BBA 45573

DIGITONIN INCUBATION OF SPINACH CHLOROPLASTS IN TRIS (HYDROXYMETHYL) METHYLGLYCINE SOLUTIONS OF VARYING IONIC STRENGTHS*

JAN M. ANDERSON** AND LEO P. VERNON
Charles F. Kettering Research Laboratory, Yellow Springs, Ohio (U.S.A.)
(Received January 23rd, 1967)

SUMMARY

Spinach chloroplasts were incubated with digitonin in various media and the resulting derived fractions separated by differential centrifugation. (1) In low-salt–Tricine, the centrifugal fractions had the same chlorophyll composition as chloroplasts. All were active in the Hill reaction with ferricyanide or $NADP^+$ and no separation of the two photosystems of photosynthesis was observed. The small particles, however, had enhanced rates of $NADP^+$ photoreduction with ascorbate as electron donor. The fractions obtained were similar to sonicated chloroplast fragments. Electron micrographs of chloroplasts in low-salt–Tricine showed a marked difference from the usual chloroplast structure. The membrane system was discernible, but there was a marked breakdown of the original tight grana structure. The fixing properties were somewhat altered. (2) By contrast, in high-salt–Tricine, or ionic buffers (phosphate or Tris) only the large particles (low chl *a*/chl *b*) were active in the Hill reaction. The smaller particles (high chl *a*/chl *b*) photoreduced $NADP^+$ at high rates, but both 2,6-dichlorophenolindophenol and ascorbate were required for maximal activity. These treatments resulted in a fractionation of the two photosystems. Electron micrographs of chloroplasts suspended in high-salt–Tricine or phosphate buffer show the usual membrane structure with intact grana. (3) Lyophilized chloroplasts gave fractions whose properties were also dependent on the ionic strength of the medium used for digitonin incubation. The Hill reaction rates were lowered, but $NADP^+$ reduction with artificial electron donors was unimpaired by lyophilization.

INTRODUCTION

The non-ionic detergent, digitonin, has been used to fragment spinach chloroplasts into particles with a fully active Photosystem 1 and which are separable from larger particles which remain attached to the grana lamellae and are enriched in

Abbreviations: Tricine, tris (hydroxymethyl) methylglycine; chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; Photosystem 1, the long-wavelength system of chloroplasts; Photosystem 2, the O_2-evolving system of chloroplasts.

* Contribution No. 270 from the Charles F. Kettering Research Laboratory. Supported in part by Research Grant GB-4797 (LPV) from the National Science Foundation.

** On leave from Division of Plant Industry, C.S.I.R.O., Canberra, Australia.

Photosystem 2 (see refs. 1–3). The larger particles, sedimentable at 10000 × *g*, contain relatively more chlorophyll *b* and manganese than chloroplasts and are capable of O_2 evolution. The smaller particles, sedimentable at 144000 × *g* contain more chlorophyll *a* and less manganese than chloroplasts; they are inactive in the Hill reaction, but are highly active in $NADP^+$ photoreduction coupled to ascorbate and 2,6-dichlorophenolindophenol (DCIP)[1].

GOOD *et al.*[4] have employed several zwitterionic buffers which appear to have many advantages over the usual buffers used in biological research. They showed that these buffers were better than ionic buffers, both with respect to inhibition of uncoupling of phosphorylation from electron transport and to the observed rates of already uncoupled electron transport reactions. Therefore, IZAWA AND GOOD[5] used the zwitterionic buffer Tricine for the isolation of a variety of chloroplast fragments for a comparative study of Hill reaction activities. The conditions of their digitonin incubation were somewhat different to those originally employed[2], in that a lower concentration of digitonin was used (0.1 rather than 0.5 %). The incubation used by IZAWA AND GOOD[5] was in Tricine with 0.4 M sucrose instead of in phosphate with 0.1 M KCl, and a preliminary high-speed spin was included to remove the excess digitonin before the differential centrifugation. They found that all the fractions isolated were active for ferricyanide reduction. This result was rather surprising, since the lack of O_2 evolution in the usual digitonin-prepared small fraction was one of the criteria which allowed its designation as a Photosystem 1 particle. Accordingly, we have investigated further digitonin fragmentation of chloroplasts in Tricine and other buffers.

This paper presents a brief survey of the chlorophyll composition and photochemical activities of fragments produced by digitonin incubation in several media. In Tricine and low-salt we have confirmed the results of IZAWA AND GOOD[5], namely, that all the fractions are active in ferricyanide reduction. Since this reaction might involve only Photosystem 2, with chloroplast fragments, activity for $NADP^+$ photoreduction was also examined, both with water as the electron donor (both Photosystem 1 and Photosystem 2 functioning) and with artificial donors such as ascorbate and ascorbate–DCIP (Photosystem 1 only). All of the low-salt–Tricine fractions catalyzed both reactions. Moreover, the fractions possessed the same chlorophyll composition as did the chloroplasts. Under these conditions then, there has been no separation of the photosystems. With digitonin incubation in high-salt–Tricine, however, the results are quite different and parallel to those obtained with phosphate or Tris, that is, there is a fractionation of the two photosystems. Electron microscopy shows different membrane conditions for the chloroplast under conditions of low or high ionic strength with Tricine.

MATERIALS AND METHODS

Chloroplasts were isolated from market spinach in 0.05 M phosphate buffer (pH 7.2) containing 0.3 M sucrose and 0.01 M KCl and were incubated with 0.5 % digitonin for 30 min at 0° as described in an earlier paper[2]. The digitonin incubation was carried out in a variety of media: 0.05 M phosphate, (pH 7.2), 0.05 M Tricine–NaOH (pH 7.4), or 0.05 M Tris (pH, 7.8) containing various salt or sucrose concentrations. The fractions were collected by differential centrifugation at the following

speeds: 1000 × *g* for 10 min, 10000 × *g* for 30 min, 50000 × *g* for 30 min and 144000 × *g* for 1 h. The pellets were resuspended in the same medium without digitonin present. Chlorophyll concentration was determined spectrophotometrically in 80% acetone using the equations of ARNON[6].

