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CHARACTERIZATION OF THREE CLASSES OF CHLOROPLASTS OBTAINED BY COUNTER-CURRENT DISTRIBUTION

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SUMMARY

Chloroplasts from spinach have been separated into three classes by counter-current distribution using a dextran–polyethylene glycol two-phase system. The three classes have been characterized with respect to ultrastructure and nitrogen/chlorophyll content. The effect of different degrees of homogenization on the relative yields of the three classes has been studied. Also different parts of the leaves as sources for chloroplast preparation have been compared. The results show that two of the classes resemble the intact (Class I) and broken (Class II) chloroplasts described earlier[3–6], while the third class is a new particle species consisting of an intact chloroplast surrounded by an extra cytoplasm-like layer. Most probably this latter class of chloroplasts is derived from special types of cells in the leaf.

INTRODUCTION

In a previous communication[1] it was demonstrated that a chloroplast preparation from spinach could be resolved into three different populations of chloroplasts by counter-current distribution using polymer two-phase systems. Since separation with this technique is dependent mainly on the surface properties of the particles[2] it was suggested that the three classes of chloroplasts would thus differ in their surface characteristics.

We have now studied the ultrastructure of the different chloroplasts and also determined their nitrogen/chlorophyll ratios. We have also compared different leaf material in addition to the effect of different degrees of homogenization on the relative yields of the different chloroplast classes. The results show that two of the classes resemble the intact (Class I) and broken (Class II) chloroplasts described earlier[3–6]. while the third class is a new particle species consisting of an intact chloroplast surrounded by an extra cytoplasm like layer. This latter type of chloroplasts is derived most probably from special types of cells in the leaf.

MATERIALS AND METHODS

Dextran was obtained from Pharmacia, Uppsala, Sweden as "Dextran 500" batch No. 4024. Similar results were obtained with batch No. 5996.

Polyethylene glycol was obtained from Union Carbide, New York as "Carbowax, polyethylene glycol 4000".

Chloroplast preparation

Spinach (Viking II; Weibulls, Landskrona, Sweden) was grown in artificial light at 15° with 16 h light (5000 lux) and 8 h darkness per day. 20 g leaves were harvested, cut into pieces with scissors and then blended in a chilled knife blendor (Turmix, Techag A.G., Zürich, Switserland) with 125 ml of a solution at pH 7.8 containing 0.4 M sucrose and 0.05 M potassium phosphate buffer. The plant material and its degree of homogenization was varied in the different preparations. (See RESULTS.) The blended material was filtered through four layers of perlon net (Monodur 31 μ, Vereinigte Seidenwebereien AG, Krefeld, W. Germany). The filtrate was then centrifuged for 10 min at 400 × g and the pellet was washed once in the same medium as used for blending, before its contents were resuspended in about 0.5 ml of this medium. All operations were carried out at 2°.

Counter-current distribution

Phase system A containing 6.3 % (w/w) dextran and 6.3 % (w/w) polyethylene glycol was prepared by mixing 63 g of 20 % (w/w) dextran, 31.5 g of 40 % (w/w) polyethylene glycol, 50 ml of 30 % (w/v) sucrose, 5 ml 0.2 M potassium phosphate buffer, pH 7.8 (ratio 1 KH_2PO_4 to 10 K_2HPO_4) and making up to a weight of 201.2 g with water. The whole mixture was shaken at 2° and allowed to separate in a funnel. The greater parts of the two phases were then isolated (interphase material discarded) and stored separately.

The chloroplast sample system for the three chambers (see below) was prepared as follows: 1.26 g of 20 % (w/w) dextran, 0.63 g of 40 % (w/w) polyethylene glycol, 0.8 ml of 30 % (w/v) sucrose were mixed and made up to 3.50 g with water. 0.5 ml of the chloroplast suspension in the preparation medium was then added. Except for the presence of chloroplasts, this mixture is now identical to phase system A. The volume ratio top/bottom of this system is 6.6/4.6. For counter-current distribution a volume ratio of 1:1 was desired. 0.66 ml of the bottom phase from phase system A was added therefore to the chloroplast sample system. The whole mixture was shaken and used for loading the counter-current distribution apparatus.

Phase system B was the same as A except that 1 ml of water was replaced by 1 ml of 1 M KCl giving a KCl concentration of 5 mM in the whole system. The chloroplast sample system for B was the same as for A except that 0.02 ml of water was replaced by 0.02 ml of 1 M KCl.

An automatic thin-layer counter-current distribution apparatus[7] with 120 chambers (IRD, Bromma, Sweden) was used. The bottom phase chamber has a capacity of 0.65 ml. Since the chloroplasts partition between the upper phase and the interface, the method of liquid interface counter-current distribution[2,8] was employed. In the experiment shown in Fig. 1 a–d each of the chambers 3–59, 63–119 were charged with 0.55 ml bottom phase and 0.55 ml top phase. Chambers 0–2, 60–62 were charged with 1.1 ml of the chloroplast phase system mixture. The settling time was 8 min and the shaking time was 30 sec. The temperature was 2°. After 57 transfers the fractions were collected and diluted three fold with the preparation medium in order to break the phase system. The absorbance of the diluted fractions was measured with a Zeiss

PMQ II Spectrophotometer. The absorbance at 550 nm ($A_{550\ nm}$) and 680 nm ($A_{680\ nm}$) were plotted against tube number.

