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DISINTEGRATION OF CHLOROPLASTS WITH DODECYLBENZENE SULFONATE AS MEASURED BY FLATTENING EFFECT AND SIZE DISTRIBUTION

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SUMMARY

New techniques for the optical measurement of the mean radius of colored particles by the flattening effect and the electric measurement of volume distribution with the COULTER counter were applied to the determination of the size of spinach chloroplasts and grana and the particles derived from them by treatment with dodecylbenzene sulfonate. The whole process of disintegration and solubilization of chloroplasts to chlorophyll holochrome was followed by these techniques and by ultracentrifugal analysis. By treatment with dodecylbenzene sulfonate, the native chloroplasts were swollen and then disintegrated into swollen grana. The electron micrograph of the swollen chloroplasts indicated that each lamella is swollen into a globular particle inside the chloroplasts. The swollen grana were disintegrated with dodecylbenzene sulfonate into smaller particles which were then transformed into a component of 2.9–3.5 S. This component by treatment with a more concentrated dodecylbenzene sulfonate dissociated into components of 1.2 S which are the smallest units obtainable with the detergent. The size of native and swollen chloroplasts and grana, determined by the new techniques, were discussed.

INTRODUCTION

Chloroplasts have a compound structure with different sizes of units; grana and stroma, lamellae and various particulates or subunits including chlorophyll holochrome or chloroplastin. The determination of the structure and the characterization of these units are essential for the correlation of these units with various reactions in photosynthesis. Such a correlation study requires a wide size spectrum of these units from the order of microns to a few Svedberg units. Extensive studies have been made on the larger units mostly by microscopy and on the smaller units by ultracentrifugal analysis as reviewed comprehensively by KUPKE AND FRENCH[1] and RABINOWITCH[2,3]. Light and electron microscopy, despite their great usefulness for the observation of structures, have limitations in the determination of particle size for the following reasons. Light microscopy entails considerable errors in the measurement of small

Abbreviation: DBS, dodecylbenzene sulfonate.

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particles such as chloroplasts and can not be applied to smaller particles. Electron microscopy requires a tedious process of fixing samples in which shrinkage or expansion of particles may occur and, in addition, the definition of particle geometry affects the result considerably.

In the present study, the process of disintegration of spinach chloroplasts by DBS was investigated by two different and new techniques: the optical measurement of the sizes of colored particles by the "flattening effect" of DUYSENS[4,5], and the electric measurement of size-distribution curves by the COULTER counter[6] which are illustrated below. The results obtained on the disintegration of chloroplasts to grana by these techniques were supplemented by electron-microscopic observations, and the successive process of solubilization of grana to chlorophyll holochrome was observed by ultracentrifugal analysis. Thus, the whole process of disintegration and solubilization was followed with special attention to the sizes of various units.

The instrument, the COULTER counter[6], is based upon the principle that each cell or particle in a suspension, when passing through a minute orifice, causes a momentary increased impedance (or pulse) to the steady flow of electric current through the orifice. By use of this instrument, the errors in counting the number of particles can be greatly reduced and, furthermore, the volume-distribution curves can be measured much more accurately and quickly than by light or electron microscopy. The instrument has been applied mostly to blood cells[6-9]. The applicability to chloroplasts was recently examined by ORTH AND CORNWELL[10], who determined the volume-distribution curve of *Zea mays* chloroplasts and found a mean volume of 22.5 μ^3.

As pointed out by DUYSENS[4,5] and RABINOWITCH[11], an absorption band of a suspension of pigment particles or light-absorbing cells is flattened as compared with the band of the same concentration of the pigment molecularly dispersed in solution, so that extraction of the pigment from the suspension with the same volume of a solvent raises the band height. This phenomenon was named the "flattening effect" by DUYSENS, who analysed the data obtained by EMERSON AND LEWIS[12] for a suspension of *Chlorella* cells. The effect arises from the heterogeneous distribution or localization of pigment in particles. Qualitatively speaking, the relative flattening is greater (a) at the wavelength where the pigment absorbs light more strongly, (b) when the pigment is localized in smaller numbers of particles, and (c) when the pigment is more concentrated in smaller particles, provided that the number of particles in the suspension is kept identical. To estimate the effect, the absorption spectra of suspensions which are generally translucent have to be measured correctly. The "opal-glass transmission method"[13-15] developed for the spectrophotometry of translucent materials was employed in the present study, and enabled us to obtain the spectra free from the errors which techniques commonly used for transparent materials entail. The flattening data thus obtained were analysed by the equations derived below.

