41. WEISS, S. P. AND E. P. KENNEDY. 1956. The enzymatic synthesis of triglycerides. J. Am. Chem. Soc. 78: 3550.
42. WEISS, S. P., S. W. SMITH, AND E. P. KENNEDY. 1956. Net synthesis of lecithin in an isolated enzyme system. Nature 178: 594.
43. WINTERMANS, J. F. G. M. 1960. Concentrations of phosphatides and glycolipids in leaves and chloroplasts. Biochim. Biophys. Acta 44: 49–54.
44. ZILL, L. P. AND E. A. HARMON. 1962. Lipids of photosynthetic tissue. I. Silicic acid chromatography of the lipids from whole leaves and chloroplasts. Biochem. Biophys. Acta 57: 573–83.

Comparative Studies of the Hill Activity of Differentially Centrifuged Chloroplast Fractions [1, 2]

M. J. Becker, A. M. Shefner, and J. A. Gross

Life Sciences Research Division, IIT Research Institute, Chicago, Illinois

Differential Hill activity of centrifugally fractionated chloroplast particles was first demonstrated in the classic investigation of Thomas et al. in 1953 (23). Their studies showed that the rate of reaction decreased with a decrease in particle size. Spherical particles of about 1×10^6 A^3 (ca. 130 A diam) retained on the order of 50 % of the original activity of the spinach grana mixture from which they had been sedimented. On the other hand, Park and Pon (17) stated that in their differentially centrifuged spinach chloroplast fractions, there was no change in Hill activity with particle size. Becker et al. (4) have presented evidence that decreased chloroplast particle size does indeed change Hill reaction rates, but that the change is not necessarily a decrease. Relatively large chloroplast fragments, which sedimented at centrifugal forces of not more than $50{,}000 \times g$ but more than $1{,}000 \times g$, exhibited higher Hill activity than smaller fragments isolated at higher centrifugal forces or than isolated intact chloroplasts. They further demonstrated that the highest activity resided in a fraction which sedimented between 20,000 and $50{,}000 \times g$ (3).

The literature cited above contains information which at present appears contradictory. It leads to the question: when the chloroplast is disrupted into fragments of various sizes, is there any correlation between particle size and Hill reaction rate?

The present report offers evidence in support of the view that there is no simple relationship between Hill activity and particle size. Our previous suggestion that activity is related not just to size but to an optimal chemical composition and molecular organization of the particle components seems sustained. In addition, this paper presents a further characterization of the fraction displaying high Hill activity.

Materials and Methods

Chloroplast Isolation. The leaves of common spinach, *Spinacia oleracea,* purchased from a local market, were used as the source of chloroplasts for these studies. Before a preparative procedure for chloroplast isolation was selected, the following variables were explored: conditions and duration of leaf storage, method of cell rupture, solution composition, osmotic conditions and conditions for chloroplast isolation and storage.

The procedure which was developed is schematized in figure 1. It is the same as that reported previously (4), except that minced leaves were homogenized for 12-second periods instead of 15-second periods. Also, chloroplasts were washed twice instead of once, since Leech and Ellis (15) had shown that 2 washings greatly decreased transaminase activity which is generally associated with mitochondrial contamination of chloroplast preparations. The washed chloroplasts were treated in the ultrasonic vibrator for 3 instead of 2 3-minute periods. The purity of our intact washed chloroplasts (WC in fig 1) was verified by microscopy.

WC were partially disrupted by suspending in dilute saline (0.015 M NaCl), which is hypotonic for chloroplasts. They were further fragmented by vibrating 35-ml volumes of the suspension in a Raytheon 9-kc, 50 w magnetostrictor at full power as described above. A 1-minute rest interval was allowed between each vibration period to maintain the temperature between 8° and 12°. No special effort was made to keep

[1] Received July 20, 1964.
[2] Supported by U.S. Air Force Systems Command, Contract No. AF 33(616)-7255.

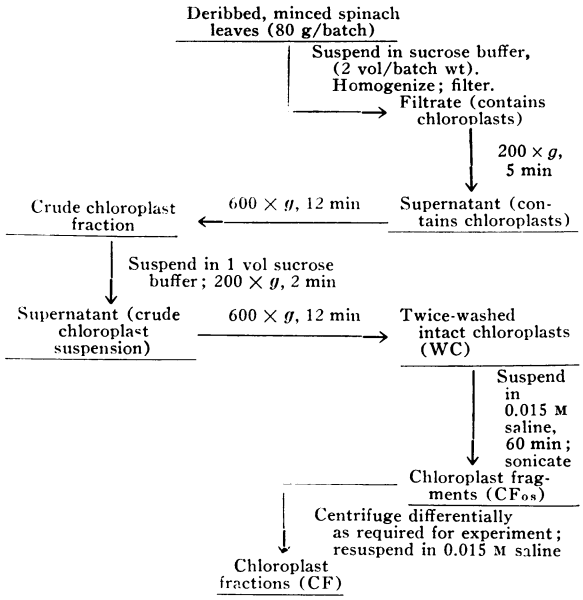

FIG. 1. Chloroplast preparation procedure.

