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THE EFFECT OF HYDROLYTIC ENZYMES ON THE PHOTOSYNTHETIC EFFICIENCY AND MORPHOLOGY OF CHLOROPLASTS

Eichanan S. Bamberger and Roderic B. Park

January 1966

THE EFFECT OF HYDROLYTIC ENZYMES ON THE PHOTOSYNTHETIC EFFICIENCY AND MORPHOLOGY OF CHLOROPLASTS[1,2]

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Evidence that the entire photosynthetic process is located in higher plant chloroplasts (3) was soon followed by assignment of partial reactions of photosynthesis to chloroplast structures. These studies showed that the light reactions and associated electron transport reactions leading from water oxidation to ferredoxin reduction are located in the internal membranes or thylakoids of the chloroplast, whereas the CO_2 fixation and other synthetic enzymes are associated with the stroma portions of the chloroplasts (14,26). Thylakoids, when observed in heavy metal shadowed preparations

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by electron microscopy, demonstrate a particulate structure (14,25). This structure, 150 to 200 Å in diameter, making up the membrane, can exist in highly ordered arrays (17) and was termed in this laboratory "quantasome" (6,15). Aggregates of small numbers of quantasomes yield Hill reaction rates comparable to those of whole chloroplasts (14). The chemical composition of these structures has been a subject of several studies from this laboratory (10,16,17). The membranes are about 50% protein and 50% lipid.

To further evaluate the relationship between energy conversion processes and thylakoid structure, one would like to employ methods which alter in specific ways both the physiological and morphological characteristics of the membranes. Detergents have often been used in this way (19). However, recent studies have indicated that hydrolytic enzymes may also serve as a useful tool for this kind of investigation (7,11,24).

This paper reports the effects of two hydrolitic enzymes on the structure and function of isolated thylakoids. The enzymes used were a crude preparation from runner bean leaves (containing galactolipases and galactosidases) and the proteolytic enzyme pronase. Physiological activity was assayed by measuring Hill reaction efficiencies (21,22) extrapolated to zero light intensities. This measure of activity produces considerably more information as to the hydrolytic enzyme effect than does measurement of saturation rate. Structural changes were followed both spectrally and morphologically. Morphological changes within the membranes were followed by use of the freeze-etch technique (12). A detailed examination of untreated thylakoids using this technique has been carried out by Branton and Park (5).

MATERIALS AND METHODS

Preparation of Chloroplasts. Intact chloroplasts and chloroplast fragments were prepared from spinach leaves obtained from local stores. In general, 125 g of leaves were homogenized for 30 seconds in 250 ml grinding medium (0.35 M NaCl, 0.02 M Tris-HCl, pH = 7.4, and 0.01 M EDTA) in a Waring Blendor. The homogenate was strained through 8 layers of cheesecloth and centrifuged according to Park and Pon (14) to isolate once washed chloroplasts or chloroplast fragments. For the spectrophotometric measurements, concentrated buffered aqueous suspensions of chloroplast fragments were diluted with deionized water in order to achieve a final absorbance between 0.3 and 1.0 at 678 mμ. Chlorophyll was determined by the method of Arnon (2).

Enzyme Preparation from Runner Bean Leaves. The runner bean leaf enzyme preparation was prepared according to the ammonium sulfate precipitation procedure of Sastry and Kates (24). Fresh primary leaves (50 g) from 3- to 4-week old runner bean plants were homogenized with 100 ml of deionized water for 2 minutes in a Waring Blendor. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 15,000 x g for 20 minutes at 0°. Seventy ml of the supernatant were centrifuged further at 100,000 x g for 60 minutes at 0°. The resulting supernatant (70 ml) was dialyzed overnight against 10 liters 0.05 M phosphate buffer (pH = 7.0). Solid ammonium sulfate was added to the dialysate. The fraction precipitating up to 70% saturation with ammonium sulfate was centrifuged at 15,000 x g for 20 minutes and dissolved in 0.05 M phosphate buffer (pH = 7.0), dialyzed overnight against 10 liters of the same buffer and diluted with buffer to 28 ml; protein concentration, 9.24 mg/ml. This preparation (RBE) was kept at -15°.

Enzymatic Degradation of Chloroplasts and Chloroplast Fragments. Concentrated buffered solution of chloroplasts and chloroplast membranes were treated with the RBE preparation or pronase (*Streptomyces griseus* proteose; B grade; 45,000 P.U.K. units/g; B grade; Calbiochem. P.U.K.: Unit of proteolitic activity of pronase measured by the Kaken Chemical Company.) The reactions were run at 30° under nitrogen and terminated by washing the chloroplasts 3 times in the same media in which they were initially suspended. Samples were diluted for the spectrophotometric assays.

Release of free galactose by RBE from the galactolipid fraction of chloroplast membranes was assayed with galactose oxidase (Galactostat, Worthington Biochemical Corp., Freehold, New Jersey). Protein nitrogen was determined by the Kjeldahl method except that $SeOCl_2$ was used as a catalyst instead of $HgSO_4$.

Hill Reaction. The DCPIP (2,6-dichlorophenolindophenol) Hill reaction activity was assayed by the spectrophotometric method described by Sauer and Biggins (21). Experiments were carried out using the Cary Model 14 spectrophotometer, modified so that the absorbance of the Hill oxidant could be monitored continuously at 580 mμ (E_{580} = 19,800 l-mole^{-1} at pH = 7.4) (1) while the sample was being irradiated from the side with longer wavelength light (650 mμ).

