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## MACROCOMPLEXES OF PHOTOACTIVE MEMBRANES IN APOLAR MEDIA: THE CHLOROPLAST ATPase

BY

**P. KERNEN\*, A. DARSZON\*\* & R.J. STRASSER\***

### ABSTRACT

Depending on the organic solvent used different amounts of spinach chloroplast proteins could be extracted as protein-phospholipid complexes into an organic phase. More than 50% of the protein from this organic phase sedimented at low g-value. The dried macrocomplexes from this heavy fraction were rehydrated to form liposomes which displayed comparable ATPase activities as the enzyme in chloroplasts. Chlb was enriched in this latter fraction and the protein/chlorophyll ratio was markedly enhanced. This approach may facilitate the study of chlorophyll-containing light-harvesting complexes. The chloroplast ATPase activity could be measured directly in an apolar media (detergent-octane-hexanol). This activity was 25% of that in an all water media and showed a pronounced dependence on the water content. This low water system may help to elucidate aspects of the catalytic process and may allow a deeper insight into the activation process which is achieved under very different conditions in all water systems.

### RÉSUMÉ

L'extraction de différentes quantités de protéines du chloroplaste de l'épinard dans une phase organique sous forme de complexes protéines-phospholipides est dépendante du solvant utilisé. Plus de 50% des protéines de la phase organique sédimentent à des basses vitesses de centrifugation. Les macrocomplexes séchés de cette fraction lourde forment des liposomes après réhydratation et montrent une activité ATPasique identique à celle de l'enzyme dans les chloroplastes. Chlb est concentrée dans cette fraction et la proportion de protéine/chlorophylle est considérablement augmentée. Cette procédure facilitera probablement des études sur les complexes d'antennes contenant de la Chl a et b. L'activité de l'ATPase du chloroplaste est mesurée directement dans un environnement apolaire (détergent - octane - hexanol). Cette activité vaut 25% de celle trouvée dans un milieu entièrement aqueux et montre une forte dépendance en fonction du contenu en eau. Ce système où l'eau est limitante devrait permettre d'étudier des aspects du processus catalytique et de mieux comprendre les phénomènes d'activation qui ont lieu dans différentes conditions dans un milieu totalement aqueux.

### INTRODUCTION

In the last years a general procedure has been established to transfer membrane proteins directly in an active form into organic apolar solvents as protein-lipid complexes. This technique was particularly successful with submitochondrial particles. The mitochondrial ATPase and isolated F<sub>1</sub>-ATPase were shown to be catalytically

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active and thermostable in organic solvents with a limited water content (GARZA-RAMOS *et al.*, 1989 and 1990). The mitochondrial cytochrome oxidase was also shown to be active in the solvent (ESCAMILLA *et al.*, 1989) or in liposomes formed after transfer through an organic solvent (AYALA *et al.*, 1986; BARRANCO *et al.*, 1981). Reconstitution of active enzymes from the inner mitochondrial membrane into liposomes after transfer into organic solvents was observed for succinate dehydrogenase, ATPase and cytochrome c oxidase (AYALA *et al.*, 1985).

On the other hand, rhodopsin-phospholipid complexes (DARSZON *et al.*, 1978 and 1979) and reaction center-phospholipid complexes (SCHÖNFELD *et al.*, 1980) were characterized spectroscopically in apolar environments with a low water content. Recently a number of photochemical studies have also been reported in reversed micelles such as photoelectron transfer reactions, photoenergy transfer, and photoionization (for a review see LUISI *et al.*, 1988 and references therein).

Inverted micellar systems as described above "may be considered to be a reasonably good model of thylakoid membranes" (LUISI *et al.*, 1988, p. 229), and may play a fundamental role, specially in plants, where the large amount of proteins and macrocomplexes make up to 50% (w/w) of the photosynthetic membranes (ALLEN *et al.*, 1972). Non-planar and non-bilayer regions or deformations in the thylakoid membrane induced by inverted micelles inside the bilayer (MURPHY, 1982; MARTINEK *et al.*, 1986) may determine the structural and functional state of such membranes. Direct observations of photosynthetic activities in water-in-oil microemulsions were reported very recently for plant cells (HOCHKOEPLER and LUISI, 1991) and for cyanobacteria (FAMIGLIETTI *et al.*). However, little is known about the behavior of proteins from photosynthetic active membranes in apolar media.