Table I. *Chlorophyll Distribution in Chloroplast Fractions*

Fraction	Maximum centrifugal force, *g*	Centrifugation time at max force, min	Avg % total chlorophyll
CF_{os}	...	...	100.0***
CF_{0-1}	1,000	7–10	5.2
CF_{1-20}	20,000	10	45.8
CF_{20-50}	50,000	10	27.3
CF_{50-70}	70,000	30	12.5
CF_{70-145}	145,000	30	4.0
$CF_{145\ spt}$*	...	...	2.5
$CF_{145\ spt-173_{30}}$**	173,000	30	1.5
$CF_{173_{30}-173_{60}}$**	173,000	60	0.9
$CF_{173_{60}-173_{120}}$**	173,000	120	0.4

* Supernatant fraction from a 145,000 × *g* spin.

** Sediments from $CF_{145\ spt}$ by centrifuging at 173,000 × *g* for 30, 60, and 120 minutes, respectively.

*** 60 mg chlorophyll (see table III).

the suspensions anaerobic during fragmentation even though Thomas et al. (23) reported loss of activity under aerobic conditions. There was no appreciable loss of Hill activity in our experiments. The osmotically shocked and sonically prepared chloroplast fraction (CF_{os} in fig 1) was centrifuged at 1,000 × *g* for 10 minutes to remove intact chloroplasts, large fragments, agglomerates, and starch. The resulting sedimented fraction was designated CF_{0-1}. Depending upon the experimental requirements, the supernatant from CF_{0-1} was differentially centrifuged at forces up to 173,000 × *g*. The subscripts appended to the fractions, CF, indicate the *g* force × 10^{-3} used to obtain the pellet. Table I shows the fractionation regimen used in our studies and the chlorophyll distribution in the fractions. All centrifugations were performed in a Spinco Model L Preparative Ultracentrifuge at 2° to 4°. A No. 30 rotor was used for attaining forces up to 105,000 × *g*, a No. 40 rotor for forces up to 145,000 × *g* and a No. SW 39 rotor for forces up to 173,000 × *g*. All CF were stored in ice in the dark until used.

Ten to 15 batches of leaves, weighing about 80 g per batch, were used in each experiment.

Total chlorophyll concentration was measured by the method of Arnon (1), which is based on the specific absorption coefficients of chlorophylls a and b in 80 % (v/v) acetone as determined by MacKinney (16). Measurements were made in a Beckman DU spectrophotometer and spectra were scanned automatically in a recording spectrophotometer.

Electron-Transfer Assay. Electron transfer was measured spectrophometrically according to the procedure of Jagendorf and Krogmann (12) and was recorded as the number of μmoles of electrons transferred per mg chlorophyll per hour. Whole chloroplasts or chloroplast fractions were resuspended in their respective media buffered at pH 6.8 with 0.05 M Sorensen phosphate buffer. In the reaction mixture the concentration of potassium ferricyanide, the oxidant, was adjusted to allow the reaction to proceed at a rate neither dependent on the oxidant concentration nor limited by the reduction step (22). Chloroplast fractions for assay were added to the reaction mixture, so that a total of 0.015 to 0.030 mg of chlorophyll was present in a total volume of 3.5 ml. The resulting oxidant-to-chlorophyll molar ratio was kept between 30 to 1 and 60 to 1. Under these conditions the measurements were linear.

The entire procedure was carried out under subdued green light, except for a 1-minute white-light illumination period of 800 ft-c from a Sylvania 30-w incandescent reflector lamp in a 15° water bath. This light intensity was not saturating, and the Hill reaction velocity-light intensity relation appeared to follow a simple rectangular hyperbola (22). Control reaction mixtures were kept in the dark. Spectrophotometric readings were made in a Bausch and Lomb Spectronic 20 colorimeter at 510 mμ 5 minutes after *o*-phenanthroline was added to produce a color complex with free ferrous ions. All assays were run in duplicate. The calibration curve used for calculation of μmoles of electrons transferred was based on potassium ferrocyanide and ferrous sulfate standards.

Oxygen Evolution Measurement. Oxygen evolution was measured manometrically in a Warburg-type illuminated respirometer. The reactions were run at 15° and were illuminated from below with 800 ft-c from Sylvania 30-w reflector lamps. Each flask contained 0.15 to 0.30 mg of chlorophyll. The oxidant, potassium ferricyanide, was adjusted to give an oxidant-to-chlorophyll molar ratio of 30:1. The total reaction volume was made up to 3.0 ml with

a buffer solution of 0.015 M NaCl and 0.05 M phosphate, pH 6.8. Both the center well and 1 sidearm of the reaction vessel contained 0.4 ml of Warburg No. 9 buffer mixture in order to maintain a constant partial pressure of CO_2.