Absorption spectra were recorded on a Cary Model 14R spectrophotometer fitted with the scattered-light transmission attachment. Fluorescence spectra were recorded on the Cary Model 14R spectrophotometer fitted with a fluorescence attachment. Excitation light of 436 mμ was provided by a Bausch and Lomb monochromator with slits at 2.0/2.0 mm (in/out). The samples in dilute phosphate buffer in a cell with 1.56 mm light path had an absorbance of 0.1 at 436 mμ. The fluorescence spectra were uncorrected.

Photoreductions of ferricyanide and $NADP^+$ were determined by recording the absorbance change at 420 mμ (ferricyanide) and 340 mμ ($NADP^+$) in a modified Bausch and Lomb spectrophotometer. A saturating intensity of red light (Corning filter, 2403) of 15×10^6 ergs·cm^{-2}·sec^{-1} was used. The temperature was 25°.

RESULTS

Distribution of chlorophyll a and chlorophyll b

Table I shows the ratio of chlorophyll *a* to chlorophyll *b* in the various centrifugal fractions obtained from spinach chloroplasts which had been incubated with 0.5% digitonin for 30 min at 0° in a variety of media. With the zwitterionic buffer Tricine and 0.01 M KCl, there was no major difference in the chlorophyll *a* and chlorophyll *b* content in the various centrifugal fractions (Table I, line 1). This is in marked contrast to the fractions produced by the usual procedure of digitonin incubation in 0.05 M phosphate and 0.01 M KCl, in which the large particles were enriched in chlorophyll *b* (chl *a*/chl *b* ratio of 2.2) and the small particles were enriched in chlorophyll *a* (chl *a*/chl *b* ratio of 4.9) as compared to chloroplasts (chl *a*/chl *b* ratio of 2.8). The effect obtained with the low-salt–Tricine was completely reproducible. Furthermore, the

TABLE I

CHLOROPHYLL CONTENT OF CHLOROPLAST FRACTIONS

Chloroplasts were incubated with 0.5% digitonin for 30 min at 0° in 0.05 M Tricine–NaOH (pH 7.4), 0.05 M phosphate (pH 7.2) or 0.05 M Tris (pH 7.8) with the addition of salt or sucrose as shown below. The mixtures were separated into fractions by differential centrifugation.

Incubation medium	*Chlorophyll a/chlorophyll b*				
*Fractions**	*1*	*10*	*50*	*144*	*144 S*
Tricine + 0.01 M KCl	3.00	2.83	2.90	3.04	3.12
Phosphate + 0.01 M KCl	2.72	2.22	3.62	4.90	3.40
Tricine + 0.4 M sucrose	2.56	2.46	2.90	2.78	3.20
Phosphate + 0.4 M sucrose	2.64	2.39	3.22	4.68	3.34
Tricine + 0.01 M KCl + 0.4 M sucrose	2.78	2.62	2.82	2.82	2.62
Phosphate + 0.01 M KCl + 0.4 M sucrose	2.72	2.31	4.04	6.34	4.70
Tricine + 0.15 M KCl	2.14	2.72	4.50	5.74	—
Phosphate + 0.05 M KCl	2.13	2.81	5.02	5.72	—
Tris + 0.35 M NaCl	2.52	2.40	4.00	4.98	3.20

* The fractions are designated according to maximum centrifugal force in $g \times 10^3$ used.

chloroplasts did not require resuspension in the low-salt–Tricine prior to the digitonin incubation. The same pattern of unchanged chl *a*/chl *b* ratios was obtained whether the chloroplasts were isolated and washed in 0.05 M phosphate and 0.01 M KCl, or were isolated in the Tricine medium. Incubation in Tricine and sucrose with or without salt gave the same result, whereas, these additions to the phosphate system resulted in the usual fractionation of chlorophyll *a* and chlorophyll *b* between the derived particles (Table I, lines 3 to 6).

A different result was obtained, however, if the digitonin incubation was carried out in high-salt–Tricine. Addition of 0.15 M KCl to the Tricine system gave a buffer with about the same ionic strength as the normal 0.05 M phosphate and 0.01 M KCl. Under these conditions, there was a difference in the chl *a*/chl *b* ratios in the various fractions, giving the same general pattern observed in phosphate (Table I, lines 7 and 8). As expected, digitonin incubation in phosphate with high-salt or in Tris with 0.35 M NaCl gave the usual chlorophyll fractionation among the particles. It has been shown previously that digitonin incubation in Tris gave the same results as in phosphate[2].

It is apparent that digitonin in low-salt–Tricine buffer gives fractions whose chlorophyll composition was essentially unaltered from that of chloroplasts. In this respect the fragments resemble those resulting from sonication. Some investigators, *e.g.* PARK AND PON[7] and KATOH AND SAN PIETRO[8] report that sonicated fragments possess the same chlorophyll composition as chloroplasts. Others including GROSS, SHEFNER AND BECKER[9], BIEHL[10] and E. GROSS AND L. PACKER (personal communication) report a slight range of difference, with the larger particles possessing slightly more chlorophyll *b* and the smaller particles slightly more chlorophyll *a*, than the chloroplasts. It has been our experience in Canberra that sonicated fragments do show these slight differences. It should be pointed out, that such slight differences were found also with the low-salt–Tricine fractions, although it may not be apparent from the limited set of values presented in Table I. The average chl *a*/chl *b* ratio

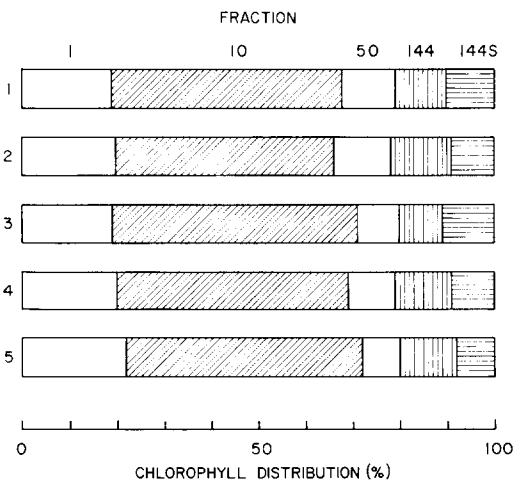

Fig. 1. The distribution of chlorophyll in the various centrifugal fractions prepared by digitonin incubation of chloroplasts in the media as listed. (1) low-salt–Tricine; (2) low-salt–Tricine *plus* 0.4 M sucrose; (3) high-salt–Tricine; (4) phosphate; (5) Tris buffer.