Since the formulation of the chemiosmotic hypothesis (MITCHELL, 1961) a rather detailed view of the coupling of the chloroplast ATP synthesis with the photoinduced electron flow has been established (HAMMES, 1983). Structural and kinetic studies of the multimeric ATPase complex, carrying out synthesis and hydrolysis, showed the highly dynamic behavior of this enzyme complex to intra- and extracellular alterations in reaction conditions (for a review see NELSON, 1982; FROMME and GRAEBER, 1990). This report provides an alternative approach to study membrane proteins and the dependence of their catalytic properties with respect to water in reverse micellar structures in apolar media where the water content can be controlled. The chloroplast ATPase, as an important component of the thylakoid membrane, is an interesting subject for low water containing systems, specially since its well known behavior in the chloroplast indicates a possible catalytic role for water in this enzyme.

## EXPERIMENTAL PROCEDURES

Broken (envelope-free) *chloroplasts* (class II, see HALL, 1972, for classification) were prepared in the cold from market spinach as follows: 80 ml of isolation buffer (50 mM tricine, 300 mM NaCl, 3 mM MgCl<sub>2</sub>, pH 7.5) were added to 18 g of fine cut leaves.

After homogenization at low and high speed (each 5 seconds) in a blender (WARING, Commercial Blender) the homogenate was filtered through 1 layer of nylon and 3 layers of gaze. The filtrate was centrifuged for 3 minutes at 3900 rpm (HETTICH, Universal/K2S). For the preparation of low-salt chloroplasts the pellet was washed with resuspension buffer (200 mM sorbitol, 20 mM tricine, 5 mM NaCl, pH 7.5). After centrifugation for 3 minutes at 3900 rpm the pellet was resuspended in 1.5 ml resuspension buffer. The resuspension buffer for high-salt chloroplasts additionally contained 10 mM  $MgCl_2$ . Aliquots of 500  $\mu$ l were stored in liquid nitrogen at a protein concentration of 30 to 40 mg/ml and a chlorophyll concentration of 3 to 4 mg/ml.

*Extraction* of chloroplast membrane proteins into organic solvents was essentially done as described elsewhere for submitochondrial particles (AYALA *et al.*, 1985). If not otherwise stated the ternary systems contained 10 mg of partially purified asolectin (KAGAWA and RACKER, 1971) per ml isooctane or hexane and 2 mg thylakoid protein were added. After sonication at 4°C during 5 minutes, 100  $\mu$ l of 1 M  $MgCl_2$  were added and vortexed during 2 minutes. After phase separation (HETTICH Rotofix II, ca. 1000 rpm, 4 min) the organic phase on top was collected. This phase contained the protein lipid complexes (PLC) and was separated into a light and a heavy fraction by centrifugation at 10000 rpm during 15 min (BECKMAN J-21, rotor JA-20, 4°C). Heavy liposomes were formed by adding 400  $\mu$ l 25 mM Tris pH 7.4 to the pellet, and light liposomes (or total liposomes) were obtained by evaporating the solvent from the supernatant (or the PLC phase) and adding of 400  $\mu$ l buffer (see DARZON and GOMEZ-PUYOU, 1982). Liposomes were formed by strong vortexing and were kept normally on ice. Protein and chlorophyll determinations were done according to LOWRY (1951) and ARNON (1949), respectively.

Chloroplasts were *transferred* into the reversed micellar phase in a quaternary system, called CHO, consisting of 0.2 M Hexadecyltrimethylammonium bromide (CTAB, cationic detergent), 9 parts n-octane and 1 part 1-hexanol (cosurfactant). If not otherwise stated the  $W_0$ -value ( $W_0=[H_2O]/[CTAB]$ ) in this apolar medium was 20, corresponding to 7.2% of water. At room temperature a typically transparent phase was obtained after short mixing under this conditions. For enzymatic activity the water phase contained 10 mM tricine pH 8.0, 30mM KAc, 12 mM  $MgAc_2$ , 12 mM Tris-ATP pH 8.0 and the desired amount of chloroplast proteins. The reaction in CHO was stopped with KOH and ADP formed was extracted with isobutanol:benzol 1:1 (saturated with  $H_2O$ ).