The cuvettes used had 4 clear sides and a rectangular internal cross-section 3 mm x 10 mm. The monochromatic measuring beam passed through the 10 mm path length, and the actinic light incident at right angles on the sample cuvette traversed a 3 mm light path in the reaction mixture. Light intensity was measured with a calibrated silicon solar cell (Hoffman, Type 120 cg) placed at the site of the cuvette; corrections for reflection losses were applied.

A stock solution of DCPIP (K & K Laboratories, Jamaica, N. Y.) was always prepared freshly on the day of the experiment. The stock DCPIP solution was prepared in 0.1 dilution of the grinding media, pH = 7.4, and DCPIP concentration was confirmed by absorbance measurements after the undissolved dye had been removed by centrifugation. The reaction mixture contained in μmoles/ml: Tris-HCl, pH = 7.4, 20; NaCl, 350; EDTA, 10; methylamine hydrochloride, 10; DCPIP, 0.03 to 0.05. Once washed chloroplasts were added in the dark at the start of each measurement to give an absorbance of 0.3 to 1.0 at 678 mμ (10 mm path). Each experiment was performed using 2 ml of reaction mixture which was divided between the sample cuvette and the reference cuvette in the spectrophotometer.

Optical Rotatory Dispersion (ORD) of Chloroplast Fragments. Chloroplast fragments were prepared from sonicated spinach chloroplasts according to the method of Park and Pon (14). The supernatant of a fraction sedimenting at 100,000 x g (20 min) in a Spinco Model L ultracentrifuge was recentrifuged, and the final precipitate at 150,000 x g (60 min) was resuspended in 0.02 M Tris-HCl buffer, pH = 7.4. ORD measurements were made at room temperature using a Cary Model 60 spectropolarimeter with a cell of 1.0 cm path length. Absorption and difference spectra of chloroplast fragments were measured by using a Cary Model 14 spectrophotometer.

Freeze-etching Technique. The Bendix-Balzers freeze-etch device and methods described by Moor (12) were used in these experiments. Once washed chloroplasts were suspended in 10% or 20% glycerol in the grinding media and then centrifuged at 2000 g for 10 minutes. Samples from the precipitate were frozen and etched for 2 minutes after which carbon platinum replica was deposited. Micrographs were taken with a Siemens Elmiskop I.

RESULTS

Effect of Enzymes on Membrane Efficiency. The effect of pronase and the RBE preparation on the biological activity of spinach chloroplasts was estimated by measuring the quantum requirement (einsteins/equivalent) for the DCPIP Hill reaction of treated chloroplasts. As shown in Figure 1, the quantum requirement of the control experiment varied between 2 and 5. The presence of 10^{-3} M methylamine in the reaction mixture greatly reduces the dependence of the quantum requirement on light intensity (22). However, pretreatment of the chloroplasts with either pronase or the RBE preparation yields a linear increase in intensity dependence of the quantum requirement. This increase in quantum requirement is dependent on the enzyme concentration and period of incubation.

Effect of the Hydrolytic Enzymes on Chemical Composition. The runner bean leaves enzyme preparation contains specific enzymes which catalyze the hydrolysis of monogalactosyldilinolenin and digalactosyldilinolenin to the corresponding free linolenic acid, free galactose and glycerol (24). The rate of release of free galactose is therefore a measure of the rate of complete hydrolysis of galactolipids by the RBE preparation.

When chloroplast fragments were incubated with the RBE preparation (Fig. 2) the rate of release of galactose was about the same at pH 7.0 (optimum pH for the activity of the monogalactosyldilinolenin enzyme) and at pH 5.6 (optimum pH for the activity of the digalactosyldilinolenin enzyme). After 4 hours of incubation at 30°, 50% of the galactose of the chloroplasts' galactolipid fraction was released into solution as free galactose. Only half of this amount was actually released by the added RBE enzyme. The second half was apparently released by endogenous enzymes present in the chloroplast preparation (see control curve, Fig. 2). The control level of galactose is initially higher than the RBE treated

material. This observation is discussed later.

The data in Table I show that in the RBE treated chloroplast preparation the ratio of chlorophyll to nitrogen decreased (1.37) with respect to the control preparation (1.78). In the pronase treated chloroplast preparation the chlorophyll to nitrogen ratio increased (2.80) over the control value. This increase represents the amount of protein digested away by pronase from the chloroplasts.

The Effect of the Hydrolytic Enzymes on Spectral Properties. The effect of pronase on the chlorophyll red absorption maximum is shown in Figure 3. The 678.5 ± 0.5 mμ peak of an aqueous chloroplast fragments suspension undergoes a blue shift after 30 minutes' incubation of the chloroplast fragments with pronase. The magnitude of the shift was not further increased by longer incubation times or by higher enzyme concentrations. The blue shift of the red absorption maximum was not observed in chloroplast fragments treated with the RBE preparation.

The complex ORD spectrum of suspension of chloroplast fragments found in our experiments (Fig. 4) is similar to the one observed by Sauer (20). Cotton effects are present throughout the visible and ultra-violet regions which are regions of strong absorption of chlorophyll *a* and *b* and carotenoids. The pronounced trough at 700 mμ in the ORD spectra of the non-treated chloroplast fragments is completely missing from the ORD spectra of the pronase treated chloroplast fragments; the rest of the ORD spectra is similar to the control spectra. The ORD spectra of the RBE treated chloroplast fragments is essentially the same as the control spectra.

