measurements. The sedimentation constants recorded were those obtained by the refractive index method.

A few runs were made with untreated leaf extracts. It was found that the total pigment sedimented at very low speeds, 2500–3000 R.P.M., and showed a purely random spread of particle sizes. This confirms the fact that the chlorophyll-protein complex of the spinach leaf is not in true solution. However, Price and Wyckoff (1938) and Loring, Osborn, and Wyckoff (1938) have reported obtaining clear green solutions from various leaves which show sharp boundaries with high but variable sedimentation constants.

1. Centrifugation of Detergents.—The experiments of McBain and his collaborators (McBain and Salmon, 1920) demonstrated that micelles are

formed in aqueous solutions of electrolytes such as soaps and other paraffin-chain salts. Sedimentation runs were therefore made with solutions of the four detergents used in these experiments. The results have already been reported elsewhere (Smith and Pickels, 1940). It was found that the non-electrolyte digitonin forms large micelles of homogeneous size in aqueous solution ($S_{20} = 5.9 \times 10^{-13}$). With the other detergents, sodium desoxycholate, bile salts, and sodium dodecyl sulfate (SDS), no detectable quantities of large micelles were found. The refractive index curves were characteristic of low molecular weight substances, there being no boundaries but only a relative decrease of concentration in the upper part of the sedimenting solution and some increase in the lower section. In the experiments with the leaf extracts, care was taken to distinguish between protein behavior and effects caused by the detergents alone.

2. Effect of Digitonin.—Chlorophyll-protein extracts in the presence of digitonin show a regularly sedimenting boundary which is equivalent in red and violet light. By comparison with the refractive index diagrams it was found that these absorption boundaries are identical with the refractive index boundary of the digitonin micelle. Thus, these boundaries are due not to the protein but to the digitonin micelle.[1] The refractive index pictures also show an additional boundary which is not represented in the absorption pictures; this boundary possesses an average sedimentation constant of 13.5×10^{-13} (Table I).

These experiments show that the pigments are detached from the protein, and are held in solution by the hydrophobic portion of the digitonin micelle. It does not seem likely that the chlorophyll remains attached to any very high molecular weight fragments since the sedimentation constant of the digitonin micelle is unaffected by the presence of the pigment. Yet the fact that no loss of pigment occurs on prolonged dialysis of a digitonin-treated solution (Smith, 1938) suggests that the pigment is still combined with particles too large to pass through a cellophane membrane.

Sedimentation of a digitonin-treated solution after prolonged dialysis indicates that some recombination of pigment and protein occurred. Comparison of absorption and refractive index diagrams shows that the pigment and protein sedimented together, but the material was quite inhomogeneous. Nevertheless, the bulk of this material did sediment at somewhat higher rates than that found for the dissociated protein particles. The refractive index diagram gave no trace of the digitonin boundary.

[1] A few preliminary runs using the light absorption method alone erroneously led us to attribute this boundary to the protein (Smith, 1938). These runs show the sedimentation constant characteristic of the digitonin micelle. One experiment which showed a double boundary was apparently due to an artefact.

When digitonin solution was added to the dialyzed preparation, the pigment was again dissociated from the protein and gave a sharply sedimenting boundary together with the digitonin micelle. The protein boundary observed on the refractive index diagram gave a sedimentation constant in line with values previously obtained. Fig. 1 shows for comparison the

TABLE I

Sedimentation Experiments with the Chlorophyll-Protein Compound in Different Detergents

Sedimentation constants were obtained from refractive-index boundaries. All runs were made at 46,800 R.P.M., equivalent to an average centrifugal force of 160,000 times gravity, at temperatures near 25°C. The optical thickness of the cell was 3 mm. Most of the solutions were buffered with 0.1 M sodium phosphate; the two alkaline ones, 8.8–8.9, contained 0.1 M borate, and the one at pH 4.85, 0.1 M acetate. The three runs made with an extract concentrated in the air-turbine concentration centrifuge are marked "concentrated preparation."