ADP formed either in a total water or in limited water system was taken as *ATPase activity* and measured spectroscopically following oxidation of NADH in an enzymatically coupled assay (PULLMAN *et al.*, 1960; COVES *et al.*, 1988). Assay conditions were 10 mM tricine pH 8.0, 30 mM KAc, 3 mM  $MgAc_2$ , 5 mM phosphoenolpyruvat and 0.22 mM NADH. Activity of thylakoids and liposomes was followed after addition of 3 mM Tris-ATP pH 8. All reactions were performed at 25°C.

*Absorption spectra* were taken with a PERKIN-ELMER lambda 3 UV/VIS spectrophotometer.

All *chemicals* used were of analytical grade. Solvents, phospholipids and detergent were obtained from FLUKA or SIGMA. Lactate dehydrogenase and pyruvate kinase were from BOEHRINGER.

## RESULTS

The efficiency of the ternary system (apolar solvent/surfactant(s)/water) to extract functional chloroplast membrane proteins was determined initially in liposomes made from the organic extracts. The extraction parameters were studied by altering protein and lipid concentration. As it can be seen in Fig. 1, the best protein recovery was achieved at around 2 mg protein per ml isooctane which contained 10 mg asolectin/ml solvent. Since different protein concentrations were added in different volumes of water the obtained curves may include a dependence of different solvent/water ratios. Optimal recovery for protein in liposomes from the total organic phase and the heavy fraction were found at the same protein concentration. Parallel results were obtained for the recovery of total chlorophyll (data not shown). Protein and chlorophyll recovery in liposomes increased with increasing asolectin concentration, saturation was obtained at 10-15 mg asolectin/ml isooctane. In the extraction usually 10 mg of asolectin/ml isooctane and 2 mg of chloroplast protein/ml solvent were used. Changes in the amount of protein added did not change significantly the specific ATPase activity in liposomes.

The extraction of chloroplast membrane proteins was rather highly influenced by the solvent used. About 50% of total protein was recovered in the total extract with isooctane while only 30% with hexane. The same behavior was found for the total chlorophyll extracted only with slightly lower percentages. Interestingly the amount of protein and chlorophyll which could be sedimented from the total organic extract at low *g*-values was markedly different for both solvents. With isooctane about 75% of the total protein in the PLC phase was found in the heavy fraction, whereas 65% could be recovered in hexane. The difference between the solvents was even more pronounced for total chlorophyll. PLC's obtained with isooctane contained a heavy fraction with 61% of the total chlorophyll but only 41% in the case of hexane, indicating that isooctane not only extracts more protein but has more chlorophyll associated to the heavy structures in the total extract.

This fact is also demonstrated in Table 1 which illustrates that both solvents showed a markedly enriched protein content in the heavy fraction compared to chlorophyll (free chlorophyll of course remains in the supernatant and led to a decreased protein/chlorophyll ratio). However, isooctane had a lower protein/chlorophyll ratio than hexane in the heavy fraction. Comparing ratios of chl<sub>a</sub>/chl<sub>b</sub> in the original chloroplast preparation with the values found in the organic extract fractions showed that chl<sub>b</sub> may be slightly enriched in the heavy fraction. This could indicate that the heavy complexes preferentially contain proteins with associated chl<sub>b</sub>, whereas the light fraction may be enriched in small complexes with more chl<sub>a</sub> (in addition to free total chlorophyll) than in the chloroplast membrane.

The specific ATPase activity, used as a marker for the transfer of active complexes through the apolar media, was preserved up to 60% (based on protein) or even to 100% (based on chlorophyll) in the heavy fraction liposomes (see Table 1). Heat-inactivated liposomes from the heavy fraction did not show ATPase activity, exactly as heat-inactivated chloroplasts.

The protein-lipid complexes were further characterized determining their absorption spectra. As Fig. 2 shows, there were no differences in the 400 to 500 nm region of the three fractions (all spectra were obtained in the solvent) with the exception of a small blue-shift (7-10nm) in the main long absorption band of the total and the light fraction. The spectra of the heavy fraction in isooctane was very similar to the one of the corresponding liposomes and with the one from the chloroplast preparation in water (see also Fig. 3).

A dramatic effect in the absorption spectra was obtained by transferring chloroplasts into CHO (octane/CTAB-hexanol/water) (see Fig. 3). All absorption bands were blue-shifted compared to the spectra in water, most obvious in the main long-wavelength band from 678 nm to 664 nm, but also about 5 nm from the 438 nm absorption band. In the 450 to 500 nm region, and between 600 and 660 nm, several bands disappeared or decreased. The CHO-spectra resembled the absorption spectra of a chlorophyll extract with 80% acetone. The CHO-system seems to disturb or influence much more absorption bands than the isooctane-phospholipid system.