Effects of the Hydrolytic Enzymes on the Morphology of Chloroplast Membranes. Branton and Park (5) have described the freeze-etch morphology of untreated *in vivo* and isolated spinach chloroplasts. A model of a

single thylakoid derived from these studies is presented in Figure 5. The model consists of a smooth inner layer on which there are large (150 A) particles. This surface is designated as B. Surface A is a view seen only within grana stacks. It represents the outer portion of the thylakoid and consists of the large particles in an embedding matrix. Surface C is the matching surface for surface B. In these experiments we have attempted to find how the thylakoid membrane, as described in Figure 5, is modified by pronase and RBE.

Control membranes were treated in the same way as the samples except that the hydrolytic enzymes were excluded from the reaction mixture. A freeze-etch picture of such a control is presented in Figure 6. Surfaces A, B and C are marked with C occurring in a lower thylakoid. Figures 7 and 8 are typical examples of membranes treated with pronase and RBE respectively. The data may be summarized as follows: Both enzyme treatments lead to decrease both in number and size of the large (150 A) particles seen on surface B in Figure 6. Moor (12) has suggested that these particles correspond to the quantasome. In pronase treated preparations (see Fig. 7) surface B is still evident, but the number of particles attached to it are less and the particles themselves are reduced in size when compared to the control. Also, the A surface is more particulate than in the control. In RBE treated preparations (see Fig. 8) the smooth layer of surface B is no longer observed and appears to be completely removed. The view in Figure 8 is representative and probably corresponds to modified C and A surfaces.

Some experiments were done in which chloroplasts were isolated from

leaves and fixed for 45 minutes in 5% glutaraldehyde. Control chloroplasts appeared similar to the pronase treated preparations in these experiments. The RBE treated chloroplasts from fixed leaves were greatly modified, however. The membrane no longer broke along discrete boundaries and appeared as layers of particles suspended in the glycerol freezing solution. The smooth layer of surface B in most RBE treated plastids was completely absent. A comparison of control and RBE treated glutaraldehyde fixed chloroplasts is shown in Figure 9 (control) and Figure 10 (RBE treated).

DISCUSSION

Galactolipids constitute a major class of lipids in photosynthetic tissues (4). In red clover they account for at least 23% of the total lipids (27) and were found to be high in linolenic acid. Chloroplast membranes are 50% protein and 50% lipids, and the contribution of the galactolipids (in moles per quantasome) is about 40% (17). The structure of galactolipids isolated from runner bean leaves was determined (23) and shown to be: 2,3-di-O-linolenoyl-1-O-β-D-galactopyranosyl-D-glycerol and 2,3-di-O-linolenoyl-1-O-(6-O-α-D-galactopyranosyl-D-galactopyranosyl)-D-glycerol.

Sastry and Kates reported (24) that runner bean leaves are rich in enzymes necessary for the complete breakdown of galactolipids to fatty acids, glycerol and galactose. Linolenic acid released by these enzymes was responsible for the changes in biochemical properties of spinach chloroplasts aged at pH = 6 or treated with runner bean leaf homogenates (11).

In confirmation of McCarty and Jagendorf (11) results, in our experiments the RBE preparation hydrolyzed about 25% of the galactolipids present in chloroplast fragments in a period of 4 hours (Fig. 2). The initial

concentration of free galactose in the supernatant of the control experiment (no RBE added) was higher than the initial concentration of free galactose found in the supernatant of the treated chloroplast membranes. This might be explained by assuming absorbance of free galactose by the added RBE preparation. Low concentration of RBE preparation yielded a linear increase in intensity dependence of the quantum requirement of the DCPIP Hill reaction by chloroplasts (Fig. 1). The order of magnitude of the quantum requirement at very low light intensities remains between 2 and 5 in all three concentrations of RBE preparation added. This suggests that one of the dark reactions in electron transport becomes rate limiting after short treatment of chloroplasts with the RBE preparation. The dependence of the quantum requirement in light intensity was observed after 30 minutes' treatment of the chloroplast with the RBE preparation. In this time interval only a very small amount of galactolipids were completely hydrolyzed (Fig. 2), suggesting that the physiological activity was altered before large chemical changes took place. In contrast to the effect of pronase, the absorbance spectra of chloroplast suspensions treated with RBE was not changed and only slight changes in the ORD spectra were observed (Fig. 4).

The *Streptomyces griseus* protease-pronase has a very broad substrate specificity and is capable of hydrolyzing various kinds of peptide bonds in protein until the majority of amino acids constructing the protein are liberated as individual amino acids. As a result, the extent of hydrolysis of proteins by this protease was estimated to reach 70 to 90% (13). The increase of the chlorophyll to nitrogen ratio (Table I) over the control value of 1.78 to 2.80 for the pronase treated chloroplasts

represents the loss of about 40* protein after 3 hours of incubation. The efficiency of the DCPIP Hill reaction is affected after only 10 minutes of incubation of the chloroplasts with pronase (Fig. 1). After treatment with pronase, the quantum requirement dependence on light intensity increases. As with the case of chloroplasts treated with RBE, the quantum requirement at very low light intensities after 10 and 30 minutes of pronase treatment remains in the order of 2 to 5. However, after one hour's incubation with pronase (1.1 and 5.5 P.U.K. units) the extrapolated quantum requirement at zero light intensity is 10 and 30 respectively. This change in intercept indicates that the efficiency of the chloroplasts is decreased even at the lowest light intensities. Thus uncoupling of light absorption from electron transport or partial loss of a component of the electron transport pathway must be invoked. After longer incubation periods with pronase no detectable DCPIP reduction was observed.

