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**« Triple » thylakoids in *Cyanidium caldarium*
« forma A » and in the *Cyanophyta*.
A hypothesis on the significance of these structures**

INTRODUCTION

In the course of an ultrastructural research on samples taken from the populations of *Cyanidium caldarium* (Tilden) Geitler of Campi Flegrei (Napoli) one of us (CASTALDO, 1968) remarked the presence of a very primitive eukaryotic organism, that had never been described previously, and she referred to it as « indefinite form » (Plate I, No. 1).

Such an organism was subsequently isolated by DE LUCA and TADDEI (1970) and temporarily named *Cyanidium caldarium* « forma A » until a more precise classification was established (DE LUCA and TADDEI, 1976).

Some subsequent ultrastructural researches on this form (CASTALDO, 1970) supplied interesting data on its nature and moreover allowed to point out the presence in its chloroplast of thylakoids with an unusual aspect, that were denominated « triple » thylakoids. Such a denomination intended to point out the peculiar tripartite structure of these lamellae, which appear as formed by three dark stripes — a thicker one in the middle and two marginal ones — separated by two clear intermediate spaces.

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LEFORT (1960) was the first Author who described this kind of thylakoids in a Cyanophyta, the *Phormidium inundatum*.

He explained the structure of such thylakoids with their lack of lumen and with the consequent tight closeness of the inner sides of the limiting membrane. He was inclined to think that was the real structure of the thylakoids of the blue-green algae *in vivo* and he suggested that the frequent presence of « classic » thylakoids, i.e. provided with lumen, in the chemically fixed cells, was due to an artefact.

More recently several AA. observed the presence of « triple » thylakoids in other Cyanophyta (RIS and SINGH, 1961; ECHLIN, 1964; ALLEN, 1968 a, 1968 b; EDWARDS et al., 1968; WHITTON, CARR and CRAIG, 1971), but it seems that no one has ever attached a particular significance to these lamellae, even if most of them agree with ECHLIN (1964) that, given the same methods of fixation, they are much more frequent in the algae grown under favourable conditions than in those grown under unfavourable ones.

The real existence of « triple » thylakoids in the Cyanophyta was confirmed by some ultrastructural works carried out by using the freeze-etching technique (COHEN and LEFORT, 1968; DREWS, 1973). It is undoubtable, on the other hand, that also normal thylakoids provided with lumen can be found in the blue-green algae (JOST, 1965; NEUSHUL, 1971; LANG and WHITTON, 1973). This might prove the existence of a structural variability in the photosynthetic apparatus of these organisms.

The aim of this work is to confirm the presence of « triple » thylakoids in the *C. caldarium* « forma A » and to study closely their analogies with the similar thylakoids observed in the blue-green algae, in order to put forward a hypothesis about their significance.

MATERIAL AND METHODS

We made use of some monoalgal cultures of *C. caldarium* « forma A » supplied by DE LUCA and TADDEI for our researches.

The material was divided into three samples, fixed as follows:

- a) with a 2% KMnO_4 unbuffered solution for 30 min. at 4°C;
- b) with a 2% KMnO_4 unbuffered solution for 1 h at 4°C;
- c) with 3% glutaraldehyde in phosphate buffer (pH 6,5) for 2 h at 4° C and postfixed in a 2 % OsO_4 solution in the same phosphate buffer for 3 h at 4° C.

The samples were rinsed in bidistilled water and in the phosphate buffer, respectively. They were then dehydrated through a graded series of ethyl alcohols and embedded in EPON 812. Thin sections were stained in saturated uranyl acetate (5 min.), poststained in lead citrate (5 min.) and viewed by a Siemens Elmiskop 1 A electron microscope, at the Electron Microscopy Center of the Faculty of Science of the University of Naples.

RESULTS

The examination of the sections obtained from the several samples of *C. caldarium* « forma A » has allowed us to verify that the « triple » structure of the thylakoids is evident both in the cells fixed with glutaraldehyde-osmium and in those fixed with permanganate and that, in the latter sample, it is substituted by the « classic » structure when the fixation is pushed beyond certain limits.

Plate II (No. 4) and Plate III (No. 6) show a detail of the chloroplast of cells coming from the sample fixed with a 2 % KMnO_4 unbuffered solution for 30 min. at 4° C. In both we can recognize some typical « triple » structure thylakoids, formed by a central highly electrondense stripe about 4 nm thick and by two marginal lighter stripes, about 2 nm thick and divided from the

medial one by a light space about 5 nm thick. Their complete lack of lumen is evident.