ANALYSIS OF FLATTENING EFFECT

Suspension of chloroplasts

DUYSENS[4,5] assumed a suspension of homogeneously dispersed particles, and derived a general equation including the effect of particle geometry. Approximations and modifications were made in the present study of the equation of DUYSENS in order

to make it applicable to chloroplasts and grana. Let us first consider a simple model system: a suspension of spherical particles, idealized chloroplasts, in which a pigment(s) is dissolved uniformly. The absorbance value of this system at a single wavelength is expressed approximately as follows:

$$E_c^* = 0.434\, dN_c\pi r_c^2(1 - e^{-x}) \quad (1)$$

where

$$x = (4/3)r_c C_c \varepsilon^* \quad (2)$$

d, the thickness of the container of the suspension; N_c, the number of chloroplasts in 1 ml of suspension; r_c, the radius of the chloroplast in cm; C_c, the molar concentration of the pigment in the chloroplasts; ε^*, the molar extinction coefficient of the pigment on the basis of natural logarithm, so that ε^* is equal to 2.303 ε.

It is assumed in the derivation that light passes through a particle without being refracted or scattered, and that the effective light-path length through the particle is equal to the average of the different path lengths which is $(4/3)r_c$. More exactly, the intensities of transmitted light beams through the particle have to be averaged. The absorbance derived by this more exact treatment is

$$E_c^{**} = 0.434\, dN_c\pi r_c^2\{x - (9/16)x^2 + (9/40)x^3 - - - - - -\} \quad (3)$$

which gives us a value close to that given by Eqn. 1. The further derivation of the equation for a more complex case is, therefore, made to the same degree of approximation as in Eqn. 1.

In the actual system of chloroplasts, pigments are concentrated mostly into grana. One may, therefore, assume that pigments are heterogeneously distributed into spherical grana, and stroma are colorless. The flattening in this case occurs owing to two different superposed localizations; the localization of pigments into grana, and the localization of grana into chloroplasts. The absorbance value of this system is

$$E_c = 0.434\, dN_c\pi r_c^2(1 - e^{-p}) \quad (4)$$

in which

$$p = (4/3)r_c N_g \pi r_g^2(1 - e^{-\beta}) \quad (5)$$

$$\beta = (4/3)r_g C_g \varepsilon^* \quad (6)$$

$$C_c = (4/3)\pi r_g^3 N_g C_g \quad (7)$$

N_g, the number of grana per ml of chloroplasts; r_g, the radius of the granum in cm; C_g, the concentration of the pigment in the grana.

Suspension of grana

If the chloroplasts in the suspension are broken and the grana are suspended in the same volume of medium as that of the suspension, the number of grana per ml of the suspension is $(4/3)\pi r_c^3 N_g N_c$. Therefore, the absorbance, E_g, of this suspension of grana is

$$E_g = 0.434\, d\pi r_c^2 N_c p \quad (8)$$

Extract

If the pigment in the suspension of chloroplasts or grana is extracted with the same volume of a solvent, one may obtain a solution of pigment concentration of $(4/3)\pi r_c^3 N_c C_c$. Therefore, the absorbance, E_e, of the extract is

$$E_e = 0.434\, d(4/3)\pi r_c^3 N_c C_c \varepsilon^* \quad (9)$$

The flattening effect

The flattening effects due to the localization of the pigment into grana and that due to the localization of grana into chloroplasts are expressed by the ratios between the absorbance values of Eqns. 4, 8 and 9;

$$E_g/E_e = (1 - e^{-\beta})/\beta = p/\alpha \tag{10}$$

$$E_c/E_g = (1 - e^{-p})/p \tag{11}$$

Various constants for chloroplasts and grana were estimated in the following manner. (a) The values of E_g/E_e and E_c/E_e were determined by the experiments of extraction, and the value of E_c/E_g was calculated from these values. The values of α, β and p were calculated from these ratios by Eqns. 10 and 11. (b) The radius, r_c, of chloroplasts was calculated from the values of p and E_g/N_c by Eqn. 8. E_g/N_c was calculated from observed values of E_c/E_g and E_c/N_c. (c) The radius, r_g, of grana was calculated with aid of the following relationship:

$$(r_g/r_c)^2 = \alpha/\beta m \tag{12}$$

in which m is the number of grana in one chloroplast; that is $(4/3)\pi r_c^3 N_g$. The value of m was determined from the change of the distribution curve from chloroplasts to grana measured by the COULTER counter, and was found to be approx. 140 for the sample chloroplasts as shown later.