Treatment of chloroplast membranes with pronase caused a slight blue shift of the absorbance peak at 678 mμ. A difference spectra between the control membranes and the pronase treated membranes shows a peak at 681 mμ and a satellite peak at 645 mμ. By integration of the area under the absorbance spectra and the difference spectra, the amount of bleaching was calculated at 5%. Pronase apparently causes a destruction of a long wavelength component of <u>in vivo</u> chlorophyll <u>a</u> (8). Otherwise this component contributes to the peak at 678 mμ (Fig. 3). The disappearance of a single cotton effect (centered around 682 mμ) in the ORD spectra of pronase treated membranes (Fig. 4) is in agreement with the postulation of a destruction of a long wave length chlorophyll <u>a</u> component by pronase. Evidence for the coexistence of two or more forms of chlorophyll <u>a</u> in green plants was given by Krasnovsky and his coworkers (8,9).

The fact that the underlying layer of surface B is quite smooth and that it disappears upon treatment with the RBE preparation suggests that the underlying layer is a galactolipid. The fact that the chlorophyll absorption and ORD spectra are not greatly affected by removal of this layer would indicate that chlorophyll is not associated with it. Chlorophyll is apparently associated with the large 150 A particles and their embedding matrix which make up the A surface. This is supported by large spectral shifts and ORD changes brought about by pronase treatment. The large particles lying on the smooth galactolipid surface appear to contain both lipid and protein since they are reduced in size but not removed by pronase treatment. The A surface appears to contain both lipid and protein since it is attacked by both enzyme preparations. The thylakoid as shown in Figure 5 appears to be built of a galactolipid layer on which large particles (150 A) in an embedding matrix are located. Chlorophyll appears to be associated with the large particles and embedding matrix and not with the galactolipid layer. The large particles protruding from the embedding matrix may correspond to the quantasome seen in shadowed preparations (18). The small particle layer on the interior of the thylakoid of *Pharbitis*, reported by Park (18), may correspond to the smooth underlying layer of the B surface which has undergone micelle formation during drying.

SUMMARY

1. Both lipase from runner beans and a protease (pronase) initially cause increased intensity dependence of the DCPIP Hill reaction of spinach chloroplasts. This is followed by an increase in the extrapolated zero intensity quantum requirement.

2. Pronase treatment of the chloroplasts causes rapid changes in absorption and ORD spectra, whereas the effect of lipase treatment is much less pronounced.

3. Long treatments of the thylakoids with the lipase and protease cause unique morphological changes within the membrane. These changes are used to assign chemical compositions to some of the structures revealed by freeze-etching.

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Table I. The Effect of 3-Hour Incubation of Once Washed Chloroplasts with RBE or with Pronase on the Chlorophyll to Nitrogen Ratio

	mg nitrogen / aliquot	mg chlorophyll / aliquot	mg chlorophyll / mg nitrogen
Control	.100	0.178	1.78
+5.5 P.U.K. Units Pronase	.075	.210	2.80
+0.069 mg RBE	.115	.158	1.37

LEGENDS

Figure 1 Quantum requirement for the DCPIP Hill reaction at 650 mμ by spinach chloroplasts treated with RBE (left) or pronase (right).

Figure 2. Release of free D-galactose from spinach chloroplast membranes treated with RBE.

Figure 3. Lower: Spinach chloroplast membranes absorption spectra from 600 mμ to 720 mμ (upper curve), and absorption spectra of pronase treated membranes (lower curve).
Upper: Difference spectrum control minus pronase treated membranes.

Figure 4. ORD spectrum of buffered aqueous suspensions of sonicated spinach chloroplasts: ----- control; ——— pronase treated (1.1 P.U.K. units for 60 min); — — — RBE treated (0.023 mg for 60 min).

Figure 5. A model for the morphology of spinach chloroplast membranes based on freeze-etch studies of Branton and Park (5).

Figure 6. Freeze-etched control thylokoid x 90,000. Surfaces A, B and C correspond to the model in Figure 5.

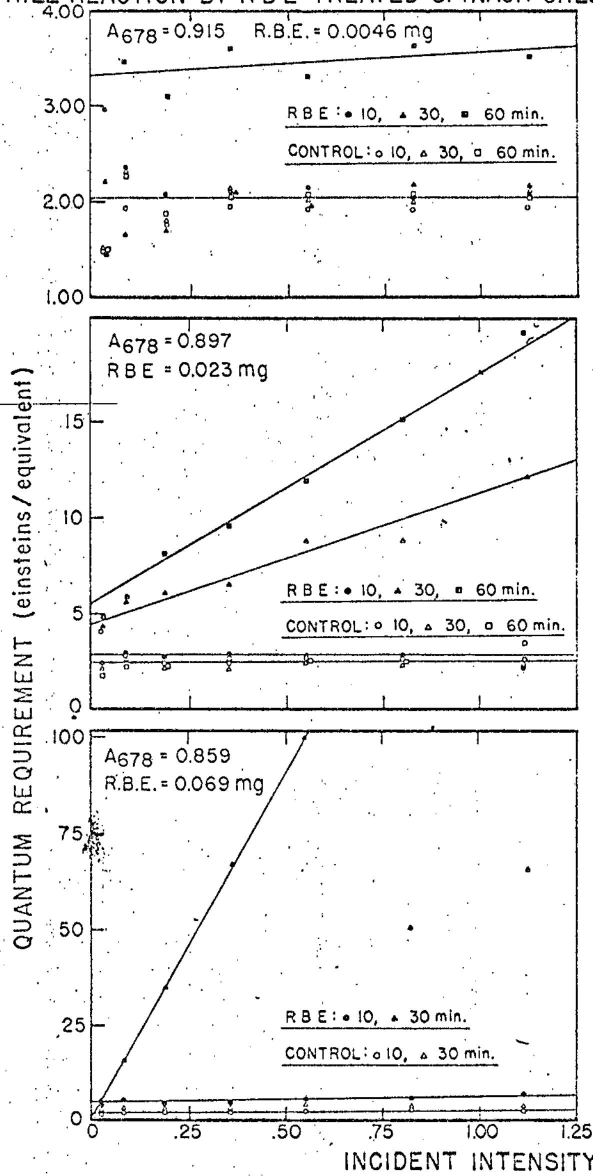
Figure 7. Freeze-etched pronase treated thylakoids x 90,000.