In the experiment of Fig. 3 only chambers 0–2 were charged with chloroplasts and 114 transfers were used instead of 57.

In the experiment with chloroplasts from the stalk and mid-vein (Fig. 4a, b) phase system B was used. A lower partition coefficient for Peak II chloroplasts is obtained[2,8,9] in 5 mM KCl and they travel together with Peak I. The chloroplasts of Peak III, however, are not affected to the same extent and can easily be separated from those of I and II. Therefore only 9 transfers were needed and chambers 1 and 2 charged with chloroplasts.

Chlorophyll determination

Chlorophyll was determined by the method of ARNON[10].

Protein determination

Protein was precipitated with an equal volume of 10 % trichloroacetic acid and centrifuged at 17000 × *g* for 20 min. The pellet was first washed with 5 % trichloroacetic acid to remove the polymers of the phase system and then with chloroform–ethanol (1:3, v/v), chloroform–methanol (1:3, v/v) and methanol–ether (3:1, v/v) to remove lipids. After resolving the protein in 0.2 ml 1 M NaOH, the protein concentration was determined by the method of LOWRY *et al.*[11].

N-determination

Nitrogen was determined by the method of Kjeldahl as described in reference[12].

Size distribution determination

Size distribution patterns of chloroplast suspensions were made using a Celloscope 302 (Lars Ljungberg Co., Sweden). This instrument utilizes the same principles as the Coulter counter, which was used by S. M. RIDLEY AND R. M. LEECH[13] for the same purpose. 100–200 μl of a chloroplast suspension was mixed with 75 ml of 0.9 % NaCl, (milliporefiltered) giving a concentration of 8000–10000 chloroplasts per ml, and measured immediately in the Celloscope.

Electron-microscopy

The suspended chloroplasts were fixed with 4 % glutaraldehyde by simply adding 0.4 ml of 25% glutaraldehyde to a 2 ml chloroplast suspension. After 2 h they were centrifuged (600 × *g* for 10 min) and the pellet was then washed with preparation medium, postfixed with 1–2 % OsO_4, in 0.1 M sodium phosphate buffer for 1 h, and then dehydrated stepwise with ethanol and embedded in Epon. All procedures except embedding done at 2°. Sections were cut on a LKB ultramicrotome and stained with uranylacetate and lead citrate.

RESULTS

Effect of degree of mechanical treatment

In order to study the effect of different degrees of homogenization, cotelydons were treated for different numbers of 2 sec periods in the blendor and the chloro-

plasts so produced were then subjected isolated and to counter-current distribution. (Fig. 1a–d). In Fig. 1a the cotelydons were treated for 3 × 2 sec only. In Figs. 1b, c and d the cotelydons were first treated by blending for 3 × 2, 9 × 2 and 12 × 2 sec, respectively and then filtered; the filtrates were discarded and the residues from the perlon-nets blended again for 3 × 2 sec. In the counter-current distribution diagram of Fig. 1a there are two peaks and in Fig. 1. b–d there are three peaks, referred to as I, II and III. (I and III are respectively the same as 1A and 1B described in ref. 1). The area under each peak was estimated and plotted as per cent of total for the $A_{680\ nm}$ diagrams in Fig. 1a–d. The plot which is shown in Fig. 2 demonstrates that the areas of Peaks II and III increase while the area under Peak I decreases as a result of the first pretreatment. After additional pretreatments the areas under Peaks I, II and III are relatively constant.

Fig. 3 shows the result of a counter-current distribution experiment with chloroplasts from primary leaves. The blending time was 10 × 2 sec, and instead of 57 transfers we used 114 transfers for better resolution. Note the small peak at tube 95. In similar experiments with full grown leaves a third peak was also obtained with the same blending time.