EXPERIMENTAL

Preparation of samples

Whole chloroplasts were isolated from leaves of *Spinacia oleracea* (spinach) by the method of ARNON, ALLEN AND WHATLEY[16], purified by repeated centrifugations, and suspended in 0.35 M NaCl solution. The molar ratio of chlorophyll *a* to *b* in this preparation was 2.8 as determined after extraction by the method of MACKINNEY[17]. Grana were prepared from the chloroplasts in the following manner. Chloroplasts were disrupted with a blendor in 0.35 M NaCl solution at 0° for 5 min. The suspension of broken chloroplasts thus obtained was centrifuged at 2800 × *g* for 10 min and the sediment was discarded. The supernatant containing grana was centrifuged at 15000 × *g* for 15 min, and the sediment was resuspended in the same saline solution.

Chloroplasts were pre-incubated with DBS in 0.04 M phosphate buffer (pH 7.0) for 5 min at 20 ± 2°, and then subjected to the measurements of the flattening effect, the Hill activity and the volume-distribution curve. The sedimentation analyses of chlorophyll holochrome were made for similar mixtures but with no buffer, because large micelles of DBS are formed by use of buffer and interfere with the observation of the sedimentation peak of chlorophyll holochrome. Samples for electron-microscopic observations were prepared by a common procedure. Chloroplasts pre-treated with DBS for 5 min were washed with phosphate buffer (pH 7.0), fixed with 1% osmium tetroxide, dehydrated with ethanol, and embedded in methacrylate resin for the preparation of thin sections.

Spectroscopic observations

Absorption spectra were observed with a Cary recording spectrophotometer model 14M, using 1.0-cm cells. For the observations of translucent suspensions of

chloroplasts or grana, the "opal-glass transmission method" was employed[13–15]. Thus, the correct absorption spectra in terms of the semi-integral attenuance could be directly recorded on the spectrophotometer. Corrections were made for the effect of reflection from the sample. As discussed in previous papers[14,15], the reflection-corrected attenuance describes the transmitting property of a translucent sample, being independent of the reflecting property of the sample as a whole, so that it is the measurement to be compared with the absorbance of the extract to estimate the flattening effect. The reflection-corrected attenuance for translucent samples will be simply called the absorbance in this paper.

Volume distribution

The volume-distribution curves of chloroplasts and grana were measured by the COULTER counter model A. A sample mixture of chloroplasts with DBS was diluted 200 times with 0.04 M phosphate buffer (pH 7.0) containing 0.01 M NaCl, and was subjected to countings. Corrections were made for the coincidence count loss and for the non-linear response of the pulse height. The volume-distribution curves are expressed in the number, n of particles *versus* log. V, in which V is the volume in μ^3. An orifice of 100 μ in diameter was used for the particles of log. $V = 1.0$–2.5, and that of 30 μ for log. $V = -1.0$–0. In the intermediate volume range observable with a 50-μ orifice, no essential change of distribution occurred in the disintegration of chloroplasts.

The Hill activity

A solution (1 ml) of 2,6-dichlorophenolindophenol was added to a sample mixture (10 ml) of chloroplasts pre-treated with DBS in the dark for 5 min. The initial rate of reduction of the dye by illumination from an incandescent lamp was estimated from the absorbance change at 610 mμ which was measured by the opal-glass transmission method.

Ultracentrifugal analysis

Sedimentation coefficients were observed with a Spinco ultracentrifuge model E at 59780 rev./min. Corrections were made for the differences in temperature and the solvent medium used in order to estimate sedimentation coefficients, $s_{20,w}$ which are expressed in Svedberg units in this report. The partial specific volume of chlorophyll holochrome was assumed to be 0.96 according to CHIBA[18].

Electron microscopy

Electron micrographs of chloroplasts were taken with an electron microscope of Japan Electron Optics Laboratory, model JEM-5G.

RESULTS

Disintegration of chloroplasts to grana

Volume distribution: Curve A in Fig. 1 shows the volume distribution of chloroplasts with 0.35 M NaCl, measured 8 min after the preparation. The curve indicates a maximum distribution at log. $V = 1.36 \pm 0.02$. In a separate experiment, intact leaves were squeezed through cheese cloth, and the distribution curve of the chloro-

plasts in the juice obtained without blending and centrifugation was observed immediately after squeezing. The curve thus obtained was quite similar in shape and position to curve A. This fact bears evidence that the chloroplasts obtained by normal procedure of preparation are in their native state in the short period of 8 min after the preparation. The chloroplasts in the saline solution, however, undergo a change in distribution when kept at room temperature. An example is shown by curve B in Fig. 1 which was observed 80 min after the preparation of the sample. The curve is shifted toward a larger volume during the incubation. The shift occurs between 20 and 60 min after the preparation. In this process, the total number of chloroplasts remains constant, so that the shift is due to swelling and not to clumping. The peak of curve B is located at log. $V = 1.64 \pm 0.03$, so that the volume of native chloroplasts is roughly doubled by swelling. It is worth noting that the saline solution commonly used for the preparation of chloroplasts causes such a marked change of volume. In subsequent experiments to study the effect of DBS, the chloroplasts kept for 80 min in the saline solution were used as a relative standard sample to obtain reproducible data free from the error due to swelling; they will be referred to as "aged chloroplasts".