Figure 8. Freeze-etched RBE treated thylakoids x 90,000.

Figure 9. Freeze-etched chloroplast from 5% glutaraldehyde fixed spinach leaves x 90,000.

LEGENDS (Cont.)

Figure 10. Freeze-etched chloroplast from 5% glutaraldehyde fixed spinach leaves after RBE treatment 90,000x.



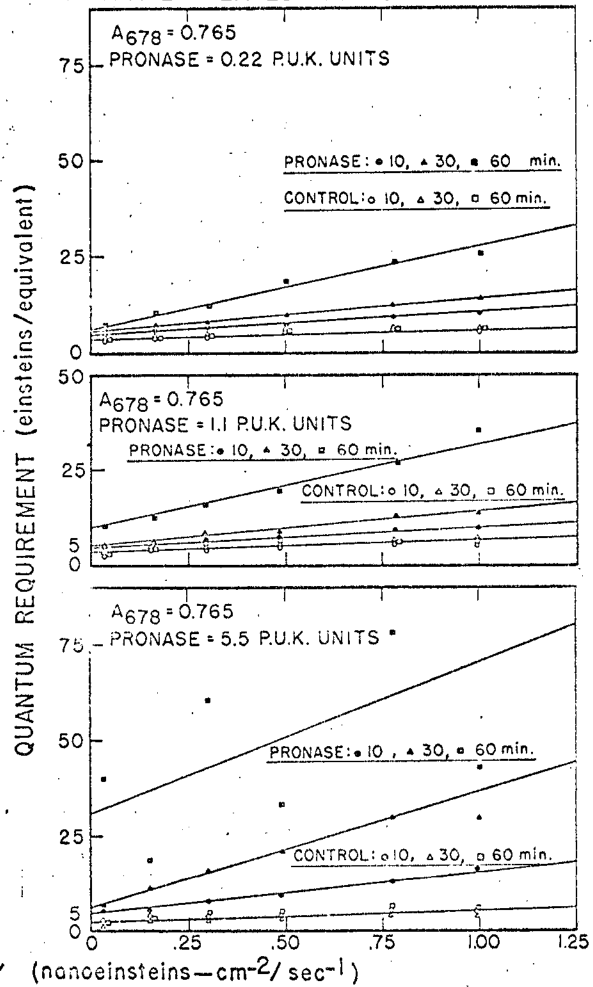


Fig. 1

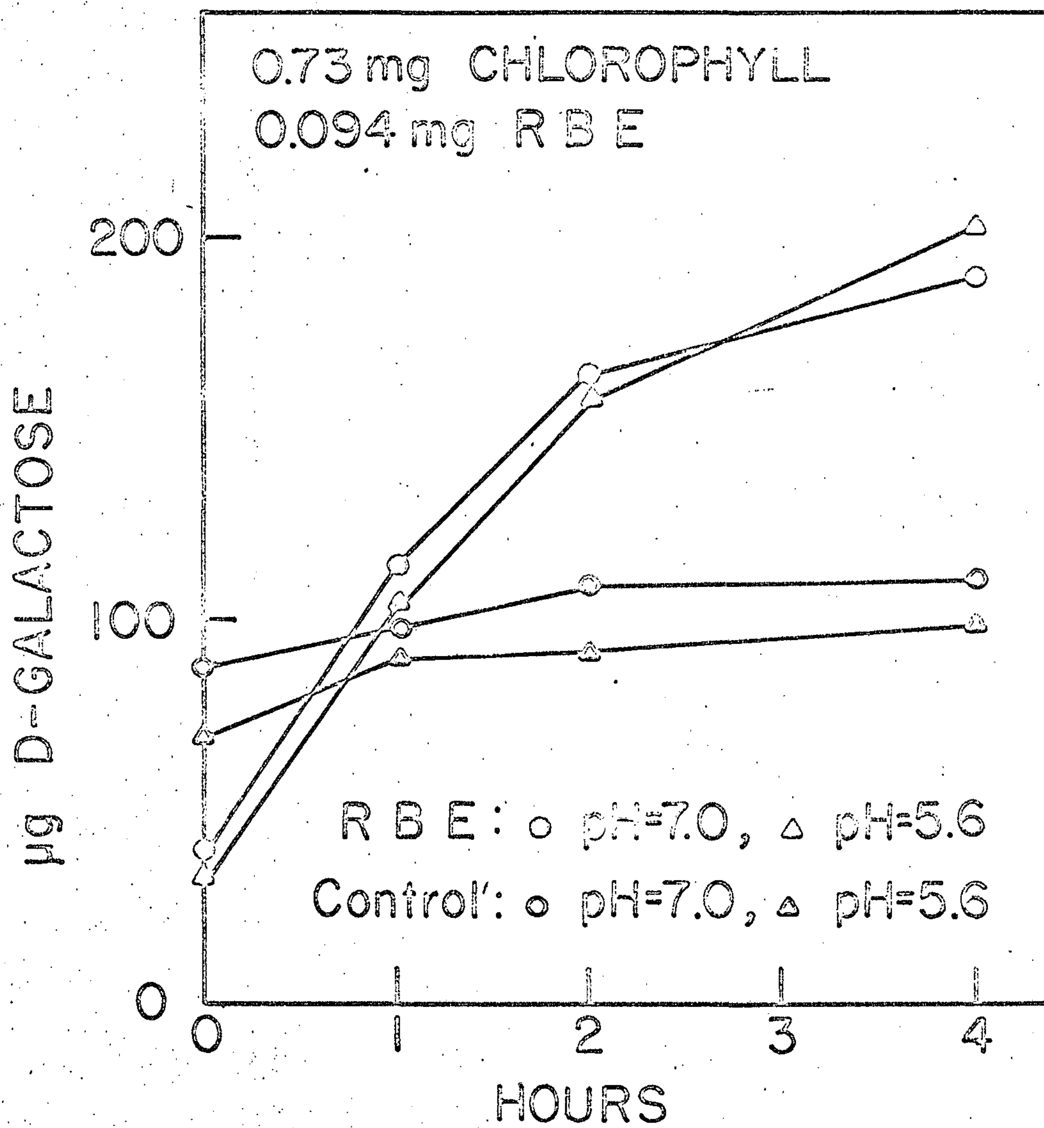


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Fig. 2