Regardless of the fact that the photosynthetic complexes appeared to be modified in CHO, substrate dependent chloroplast ATPase activity was obtained in this system. As Fig. 4 demonstrates, there was an almost linear increase in ADP formation with increasing ATP concentration up to 12 or 24 mM ATP. Higher concentrations had an inhibitory effect on the enzymatic activity. The enzyme activity was linear for up to 90 minutes in the restricted water system. A linear increase in ATPase activity was also observed by increasing the protein and chlorophyll concentration up to 70  $\mu\text{g}$  chlorophyll/ml CHO, corresponding to 640  $\mu\text{g}$  protein/ml CHO (data not shown). Higher values had an inhibitory effect.

The activity of the transferred chloroplast ATPase was strongly dependent on the water content present in the CHO system. Reducing the water content from 7.2% to 3.6% decreased the specific activity by 60 to 70%, indicating that water may play an important regulatory role in the catalytic reaction.

## DISCUSSION

We report here that chloroplast ATPase can be transferred into an apolar organic phase containing limited amounts of water via protein-phospholipid complexes, and back to a water phase into liposomes maintaining their functional state. Under the best phospholipid and protein conditions, the ATPase activity of liposomes formed from the heavy fractions of hexane and isooctane extracts was similar to that of chloroplasts.

The heavy fraction of the isooctane extract had a significantly higher protein/chlorophyll ratio than the total organic extract and close to that found in chloroplasts (even larger, see Table 1). These results suggest the enrichment of certain protein-lipid complexes in the heavy fraction. In addition, the overall spectral characteristics of the heavy fraction are close to those of chloroplasts. Thus, some of these complexes could be photochemically active. On the other hand, a fair amount of bound or protein-related chlorophyll could be isolated in the heavy fraction of the organic extract containing phospholipids. The formed chlorophyll – protein – lipid complexes seem to contain antenna chlorophylls (e.g. only LHC-II contains chl<sub>b</sub>) non-covalently bound to specific chlorophyll a/b-binding proteins (see GREEN *et al.*, 1991), and may resemble membrane complexes engaged in harvesting and transferring light energy to the photochemical reaction centers in the chloroplast. In this regard, the isooctane PLC's may allow an alternative approach to isolate and study these complexes.

Another very interesting question that has emerged from the present studies focuses on the structure of the protein-lipid complexes in these ternary systems. As discussed above, when the chloroplast extracts were prepared using phospholipids as surfactants in hexane, octane, toluene or isooctane, centrifugation at 8000xg yielded a pellet containing > 50% of the total protein, and its spectral properties were close to the native chloroplast spectra. In contrast, under identical centrifugation conditions no pellet was obtained in CHO. This is in spite of the fact that the transfer into CHO involves no sonication and the mildest mechanical procedure. More so, the CHO extracts displayed obvious alterations in the absorption bands of the carotenoid region between 450 and 500 nm. Nevertheless the CHO system allowed the measurement of ATPase activity in the ternary system. Apparently the chloroplast membrane proteins are solubilized in a reverse micellar phase forming significantly smaller structures than in the phospholipid system where even whole membrane fractions could be housed. This is consistent with recent reports of whole cell solubilization in ternary systems preserving cell structure (Darszon and Shoshani, 1992).

The ATPase was also active in an apolar environment at a low water content. In CHO at  $W_0=20$ , its specific activity was 25% of that found in an all water system. As expected, the ATPase activity in CHO was dependent on  $W_0$ . In contrast, this activity could not be measured using phospholipids either in hexane or isooctane. This was possibly due to the fact that the phospholipid system has a limited capacity to accommodate water (~1%, AYALA *et al.*, 1986). The water dependence of the ATPase activity in CHO could also reflect the special role water plays in the catalytic cycle of this enzyme. A recent study showed a photophosphorylation dependent transfer of membrane-bound tritium to isolated CF<sub>1</sub> (ZOLOTAREVA *et al.*, 1990) indicating the possible direct influence of water on catalysis. In this respect it has been reported that under specific conditions the alteration of the structure of water may lead to spontaneous ATP synthesis by the mitochondrial F<sub>1</sub> (GOMEZ-PUYOU *et al.*, 1986) and to changes in orders of magnitude of the equilibrium constant for pyrophosphat hydrolysis (DE MEIS, 1984).