Each flask was flushed for 15 minutes with oil-pumped nitrogen and shaken before an experimental run. It was then equilibrated for an additional 15 minutes before the oxidant was added, and for 5 minutes more before a zero reading was taken, and the lights were turned on. Readings were then taken every 3 minutes without cessation of shaking. The reaction was carried out for as long as 24 minutes, but the rate of O_2 liberation generally decreased after 18 minutes. Calculations for the rate of O_2 liberation were based upon the rate of the reaction between 6- and 18-minute readings where the relationship was linear, and were corrected for changes in the thermobarometer and the respective dark controls.

Electron Microscopy. Formvar-coated 200-mesh copper grids were used for mounting specimens. The mounts were prepared according to Hall (10), by placing a droplet of particle suspension on a dust-free grid. The excess solution was absorbed with a piece of filter paper, and the preparation was gently washed with successive droplets of distilled water. Finally, the grid, resting on a clean glass slide in a petri dish, was dried overnight in a desiccator. In some experiments polystyrene latex particles 880 A in diameter were mixed with the suspension before mounting.

Untreated and metal-shadowed specimens were examined. Germanium, or gold and palladium alloy, or gold and nichrome alloy were used for metal-shadowing. An Hitachi HS-6 electron microscope was used for some observations. To obtain better resolution, an Hitachi HU-11 was employed.

Experimental Results

Hill Reaction Rate. To determine whether Hill activity changed during the time required to obtain all fractions, several fractions from the same batch of spinach were assayed shortly after the fraction was first obtained, again after the last fraction was isolated, and once more on the following day. The relative electron transfer activity of the fractions remained the same even after storage for 24 hours at 2° to 4° in the dark. However, the rate of activity in each fraction changed with time and depended on the particular fraction as well as on fraction storage conditions.

Fractions suspended in dilute saline were tested 7 hours and 24 hours after isolation of WC. The pH during storage was 6.2 to 6.4. Table II shows these results. In contrast to Thomas et al. (23) who reported a 2-fold drop in activity in about 2 hours, we found that the fractions remained relatively stable for the entire test period. We have designated the zero point in our studies as the time of isolation of WC; the zero point in Thomas's studies is unidentified. The reported activity decay might be more meaningful were the zero point known.

To correlate O_2 evolution and electron transfer activity, fractions were assayed manometrically and spectrophotometrically.

The theoretical relationship between electron transfer activity and O_2 evolution is such that 4 moles of electrons should cause the evolution of 22.4 liters (1 mole) of O_2, a relationship involving a numerical factor of 5.6. As shown in table II, for any fraction, this relationship is never exceeded. In CF_{20-50}, the theoretical value was not attained, but it was almost reached in the other fractions measured. Photosynthetic cofactors were not added in any of the described experiments.

Table II also shows that O_2 evolution in the chloroplast fractions followed the trend observed for electron transfer in that the activity of CF_{20-50} was always higher than that of any other fraction. Compared with other fractions, specific electron transfer activity of CF_{20-50} was more stable and yielded more reproducible results as indicated by the low standard error.

Table II. *Comparison of Electron Transfer and Oxygen Evolution in Chloroplast Fractions*

Fraction	Electrons transferred ± SE* μmoles/mg chlorophyll/hr		Oxygen evolved μl/mg chlorophyll/hr	
	7 hr**	24 hr**	7 hr**	24 hr**
WC	50	40	250	200
CF_{os}	101 ± 6.5	89 ± 5.1	380	438
CF_{0-1}	74 ± 6.1	85 ± 11.5	...	...
CF_{1-20}	119 ± 17.7	135 ± 17.0	...	...
CF_{20-50}	141 ± 6.1	167 ± 10.0	522	563
CF_{50-70}	...	81	...	453
CF_{70-145}	46	51	...	...
$CF_{145\,spt}$	28	...	...	...
$CF_{145-173_{30}}$	41	...	...	...
$CF_{173_{30}-173_{100}}$	39	...	...	...

* SE, standard error; data from replicate experiments.

** Time after isolation of WC.

Table III. *Hill Activity Balance*

Fraction	Wt of chloro-phyll* mg	Total activity** μmoles electrons/hr 7 hr***	24 hr***
CF_{os}	60.0*	6060	5340
CF_{1-0}	3.2	236	271
CF_{1-20}	27.4	3260	3695
CF_{20-50}	16.4	2320	2745
CF_{50-70}	7.5	(608)	608
CF_{70-145}	2.4	111	122
$CF_{145\,spt}$	1.5	42	(42)
Total recovery	58.4	6577	7483
Total recovery, %	97.3	112	140
Total recovery corrected for chlorophyll loss, %	100.0	115	144

* An average yield from 10 to 12 batches of spinach leaves.
** Figures in parentheses are estimated values.
*** Time after isolation of WC.