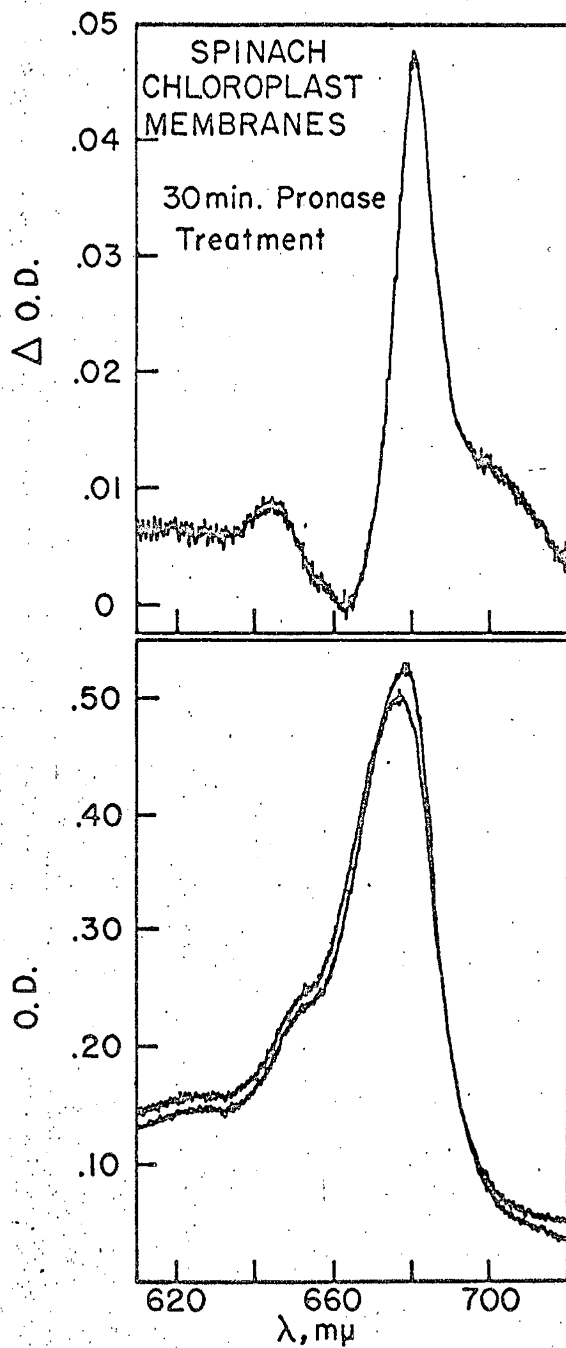

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Fig. 3

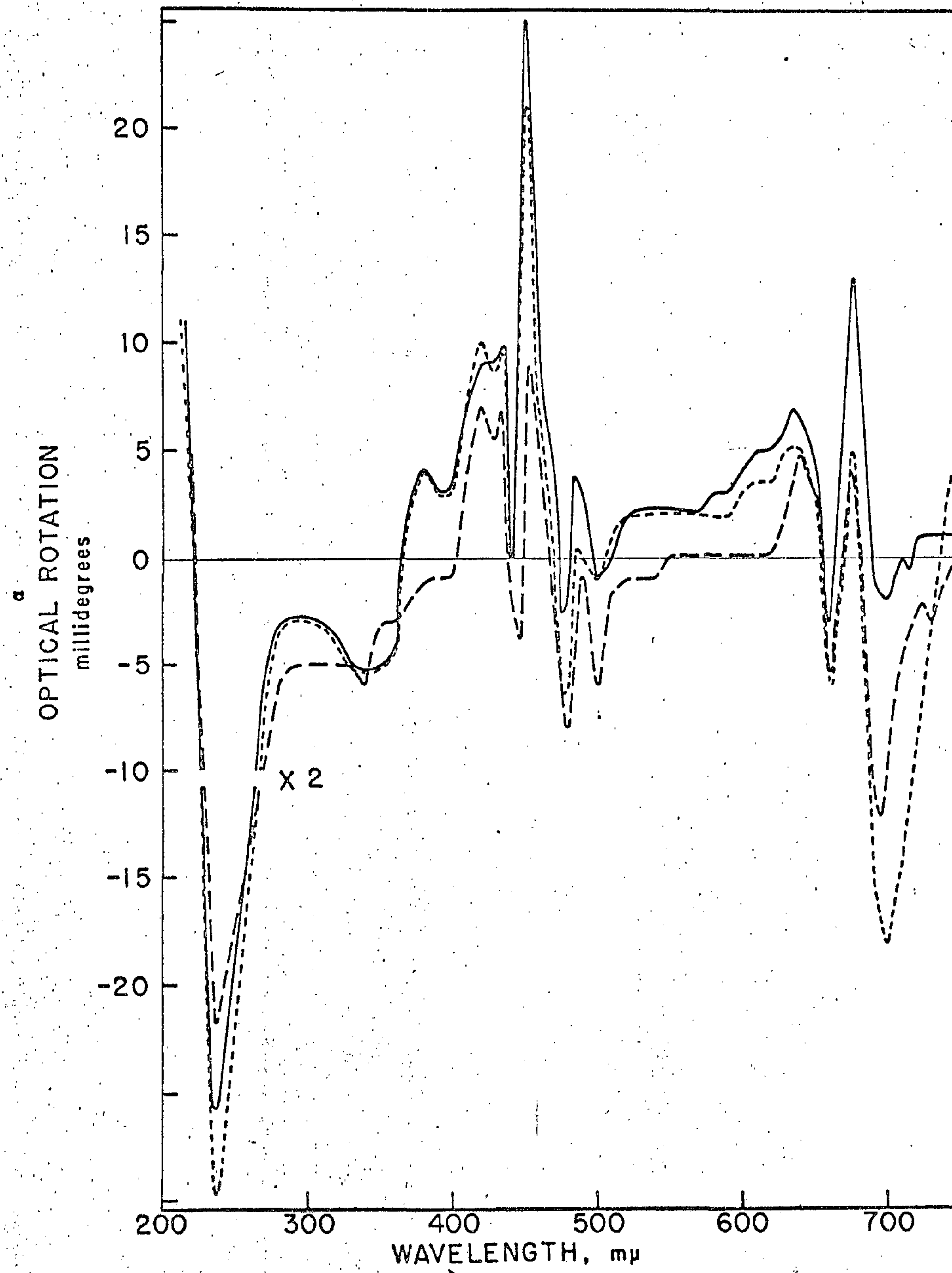

Fig. 4

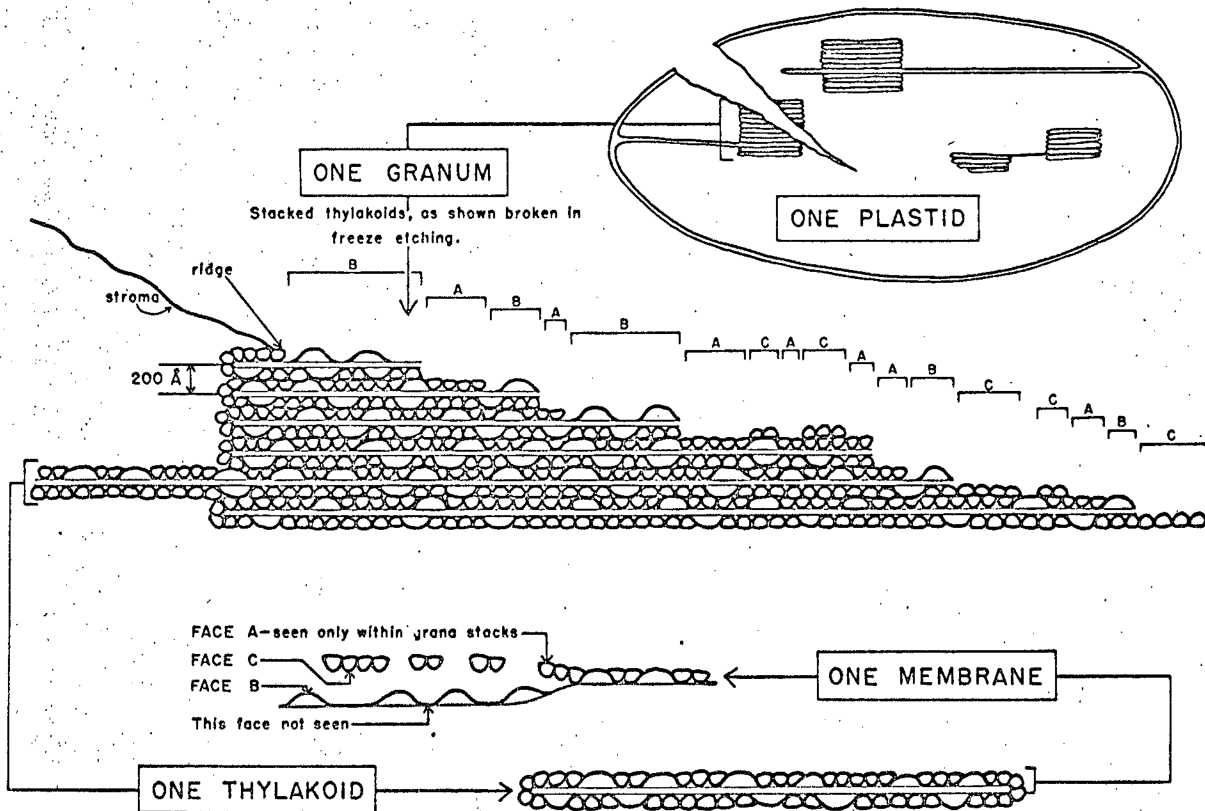

Fig. 5