(calculated from all the experiments performed) for the 10000 × *g* fraction was 2.81 and for the 144000 × *g* fraction was 3.21, in contrast to a chloroplast average of 2.98.

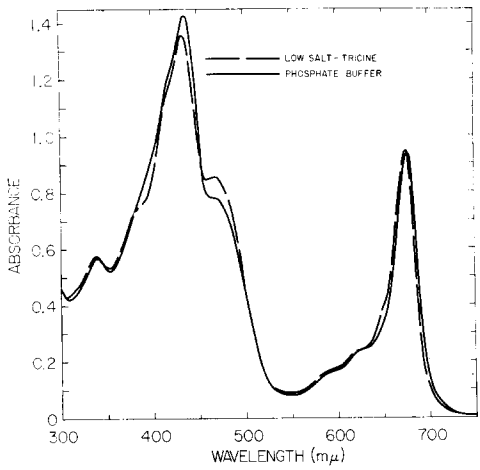

Fig. 2. Absorption spectra of the 144000 × *g* fractions prepared from digitonin incubation in 0.05 M phosphate (pH 7.2) *plus* 0.01 M KCl (designated 144 P) and 0.05 M Tricine (pH 7.4) *plus* 0.01 M KCl (designated 144 T). The fractions were suspended in the above media to give a total chlorophyll content of 16 μg/ml (phosphate) and 19 μg/ml (Tricine).

Regardless of the incubation medium used (with the exception of those media which included sucrose) the distribution of total chlorophyll in the various fractions was approximately the same (Fig. 1). Thus, in the low-salt–Tricine media where the chl *a*/chl *b* ratios were unaltered in the various fractions, the distribution of total chlorophyll was the same as in the media where the chl *a*/chl *b* ratios increased with decreasing size of the fragments.

The absorption spectra of the 144000 × *g* fractions isolated after digitonin incubation in 0.05 M Tricine *plus* 0.01 M KCl (144 T) and in 0.05 M phosphate *plus* 0.01 M KCl (144 P) are shown in Fig. 2. The chlorophyll *b* maxima *in vivo* at 650 mμ in the red and 475 mμ in the Soret region are much higher in the 144 T compared to 144 P. The chl *a*/chl *b* ratio of 144 T was 2.81 and of 144 P was 4.91. Absorption in the region around 700 mμ was higher in 144 P than in 144 T, which suggests that 144 T contains less of P 700, the reaction centre chlorophyll of Photosystem 1 (see ref. 11). The 144 P particle is enriched in P 700 and contains one P 700 per 200 chlorophylls instead of one P 700 for some 430 chlorophylls as in chloroplasts[12]. A difference spectrum of 144 T *minus* 144 P normalized at the red maxima shows that 144 T contains more chlorophyll *b* and more of the lower wavelength chlorophyll *a* than 144 P, which contains more of the longer wavelength forms of chlorophyll *a*. The absorption spectrum of 144 T is similar to that of chloroplasts.

Another parameter which relates to chlorophyll differences *in vivo* is fluorescence. Incubation of chloroplasts with digitonin in phosphate medium gave large particles with an increased fluorescence yield at 20°, while the small particles were much less fluorescent[13]. There is evidence to suggest that the fluorescence band at 684 mμ arise primarily from chlorophyll *a* 672, that is from Photosystem 2, and the

far-red fluorescence band at 730 mμ originates primarily from Photosystem I (see refs. 13 and 14). As would be expected, a comparison of the fluorescence of 144 P and 144 T revealed differences in the ratios of these two bands. 144 T was more fluorescent than 144 P, and the ratio of 735 mμ to 684 mμ fluorescence was lower for the 144 T particles (Table II). Thus both the absorption and fluorescence properties of 144 T indicate that its chlorophyll composition *in vivo* is similar to the chloroplast. The 144 P particle, however, has a higher relative content of chlorophyll *a* 684 and P 700, consistent with its designation as a Photosystem I particle.

TABLE II

COMPARISON OF FLUORESCENCE INTENSITIES OF 144000 × *g* FRACTIONS

Fractions 144 T and 144 P were prepared as in Fig. 2.

Fraction	*Temp.*	*Fluorescence*			*chl a/chl b*
		684 mμ	*735 mμ*	*735 mμ/ 684 mμ*	
144 T	20	35.0	5.4	0.16	2.81
144 T	−196	90	110	1.22	
144 P	20	26.5	9.0	0.34	4.91
144 P	−196	60	115.5	1.93	

Photochemical activities

The Hill reaction activities for the various fractions obtained after digitonin incubation in a variety of media were measured by ferricyanide reduction. This reaction requires a functional Photosystem 2. Typical activities are shown in Fig. 3 by the unshaded bars. The rates for chloroplasts were about 50 μmoles of ferricyanide reduced per mg chlorophyll per h. The activities of the fractions were normally higher