Total activity (table III) was calculated on the basis of chlorophyll recovery data (table I) which was consistently near 100% of that in the control fragment mixture (CF_{os}) and from data on the rates of electron transfer (table II). The summed activity of the fractions was greater than that of CF_{os} and increased during storage in dilute saline. The enhancement was due chiefly to increased activities of CF_{1-20} and CF_{20-50}.

CF_{20-50}, the fraction with the highest Hill reaction rate, and CF_{70-145}, which is similar to fractions variously reported to be composed of quantasomes or quantasome aggregates (18, 20, 21) were of special interest. CF_{20-50} was usually isolated 3 to 4 hours after WC, while CF_{70-145} was generally isolated 6 to 8 hours after WC. To determine whether the lower activity of CF_{70-145} was due to the extended time required for its isolation, it was obtained within 4 hours after WC by eliminating the intermediate centrifugal fractionations, i.e., by centrifuging CF_{os} directly at $70,000 \times g$. The resulting supernatant was immediately centrifuged at $145,000 \times g$, the sediment being rapidly isolated CF_{70-145} [$CF_{70-145(r)}$]. The sediments were resuspended and stored in dilute saline.

The data in table IV demonstrate that preparation time influences activity. When assayed about 4 hours after isolation, $CF_{70-145(r)}$ had a higher activity than CF_{70-145}. The activity, however, remained lower than that of CF_{20-50}. After another 24 hours the activities of the slowly and rapidly prepared CF_{70-145} declined to the same level. It appeared that time of exposure to the supernatant had an effect on the activity of the fractions. Prolonged exposure of CF_{70-145} to the supernatant might have induced the decreased activity.

In order to test this hypothesis, $CF_{70-145(r)}$ was suspended in dilute saline and split into 2 aliquots. Each aliquot was recentrifuged at the speed originally used to obtain the fraction. One aliquot was then

Table V. *Effect of $CF_{145\,spt}$ on Hill Activity* of CF_{20-50} and CF_{70-145}*

Fraction	Assay time, hr after isolation	Storage medium	
		0.015 M NaCl	$CF_{145\,spt}$
CF_{20-50}	3–4	141	147
	24	167	100
$CF_{70-145(r)}$	3–4	110	120
	24	48	90

* μmoles electrons transferred per mg chlorophyll per hour.

Table IV. *Hill Activity in Relation to Time of Isolation and Assay*

Fraction*	Isolation time (hr after WC)	Assay time (hr after isolation)	Specific activity, μmoles electrons/mg chlorophyll/hr	
			Individual	Avg
CF_{20-50}	3–4	3–4	129	169
			164	
			171	
			191	
		20–24	155	171
			168	
			189	
$CF_{70-145(r)}$	3–4	3–4	104	107
			110	
		20–24	36	42
			41	
			48	
CF_{70-145}	6–8	3–4	33	48
			62	
		20–24	26	54
			81	

* Fractions resuspended in 0.015 M NaCl.

resuspended in dilute saline; the other aliquot was resuspended in $CF_{145\ spt}$. The Hill reaction was measured 3 to 4 and 24 hours later. No measurable difference occurred in either aliquot in the first 3 to 4 hours. After 24 hours the activity of $CF_{70-145(r)}$ in supernatant was twice that in saline. Although in both aliquots the activity declined from the starting level, the rate of decay was retarded by exposure to the supernatant.

If the activity differential observed between slowly and rapidly isolated CF_{70-145} (table IV) was caused by extended contact with the supernatant one would expect that reexposure of the rapidly isolated fraction to the supernatant from which it had been separated would cause an activity decline. This was not the case. As a matter of fact, the supernatant apparently exerted a protective effect against activity decay (table V).

To determine whether this might be a general effect of the supernatant, the experiment was repeated using CF_{20-50} as a test fraction. Clearly, anything which could increase the activity of this fraction which already was a more optimal fraction than any other one isolated, would be desirable. Table V also shows the results of this experiment. Again, no difference was noticed after 3 to 4 hours, but after 24 hours, the activity of the aliquot exposed to supernatant was only 60 % of that of the aliquot in saline. There is no satisfactory explanation for these effects at this time. It is possible, however, that the answer resides in the slightly different nature of the supernatants to which the 2 fractions were exposed.

Effects of Chloride and Phosphate Anions on Hill Reaction. Jagendorf and Krogmann (13) have shown that whole chloroplasts suspended in 0.4 to 0.5 M sucrose and diluted with 0.35 M NaCl at pH 6.3 have an electron-transfer activity 5 to 10 times that of whole chloroplasts in sucrose alone. The effect is thought to be related to permeability and to an uncoupling of Hill activity from photophosphorylation (11). Clendenning and Gorham (6) studied activity of whole chloroplast and grana preparations of various plants and showed specific requirements for, and specific effects of, anions on photoactivity. Their studies also revealed a time-course effect. Gibbs and Calo (7) demonstrated an optimal concentration range of phosphate for CO_2 fixation in whole chloroplasts; outside the optimal range phosphate was shown to have adverse effects and the measured reaction became minimal.