Thus, three peaks in the counter-current distribution diagram are obtained for

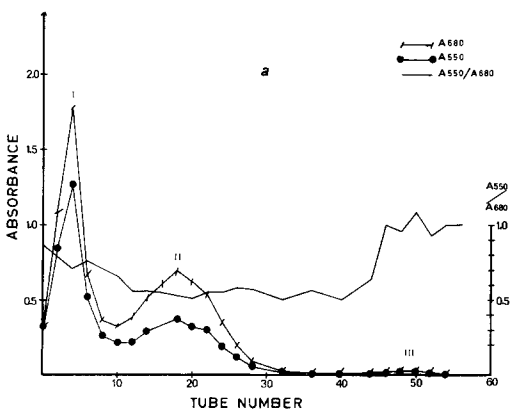

Fig. 1

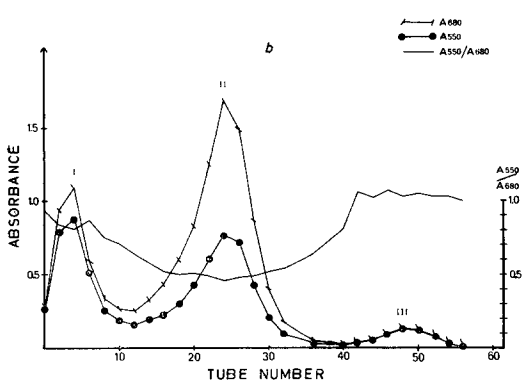

Fig. 1

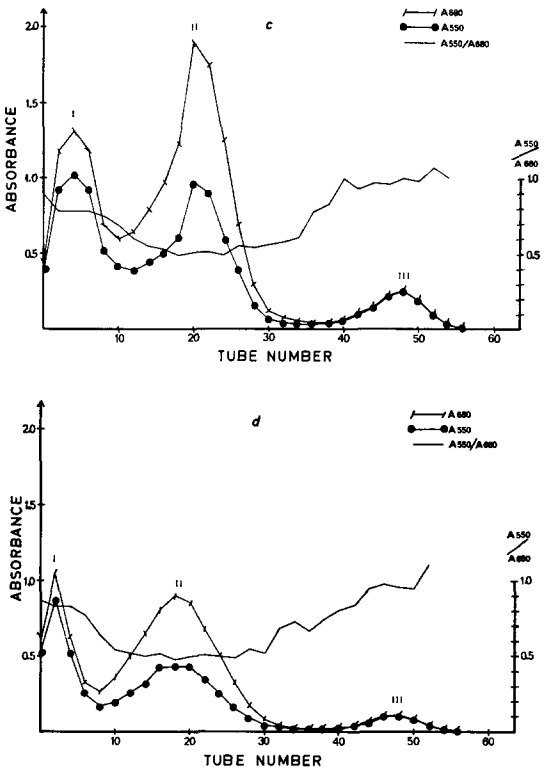

Fig. 1. Counter-current distributions of chloroplasts obtained after different degrees of homogenization of the leaf material. Cotelydons were blended for 3 × 2 sec after the following pretreatments in the blendor: (a) pretreatment 0 sec; (b) pretreatment 3 × 2 sec; (c) pretreatment 9 × 2 sec; (d) pretreatment 12 × 2 sec.

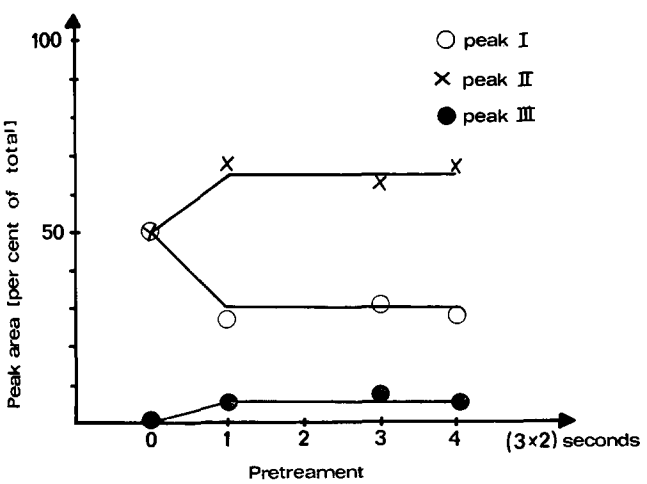

Fig. 2. Yield of different fractions of chloroplasts as function of time of pretreatment of cotelydons. The area under each $A_{680\ nm}$ peak from the diagrams of Fig. 1. (between two adjacent minima) as per cent of total was plotted.

both cotelydons, and normal leaves provided, the blending time is long enough (compare with results in ref. 1).

In the phase contrast microscope the chloroplasts of Peaks I and III look intact, that is, have an opaque appearance, while Peak II seems to consist of broken chloroplasts with clearly visible grana[3–6]. The ratio $A_{550\ nm}/A_{680\ nm}$, which is plotted in

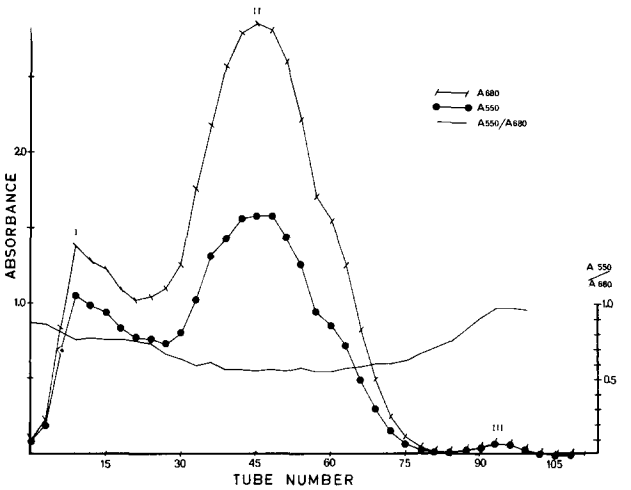

Fig. 3. Counter-current distribution of chloroplasts obtained after blending primary leaves for 10 × 2 sec. Note the third peak at Tube 95.