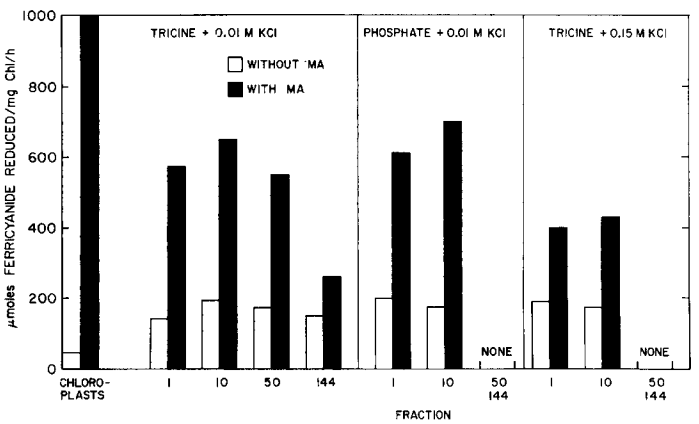

Fig. 3. Comparison of Hill reaction activities of the various centrifugal fractions prepared by incubation of chloroplasts with digitonin in 0.05 M Tricine (pH 7.4) *plus* 0.01 M KCl or 0.05 M phosphate (pH 7.2) *plus* 0.01 M KCl respectively. The reaction mixture contained in 3 ml, chloroplast fraction containing 30 μg chlorophyll and (in μmoles): Tricine–NaOH (pH 7.2), 150; sucrose, 500; $K_3Fe(CN)_6$, 1.5; methylamine hydrochloride (MA) (if added), 150.

than this. A striking difference between the fractions was noted. All of the fractions from the low-salt–Tricine incubations were capable of ferricyanide reduction. With the phosphate system, however, only the larger particles were able to reduce ferricyanide. A quite different result was obtained when the digitonin incubation was in the high-salt–Tricine. The smaller fractions were completely inactive in this case.

When electron transport is uncoupled from the accompanying photophosphorylation, the rate is considerably enhanced. The shaded bars in Fig. 3 represent the ferricyanide reduction in the presence of methylamine, an effective uncoupler for ferricyanide reduction. With chloroplasts, an unusually high value of 1600 μmoles ferricyanide reduced per mg chlorophyll per h was obtained for chloroplasts prepared during one month. However, with other batches of spinach the activity was routinely 800 to 1000 μmoles per mg chlorophyll per h. One serious difficulty in the measurement of uncoupled ferricyanide reduction is the rapid decline of activity with time after isolation of the chloroplast. Chloroplasts which were capable of 1600 μmoles ferricyanide reduced per mg chlorophyll per h immediately after resuspension, had declined to 75 % of that value within 30 min. Thereafter, the decline was slower. After 3.5 h (the time taken for the digitonin incubation and subsequent centrifugations) the activity was about 60 % of the original value. In addition to this, digitonin itself causes a decrease in the rate of uncoupled electron transport. Concomitant O_2 evolution linked to ferricyanide reduction was measured by a macro-O_2 electrode assembly. The theoretical amount of 4 moles of ferricyanide per mole of O_2 evolved was found for all of the fractions.

Chloroplasts are capable of $NADP^+$ photoreduction with water as the electron donor, in a typical Hill reaction in which both Photosystem 1 and Photosystem 2 are involved. With an alternate source of electrons, such as DCIP and ascorbate, it is possible to measure the activity of Photosystem 1 only[15]. With water as the electron donor, the rates of $NADP^+$ reduction paralleled those of ferricyanide reduction. Only in the case of the low-salt–Tricine media were all the fractions capable of $NADP^+$

TABLE III

REQUIREMENTS FOR $NADP^+$ PHOTOREDUCTION WITH 144000 × *g* FRACTIONS

144 T and 144 P fractions were prepared as in Fig. 2. The reaction mixture contained in 3 ml, chloroplast fraction with a chlorophyll content of 22 μg and (in μmoles): phosphate (pH 7.0), 150; sucrose, 300; $MgCl_2$, 40; $NADP^+$, 0.5; (if added) DCIP, 0.1; ascorbate, 8. Saturating amount of ferredoxin and ferredoxin–$NADP^+$ reductase were added together with 4 mμmoles of plastocyanin. The reactions were carried out anaerobically.

	μmoles $NADP^+$/mg chlorophyll per h	
	144 T	*144 P*
Complete reaction mixture	200	333
Reaction mixture without		
DCIP	187	130
DCIP and ascorbate	98	0
Ferredoxin	0	0
Plastocyanin	30	101
$NADP^+$ reductase	130	80

reduction. The values obtained were rather low and declined with illumination. With the fractions isolated from the phosphate, or high-salt–Tricine media, only the 1000 and 10000 $\times$ *g* fractions gave $NADP^+$ reduction and the smaller fractions were completely inactive. The values were lower than with whole chloroplasts. It has been shown that the percentage loss of activity of chloroplasts in digitonin is much greater for $NADP^+$ reduction than for coupled ferricyanide or DCIP reduction[2], and this may partly account for the low values of $NADP^+$ reduction of the fractions.

The component requirements for 144000 $\times$ *g* fractions are compared in Table III. Only 144 T was capable of $NADP^+$ photoreduction with no electron donor. This rate was sustained for only half a minute and then began to decline. With the addition of ascorbate, or ascorbate and DCIP, the rates were uniform until the $NADP^+$ concentration became limiting. A further distinction between the fractions was observed. Ascorbate was almost as effective an electron donor as ascorbate and DCIP with the 144 T fraction, but with 144 P both ascorbate and DCIP were required for maximal activity, as had been demonstrated earlier[2].

DAVENPORT[16] reported that the requirement for DCIP as an intermediate electron carrier between ascorbate disappeared on sonication of chloroplasts. KATOH AND SAN PIETRO[8] found the absence of DCIP resulted in a slightly decreased activity with small fragments obtained by sonication of chloroplasts. VERNON, SHAW AND KE[17] reported much higher rates in the presence of both dye and ascorbate than with ascorbate alone for the small particle P-D10 obtained with Triton X-100.

Both 144 T and 144 P required the addition of ferredoxin, plastocyanin and ferredoxin–$NADP^+$ reductase. The rates of $NADP^+$ photoreduction previously reported by us[1,2] with 144 P were low because the carrier, plastocyanin was not added to the reaction mixture. Ample evidence that this protein is needed has been provided by many investigators[17–20].