The effect of ion concentration on differentially centrifuged and isolated chloroplast fractions has not been previously reported. Hill activity of intact chloroplasts is known to be stimulated by 0.35 M NaCl (2, 11, 13). We found that CF may be activated or inhibited by high salt (0.35 M NaCl) concentration depending on particle size and saline level of storage and assay media.

Upon storage for 24 hours in 0.35 M NaCl, precipitation occurs and Hill activity declines in WC as well as CF. The fractions are more stable when stored in dilute saline.

If stored in 0.015 M NaCl and assayed in 0.35 M NaCl, Hill activity of CF containing large fragments is inhibited while activity of CF containing small particles is stimulated. However, if stored in 0.35 M NaCl and assayed in 0.35 M NaCl, although overall activity is low, CF containing large particles is stimulated while CF containing small particles is inhibited.

With pH at 6.8, phosphate level in the assay buffer was varied. It was found that Hill activity of all fractions that were tested were inhibited by 0.005 to 0.01 M phosphate, while below and above this range the reaction rate was stimulated. Maximum activity occurred at the order of 0.05 to 0.10 M phosphate.

This pattern cannot be explained satisfactorily at this time. Previously, Jagendorf (11) and Good (8) also reported that certain ion concentrations are optimal for the Hill reaction in whole chloroplasts. Neither investigator, however, was able to suggest a logical explanation of his results.

Effect of pH on Hill Reaction. In our standard procedure we utilized an isolation medium of unbuffered 0.015 M saline and an assay medium of 0.05 M phosphate buffer (pH 6.8) containing 0.015 M NaCl. The variable response of the fractions to phosphate and NaCl at pH 6.8 led us to study the effect of the pH per se in 2 buffer systems. The most active fraction, CF_{20-50}, and the less active, CF_{70-145}, were used.

The results of assays on both fractions in Tris-maleate and phosphate buffers over the pH range 5.8 to 8.3 showed that Hill activity was always higher in CF_{20-50} than in CF_{70-145}. Comparison of activity levels in the 2 buffers indicated that phosphate is preferred.

A pH of 6.8 was optimal for Hill activity of CF_{20-50}. For CF_{70-145}, pH 7.8 was only slightly more favorable than pH 6.8.

Good (8) showed that 0.01 to 0.03 M Tris-hydrochloride was more inhibitory than tricine (a glycine derivative of Tris). In addition, Good showed that Hill activity of whole chloroplasts isolated in Tris-hydrochloride at pH 7.5 was variable in the presence of phosphate, sulfate, citrate, or lactate. The response was not in a direct relationship to the concentration of the different anions. Our results with fractions of chloroplasts support those of Vennesland and Stiller (24) and Good (8) who reported that the medium used for chloroplast isolation affected Hill reaction rate as well as photophosphorylation. There is also a pH dependence of CO_2 fixation for whole and broken chloroplasts (14).

Thus, the variable influence of pH on photosynthetic reactions observed by others is confirmed by our results. We have also shown that different fractions have different optima. The efficiency of the Hill reaction under different ionic conditions may be a reflection of the chemical and structural relationships of the macromolecules composing the fragments in each fraction.

Stability of CF_{20-50} Fraction. The effects of dialyzing CF_{20-50} against distilled water and of adding sucrose to the assay system to induce sudden osmotic changes were tested over a 48-hour period. The re-

Table VI. *Hill Activity of CF_{20-50} before and after Dialysis*

Condition	Assay solution	Activity, μmoles electrons/mg chlorophyll/hr			
		0 hr*	3 hr*	24 hr*	48 hr*
Before dialysis	Standard	159	148	132	141
	Standard plus 0.06 M sucrose	...	160	126	144
After dialysis	Standard	...	153	136	130
	Standard plus 0.06 M sucrose	...	123	153	150

* Assay time, hour after isolation.

sults shown in table VI demonstrate the extreme stability of this fraction to dialysis and to storage.

Particle Size Estimation. Particle size was estimated from sedimentation time by assuming that all particles in each centrifuged fraction were typical spherical proteins (table VII).

Particle size was also measured from electron micrographs of metal-shadowed, dried suspensions of chloroplast fractions. Figures 2a, b, and c are representative electron micrographs of CF_{20-50}; figures 3 and 4 are micrographs of CF_{70-145} and $CF_{145\ spt}$, respectively. Polystyrene latex beads of known dimensions were included in the fragment suspensions to facilitate measurement of particle sizes directly from photographs. From these figures and a number of similar photographs, the dimensions of the chloroplast fragments found in each fraction were measured (table VII).