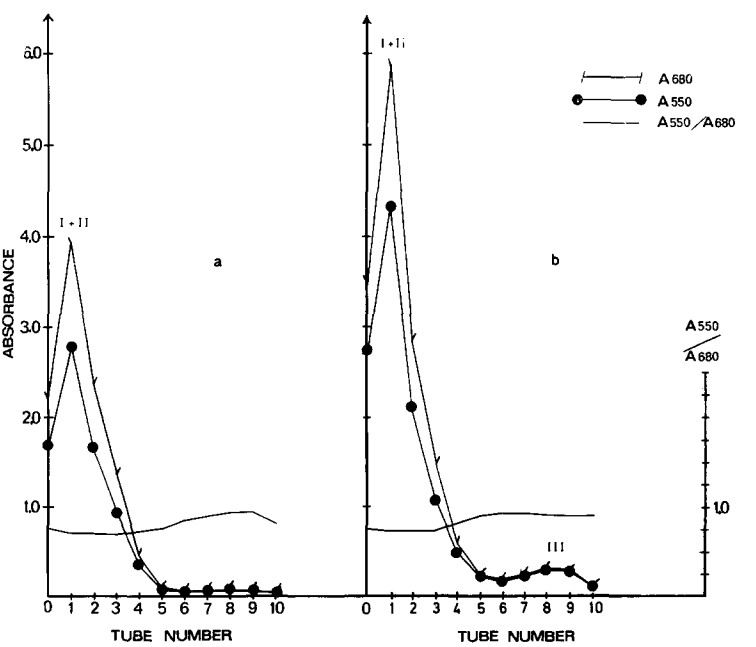

Fig. 4. Counter-current distribution of chloroplasts from (a) full grown leaves *minus* stalks and mid-veins, (b) stalks and mid-veins only. In this run chloroplasts of Peak I and II in Figs. 1 and 3 are found together under the left main peaks, while Peak III chloroplasts are present in tubes No. 6–10.

Figs. 1, 3 and 4, varies linearly with the percentage of intact chloroplasts. A suspension of only broken chloroplasts gives a ratio of 0.5 and a suspension of 100 % intact chloroplasts would give a ratio of about 1.0 (Ref. 14).

Chloroplasts from leaf stalks and mid veins

Leaves from full grown plants were divided into two parts before treatment in the blendor. One part consisted of the leaf-stalks and the mid-veins and the other part the rest of the leaves. Each part was blended for 5 × 2 sec, filtered, the residue blended for 5 × 2 sec, again filtered and the two filtrates pooled. Fig. 4 shows a comparison of the counter-current distribution diagrams obtained with chloroplasts from the two leaf parts. Chloroplasts from the stalks and mid-veins gave a dinstinct peak of chloroplasts (Fig. 4b) corresponding to those of Peak III in Figs. 1 and 3. About 7 % of the total population is of this type as judged from the peak area. A chloroplast preparation from the remaining parts of the leaf contains much less of Peak III (Fig. 4a) estimated as about 2 % of the total.

Protein and nitrogen content

Protein, nitrogen and chlorophyll was determined on the different chloroplast classes (Table I). For both the protein/chlorophyll and nitrogen/chlorophyll ratios Peak III chloroplasts have a higher, and Peak II chloroplasts a lower value than Peak I. The chlorophyll a/b ratio is not significantly different for the chloroplasts of the three peaks.

TABLE I

PROTEIN/CHLOROPHYLL AND NITROGEN/CHLOROPHYLL RATIOS OF CHLOROPLASTS FROM DIFFERENT PEAKS OBTAINED BY COUNTER-CURRENT DISTRIBUTION

Peak	*μg protein*/ μg chlorophyll*	*μg N/ μg chlorophyll*	*μg protein**/ μg chlorophyll*	*chlorophyll a/ chlorophyll b*
I	28	6,2	39	2.4–2.8
II	11	2.7	17	2.4–2.8
III	36	10.6	66	2.4–2.8

* As measured by the method of LOWRY *et al.*[11].

** Calculated from the nitrogen content assuming all nitrogen is derived from protein with a nitrogen content of 16 %.

Size distribution differences

Fig. 5 shows an example of size distributions of the chloroplasts from the different peaks. The chloroplasts of Peaks I and III have narrower distributions than those of Peak II. The chloroplasts of Peak III have a somewhat smaller (about 10 %) average diameter compared to those of Peak I. This has been the trend in all experiments, even if the absolute values have varied. The Celloscope 302 measures the excluded volume of electrolyte in a small capillary. The diameter values are calculated assuming all chloroplasts were spheres.

