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Rapid Separation of the Plastid, Mitochondrial, and Cytoplasmic Fractions from Intact Leaf Protoplasts of *Avena*

Determination of In Vivo ATP Pool Sizes during Greening

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Abstract. Purified intact protoplasts were isolated from etiolated and greening leaves of *Avena sativa*. They were ruptured by forcing them through a 20-μm aperture nylon net and immediately thereafter fractionated into a pure pellet of plastids (well above 70% of total plastids), a layer of mitochondria only slightly contaminated by other cellular constituents (about 50% of total mitochondria), and a cytoplasmic supernatant. This was achieved within 60 s by an integrated method of homogenation of protoplasts and centrifugal filtration of the homogenate on a gradient of silicone oils, contained together with the nylon net in 450 μl microtubes, and verified by comparing the levels of activity of specific markers within the three fractions obtained. With appropriate modifications to immediately quench metabolic reactions within the fractions, this method allows the determination of metabolite levels within plastids, mitochondria, and the cytoplasmic compartment of intact protoplasts. The applicability of this technique is demonstrated by the determination of ATP in the plastids, mitochondria, and the cytoplasm of protoplasts obtained from etiolated and greening primary leaves of *Avena*. The levels of ATP, corrected for contamination of the fractions by each other, exhibit a pronounced transient increase during greening, especially within the cytoplasm.

Key words: *Avena* – ATP pool sizes – Chloroplast development – Protoplast fractionation.

Abbreviations: BSA = bovine serum albumin; Cyt c = cytochrome c; EDTA = ethylenediamine tetraacetic acid; HEPES = N-2-hydroxyethyl-piperazine-N′-2-ethane sulphonic acid; MES = 2(N-morpholino) ethane sulphonic acid; PGA = 3-phosphoglyceric acid; PEP = phosphoenol pyruvic acid; RuBP = ribulose-1.5-bisphosphate

Introduction

Isolated plant organelles, mainly plastids and mitochondria, are commonly used to investigate biochemical aspects of, e.g., photosynthesis and respiration. Usually, the tissue is homogenized by mechanical grinding and the organelles are separated from the resulting homogenate by differential or density gradient centrifugation. In such procedures only a small amount of organelles is recovered which, in addition, is subject to a lot of artefacts due to the time interval between the beginning of the homogenation of the tissue and the investigation of the more or less contaminated organelles. This is especially true for mitochondria preparations. In addition, it is not possible to study the interaction and compartmentation of metabolic processes which occur in the cytoplasm, in mitochondria, or in plastids, like the regulation of the energy charge of the cell during light-dark transition or developmental processes, as well as protein, lipid, and carbohydrate metabolism.

Recent work indicates that the problems involved in isolating chloroplasts in a more or less undamaged state by mechanical disruption of tissue can be reduced by the preparation of protoplasts (Kanai and Edwards 1973; Edwards et al. 1978; Hampp and Ziegler 1980). When these protoplasts are gently ruptured a large proportion of the organelles remains intact, and efforts have been made to separate intact chloroplasts and other organelles from the cytoplasm and each other by sucrose density gradient centrifugation of protoplast extracts (Nishimura et al. 1976; Nishimura and Beevers 1978; Haas et al. 1979). While this method may be convenient for investigations concerning the compartmentation of enzymes within the cell, it is far too slow to study the distribution of metabolites between different cell compartments.

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Recently, it was demonstrated that relatively pure chloroplast fractions can be separated from intact leaf protoplasts within seconds by an integrated process of protoplast homogenation and selective silicone oil filtration of plastids (Robinson and Walker 1979; Wirtz et al. 1980). It appeared therefore worthwhile to investigate whether this method can be improved in such a way that it delivers mitochondria in addition to pure plastid fractions and a cytoplasmic supernatant at the same time. This was of particular interest with respect to the compartmentation and regulation of chloroplast development (Hampp and Schmidt 1976; Hampp and Wellburn 1976; Hampp 1978; 1979).

In this paper a microgradient method is presented that meets the requirements for a rapid separation of mitochondria and plastids from protoplast homogenates with acceptably low levels of cross contamination, as determined by the use of specific markers. As documented by the determination of ATP pool sizes, this method further offers the possibility for rapid quenching of metabolic reactions within all fractions in order to prevent any significant changes in the metabolite levels during the course of cell fractionation.

The results show that with slight modifications of the microgradient employed, this method can be applied to all stages of the etioplast-chloroplast transition associated with the process of the greening of etiolated primary leaves of *Avena*.