TABLE 1

Ratios<sup>1</sup> found in liposomes of different fractions obtained by extraction of 2 mg chloroplast protein in 10 mg asolectin/ml isooctane or hexane and corresponding ATPase activity<sup>2</sup>.

	<i>Protein/Chrolophyll</i>	<i>Chla/Chlb</i>	<i>ATPase</i>
Chloroplast	9.3 ± 0.3	2.4 ± 0.1	17.8 ± 4.3 168 ± 42
Total PLC phase:			
isooctane	10.5 ± 1.5	2.4 ± 0.2	3
hexane	10.0 ± 0.9	2.5 ± 0.2	3
Light fraction:			
isooctane	6.5 ± 1.0	3.4 ± 0.4	4
hexane	5.5 ± 0.7	2.9 ± 0.6	4
Heavy fraction: <sup>5</sup>			
isooctane	12.9 ± 1.1	2.0 ± 0.6	10.9 ± 2.5 178 ± 29
hexane	15.0 ± 0.4	1.8 ± 0.1	9.6 ± 0.6 147 ± 8

<sup>1</sup> Mean ± SEM. Number of experiments varied between 2 and 8.

<sup>2</sup> ATPase activity is expressed in nmoles ADP formed min<sup>-1</sup> (mg protein)<sup>-1</sup> or *nmoles ADP formed min<sup>-1</sup> (mg Chl)<sup>-1</sup>*.

<sup>3</sup> Not determined.

<sup>4</sup> With equal protein concentration no ATPase activity was detectable.

<sup>5</sup> Heat-inactivated (3min, 80°C) chloroplasts and heavy liposomes showed no ATPase activity.

Further studies using low water systems described here may help to gain a more detailed insight in the catalytic process of the chloroplast ATPase. The approach used may also allow the study of membrane-located photosynthetic reactions.

#### ACKNOWLEDGMENTS

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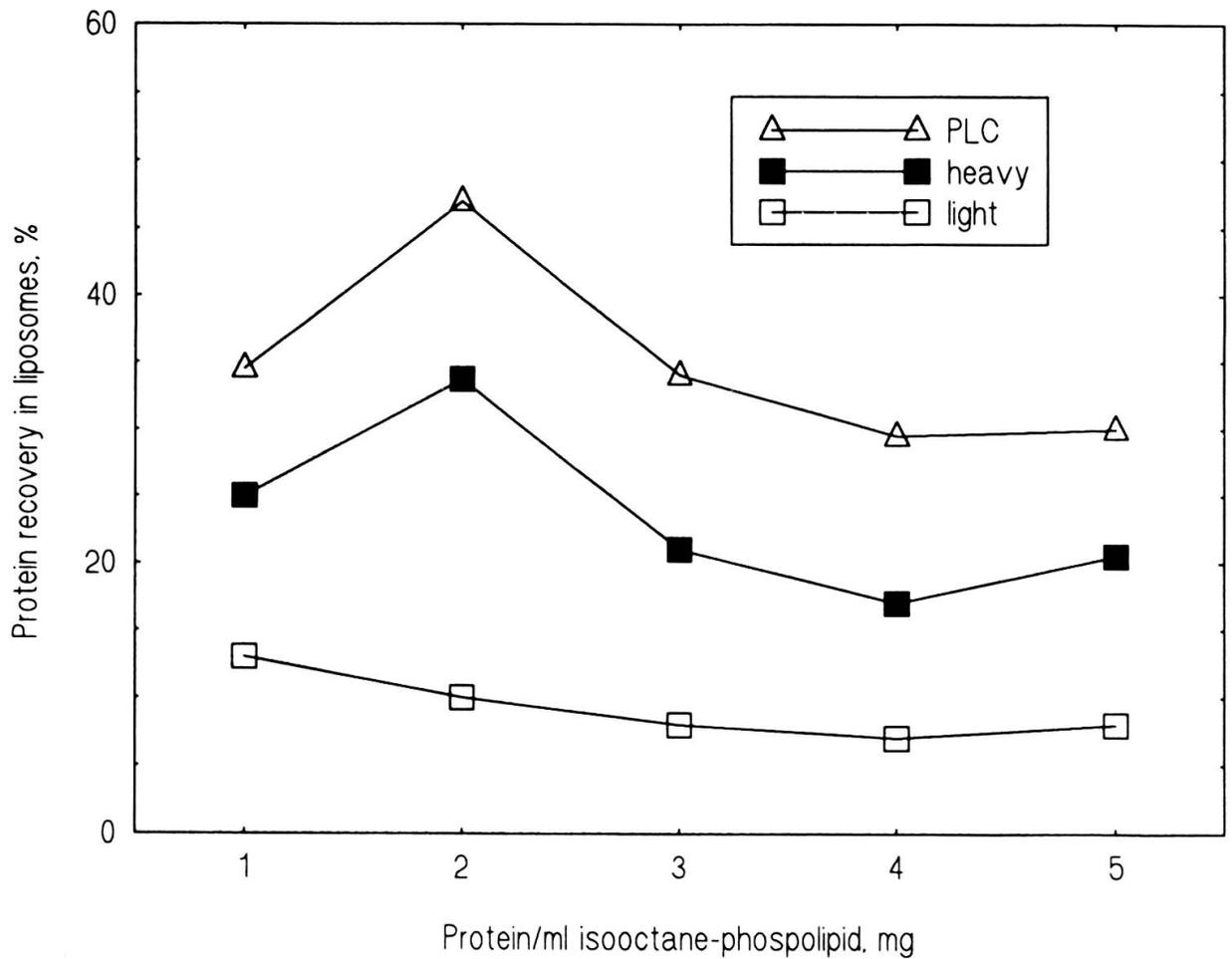