Instead we can see the complete absence of « triple » structures in the chloroplast of cells drawn from the sample fixed with a 2 % KMnO_4 unbuffered solution for 1 h at 4° C: all the thylakoids, in fact, have their classical flattened vesicle aspect, with a very evident lumen (Plate I, No. 2 and Plate II, No. 3).

At last Plate III (No. 5) shows a portion of the chloroplast of cell fixed with 3 % glutaraldehyde in phosphate buffer (pH 6,5) for 2 h and postfixated in 2 % OsO_4 in the same buffer for 3 h at 4° C. The thylakoids show a « triple » structure slightly different from the one that we can observe in the samples fixed with KMnO_4 , to be seen in Plates II (No. 4) and III (No. 6), for a lesser opacity of the two marginal stripes. But, on the whole, the « triple » structure of the thylakoids is quite recognizable.

These results lead us to think that the « triple » thylakoids are structures really present in the cells of *C. caldarium* « forma A » and that the appearance of « classic » thylakoids should be generally due to the alterations caused by the fixatives. The examined material allowed us to observe, however, that the « classic » structure of the thylakoids is particularly frequent in those chloroplasts which are characterized by a less developed lamellar apparatus (Plate III, No. 7). Contrarywise, the « triple » structure is typical of those chloroplasts which contain a great number of thylakoids (Plate II, No. 4 and Plate III, Nos. 5, 6).

All this induced us to take into account the possibility that both the « triple » structure and the « classic » one are two different functional states of the thylakoids and that the passage from the former to the latter is the morphological manifestation of an adaptation of the cells to different physiological conditions.

In the following discussion we shall dwell, therefore, upon some data gathered from bibliography, which are particularly useful to explain the two structures as we suggested.

DISCUSSION

The first observations that aroused our interest as to the above mentioned problem are those reported by HOMANN and SCHMID (1967), who ascertained that in a tobacco mutant with variegates leaves (N. C. 95 var.) the chloroplasts of the green portions carry out normal photosynthesis and show the typical granal structure, while about 80 % of the cells in the yellow portions contain chloroplasts which have only large single thylakoids. These chloroplasts carry out the ADP cyclic photophosphorylation but are unable to evolve oxygen. From such data the AA. deduced that the chloroplasts of the yellow portions possessed an active PS I, but lacked PS II, and concluded that the PS II activity was dependent on the presence of close packed thylakoids, such as the ones that constitute the grana.

GYLDENHOLM and WHATLEY (1968) came to similar conclusions. They proved that in the etioplast of *Phaseolus vulgaris* the cyclic photophosphorylation appears after about 10 h of illumination, whereas the noncyclic photophosphorylation with water as donor and NADP⁺ as electron acceptor (requiring PS I, PS II and the complete electron transport chain) appears between 15 and 20 h, with the first granal structures.

Woo et al. (1970), too, showed that the bundle sheath chloroplasts of maize and sorghum, which are characterized by the absence of granal structures, possess an active PS I but lack PS II.

The examination under the electron microscope of granal chloroplasts appropriately fixed reveals that the tight closeness of the thylakoids produces a peculiar structure in the contact area, which appears, at very high magnification, as a greatly electron-dense stripe with a granulose aspect.

WEIER (1961) proposed the term « partition » to designate the above mentioned structure and the terms « margins », « loculus » and « end granal membranes » to designate, respectively, the ends and the lumen of the granal thylakoids and the uncoupled membranes of the two outermost thylakoids of each granum. This

distinction in the lamellar apparatus of the granal chloroplasts was later on confirmed by WEIER and BENSON (1967), who assumed that the above mentioned regions had different chemical compositions, structures and functions, on the basis of electron microscopy and X-ray studies.

Some subsequent ultrastructural works (SANE, GOODCHILD and PARK, 1970; GOODCHILD and PARK, 1971; PARK and SANE, 1971) seem to demonstrate, in effect, that the two photosystems are morphologically represented by two kinds of particles with different sizes and distribution. Particularly, the PS I would correspond to particles about 12 nm in diameter, diffused in the whole lamellar apparatus. The PS II, on the contrary, would be represented by particles about 17,5 nm in diameter, located exclusively in the partitions.