Chloroplast structure

The structure of chloroplasts suspended in phosphate buffer or in Tricine buffer with the different regimes of ionic strength are shown in Fig. 4. These electron micrographs show a clear distinction between the two types of chloroplast material which give the different responses when fragmented with digitonin. Figs. 4A and B, which represent chloroplasts suspended in high-salt–Tricine and in phosphate buffer, clearly show the usual membrane configuration of chloroplasts, with the appearance of normal grana. Both chloroplast preparations contain some unbroken as well as broken chloroplasts, but the membrane structure is retained even in the broken chloroplasts. This structure corresponds to the condition which allows digitonin to produce fragments of differing chlorophyll ratios and photochemical activities.

Fig. 4C shows a chloroplast suspension in low-salt-Tricine. In this case the membrane system is different from that observed in phosphate or in high-salt-Tricine buffers. Under the conditions of low-salt-Tricine, the whole membrane system is rather ill-defined, and exhibits somewhat different fixing properties. This structure correspond to the conditions under which digitonin produces fragments which have the same chlorophyll composition as the chloroplasts, and all of the fragments possess both Photosystem 1 and Photosystem 2 activities.

Lyophilized chloroplast fractions

The experiments were repeated with lyophilized chloroplasts which were prepared by isolation in 0.05 M phosphate (pH 7.2) containing 0.3 M sucrose and 0.01 M KCl, once washed and lyophilized. Lyophilized chloroplasts were suspended in the various media shown in Table I by gentle homogenization with a tissue grinder and left for 10 min prior to the digitonin incubation. The chlorophyll composition and the photochemical activities of the fractions isolated, paralleled the results shown in Table I, *viz.*, all the fractions from the low-salt–Tricine media had unaltered chl *a*/chl *b* ratios and ferricyanide Hill activity, while the fractions from the other media showed the usual range of chlorophyll *a* to chlorophyll *b* ratios and a separation of ferricyanide Hill activity. The distribution of total chlorophyll between the various fractions was virtually unchanged regardless of the media employed, which was similar to the data reported in Fig. 1. Also, the ability to sustain $NADP^+$ reduction with water as the electron donor was rather poor with lyophilized preparations. This reaction, which is even lower than might be expected in intact chloroplasts, at least compared to electron transport with ferricyanide or DCIP, appears to be extremely sensitive to any disruption of the chloroplasts (*e.g.* treatment with digitonin[2] or sonication[8]). However, the rugged nature of Photosystem 1 was again demonstrated with lyophilized fractions. The activity for $NADP^+$ reduction with ascorbate and DCIP was unaltered; the values and component requirements for the lyophilized small fractions were identical to fresh fractions. This Photosystem 1 reaction withstands digitonin and Triton treatments[17], sonication[8] or aging of the chloroplasts[15] also.

DISCUSSION

The fractions obtained from fragmentation of spinach chloroplasts by several methods fall into two distinct categories. (i) Fractions which have chlorophyll composition similar to the original chloroplasts and which are all capable of both Photosystem 1 and Photosystem 2 photoreactions. These are derived from chloroplasts with markedly altered membrane systems as seen by electron microscopy. (ii) Fractions with altered chlorophyll *a* to chlorophyll *b* ratios and which may exhibit only one of the photosystem reactions. These are derived from chloroplasts which have the usual membrane system with tight, well-ordered grana. It is of interest that the simple spectrophotometric determination of chlorophyll *a* and chlorophyll *b* is extremely useful as a first clue to whether or not any fractionation of the photosystems has been achieved.

Fragments produced by digitonin incubation in low-salt–Tricine or sonication of chloroplasts belong to category (i). These treatments produce particles of varying size but their chlorophyll composition is not much different from that of the original chloroplasts. In both cases the ability to reduce ferricyanide or to photoreduce $NADP^+$ are retained by all of the fractions obtained by centrifugation although some inactivation is observed. Fragmentation itself and digitonin appear to cause a certain amount of uncoupling. Both the small sonicated fragments of KATOH AND SAN PIETRO[8] and the small, low-salt–Tricine fractions, are very active for Photosystem 1 mediated $NADP^+$ photoreduction. In contrast to the small fractions of category (ii), however, ascorbate alone was almost as effective an electron donor as ascorbate