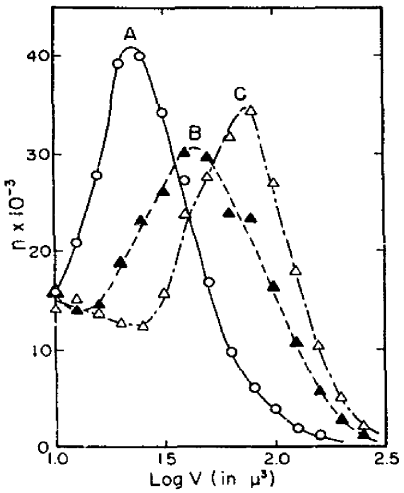

Fig. 1. Volume-distribution curves of native, aged and DBS-treated chloroplasts. Curve A, chloroplasts in 0.35 M NaCl solution, 8 min after the preparation; curve B, the same sample but 80 min after the preparation; curve C, treated with $2.29 \cdot 10^{-4}$ M DBS for 5 min.

When DBS is added to a suspension of aged chloroplasts, they swell further and the distribution is sharpened. Maximal swelling occurs with $1\text{–}2 \cdot 10^{-4}$ M DBS, and the peak of distribution shifts to log. $V = 1.88 \pm 0.02$ as shown by curve C obtained with $2.29 \cdot 10^{-4}$ M DBS. This interpretation of swelling is supported by electron-microscopic observations. The micrograph of an aged chloroplast shown in Fig. 2A is normal and shows grana with lamellar structures. By treatment with $1.14 \cdot 10^{-4}$ M DBS (Fig. 2B), the outer surface of the chloroplast is expanded markedly, and each lamella swells into a globular particle, which looks like an air mattress being inflated. This phenomenon confirms the view[19–23] that each lamella is made up of two sheets of material superposed and connected together at the ends.

With DBS concentration increasing above $2 \cdot 10^{-4}$ M, a different change occurs in