Such observations seem to demonstrate clearly the necessity of the presence of partitions for a normal development of the photosynthetic processes. The reasons are still not very clear. But, in our opinion, the peculiar location of the PS II might be related to the necessity of isolating this photosystem in a zone where the pH might kept at values higher than the acid ones found in the thylakoid lumen (see TREBST, 1974). As it's known, in fact, the potential required for the oxidation of water — at pH 7 — is higher than 0,82 V, whereas the researches carried out so far didn't allow to identify an oxidative system with such a high potential in the PS II. The potential necessary for oxidizing water, however, decreases by 0,06 volts per each pH unit above neutrality.

On the basis of these observations, GREGORY (1971) suggested the existence of a hydrogenionic pump in the PS II to keep the pH of this photosystem around basic values. We think that the partitions could be the seat of such a mechanism.

Such a hypothesis, if confirmed, would supply a sound explanation of the frequent tendency of the thylakoids of most algae to be stacked in variable numbers and more or less closely (DODGE, 1973; BISALPUTRA, 1974).

The only algae in which no association between thylakoids has been found, as KIRK (1971) too observed, are essentially the Cyanophyta and the Rhodophyta. Although they are provided with both photosystems and can transfer electrons from water to NADP^+ , they always show individual thylakoids freely scattered either in the protoplasm (Cyanophyta) or in the plastidial stroma (Rhodophyta) (FOGG et al., 1973; DODGE, 1973; BISALPUTRA, 1974). We observe the same feature in the chloroplast of *C. caldarium* « forma A » and « forma B » (CASTALDO, 1968, 1970).

Particles of two different kinds, likely corresponding to the ones described in the granal chloroplasts, seem to be present, on the other hand, both in the Cyanophyta and Rhodophyta (NEUSHUL, 1971).

The presence of the « triple » thylakoids in the Cyanophyta and in the C. caldarium « forma A » appears therefore extremely interesting, in this context, since the absence of the lumen could be interpreted, in our opinion, as an adaptation aiming at establishing a structure similar to the granal partitions in every single thylakoid.

The main function of such a structural organization would be the creation of the conditions necessary to secure the PS II activity and to allow a precise cooperation of the PS II with the PS I in order to carry out the photoreduction of NADP^+ as well as the simpler process of ADP cyclic photophosphorylation.

This hypothesis is supported by the results obtained by LAURITIS et al. (1975) that demonstrated a photosynthetic PS II-linked DAB oxidation in the inner electrondense stripe of the « triple » thylakoids in three species of Cyanophyta. Furthermore, as far as we know, no thylakoids with a « triple » structure have ever been observed in the heterocysts, in which only the PS I seems to be in a functional state (BRADLEY and CARR, 1971; DONZE et al., 1972).

On the ground of the data available so far we cannot prove the physiological reversibility between the « triple » structure

and the « classic » one in the thylakoids of *C. caldarium* « forma A ».

Nevertheless, as we already remarked, in that organism the lamellae supplied with lumen as a rule are rather fewer (three-six in each chloroplast) than the « triple » ones (Plate III, No. 7). Therefore, the chloroplasts containing « classic » thylakoids seem to be scarcely functional, especially if compared with the « triple » thylakoids chloroplasts.

On the other hand, it's well known that the reduced pyridine coenzymes, which are greatly required in the growing cells, are necessary only in very small quantities in the cells with a slackened metabolism. In the latter, therefore, the synthesis of those coenzymes is more or less repressed, either if it proceeds by oxidative or photochemical pathway.

We think that the results obtained by ELEY (1971) are interesting in this connection. This Author demonstrated that some fragments of thylakoids extracted from cells of *Anabaena variabilis* grown for a long time without CO₂ show a clean decrease in ability to transfer electrons from water to NADP⁺, whereas they carry out the cyclic photophosphorylation normally. ELEY ascribed the origin of this phenomenon to a repression of the synthesis of PS II components.

We do not exclude therefore the possibility of a structural variability in the thylakoids of *C. caldarium* « forma A », that could represent the morphological expression of such a mechanism of autoregulation, as well as possibly in the Cyanophyta.

As regards the Rhodophyta it does not seem that any « triple » structure has ever been described up to this time for the lamellae of these algae, in which one should expect to find it as well. Some profound affinities exist, in fact, between the photosynthetic apparatus of the red algae and those of *C. caldarium* and of the Cyanophyta, especially regarding the isolated disposition of the thylakoids and the nature and arrangement of the photosynthetic pigments (BOGORAD, 1975).