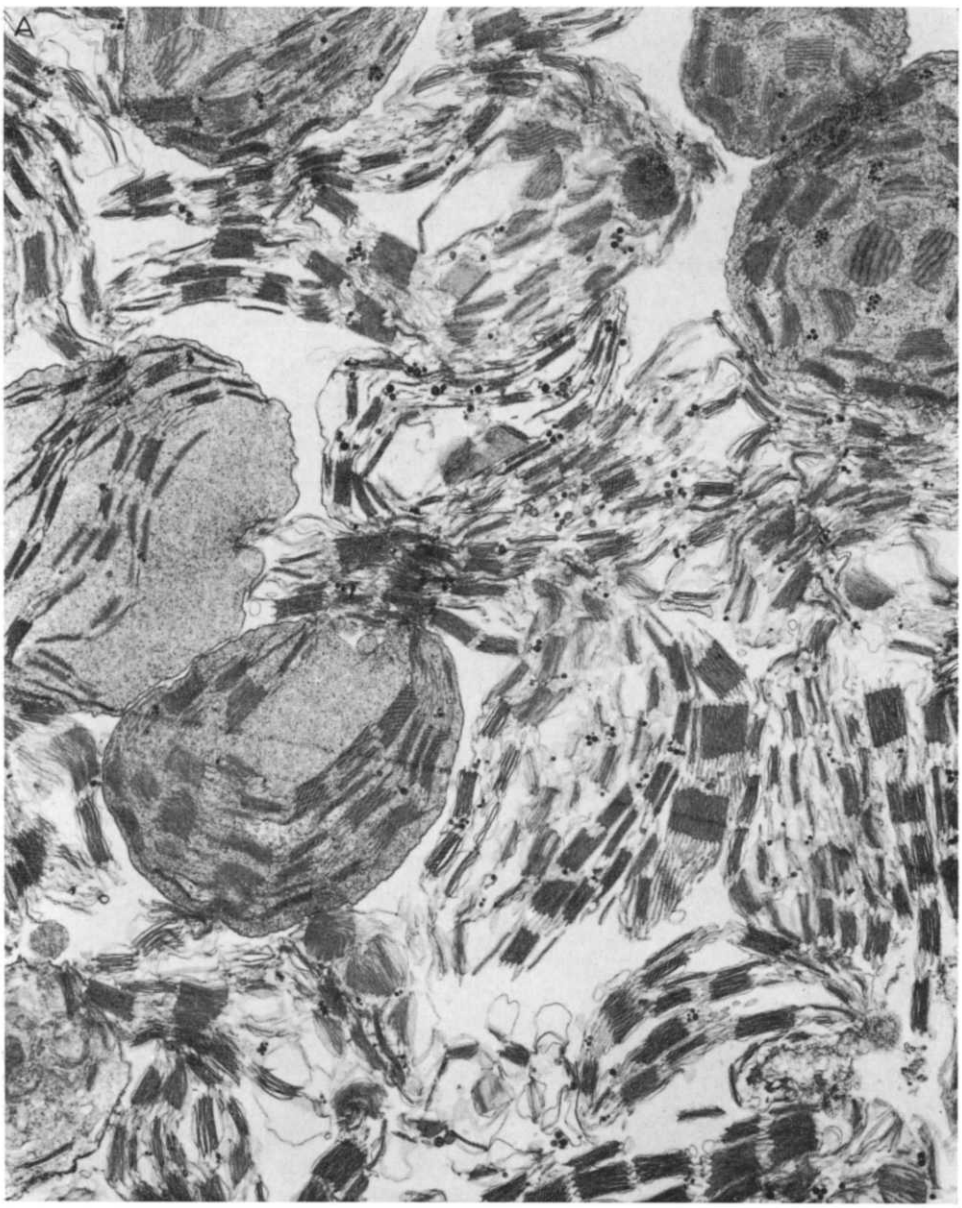

Fig. 4. Electron micrographs of spinach chloroplasts suspended in media of different composition and ionic strength. The chloroplasts were prepared as usual, washed once and resuspended in 2.0 ml of the following media: (A) 0.05 M Tricine (pH 7.4) *plus* 0.15 M KCl. (B) 0.05 M phosphate (pH 7.2), 0.01 M KCl and 0.3 M sucrose. (C) 0.05 M Tricine (pH 7.4) *plus* 0.01 M KCl. The chlorophyll contents varied from 0.6 to 0.8 mg/2 ml. Chloroplasts were fixed for 1 h in 2.5% glutaraldehyde, washed in five changes of buffer at 10-min intervals, and post-fixed in 1.0% OsO_4 for 2 h. A 0.05 M sodium phosphate buffer at pH 7.0 was used for all steps. Following fixation in OsO_4, the chloroplasts were dehydrated in an ethyl alcohol series followed by three changes in 100% acetone. Embedding was in an Eponaraldite epoxy resin mixture.

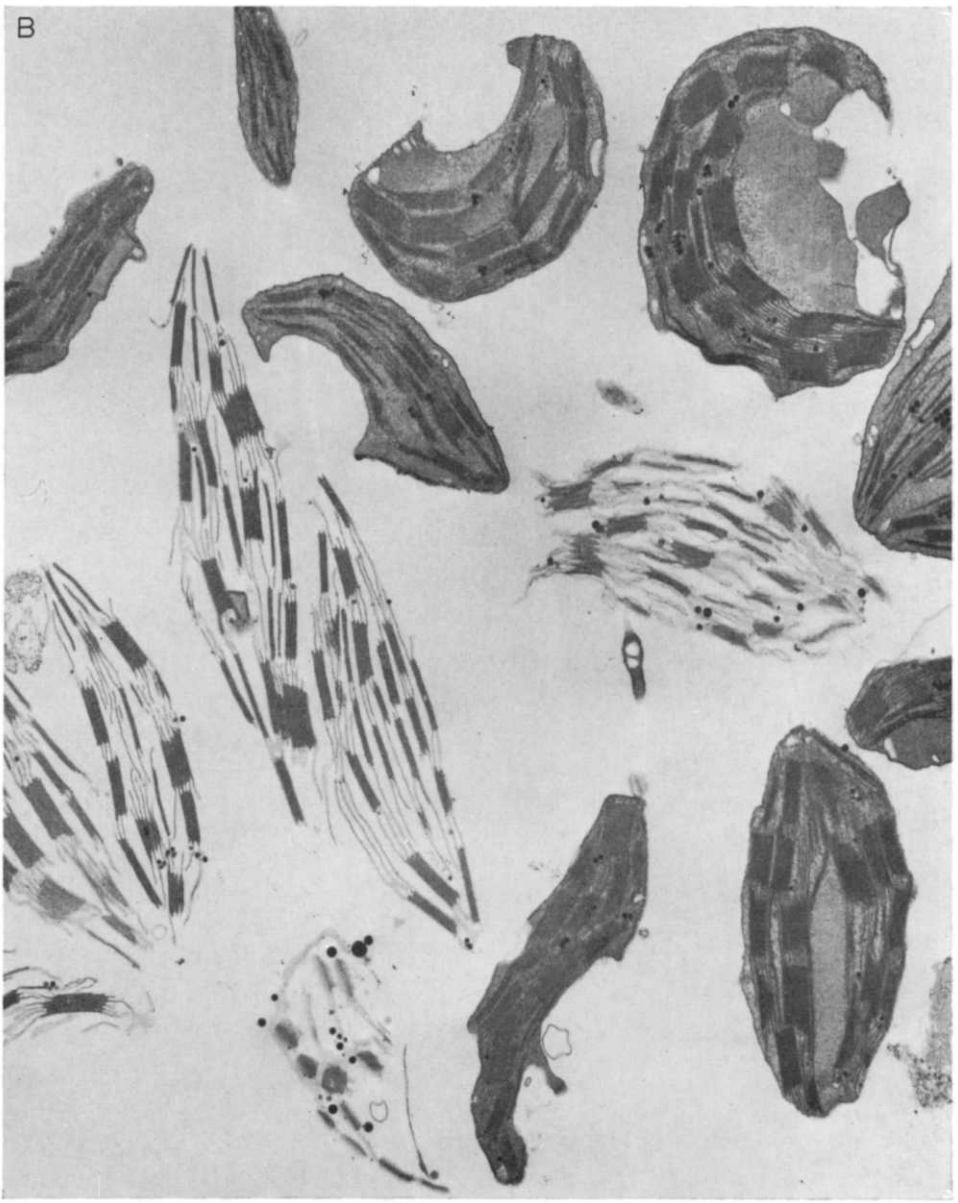

Fig. 4 B.