It was predicted from sedimentation times and the consideration that the particles were typical spherical proteins (table VII) that the size range in CF_{20-50} would vary from 1500-A-diameter particles, which are the smallest particles which theoretically could be completely sedimented, to particles no larger than 2300 A. Larger fragments would have sedimented during the previous centrifugation at $20{,}000 \times g$ (CF_{1-20}). Yet, electron micrographs (fig 2) indicated a much wider range of fragment sizes. Since it is unlikely that particles much smaller than 1000 A and virtually impossible that particles larger than 2300 A were originally sedimented in CF_{20-50}, these must have formed aggregates either because of a natural affinity or as an artifact of the method of preparing the specimens. Nevertheless, the large fragments appear to be aggregates of the smaller particles in the suspension. Similar aggregation was observed for the particles in CF_{70-145}.

Data in table VII show good agreement between estimates of average particle size from sedimentation times and measurements from electron micrographs, despite the fact that the particles are not typical spheroids. The particle sizes in $CF_{145\ spt}$ compare fairly well with the measurements reported by Thomas et al. (23), although his particles ranged from 30 to more than 330 A in diameter. Thomas's starting material, however, was not isolated chloroplasts but leaf homogenate. It is, therefore, possible

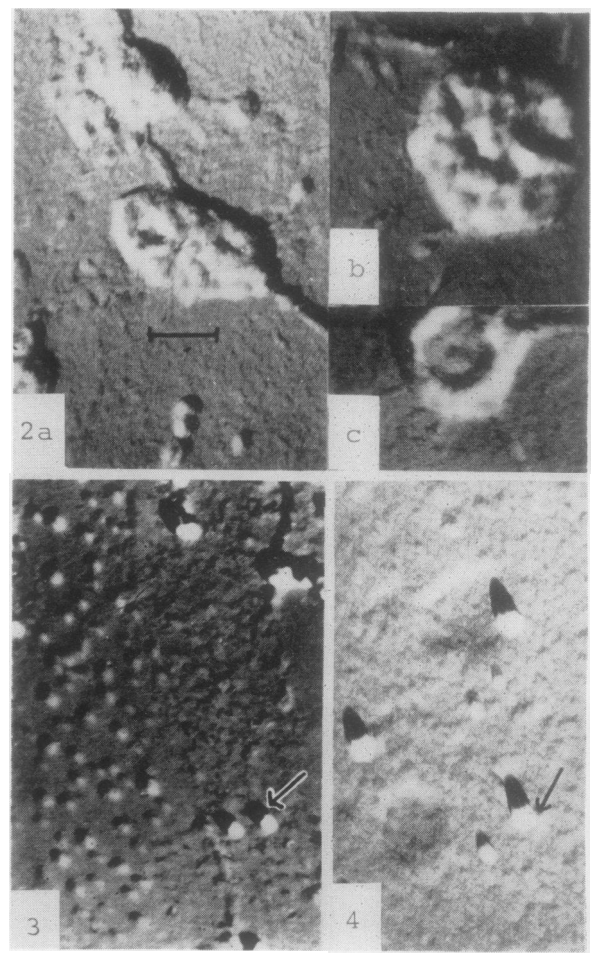

FIG. 2–4. Electron micrographs of metal-shadowed particles from chloroplast fractions. Figure 2. CF_{20-50}, 0.5 μ marker; a, several 1500-A particles separated from larger aggregates; b, large aggregate composed of 1500-A subunits; c, an aggregate crater showing area previously occupied by subunits. Figure 3. CF_{70-145} showing double-layered particles as indicated by double shadows; average particle diameter is 600 A. Figure 4. $CF_{145\ spt}$ exhibiting several isolated 200-300-A particles. Arrows in figures 3 and 4 indicate 880-A polystyrene latex particles.

that the smaller particles in the suspensions which he observed and measured by electron microscopy represented fragments of cytoplasmic components other than chloroplasts. The photoactivity measurements reported in Thomas's studies could also have been influenced by the presence of non-chloroplastic particles.

Table VII also reveals that there was greater variability in the mean height of large particles (CF_{20-50}) than of smaller particles (CF_{70-145}). The ratio of mean diameter to mean height suggests that all the fragments have a discoid or oblate spheroid shape. The smaller fragments are more nearly spherical, as indicated by the approach of the ratio to unity. Furthermore, the particles in CF_{70-145} (fig 3) can be seen to show a double shadow. We interpret this to mean that these units are double layered structures in which the lower layer projects some distance beyond the upper layer.

Discussion and Conclusions

The major point which emerges from this investigation is that a fraction with high Hill activity, CF_{20-50}, can be differentially centrifuged between 20,000 and 50,000 $\times$ *g* from a mechanically ruptured spinach chloroplast suspension. Its high Hill reaction rate relative to other fractions is maintained regardless of ion concentration in either storage or assay media, solution tonicity, pH, or time of isolation. It is a particularly stable fraction whose physiological properties are probably dependent upon its specific chemical composition, e.g. the ratios of chlorophyll to protein to plastoquinone (3) and its physical properties.