Such affinities led many AA. (ALLSOPP, 1969; FREDRICK, 1971; SECKBACH, 1972) to assume the Rhodophyta might have derived from Cyanophyta and induced one of us (CASTALDO, 1968) to include *C. caldarium* in a possible group of very primitive eukaryotic algae, constituted by intermediate forms between Cyanophyta and Rhodophyta.

On the basis of the explained facts, we could come to the conclusion that, in our opinion, the « triple » thylakoids of Cyanophyta and *C. caldarium* « forma A » may represent a structure analogous to granal partitions. In our opinion, that is, the « triple » thylakoids should be interpreted as *intrathylakoidal partitions*, as against the *interthylakoidal partitions* which characterize the chloroplasts of the other photosynthetic organism. The presence of « triple » thylakoids, as well as of *interthylakoidal partitions*, would be closely related to the development of a normal photosynthetic activity.

The disappearance of the « triple » structure, that seems to take place spontaneously in the old cells or in those grown under unfavourable condition, could be connected with the repression of the synthesis of some thylakoidal membrane components, among which the PS II ones.

From this point of view the « triple » thylakoids acquire a particular functional and phylogenetic significance.

Outstanding researches aim at verifying the hypothesis that we have presented here.

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RIASSUNTO

La ricerca conferma l'esistenza di tilacoidi privi di lume e caratterizzati da una peculiare struttura tripartita nel cloroplasto di un organismo termale eucariotico, temporaneamente denominato *Cyanidium caldarium* « forma A ».

In accordo con numerosi dati noti in letteratura gli AA. suggeriscono la possibilità di un'analogia funzionale di tali tilacoidi (che essi indicano come tilacoidi « tripli ») con le partizioni esistenti nei cloroplasti della maggior parte degli organismi fotosintetici. Tale ipotesi viene inoltre estesa alle Cyanophyta, nelle quali pure sono stati frequentemente osservati tilacoidi « tripli ».

SUMMARY

The research confirms the existence of thylakoids devoid of lumen and with a distinctive tripartite structure in the chloroplast of a thermal eukaryotic organism, temporarily named *Cyanidium caldarium* « forma A ».

In accordance with several data found in literature the AA. suggest a possible functional analogy of such thylakoids (which they denominated « triple » thylakoids) with the partitions extant in the chloroplasts of most photosynthetic organisms. Such a hypothesis is extended by the AA. to the Cyanophyta, in which too « triple » thylakoids have been frequently observed.

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P L A T E S

PLATE I

Cyanidium caldarium « forma A ».

No. 1. Fixation with 2% KMnO₄ for 1 h.

No. 2. A detail of the chloroplast of the same cell. × 100,000.

Abbreviations: Ch, chloroplast; ChMe, chloroplast membrane; CW, cell wall; M, mitochondrion; N, nucleus; Pl, plasmalemma; Th, thylakoids.