and DCIP. These two treatments appear to have cleaved the chloroplast lamellae into fragments in a random fashion so that no units of only Photosystem 1, or Photosystem 2 are obtained.

Fragments of category (ii) are produced by the following treatments: digitonin incubation in high-salt–Tricine systems, in phosphate, or in Tris media. In these cases fractions with different chlorophyll composition relative to that of the chloroplast

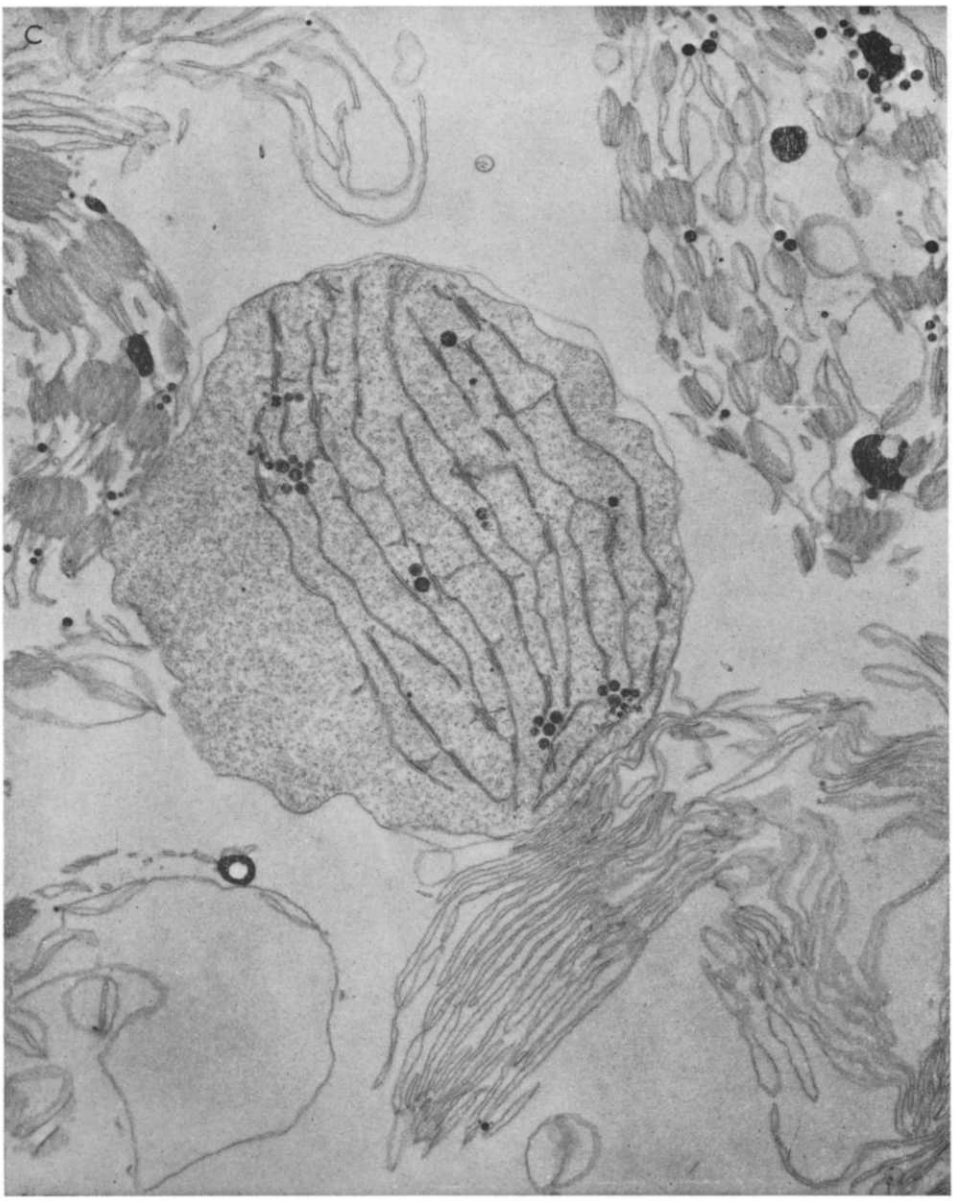

Fig. 4 C.

are obtained. The large particles are enriched in chlorophyll *b* and manganese and are capable of O_2 evolution. By considering the photochemical activities[2] and the amount of P 700 in the fraction[11] they appear to consist of three parts of Photosystem 2 and one part of Photosystem 1. The small particles produced by these methods display all the properties of functional Photosystem 1 particles with no signs of any Photosystem 2 activity[1,2]. They possess a higher relative content of

chlorophyll *a* 684 and P 700 and a lower relative content of chlorophyll *a* 672 and chlorophyll *b* 650. Triton X-100 treatment also provides different particles as shown by VERNON, SHAW AND KE[17]. The large particle does not retain O_2-evolving ability or the ESR signal associated with Photosystem 2, but its composition is similar to the digitonin large particle. The small Triton particle, P-D10, is very active for Photosystem 1 activity[17]. Sodium dodecyl sulphate treatment yields two distinct pigment proteins which are separable on polyacrylamide gels as shown by OGAWA, OBATO AND SHIBATA[21]. They were inferred to be the pigment proteins responsible for Photosystem 1 and Photosystem 2 activity. All of the treatments in category (ii) result in partial separation of the two photosystems. It is well to remember though, that knowledge of all of the components of the electron transport chain is still very limited and while these explanations are useful working hypotheses, they are perhaps rather naive.