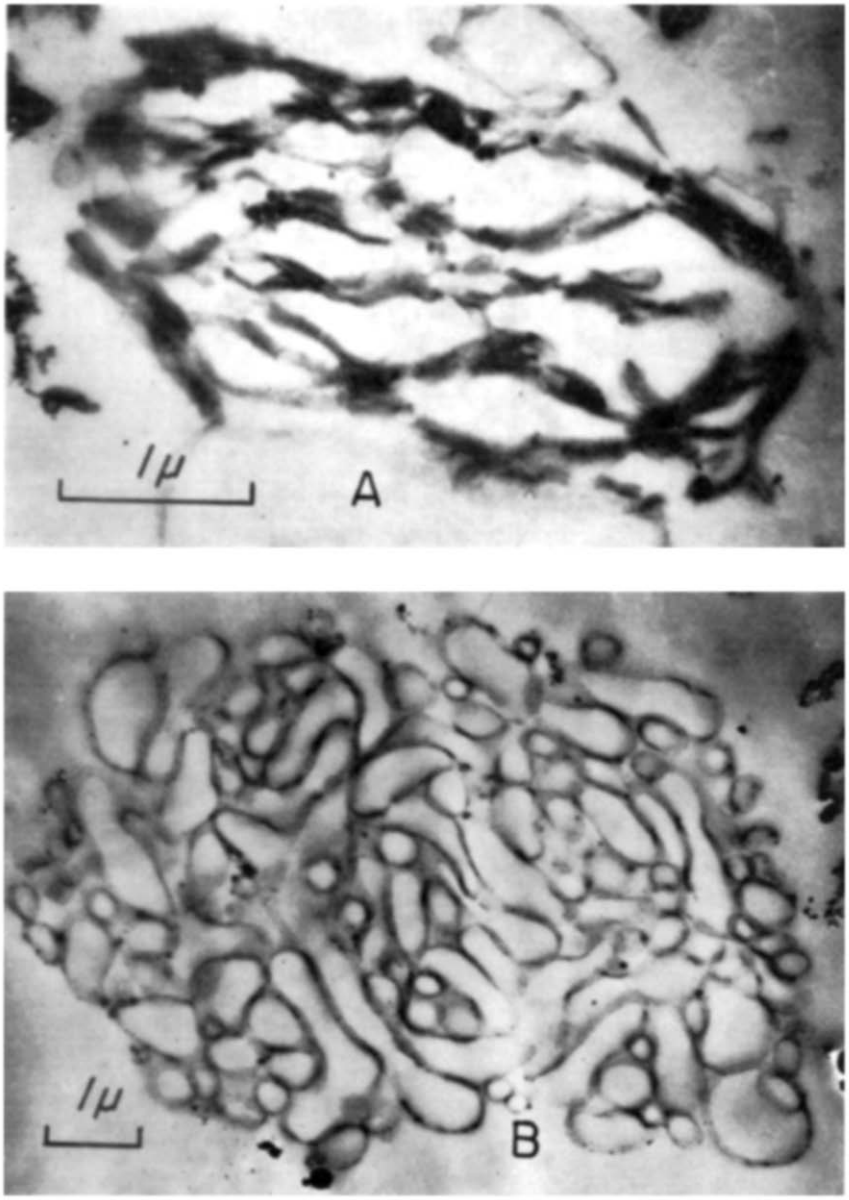

Fig. 2. Electron micrographs of thin sections of an aged (A) and DBS ($1.14 \cdot 10^{-4}$ M)-treated (B) chloroplast, each fixed with 1% OsO_4 in 0.07 M veronal–acetate buffer (pH 7.4) and embedded in methacrylate.

the distribution. The peak for the swollen chloroplasts is lowered and a new peak appears in much smaller volume range as seen from curves D, F and G in Fig. 3. From the change in relative height of the two peaks in the transformation, the number of small particles formed from one chloroplast was estimated to be 140 ± 30. This number is in approximate agreement with 50–200, the number of grana in one chloro-

plast estimated by RABINOWITCH[3] from the electron micrographs of the chloroplasts of various higher plants.

The distribution curve of native grana prepared by mechanical disruption of chloroplasts was observed similarly, but the size was found to be below the observation limit of the instrument. However, the grana when treated with $1.14 \cdot 10^{-4}$ M DBS show a peak at log. $V = -0.76 \pm 0.03$ (curve H in Fig. 3), which agrees roughly with the peak positions of curves D, F and G. This implies that the small particles formed by treatment of chloroplasts with DBS are the grana swollen with DBS.

The volumes of various states of chloroplasts and grana at the distribution maxima are listed in Table I together with the radii calculated from the volumes on the assumption that they are spherical. Comparison will be made later between these data and those measured by the flattening effect.

TABLE I

VOLUMES AND RADII OF VARIOUS STATES OF CHLOROPLASTS AND GRANA AS MEASURED BY THE COULTER COUNTER

Sample	*State*	*Log. V*	*V* (μ^3)	*r* (μ)
Chloroplasts	native	1.36 ± 0.02	23	1.8
	aged	1.64 ± 0.03	44	2.2
	swollen	1.88 ± 0.02	76	2.6
Grana	swollen	-0.76 ± 0.03	0.17	0.34

The flattening effect: The absorption spectrum of a chloroplast suspension is greatly changed by treatment with DBS. By the action of DBS of less than $6 \cdot 10^{-4}$ M, the absorption is intensified over the entire visible region (curves A and B in Fig. 4). With this intensification, the red band shifts only slightly from 678 to 675 mμ and the position, 437 mμ, of the Soret band is unaltered, so that the spectral change may be regarded as the reverse process of the flattening of DUYSENS. Above $6 \cdot 10^{-4}$ M DBS, a successive and different change occurs; the Soret band is transformed into a band

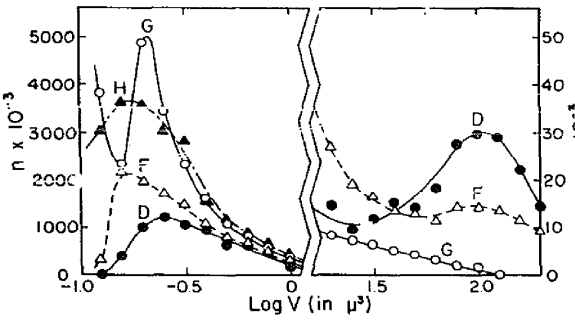

Fig. 3. Volume distribution curves of DBS-treated chloroplasts. Curves D and F, treated with $3.43 \cdot 10^{-4}$ M DBS for 5 and 30 min, respectively; curve G, treated with $5.72 \cdot 10^{-4}$ M DBS for 5 min; curve H, grana treated with $1.14 \cdot 10^{-4}$ M DBS for 5 min.