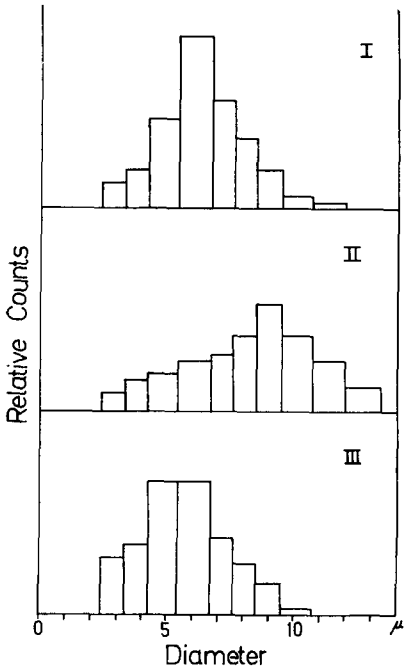

Fig. 5. Size distributions of Peak I, II and III chloroplasts.

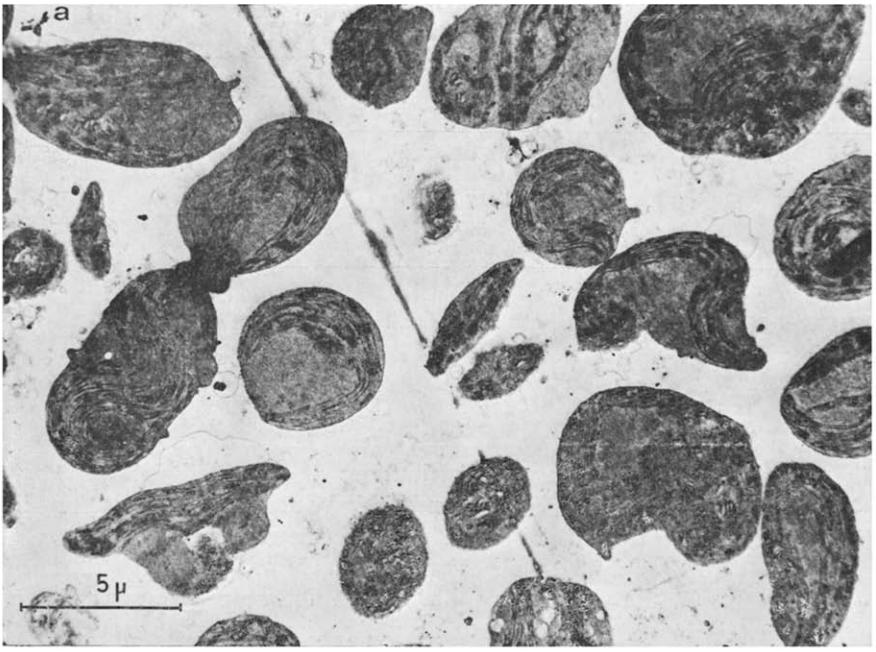

Fig. 6

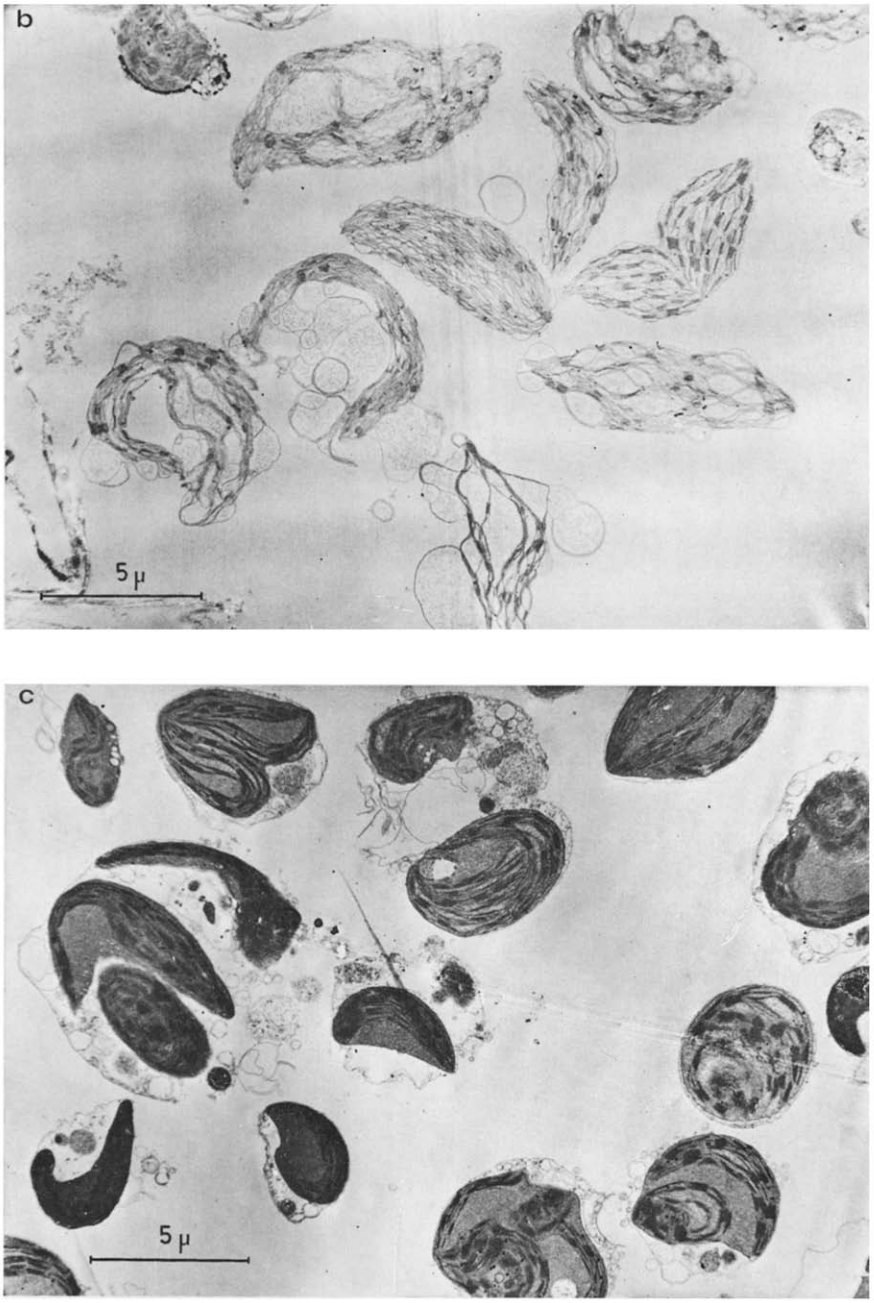

Fig. 6. Electron micrographs of chloroplasts from the three peaks in the counter-current distribution diagram. (a) Peak I. The chloroplasts are rich in stroma material and surrounded by an outer membrane, the chloroplast envelope. (b) Peak II. The chloroplasts are stripped and lack outer membranes but still containing small amounts of stroma material trapped by the lamellae systems. (c) Peak III. The chloroplasts are each surrounded by an extra membrane, similar to a cytoplasmic membrane, which is more or less intact, and in the space between the chloroplast envelope and the extra membrane there is cytoplasmic material, such as mitochondria, peroxisomes and ribosomes.

Electron microscopy pictures

Figs. 6a, b, c are low magnification pictures of chloroplasts from Peaks I, II and III respectively. Fig. 6a shows morphologically intact chloroplasts (Class I), rich in stroma material, and each surrounded by an envelope. Fig. 6b shows stripped chloroplasts without envelopes (Class II), but still containing small amounts of stroma material trapped by the inner lamellae system. In Fig. 6c the chloroplasts not only have their envelopes and stroma material intact, but each is also surrounded by an extra membrane similar to a cytoplasmic membrane. Furthermore the space between the chloroplast envelope and the extra membrane contains cytoplasmic material, such as mitochondria, peroxisomes and ribosome-like particles. Often there seems to be more than one chloroplast inside such a cytoplasmic coat. Figs. 7a, b, c are examples of chloroplasts from Peak III.

The thickness of the coats varies considerably, in some cases the "cytoplasmic" membrane is close to the chloroplast envelope, in other cases they are widely separated and the intervening space contains other cell organelles. There is a large number of vesicles in the surrounding layer.

DISCUSSION

The present work shows that the three different classes of chloroplasts are very different morphologically and that the relative amounts of the two "intact" classes of Peaks I and III depend on the degree of homogenization and from which part of the leaf the chloroplasts are prepared. There are also size and chemical differences between