Detergent	Concentration	pH	$S_{20} \times 10^{-13}$	Remarks
	per cent			
Digitonin	2.5	7.61	12.8	
	2.5	8.80	13.3	
	2.5	7.80	13.4	Concentrated preparation
	0.63	7.87	14.5	
	2.5	7.83	13.7	Digitonin added to solution after initial removal of digitonin by dialysis
			Average 13.5	
Sodium desoxycholate	0.5	7.59	13.8	
	0.25	7.60	13.8	
Bile salts	3.0	7.50	13.5	Concentrated preparation
Sodium dodecyl sulfate	0.25	8.89	2.53	Prosthetic group is chlorophyll, solution is green
	0.25	4.85	2.84	Prosthetic group is phaeophytin, solution is yellow
	0.25	7.52	2.32	Solution initially green, at end of run some phaeophytin formation
			Average 2.56	
	2.5	7.76	1.69	Concentrated preparation

absorption photographs obtained with *a*, the dialyzed preparation, and *b*, the same solution to which digitonin has been added. The data for these experiments are included in Table I.

3. Effect of Sodium Desoxycholate and Bile Salts.—With these solutions the absorption photographs show no sedimenting boundaries whatsoever, indicating that the chlorophyll is dissociated from the protein. The refractive index photographs clearly show only one sedimenting boundary

similar to that found with digitonin and possessing the same sedimentation constant (values in Table I). Digitonin and these two detergents therefore act in the same way on the chlorophyll-protein compound, dissociating the pigments from the protein and breaking the material into particles of the same size. In the experiment with bile salts using a concentrated

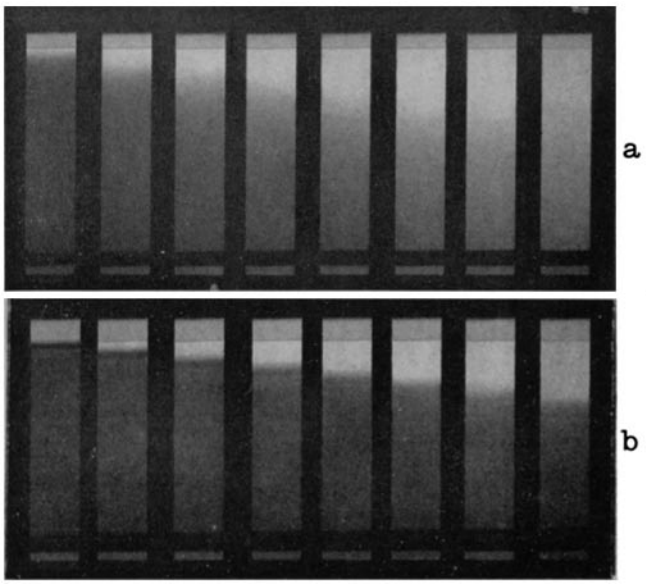

FIG. 1. Sedimentation photographs of *a*, a dialyzed digitonin-treated extract, and *b*, the same solution with digitonin again added. The dialyzed extract shows the inhomogeneous and comparatively rapid sedimentation of the pigment after removal of the digitonin. When digitonin was again added equivalent to the initial concentration (2.5 per cent), a sharply defined boundary (*b*) was obtained which was coincident with that of the colorless digitonin micelle ($S_{20} = 5.9 \times 10^{-13}$) observed refractometrically. This coincidence is also shown in the first absorption pictures by the presence of a dark band; this band corresponds to the micelle boundary which has caused a deviation of light beyond the aperture of the camera lens. Both sets of photographs were taken with red light at 10 minute intervals. The solutions contained 0.1 M phosphate at pH 7.8.

leaf extract, a large part of the protein sedimented irregularly and inhomogeneously. These particles had a wide variation in size; some were smaller and some larger than the principal component.

A single experiment with a concentrated urea solution as the solvent was performed using only the light absorption method. No sedimentation of the pigment occurred showing that in this solvent also, the chlorophyll was dissociated from the protein.

4. Effect of Sodium Dodecyl Sulfate.—It has already been shown (Smith, 1941 *b*) that sodium dodecyl sulfate (SDS) alters the nature of the prosthetic group of the chlorophyll-protein compound. In weakly acid solutions, magnesium is rapidly eliminated from the chlorophyll, converting it to phaeophytin; in alkaline solutions this reaction takes place very slowly. It was therefore of considerable interest to study the effect of pH on the sedimentation constant. Three runs were carried out in 0.25 per cent SDS,