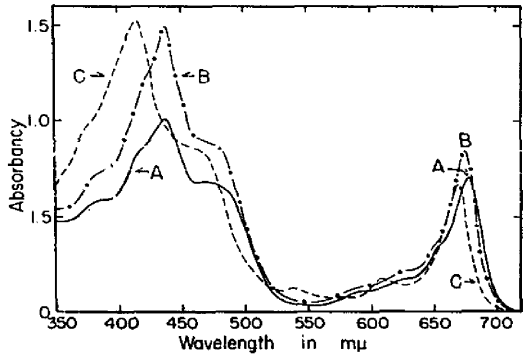

Fig. 4. Absorption spectra of suspensions of chloroplasts with DBS. Curve A, aged chloroplasts; curve B, treated with $5.72 \cdot 10^{-4}$ M DBS for 5 min; curve C, treated with $2.86 \cdot 10^{-3}$ M DBS for 30 min. Each suspension contains an equal amount of chlorophyll.

at 415 mμ and the red band is shifted to 670 mμ. The resultant spectrum is similar to that of chloroplasts treated with sodium dodecyl sulfate observed by SMITH[24] who interpreted it to be the spectrum of pheophytin.

The intensification of absorption was observed more precisely as a function of DBS concentration at 437 mμ and at 675–678 mμ, the absorption maximum of the red band, and the results are shown by curves A and B (Fig. 5), respectively. The relative absorbance in the figure stands for the ratio of the absorbance value of a suspension of DBS-treated chloroplasts to the value observed for the control, a suspension of the same concentration of aged chloroplasts without DBS. The figure indicates that the intensification proceeds in two steps. In the first step, which is observed between 0 and $1.5–2.0 \cdot 10^{-4}$ M DBS, the relative absorbances at 437 mμ and at 677–678 mμ increase to intermediate levels of 1.21 and 1.11, respectively. The DBS concentration for completion of the first step agrees nicely with the concentration for the maximal swelling estimated from the distribution curves, so that the above values of relative absorbance are those of the suspension in which the chloroplasts are swollen to the maximal extent.

In a separate experiment, the Hill activity was observed as a function of DBS concentration together with the absorbance change at 437 mμ (Fig. 6). The activity drops markedly below $5 \cdot 10^{-5}$ M DBS, and is practically zero at $8.5 \cdot 10^{-5}$ M. The activity is inhibited completely before the first step of absorbance change proceeds to completion or below the concentration for the maximal swelling. The concentration for complete inhibition is only 6–7 times the chlorophyll concentration, $1.3 \cdot 10^{-5}$ M, of the chloroplast suspension measured. The significance of the smallness of the concentration was discussed by KE AND CLENDENNING[25], who observed the effects of various detergents on the Hill reaction and found the concentrations for the inhibition to be much lower than those required for chloroplast solubilization as assessed by ultrafiltration.

Upon increasing DBS concentration above $2 \cdot 10^{-4}$ M, the relative absorbance increases again and attains a maximum value at $6 \cdot 10^{-4}$ M DBS. This concentration agrees with the concentration, at which the distribution peak for swollen chloroplasts

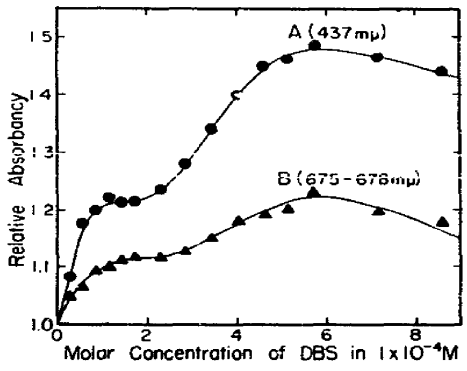

Fig. 5. Relative change of absorbancy at the Soret (curve A) and red (curve B) bands of the suspension of chloroplasts as a function of DBS concentration.

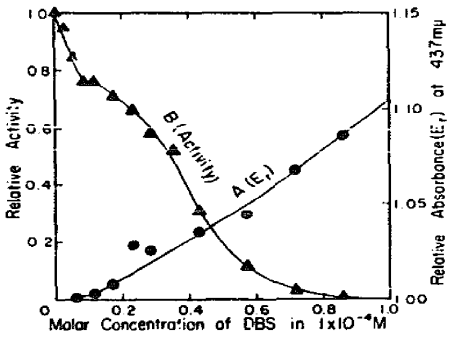

Fig. 6. Relative change of absorbancy at he Soret band (curve A) and the Hill activity (curve B) of chloroplasts as a function of DBS concentration. The relative activity of chloroplasts without DBS is taken as unity.

is completely transformed into the peak of swollen grana. The second step of intensification may, therefore, be interpreted as due to the disintegration, so that the values of relative absorbance, 1.48 at 437 mμ and 1.23 at 675–677 mμ, on the maximal levels are those of the suspension of swollen grana. The absorbance drops from these levels above $6 \cdot 10^{-4}$ M DBS, and the phenomenon is due to the transformation of bands (curve C in Fig. 4).