Certainly, CF_{20-50} particles are too large (table VII) to be considered fundamental photosynthetic units themselves. However, their size and other physical and chemical properties permit some speculation about their construction from subunits. Several assumptions, which are subject to a certain amount of criticism, can be made. The average CF_{20-50} particle appears to have a disc-like shape and to be 1500 A in diameter and about 360 A in height (fig 2; table VII). These dimensions do not take into account the relatively heavy metal layer; the actual particles are undoubtedly smaller. The size of a CF_{20-50} particle suggests that it was derived from a double lamellar layer of a chloroplast granum and would consequently be pigment-lipid coated on upper and lower surfaces. The total pigment-lipid surface area available can be calculated to be 35.3×10^6 A^2. If the dimension of the porphyrin head of a chlorophyll molecule is taken as 15 A $\times$ 15 A (25), then the geometry of the CF_{20-50} particle would permit it to accommodate some 16,000 chlorophyll molecules with their heads parallel to the plane of the surface. Up to twice that many chlorophylls could fit this area if the heads were tilted at 45°. In CF_{20-50}, chlorophyll has been reported to represent 10 % of the dry weight (9). Assuming a molecular weight of 900 for chlorophyll, the fractional molecular weight of this pigment in CF_{20-50} would be 14.4×10^6 and the total weight of an average CF_{20-50} particle would be 14.4×10^7. Another approximation of the particle molecular weight is possible by calculating from the sedimentation constant of 800 S (9) and assuming a particle density of 1.17 like that of the quantasome (19) and an estimated diffusion constant of unity. These calculations yield a value of 13.4×10^7. A third approximation can be derived from the reported molecular weight of 1.92×10^6 for the quantasome (19) and an assumed area of 200 A $\times$ 200 A required for each quantasome. This requirement takes into account that a 200-A-diameter oblate spheroid (5, 17, 18) would occupy the same space as a square measuring 200 A on a side. Thus, the available surface area of a CF_{20-50} discoid could accept up to 88 quantasome-sized particles with a total molecular weight of 16.9×10^7.

Similarly the particles in CF_{70-145} which, by virtue of their sedimentation characteristics (9) and size (table VII), are similar to quantasome aggregates (18, 20, 21) could contain about 2400 chlorophyll

Table VII. *Particle Size in Chloroplast Fractions*

Fraction	Estimated* diameter, A	Measurements from electron micrographs		
		Mean diameter, A	Mean height, A	Ratio of mean diam to mean height
CF_{os}	...	2500	...	...
CF_{0-1}	> 20,000	...	...	...
CF_{1-20}	2,300–20,000	...	...	...
CF_{20-50}	1,500–2,300	1500	360	4.17
CF_{50-70}	1,000–1,500	...	...	...
CF_{70-145}	470–1,000	600	350	1.43
$CF_{145\ sdt}$	< 470	300	250	1.20
$CF_{145-173_{30}}$	320–470	...	...	...
$CF_{173_{30}-173_{100}}$	150–320	...	...	...

* Estimated from sedimentation rate according to Beckman Model L Instruction Manual, Memo II-B, 1.0, fig. 503, 1959. Particles considered as average biological particles. If not spherical or typical, the actual diameters would be larger than estimated.

molecules, comprise about 10 quantasome-type subunits and have a molecular weight of the order of 1.5 to 2.0 $\times 10^7$.

We have also shown that chloroplast particles smaller than CF_{70-145} still exhibit measurable Hill activity. These fragments, obtained by successively centrifuging $CF_{145\ spt}$ for 30 and 100 minutes at 173,000 $\times g$ (tables I and II), would be quantasome-sized or larger (table VII) depending upon particle density. They have been likened to a chlorophyll-associated 38 S sedimenting component in $CF_{145\ spt}$ (9). A first approximation of the molecular weight of 38 S is 3.5×10^6. Thus, the CF_{173} particles, like 38 S, could be small quantasome aggregates of 2 or 3 subunits or might themselves be considered the fundamental subunits for photosynthetic electron transfer (9). In fact they are at present the smallest units unequivocally demonstrated to show Hill activity. We propose that these 38 S units be termed "polyquantasomes." On this basis, CF_{20-50} particles would comprise 30 to 40 functional subunits of photosynthesis. It is this construction which appears to endow CF_{20-50} with high electron transfer capacity.

Further correlative studies of the chemistry and structure with Hill activity of the centrifuged chloroplast fractions are essential to substantiate or refute the concept that more than 1 type of basic photosynthetic unit may exist and that functional capacity depends upon the molecular balance and orientation in these units as well as the number and arrangement of the units in relation to each other.