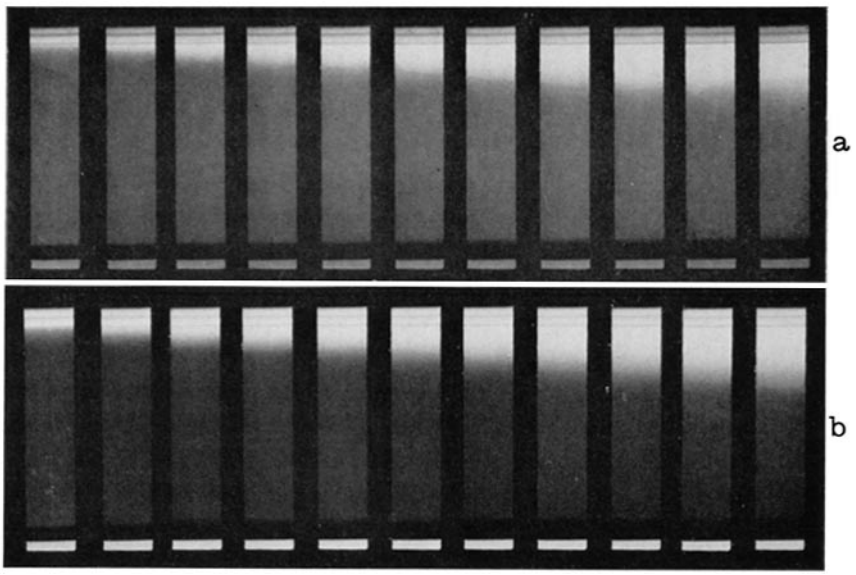

FIG. 2. Absorption photographs taken alternately at 5 minute intervals with *a*, red, and *b*, violet, light, showing sedimentation of the chlorophyll-protein in 0.25 per cent sodium dodecyl sulfate at pH 8.9. No differences are detectable in the two regions of the spectrum. The common sedimenting boundary was coincident with the protein boundary ($S_{20} = 2.6 \times 10^{-13}$) detected by the refractive index method, showing attachment of the pigment to the protein.

at pH 4.85 where phaeophytin formation is complete in a few minutes, at pH 7.52 where the reaction requires many hours for half completion, and at pH 8.89 where only barely detectable changes take place in 24 hours.

The results demonstrate that at all three pH values, the prosthetic group remains attached to the protein component. Fig. 2 shows absorption photographs taken alternately in red and in violet light at pH 8.89. The refractive index curves show only a single sedimenting boundary corresponding to the sedimentation of the pigment shown in the absorption pictures. The absorption photographs show that the single boundary is identical in

the two regions of the spectrum, and demonstrate that the carotenoid and chlorophyll pigments remain attached either to identical particles or to particles of the same size. The same sedimentation constant (Table I) was found at all three pH values; the average is 2.56×10^{-13}. It is apparent that the average size of the particles is not affected by the presence or absence of magnesium in the chlorophyll. The effect of SDS differs strikingly from the other detergents; not only does the prosthetic group remain attached to the protein, but the particles are split into apparently homogeneous fragments of lower sedimentation constant and are therefore of smaller size.

A single run was carried out in the presence of 2.5 per cent SDS using a more concentrated preparation of the chloroplast material. In the presence of the ten times higher concentration of SDS, the prosthetic group still remained attached to the protein, but the protein was further reduced in size yielding an S_{20} value of 1.69×10^{-13}. A photograph of one of the refractive index curves is shown in Fig. 3. Since the photographs were taken with visible light (mercury line of 546 mμ) the boundary on the refractive index curve shows the absorption of the pigments to correspond exactly with the schlieren pattern.

If it is assumed that the other physical characteristics of the particles and solvents are roughly the same for the two different concentrations of SDS, the approximate relationship between the size of the particles is as the ratio of the three-half powers of the sedimentation constants, or $S_1^{3/2}/S_2^{3/2} = M_1/M_2$. The ratio found was 0.54 using the average value of 2.56 for S_2 and 1.69 for S_1. The ratio suggests that at the higher SDS concentration, the particles are split into approximately half the size. A similar comparison between the sedimentation constant in digitonin ($S=$ 13.5) and in 0.25 per cent SDS, gives a size ratio of 12 to 1.