The relative absorbance of a suspension of native grana prepared by mechanical disruption of chloroplasts and that of the extract with 80 % acetone from a suspension of aged chloroplasts were measured at the two wavelengths. The results are listed in Table II together with the values estimated above for the swollen chloroplasts and grana. The radii, r_c and r_g, of the aged and swollen chloroplasts and grana were calculated by the procedure illustrated above from these absorbance values and N_c/E_c and m. The radii thus calculated are listed in the same table with the values of E_g/E_e, E_c/E_g, α, β and p.

TABLE II

RADII OF AGED AND SWOLLEN CHLOROPLASTS AND GRANA AS ESTIMATED FROM THE FLATTENING EFFECT

Wavelength	*437 mμ*		*675–678 mμ*	
State	*aged*	*swollen*	*aged*	*swollen*
E_c	1.00	1.21	1.00	1.11
E_g	1.33	1.48	1.25	1.23
E_e	1.93	1.93	1.55	1.55
$(N_c/E_c) \times 10^{-7}$	2.43	2.43	3.55	3.55
m	140	140	140	140
E_g/E_e	0.689	0.767	0.806	0.794
E_c/E_g	0.752	0.818	0.805	0.902
α	0.87	0.55	0.57	0.27
β	0.79	0.55	0.45	0.48
p	0.60	0.42	0.46	0.21
r_c (μ)	2.6	3.3	2.4	3.5
r_g (μ)	0.23	0.28	0.23	0.22

Solubilization of grana

When DBS concentration is raised above $6 \cdot 10^{-4}$ M, the distribution peak for swollen grana shown in Fig. 3 disappears. This fact indicates the disintegration of swollen grana into smaller particles. The size of the particles formed is, however, below the observation limit of the COULTER counter, and the flattening effect could not be applied for the determination because of the transformation of bands. Ultracentrifugal analysis made for the suspensions with $6–25 \cdot 10^{-4}$ M DBS showed no sedimentation peak. The particles appear to be too large to be observed by the Schlieren optical system, since a clearly defined peak of 3.5 S is observed when the concentration is raised to $2.9 \cdot 10^{-3}$ M. The sedimentation coefficient of this peak decreases gradually from 3.5 S to 2.9 S as the concentration increases from $2.9 \cdot 10^{-3}$ to $2.0 \cdot 10^{-2}$ M as shown in Table III. During centrifugation, the peak moved with a yellowish green boundary and the supernatant was colorless, so that this component is inferred to carry pigments. The coefficient, 2.9–3.5 S is slightly larger than but is close to 2.6 S

of a colored component observed by SMITH[26] and SMITH AND PICKELS[27] with 0.25 % sodium dodecyl sulfate as the solubilizing reagent. The coefficient is, however, smaller than 13.5–13.8 S of the component obtained with digitonin, sodium deoxycholate and bile salts[25–28], and 5.4–5.5 S of the component observed with Nacconal NRSF[28] or a mixture of Duponol C and Span 80[18].

TABLE III

SEDIMENTATION COEFFICIENTS IN SVEDBERG UNITS FOR THE COMPONENTS IN THE MIXTURES OF CHLOROPLASTS AND DBS

DBS concentration ($M \cdot 10^3$)	*Larger component*	*Smaller component*
2.86	3.5	
5.72	3.3	
8.58	3.2	
11.4	3.1	
14.3	3.1	
17.2	3.0	
20.0	2.9	
22.9	2.6	1.1
25.7	2.1	1.4
28.6		1.0
34.3		1.2
40.0		1.2

SMITH made a run of sedimentation experiments with a more concentrated sodium dodecyl sulfate of 2.5 % and observed a peak of a lower coefficient of 1.7 S, which suggested the possibility of dissociation or splitting of the 2.6-S component into the smaller units. With DBS as the reagent, the dissociation occurs in the concentration range of $2.3–2.6 \cdot 10^{-2}$ M as seen from the data in Table III. The sedimentation patterns in the range showed two peaks; one corresponding to the 2.9–3.5-S component and the other having an average coefficient of 1.2 S. Above $2.6 \cdot 10^{-2}$ M, only the 1.2-S component was observed. A boundary due to yellowish green color moved with this peak, and the supernatant was faint greenish yellow.