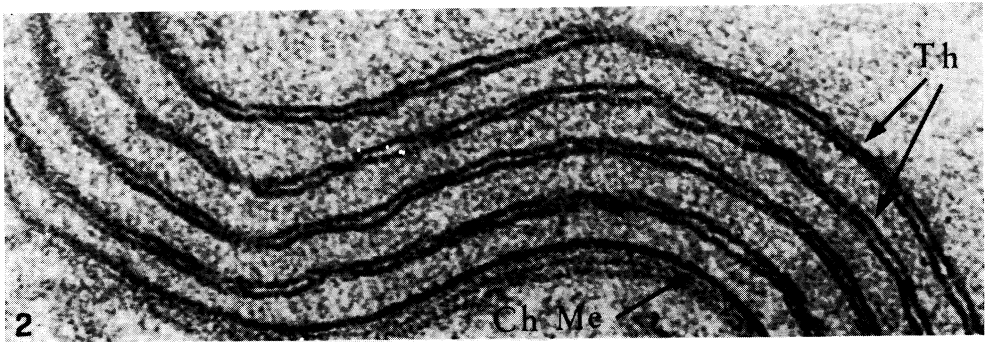
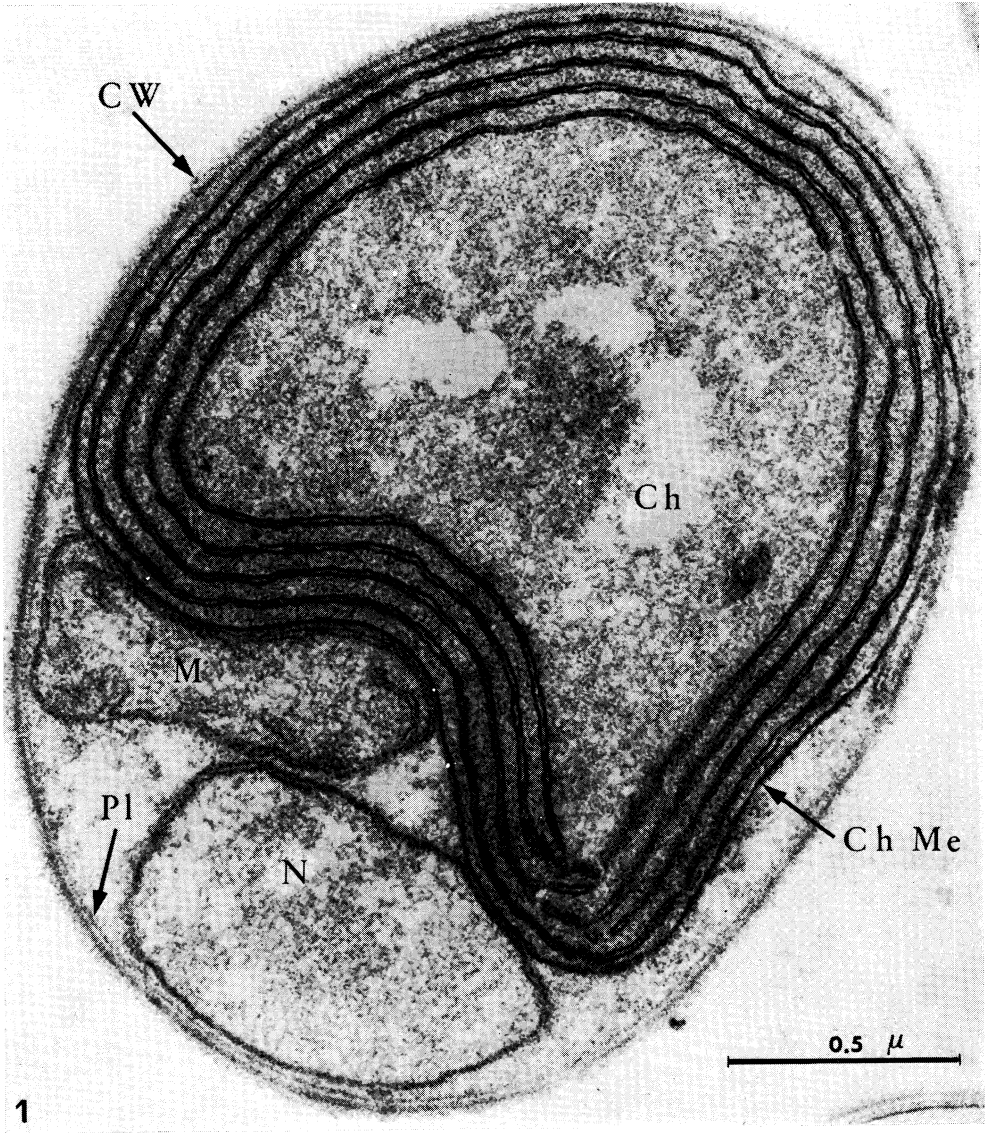


PLATE II

Details of the chloroplast of *C. caldarium* « forma A »:

No. 3. Fixation with 2 % KMnO_4 for 1 h. \times 110,000.

No. 4. Fixation with 2 % KMnO_4 for 30 min. \times 105,000.

Abbreviations: ChMe, chloroplast membrane; CW, cell wall; RG, reserve granules; Th, thylakoids.