FIG. 1.

Dependence of protein recovered in liposomes on the total amount of chloroplast protein (1-5mg) added to 1ml of isooctane containing 10mg asolectine. Extraction and liposome formation was done as described in Experimental Procedures. Liposomes were formed from the total organic extract (PLC), the supernatant (light) and pellet (heavy) obtained after low speed centrifugation of the PLC-phase. Protein was determined in each fraction separately and expressed as % of initial amount.

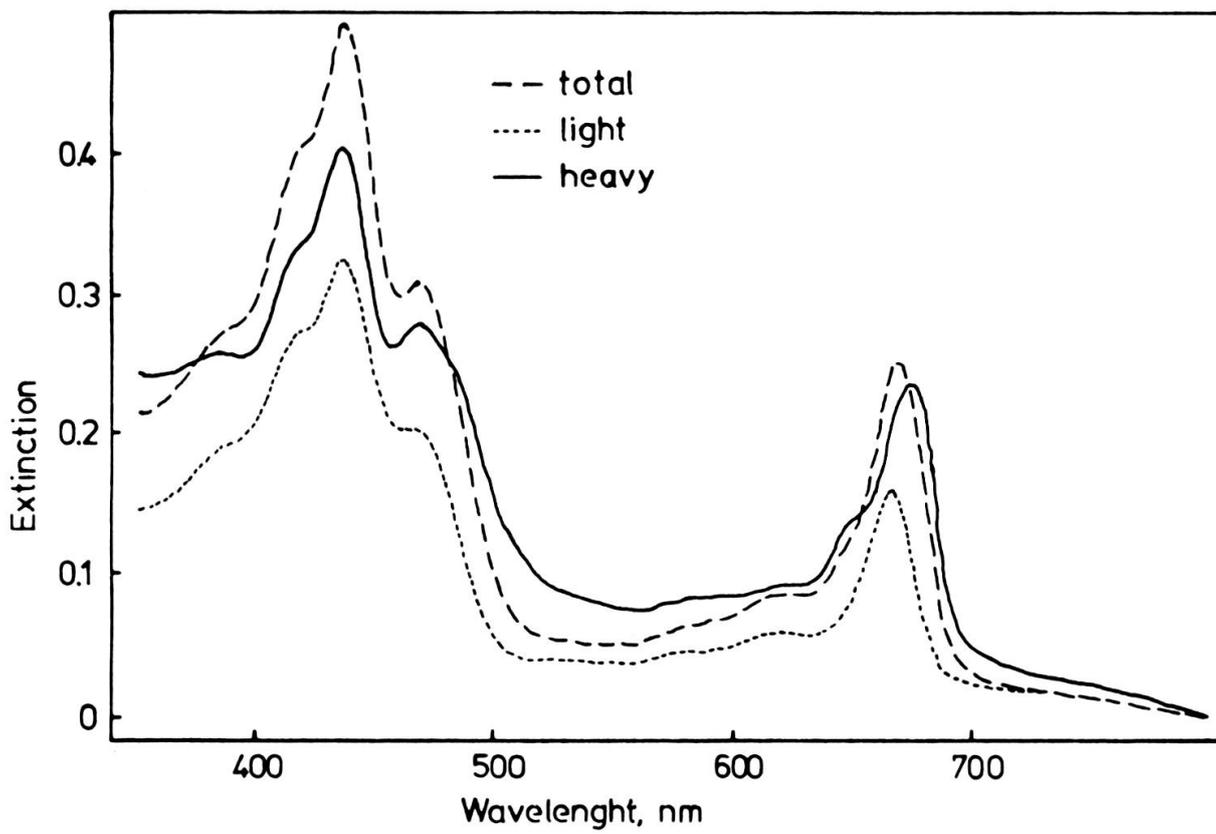


FIG. 2.

Absorptiospectra of fractions obtained by isooctane extraction. Equal volumes of total organic phase (PLC), supernatant (light) and pellet resuspended in isooctane (heavy) were recorded.

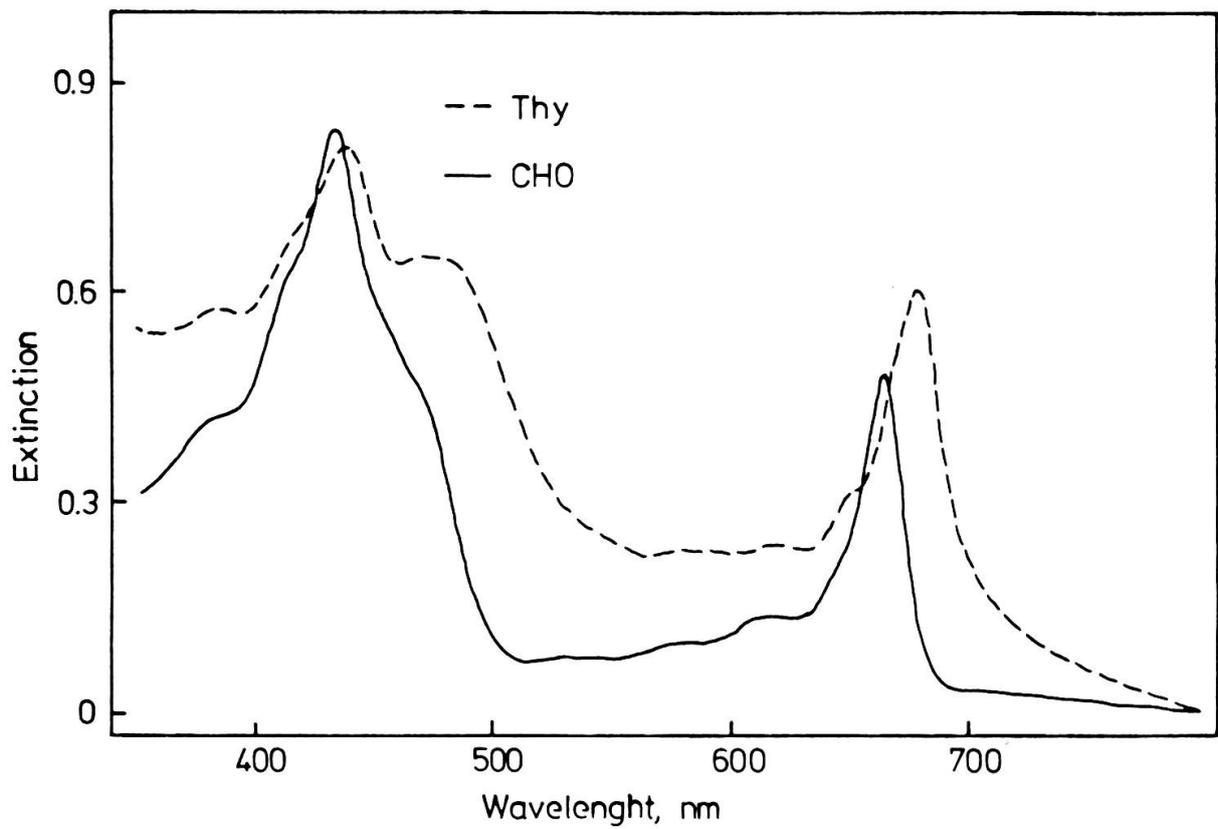


FIG. 3.