Summary

A fraction with high Hill activity has been differentially centrifuged between 20,000 and 50,000 $\times g$ from sonically ruptured isolated spinach chloroplasts. This fraction has been compared under a variety of conditions with other fractions isolated at higher and lower centrifugal forces. It has been characterized physiologically and by particle size and has been shown to be extremely stable. It appears from centrifugal fractionation and electron microscopy to be composed of photoactive subunits. These subunits are larger than those presumed by the present quantasome model and are thought of as fundamental functional units of photosynthesis. Hypothetically, the fraction is a highly efficient oxygen-evolving, structured complex composed of functional subunits, which, when separated from the organized structure, are less efficient.

Literature Cited

1. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
2. Arnon, D. I., M. B. Allen, and F. R. Whatley. 1956. Photosynthesis by isolated chloroplasts. IV. General concept and comparison of three photochemical reactions. Biochim. Biophys. Acta 20: 449–61.
3. Becker, M. J., J. A. Gross, and A. M. Shefner. 1962. Hill activity and the plastoquinone content of spinach chloroplast fractions. Biochim. Biophys. Acta 64: 579–81.
4. Becker, M. J., A. M. Shefner, and J. A. Gross. 1962. Hill activity of isolated fractions of broken chloroplasts. Nature 193: 92–93.
5. Calvin, M. 1962. The path of carbon in photosynthesis. Science 135: 879–89.
6. Clendenning, K. A. and P. R. Gorham. 1952. Anionic stimulation of the Hill reaction in isolated chloroplasts. Arch. Biochem. Biophys. 37: 199–223.
7. Gibbs, M. and N. Calo. 1960. Studies on carbon dioxide fixation by chloroplasts. The effect of arsenite and other factors. Biochim. Biophys. Acta 44: 341–47.
8. Good, N. E. 1961. Uncoupling of the Hill reaction from photophosphorylation by anions. Plant Physiol. Proc. (Suppl.) 36: p 11.
9. Gross, J. A., M. J. Becker, and A. M. Shefner. 1964. Some properties of differentially centrifuged particulate fractions from chloroplasts. Nature 203: 1263–65.
10. Hall, C. E. 1953. Introduction to Electron Microscopy. McGraw-Hill, New York.
11. Jagendorf, A. T. 1961. Characteristics of ATP formation by chloroplasts. Symposium on Photosynthesis. Am. Soc. Plant Physiol., Southern Sect. 12–34.
12. Jagendorf, A. T. and D. W. Krogmann. 1957. A spectrophotometric assay of the Hill reaction with ferricyanide. Plant Physiol. 32: 373–74.
13. Jagendorf, A. T. and D. W. Krogmann. 1959. Comparison of ferricyanide and 2,3,6-trichlorophenol indophenol as Hill reaction oxidants. Plant Physiol. 34: 277–82.
14. Kandler, O. and H. Elbertzhagen. 1962. Differences in the pH optimum of the photosynthetic fixation of carbon dioxide in isolated whole and broken chloroplasts. Nature 194: 312–13.
15. Leech, R. M. and R. J. Ellis. 1961. Coprecipitation of mitochondria and chloroplasts. Nature 190: 790–92.
16. MacKinney, G. 1941. Absorption of light by chlorophylls. J. Biol. Chem. 140: 315–22.
17. Park, R. B. and N. G. Pon. 1961. Correlation of structure with function in *Spinacia oleracea* chloroplasts. J. Mol. Biol. 3: 1–10.
18. Park, R. B. and N. G. Pon. 1963. Chemical composition and the substructure of lamellae isolated from *Spinacia oleracea* chloroplasts. J. Mol. Biol. 6: 105–14.
19. Park, R. B. and J. Biggins. 1964. Quantasome: Size and composition. Science 144: 1009–11.
20. Sauer, K. and M. Calvin. 1962. Molecular orientation in quantasomes. I. Electric dichroism and electric birefringence of quantasomes from spinach chloroplasts. J. Mol. Biol. 4: 451–66.
21. Sauer, K. and M. Calvin. 1962. Absorption spectra of spinach quantasomes and bleaching of the pigments. Biochim. Biophys. Acta 64: 324–39.
22. Spikes, J. D., H. Eyring, and R. Lumry. 1954. Photosynthesis. Ann. Rev. Plant Physiol. 5: 271–340.
23. Thomas, J. B., O. H. Blaauw, and L. N. M. Duysens. 1953. On the relation between size and photochemical activity of fragments of spinach grana. Biochim. Biophys. Acta 10: 230–40.
24. Vennesland, B. and M. Stiller. 1961. Photophosphorylation and the Hill reaction. Nature 191: 677–79.
25. Wolken, J. J. and F. A. Schwertz. 1953. Chlorophyll monolayers in chloroplasts. J. Gen Physiol. 37: 111–20.