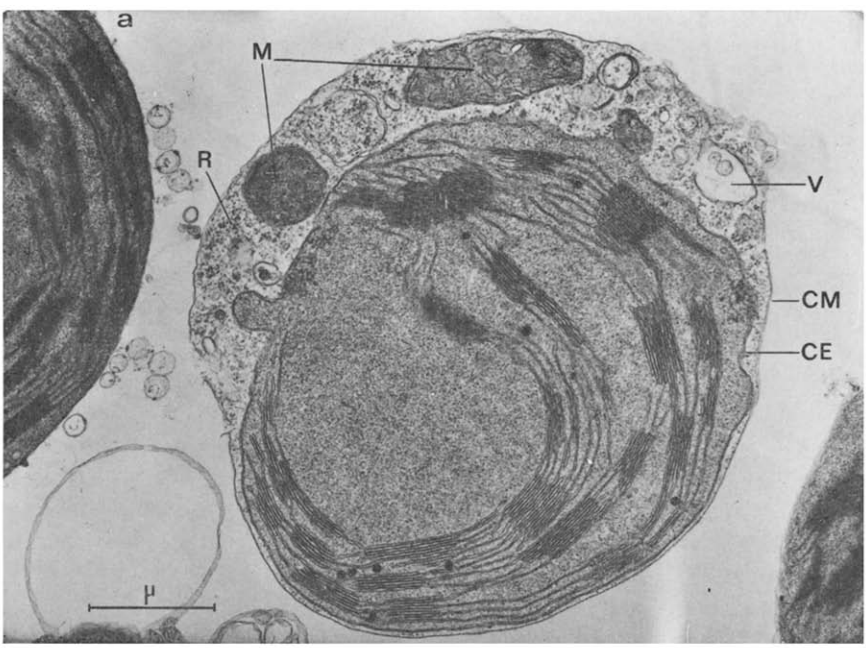

Fig. 7

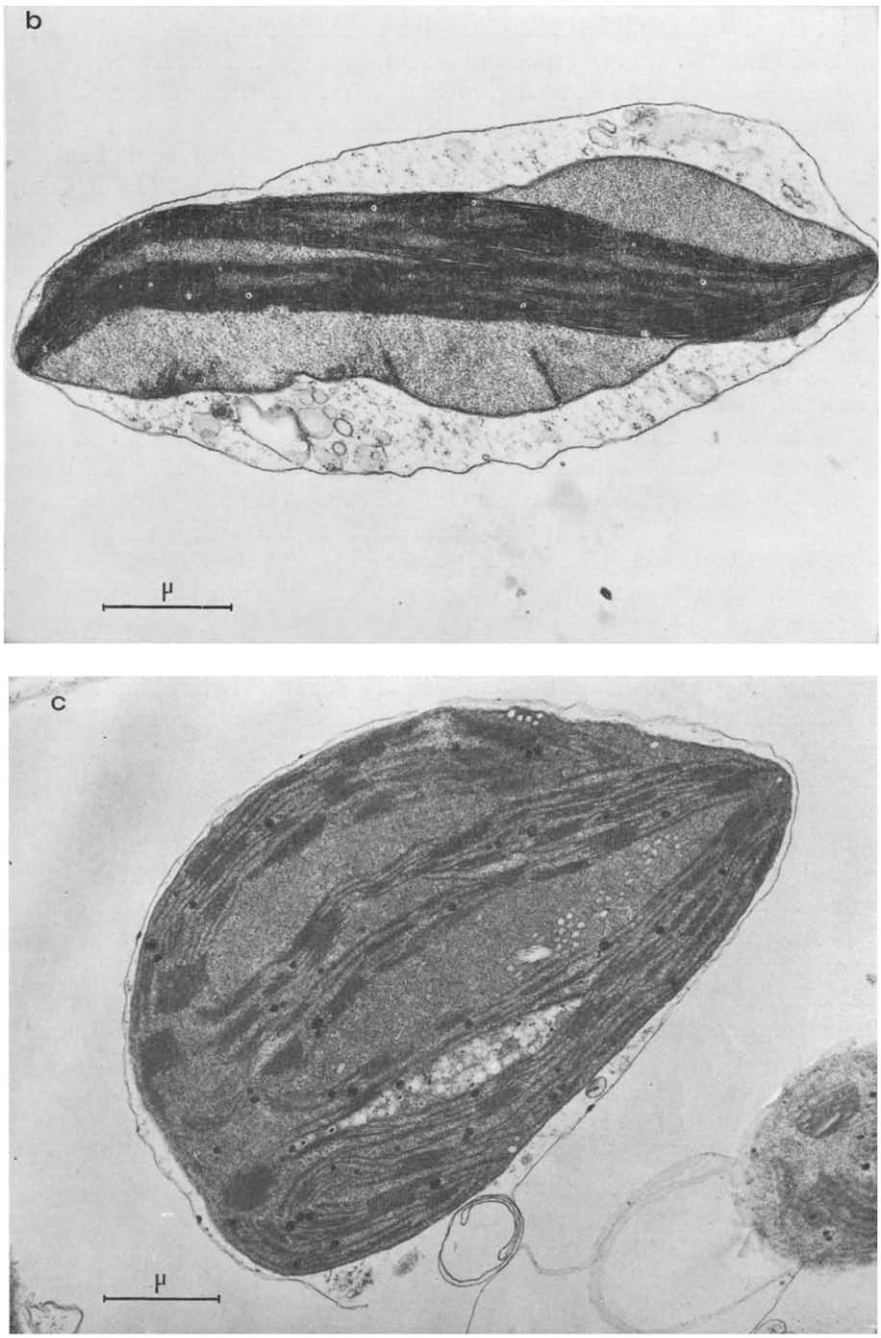

Fig. 7. Chloroplasts from Peak III. These figures show some variations in the cytoplasmic material coat surrounding the chloroplast. In (a) there are ribosome like particles and mitochondria included in the cytoplasmic material. The chloroplast in (b) is surrounded by a thicker cytoplasmic coat compared to the chloroplast in (c). It should be noted that all the coats contain filled and empty vesicles. CE, chloroplast envelope; M = mitochondrion; CM = cytoplasmic membrane; V = vesicle; R = ribosome like particles.

the different classes of chloroplasts. The question then arises as to the origin of the chloroplasts of Peak III. We have considered the following alternatives:

(1) Peak III chloroplasts come from special cells different from those containing Peak I chloroplasts. This is supported by the experiments described in Fig. 1 where it is shown that a stronger mechanical disintegration of the leaves gives more Peak III chloroplasts in the preparation. If the chloroplasts are obtained from leaves cut only by razor blades no third peak appears in the counter-current distribution diagram. (This is not shown here, but the result is very similar to that in Fig. 1a). Only after a relatively harsh blendor treatment they do appear. It would thus appear reasonable to suppose that the Peak III chloroplasts come from cells which have a high resistance to breakage. What kind of cells these are is difficult to ascertain but it seems very probable they are associated with the vascular bundle such as phloem parenchyma or companion cells. Light microscopy of the residue on the perlon net shows that these cells are the most resistant to breakage. Even after severe blending (12 × 2 sec) many of the cells adjacent to the vascular bundles are fairly intact. These cells are smaller and more compact than the mesophyll cells. As revealed by electron microscopy the vacuole usually is absent or only small, and the chloroplasts are more uniformly distributed in the cytoplasma and do not lie close to the cell wall as in the mesophyll cells. In this respect our observations of the leaf structure agree with those described for sugar beet[15].

Our interpretation, that Peak III chloroplasts come from special cells is also strongly supported by the experiments described in Fig. 4, which show that a chloroplast preparation from stalks and midveins gives relatively more of Peak III (7 %) than a preparation from the rest of the leaves (2 %). A considerable difference in resistance towards breakage between mesophyll cells and vascular bundle sheath cells of Atriplex Rosea was demonstrated by BJÖRKMAN AND GAUHL[16].

(2) Both Peak I and III particles come from the same cell types but Peak III have retained part of the cytoplasm surrounding the chloroplast *in vivo*. Thus a piece of the cytoplasm has been cut off and the coat around the chloroplast contains therefore cytoplasmic material such as ribosomes, mitochondria and peroxisomes, which often are seen lying adjacent to the chloroplast in thin sections of whole cells. The electron microscope pictures support this explanation.

The experiments on different mechanical treatment, Fig. 1, and on different leaf material, Fig. 4, do not, however, support this. On the other hand the Peak III chloroplasts might then be chloroplasts which are more firmly stuck to the cell walls and would therefore be released only after stronger mechanical treatment.

(3) Chloroplasts of Peak III are in a special stage in development. If we assume that chloroplasts pass through a growth cycle involving fission, the chloroplasts of Peak III could be in a special stage of this growth cycle. The surrounding coat might then be a structure in functional cooperation with the chloroplast during its division. The large number of "twins" in the Peak III chloroplasts would favour this interpretation. However, we feel this alternative is unlikely because of the experiments shown in Figs. 1 and 4.

(4) A fourth alternative is that the Peak III chloroplasts are artificial products formed during the isolation procedure. For example, the coat around the chloroplasts could be material attached during the separation procedure. However, precipitation of material on to the particles would probably cause intermediate forms and not give

a distinct fraction as Peak III. We therefore consider this alternative to be highly unlikely. The Peak III chloroplasts are also not a product of the polymer phase system. They can be found in electron microscope pictures of chloroplast-preparations, which have had no contact with the polymers.

Considering the experimental data obtained so far, we favour the first interpretation, that is the Peak III chloroplasts come from special cells, and most likely from cells associated with the vascular bundles. But other cell-types can not be excluded. The question then arises why the chloroplasts are surrounded by a cytoplasmic coat when isolated from these cells. One explanation could be that the cytoplasma of these cells is more rigid.

It may seem paradoxical that the Peak III chloroplasts, in spite of their cytoplasmic coat, and often containing two chloroplasts together, are smaller than the Peak I chloroplasts. If the two types of chloroplasts come from different types of cells, this might provide the explanation. Or, on the other hand, Peak III chloroplasts might simply be less swollen than the Peak I chloroplasts, because of the protection from the surrounding medium by the cytoplasmic coat. Furthermore it is difficult to know what exactly is measured with the method of size distribution determination used here.

The ratio between chlorophyll *a* and *b* is the same for all classes of chloroplasts. Also the inner lamellae system does not seem to differ between chloroplasts of Peak I and III.

The differences in nitrogen — and protein content — agrees well with our electron microscope pictures. The Peak II chloroplasts have, not unexpectedly as they have lost most of their stroma material, in the case of both nitrogen and protein less than half the content relative to chlorophyll with the Peak I chloroplasts. Of the two types of intact chloroplasts those of Peak III have higher nitrogen and protein/chlorophyll ratios. This is what one would expect, as the cytoplasmic coat probably contains no chlorophyll.

All values for protein/chlorophyll ratios obtained by the Lowry method are lower than those calculated from the nitrogen/chlorophyll ratios. This is to be expected as the Kjeldahl method measures also nonprotein nitrogen. Further, the Lowry method can be disturbed by various materials in the samples. The discrepancy between the two methods, however, is within reasonable limits.

Whatever the explanation for the origin of the Peak III chloroplasts is, these should offer interesting particles for studying various biochemical and metabolic properties of the chloroplasts. In these particles the chloroplasts would be expected to be embedded in an almost *in vivo* environment. In fact many of the Peak III particles may be considered as multiorganelle complexes containing chloroplasts, mitochondria and other cell components and would allow a study of the cooperation between different cell organelles.

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