Absorption spectra of isolated chloroplasts in buffer (Thy) and in low water system of CHO with 7.2% of water (CHO). The total water and the CHO spectra contained 2.7  $\mu\text{g}$  Chl/ml and 6.1  $\mu\text{g}$  Chl/ml total volume, respectively.

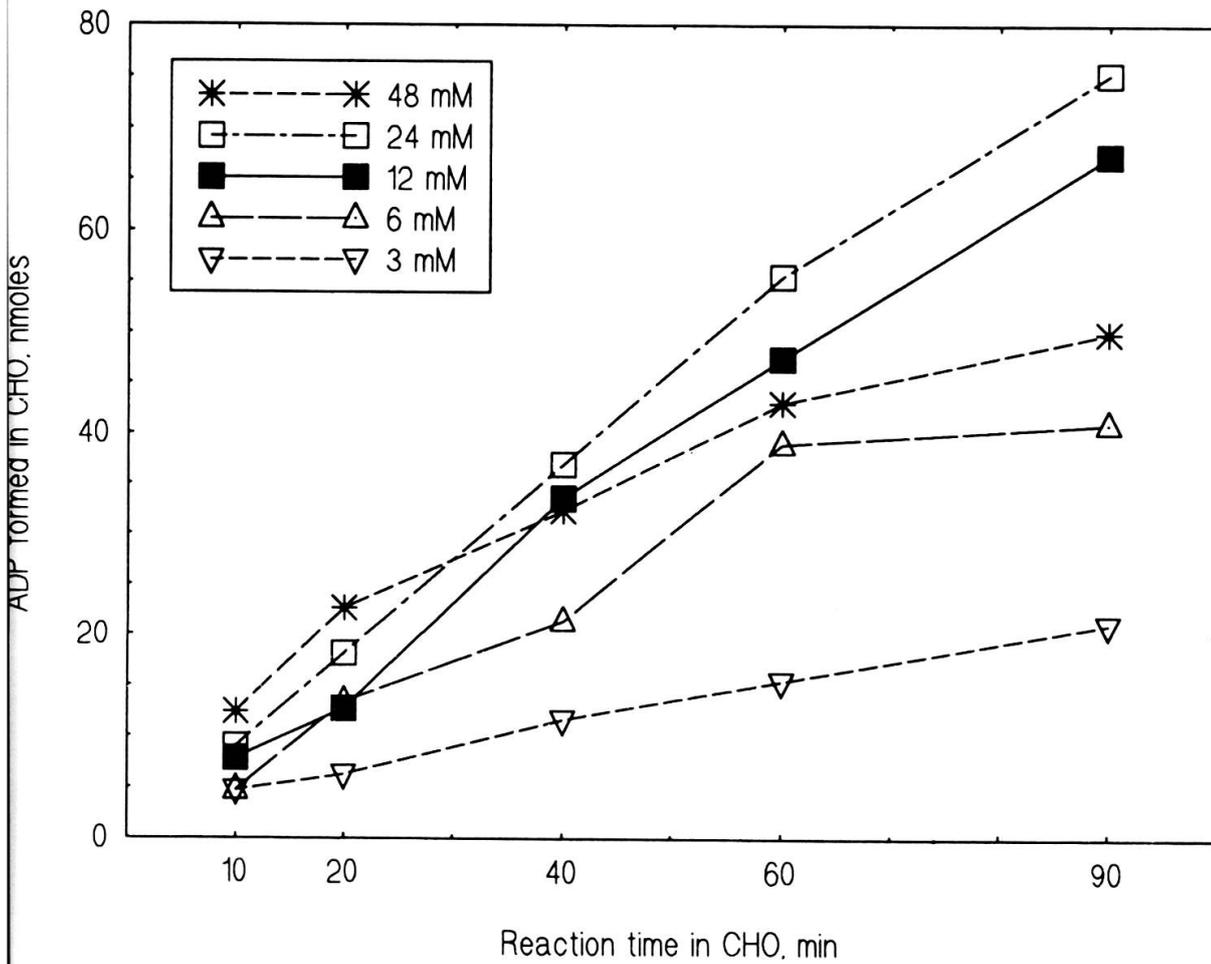


FIG. 4.

160  $\mu\text{g}$  chloroplast protein (17.5  $\mu\text{g}$  chlorophyll) were transferred in 1ml CHO at  $W_{\text{O}}=20$  (7.2% of water). Time courses of ATPase activity (see Experimental Procedures) were measured with different concentrations of ATP in the water pool (final concentrations are indicated).

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