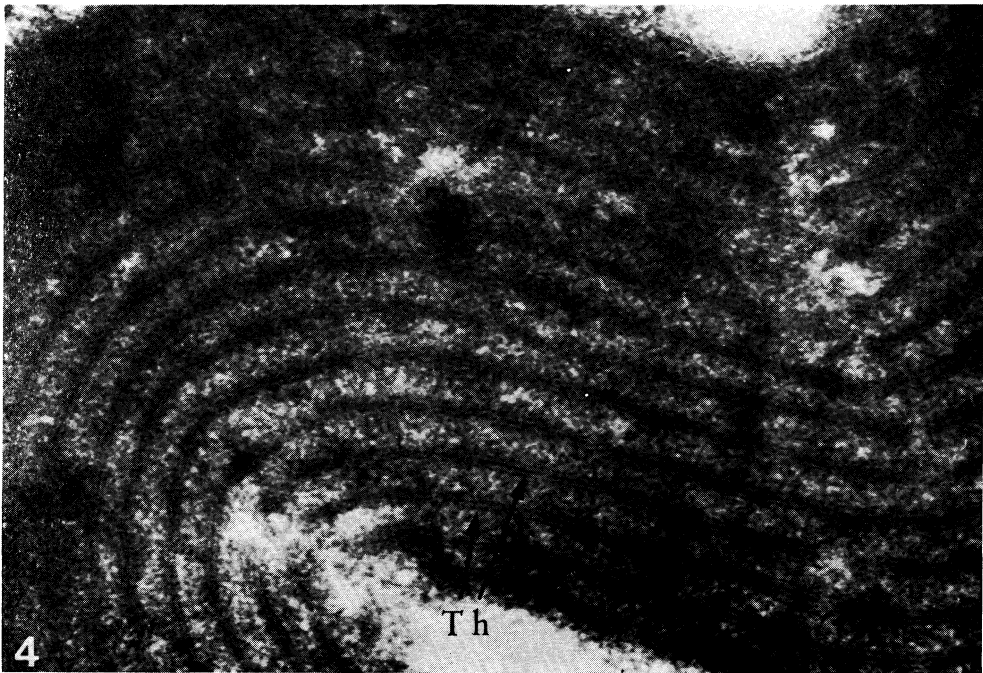
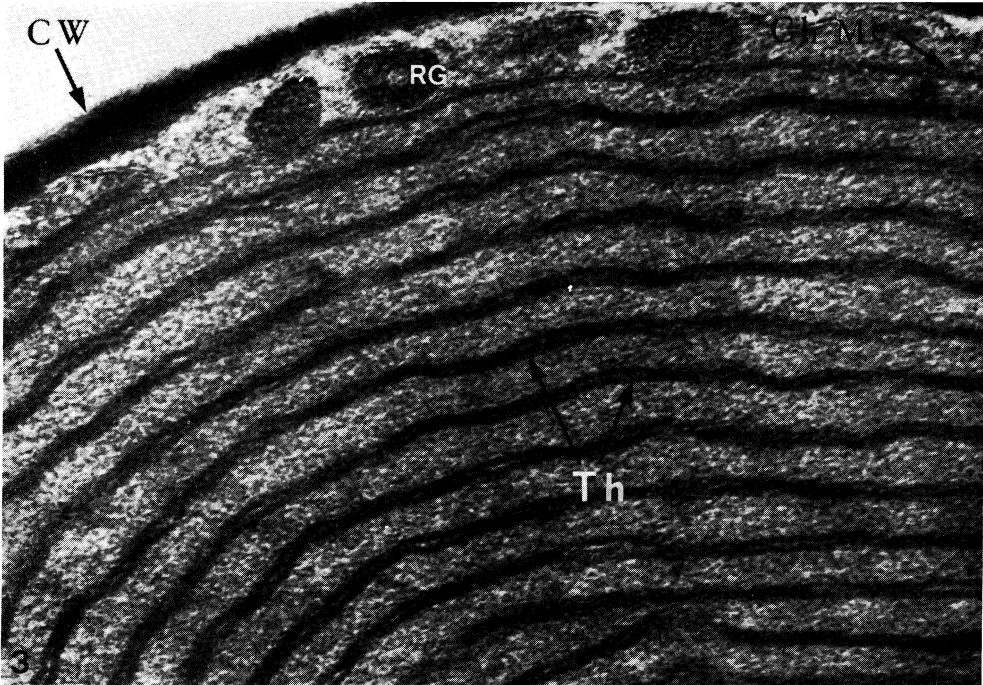


PLATE III

Details of the chloroplast of *C. caldarium* « forma A »:

No. 5. Fixation with 3 % glutaraldehyde + 2 % OsO₄ × 80,000.

No. 6. Fixation with 2 % KMnO₄ for 30 min. × 105,000.

No. 7. Fixation with 2 % KMnO₄ for 1 h. × 80,000.

Abbreviations: ChRi, chloroplast ribosomes; Th, thylakoids.