When the areas under the boundaries of the refractive index curves were measured, it was found that the apparent concentration of the sedimenting material in SDS was higher than that found by spectrophotometric measurement. The concentration of the chlorophyll-protein compound was estimated spectrophotometrically by measuring the height of the main absorption band in the red end of the spectrum. In the experiment with 2.5 per cent SDS using the values previously determined (Smith, 1941 *a*) from the average chlorophyll content of 7.9 per cent, the concentration of the chloroplast material was 1.3 per cent; using the chlorophyll content of 16 per cent in relation to the protein alone, the concentration was 0.63 per cent. Assuming the usual refractive index value for proteins, the concentration of sedimenting material was 2.4 per cent. The appreciably

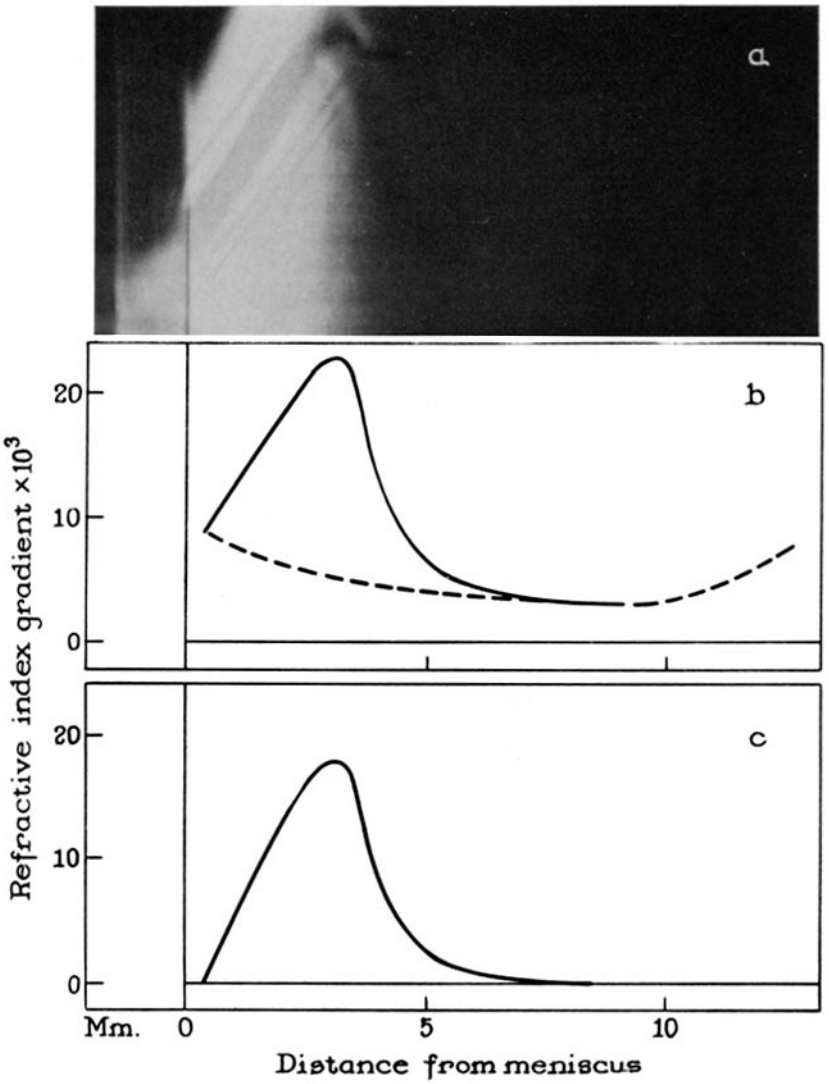

FIG. 3. Refractive index gradient curve, obtained after 3 hours, of a chlorophyll-protein solution in 2.5 per cent sodium dodecyl sulfate at pH 7.8. The sedimentation constant was approximately 1.7×10^{-13} cm./sec./dyne. A very high concentration of chlorophyll-protein was used to increase the size of the index curve. Although green light was used, a 15 minute exposure was necessary; this caused a reduction in the photographic definition of the curve *a*. That protein and pigment sedimented together is evident from the coincidence of the absorption and index boundaries *a*. The dotted baseline in *b* is the index curve for 2.5 per cent SDS alone. Curve *c* represents the difference between the dotted and smooth curves of *b*. Measurement of its area shows too high a concentration to be explained by the protein alone, indicating possible association with SDS. The unsymmetrical shape of the difference curve *c* is to be expected for very low sedimentation rates.

higher concentration of sedimenting material suggests that a considerable amount of SDS was sedimenting together with the protein, giving the effect of an increase in concentration of protein. Yet it is difficult to assess what effect this had on the apparent sedimentation constant. If it tends to increase the size of the sedimenting particles, the true size of the particles in 2.5 per cent SDS would be even smaller than half that in 0.25 per cent solution.

In one experiment with digitonin as the detergent and the same protein solution, the concentration of protein from the area of the refractive index curve was 0.7 per cent as compared with 1.3 and 0.63 per cent as estimated spectrophotometrically. Here there does not seem to be any appreciable quantity of detergent influencing the concentration of sedimenting material. This is to be expected since the total digitonin sediments as apparently homogeneous micelles.