DISCUSSION

The volume, 23 μ^3, of the native spinach chloroplasts determined with the COULTER counter coincides with the mean volume, 22.5 μ^3, of *Zea mays* chloroplasts measured by ORTH AND CORNWELL[10] with the same instrument. The complete agreement is, of course, fortuitous because our value is the volume at the distribution maximum and their value is the mean volume. The volume is also similar to 20–50 μ^3 estimated by RABINOWITCH[3] and other data calculated from particle dimensions[29–34]. Attempts to estimate the mean volume from the distribution curve were unsuccessful. This is because the result depends upon the volume range where the volumes are to be averaged. The fractional error in number in the plot of number *versus* volume varies considerably along the axis of volume, whereas the error in the plot of number *versus* log. V is nearly constant along the axis of log. V. Therefore, the average of the values of log. V would be more reliable than the average of the volumes. The average of log. V appears to be close to the value of log. V at the distribution maximum, since the distribution curve is approximately symmetrical.

The electron micrograph of aged chloroplast in Fig. 2A shows grana with a disk shape approx. 0.3 μ in diameter and 0.1 μ in height. The volume of the grana is, therefore, calculated to be 0.07 μ^3, which is smaller than the volume of the swollen grana (0.17 μ^3) at the distribution maximum. If we can assume that the grana are swollen by the same factor of 76/23 as observed for chloroplasts, the volume of native grana is to be 0.05 μ^3, being in rough agreement with the volume estimated from the electron micrograph. RABINOWITCH[3] estimated the average volume of the grana of higher plants from a number of electron-microscopic data, and found it to be about 0.05 μ^3, which agrees with the value estimated above. Since the sample spinach chloroplast contains 140 grana, the total volume of the grana in one chloroplast amounts to 7 μ^3. This volume is 30 % of the volume of the native chloroplast, which is in the range between 15 and 50 % estimated by RABINOWITCH[3] and FREY-WYSSLING AND MÜHLETHALER[35] for varieties of chloroplasts.

The radius of aged or swollen chloroplasts determined from the flattening effect at 437 mμ agrees well with the radius of the same chloroplasts determined at 675–678 mμ. The constancy of the estimated radius is an indication that the equations derived above are applicable for the analysis of the flattening effect. The radii, 2.4–2.6 and 3.3–3.5 μ of aged and swollen chloroplasts are, however, appreciably larger than the corresponding radii, 2.2 and 2.6 μ, estimated from the distribution maxima. This difference may be accounted for at least partly by the difference between the observed quantities; the radius averaged optically in the flattening effect and the radius at the distribution maximum.

Similarly, the radii of aged grana determined at the two wavelengths agree with each other. On the other hand, the radius of the swollen grana determined at 675–678 mμ differs considerably from that determined at 437 mμ. The smaller value of 0.22 μ is, probably, erroneous because it is even smaller than the value obtained for the aged grana. The erroneous value would arise from the low value (1.23) of E_g for the swollen grana, the value being smaller than 1.25 for aged grana. Repeated measurements gave similar low values of E_g, which may be due to the effect of band transformation occurring before the disintegration into grana is completed. The effect would be appreciable at 675–678 mμ and may be small at 437 mμ as judged from the shapes of the curves in Fig. 5. The larger value of 0.28 μ is, probably, the correct average radius for the swollen grana. This value is, however, smaller than the radius (0.34 μ) estimated from the distribution maximum. This suggests the presence of a considerable number of particles carrying pigments, which could not be observed by the COULTER counter but were observed optically as the flattening effect.

It may be concluded from these results that the flattening effect is a useful tool to determine the average radius of particles, and that the COULTER counter is invaluable for determining the size at the distribution maximum. The flattening effect records the particles carrying pigments, whereas the counter scans all the colored and colorless particles in a suspension. These characteristics are advantageous or disadvantageous, depending upon the purpose of the experiment.

The process of disintegration and solubilization of chloroplasts with DBS may be summarized by the following scheme:

$$\text{Native chloroplast} \xrightarrow{\text{NaCl}} \text{aged chloroplast} \xrightarrow{\text{DBS}} \text{swollen chloroplast} \xrightarrow{\text{DBS}} \text{swollen grana} \rightarrow$$
$$\xrightarrow{\text{DBS}} \text{particles of unknown size} \xrightarrow{\text{DBS}} \text{2.9–3.5-S component} \xrightarrow{\text{DBS}} \text{1.2-S component}$$

The disintegration from native chloroplasts to swollen grana could be followed quickly and reproducibly by the flattening effect and by the electric measurement with the COULTER counter. The swollen grana were found to be disintegrated into particles of unknown size, and then transformed into the 2.9–3.5-S component. Application of other techniques such as density-gradient centrifugation or exploration of new techniques is needed to detect these particles and to determine their size. The 2.9–3.5-S component was found to dissociate further into smaller components of 1.2 S. The determination of the molecular weight and other physico-chemical and chemical properties of this component as the smallest unit derived from chloroplasts may be of great interest.

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