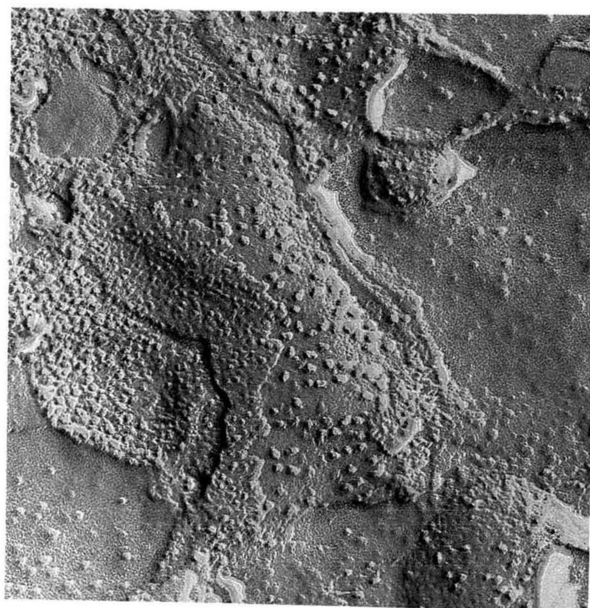

Fig. 6

Fig. 7

LRL

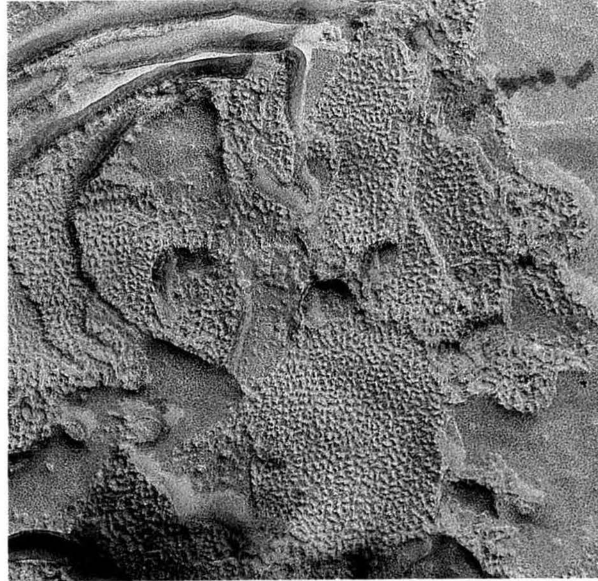

Fig. 8

Fig. 9

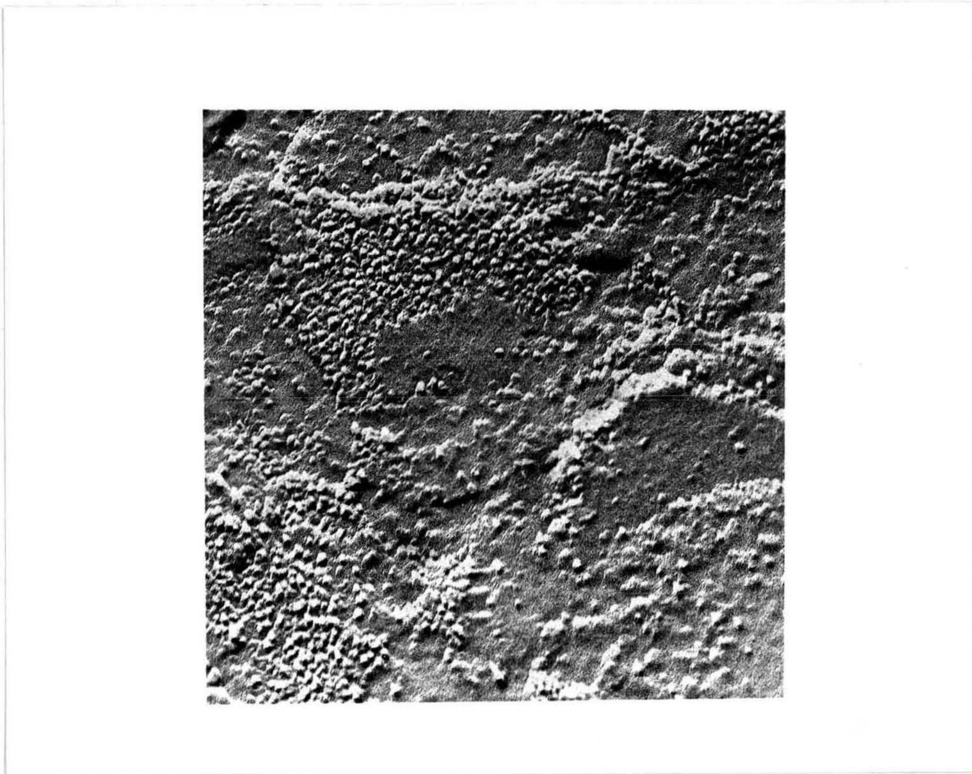

Fig. 10

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