IV

DISCUSSION

It has been known for some time that proteins can be split by various substances into fragments of lower molecular weight. The studies of Svedberg and his collaborators (Svedberg, 1937) have shown that acid or alkaline solutions cause the splitting of many proteins into particles smaller than those found over the range of pH near neutrality. Various proteins have been shown to be split by urea (Burk and Greenberg, 1930; Burk, 1937). Steinhardt (1938) has observed that while horse hemoglobin is split by concentrated urea solutions into halves, the absorption spectrum and oxygen binding capacity are not changed. The action of urea in splitting proteins is not unique and there are undoubtedly numerous reagents which will split proteins but these have been studied little as yet. Sreenivasaya and Pirie (1938) found that the tobacco mosaic virus is inactivated by SDS with the separation of nucleic acid and splitting of the protein into fragments. However, these investigators did not study the size of the split particles.

The action of the detergents studied in the present investigation shows that different reagents produce quite different effects on the same protein. SDS is unique among those studied since it is capable of converting chlorophyll into phaeophytin in neutral solutions, and it does not detach the prosthetic group from the protein. The other detergents attack different linkages since the chlorophyll is liberated or remains combined with only small fragments of the protein which are not sedimentable at the gravitational forces used. The latter possibility seems the more probable since pigment

is not lost on dialysis showing that the particles are still too large to pass through an ordinary cellophane membrane.

Not only do the various splitting agents produce different effects on the same protein but their action is likely to be different on other proteins. Urea, for example, does not affect the molecular weight or catalytic activity of pepsin (Steinhardt). With visual purple, a conjugated carotenoid-protein (Wald, 1935), the bleaching properties of the pigment are not affected by digitonin, bile salts, or sodium desoxycholate. However, after bleaching by light, regeneration *in vitro* takes place in the presence of the first two detergents but not in solutions of sodium desoxycholate (Chase and Smith, 1939). Moreover, dilute solutions of SDS instantaneously bleach visual purple in the dark.[2] It is clear that no rule can be laid down for the probable action of these detergents.

True molecular weight values for the chlorophyll-protein compound of spinach cannot be assessed as yet. The observations of Price and Wyckoff, and Loring, Osborn, and Wyckoff on the leaves of other plants suggest very high molecular weights of the same magnitude as those of the various hemocyanins. Since the protein of the spinach leaf is insoluble, no comparison is possible. The particle sizes found in the various detergents do suggest certain units which may be of importance. The sedimentation constant of 13.5×10^{-13} found in three detergents is equivalent to a molecular weight of at least 265,000. This molecular weight value is of the approximate order of magnitude previously found for many plant proteins such as phycocyanin, phycoerythrin, and many seed globulins (Svedberg, 1937). For the spinach leaf, 265,000 represents the minimum size of the protein in native form.

In all of the experiments, a careful examination was made for evidence of differential behavior of the various chloroplast pigments. No differences were found for chlorophylls *a* and *b*, and the carotenoids always followed the chlorophylls. This shows a strong association of all these pigments, and suggests that the carotenoids as well as the chlorophylls are chemically bound to the chloroplast protein.

SUMMARY

1. The chlorophyll-protein compound of the spinach leaf has been studied in the air-driven ultracentrifuge using the Svedberg light-absorption method, and a direct-reading refractive index method.

2. When the untreated extracts are centrifuged at low speeds, the green

[2] Unpublished observations by one of us (Smith, 1939).

protein sediments with a purely random spread of particle sizes confirming the fact that the protein is not in true solution.

3. In the presence of digitonin, bile salts, and sodium desoxycholate, the extracts are clarified. These detergents split the chlorophyll from the protein and the protein itself shows a sedimentation constant of 13.5×10^{-13} equivalent to a molecular weight of at least 265,000 as calculated from Stokes' law. This probably represents the minimum size of the protein in native form.

4. Sodium dodecyl sulfate, a detergent which also clarifies the leaf extracts, shows a different behavior. The prosthetic group remains attached to the protein but the protein is split into smaller units. In 0.25 per cent SDS, S_{20} is 2.6×10^{-13} over a pH range of 5 to 9, although at the acid pH chlorophyll is converted to phaeophytin. In 2.5 per cent SDS, S_{20} is 1.7×10^{-13} suggesting a further splitting of the protein.

5. No differences in behavior were found for the various chloroplast pigments.

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