The question arises as to why the action of digitonin should be so different in low-salt–Tricine. While there is no definitive explanation at this point, possible answers might lie in one of two directions, or possibly a combination of both. One could envisage that the digitonin itself might undergo some changes in physical properties which prevents it from functioning in the usual way. Digitonin is a non-ionic detergent, with a hydrophobic steroid linked to a hydrophilic, 5-carbon sugar, glycosidic chain. In aqueous solutions, digitonin forms a large colloidal complex, *i.e.* a micelle with a minimum molecular weight of 75000 (see refs. 22 and 23). The micelle, therefore, consists of a least 60 molecules of digitonin packed with the steroid portions to the centre of the cluster surrounded by the polar tails exposed to the medium. The effect of ionic strength on digitonin micelle formation has not been reported. The presence of salts should not exert as profound an influence on non-ionic detergents as they do on cationic or anionic detergents. However, the environment around the polar tails might be somewhat different with varying ionic strength.

Another explanation, which we prefer, is that the chloroplast's lamellar structure itself is altered and the digitonin functions in another way in this different environment. The forces holding together chloroplast lamellae may be drastically altered. In a remarkable series of electron micrographs, IZAWA AND GOOD[24] showed that chloroplasts in the low-salt–Tricine exhibited quite a different structure than those seen *in situ*. These chloroplasts possessed none of the ordered lamellar stacks, *i.e.* grana normally seen in conventionally isolated chloroplasts. Instead the chloroplast lamellae under their conditions consisted of continuous, or almost continuous strands which appeared to be rather loosely held together at the edges of the chloroplast. The distance between lamellar strands in this case was very large indeed. Even more astounding, they showed that on addition of salts to the Tricine buffer, the strands appeared to become cemented together, sometimes in a random fashion and sometimes in passable imitation of reconstituted grana.

In our hands, chloroplasts in low-salt-Tricine show markedly different structural properties from those isolated in phosphate or high-salt-Tricine. We have not always observed the transition from the grana structure to the long membrane strands observed by IZAWA AND GOOD[24], but see a general breakdown of the grana structure to a less well-defined membrane system. However, the present data show that a rather significant change takes place in the biochemical and physical properties of the chloroplast lamellar system in low-salt-Tricine buffer. An interesting implication

arises from these studies; it is possible that digitonin will not be able to release Photosystem I particles from chloroplasts which do not possess grana, *in vivo*.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the contribution of Dr. HILTON MOLLENHAUER who carried out the electron microscopic investigation of the chloroplast preparations. One of us (J.M.A.) is grateful for discussions with Dr. BERGER MAYNE and Prof. GOOD (M.S.U.); also for the cheerful welcome of ELWOOD SHAW and DOROTHY LIMBACH, with whom I shared laboratory space.

REFERENCES

1 N. K. BOARDMAN AND J. M. ANDERSON, *Nature*, 203 (1964) 166.
2 J. M. ANDERSON AND N. K. BOARDMAN, *Biochim. Biophys. Acta*, 112 (1966) 403.
3 J. M. ANDERSON, N. K. BOARDMAN AND D. J. DAVID, *Biochem. Biophys. Res. Commun.*, 17 (1964) 685.
4 N. E. GOOD, G. D. WINGET, W. WINTER, T. N. CONNOLLY, S. IZAWA AND R. M. M. SINGH, *Biochemistry*, 5 (1966) 467.
5 S. IZAWA AND N. E. GOOD, *Biochim. Biophys. Acta*, 109 (1965) 372.
6 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
7 R. B. PARK AND N. G. PON, *J. Mol. Biol.*, 3 (1961) 1.
8 S. KATOH AND A. SAN PIETRO, *J. Biol. Chem.*, 241 (1966) 3575.
9 J. A. GROSS, A. M. SHEFNER AND M. J. BECKER, *Nature*, 209 (1966) 615.
10 B. BIEHL, *Z. Naturforsch*, 21b (1966) 501.
11 B. KOK, *Biochim. Biophys. Acta*, 48 (1961) 527.
12 J. M. ANDERSON, D. C. FORK AND J. AMESZ, *Biochem. Biophys. Res. Commun.*, 23 (1966) 874.
13 N. K. BOARDMAN, S. W. THORNE AND J. M. ANDERSON, *Proc. Natl. Acad. Sci.*, 56 (1966) 586.
14 GOVINDJEE AND L. YANG, *J. Gen. Physiol.*, 49 (1966) 763.
15 L. P. VERNON AND W. S. ZAUGG, *J. Biol. Chem.*, 235 (1960) 2728.
16 H. E. DAVENPORT, in A. SAN PIETRO, *Non-heme iron proteins, role in energy conversion*, Antioch, Yellow Springs, 1965, p. 115.
17 L. P. VERNON, E. SHAW AND B. KE, *J. Biol. Chem.*, 241 (1966) 4101.
18 S. KATOH AND A. TAKAMIYA, *Biochim. Biophys. Acta*, 99 (1965) 156.
19 J. S. C. WESSELS, *Biochim. Biophys. Acta*, 109 (1965) 614.
20 A. TREBST AND E. ELSTNER, *Z. Naturforsch.*, 20b (1965) 925.
21 T. OGAWA, F. OBATO AND K. SHIBATA, *Biochim. Biophys. Acta*, 112 (1966) 223.
22 E. L. SMITH AND E. G. PICKELS, *Proc. Natl. Acad. Sci.*, 26 (1940) 272.
23 R. HUBBARD, *J. Gen. Physiol.*, 87 (1954) 381.
24 S. IZAWA AND N. E. GOOD, *Plant Physiol.*, 41 (1966) 544.