Material and Methods

Materials. Seedlings of *Avena sativa* L. (var. Arnold) were grown in moist peat at 25° C for 7 d in the dark. Different periods of greening were achieved by partial illumination (Osram HQLS, 400 W) during the later stages of growth (8.9 W m^{-2} at seedling level). When green plants were required, seedlings were grown in a greenhouse.

Cellulysin was obtained from Calbiochem (Paesel, Frankfurt, FRG). All other biochemicals were from Sigma (München, FRG). The nylon nets (200 µm and 20 µm mesh) were ordered from Henry Simon Ltd., Stockport, U.K., silicone oils (Type AR and CR, viscosity and density adjusted by mixing AR 20/200 and CR 50/100 oils, respectively, see Results) were from Wacker Chemie (München, FRG).

Isolation of Protoplasts. The preparation of protoplasts was carried out essentially as described recently (Hampp and Ziegler 1980) by incubation of leaf segments (1 mm in width) in 2% cellulysin, 0.6 M mannitol, 1 mM $CaCl_2$, 0.5% BSA, and 5 mM MES-KOH (pH 5.6) for 2 h at 30° C. The resulting protoplast suspension was purified on a sucrose-sorbitol gradient, pelleted and resuspended in 0.5 M sorbitol containing 0.5% BSA, 7.5 mM $CaCl_2$, and 20 mM HEPES-KOH (pH 7.6). The density of the protoplast suspension was adjusted to about $0.5 \cdot 10^6$ cells ml^{-1}.

Fractionation of Protoplasts. Purified protoplasts were fractionated into plastids, mitochondria, and the rest of the cellular homogenate employing polypropylene microtubes (450 µl, Beckman) which contained; starting from the tip (see Fig. 1), 20 µl 0.6 M sucrose; 30 µl silicone oil (b) (CR Type in different mixtures of CR 50 and CR 100, see Results); 50 µl 0.4 M sucrose, containing 10 mM EDTA, 50 µl silicone oil (a) (AR 150, density 1,030 at 25° C); an air space of about 70 µl; and a 20 µm nylon mesh which was kept in position by a 6 mm length of polypropylene tubing (outer/inner diameter: 4/2 mm; see also Wirtz et al., 1980). Into the tube space above the nylon net were pipetted 50 µl of the protoplast suspension (diluted to 0.39M sorbitol). The tubes, 4 parallels each time, were centrifuged for 30 to 180 s (see Results) in a Beckman microfuge 152 at 8° C. Immediately after centrifugation the microtubes were cooled on ice and the three fractions (supernatant: S; middle layer: M; pellet: P; Fig. 1) collected by slicing through the silicone layers and the remaining air space between the supernatant and the nylon net (see Fig. 1). Each of the combined P, M, and S fractions were thoroughly mixed with 1 ml HEPES-KOH (20 mM, pH 7.6) plus 8 mM $MgCl_2$ and carefully removed from the remainder of the tubes and the silicone. For comparison, the whole protoplast extract was measured as obtained by homogenation of protoplasts without any further fractionation (Fig. 1). To correct for contamination of the protoplast suspension by broken protoplasts, intact protoplasts were filtered through silicone AR 140, layered on 25% sucrose (20 s, 16,000 *g*, 8° C). Levels of marker activity in the resulting supernatant above the oil were subtracted from those found in the supernatant of the gradient. Phase contrast microscopy of the protoplast layer banding at the oil-sucrose interphase revealed intact cells only.

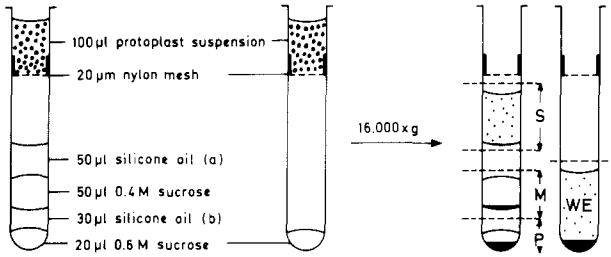

Fig. 1. Schematic presentation of the procedure of homogenation and fractionation of isolated intact protoplasts. Protoplast suspensions were pipetted onto a 20 µm aperture nylon net, fixed inside the microtube by a polypropylene tubing. By centrifugation (16,000 *g*, 30 to 180 s, 8° C), the protoplasts are forced through the nylon net and thereby ruptured. The resulting protoplast homogenate is then fractionated on a density gradient, formed by layers of silicone oils and sucrose (left), or collected without further separation (tubes with 0.6 M sucrose only; control). After the end of the run the fractions are recovered by slicing through the microtubes at the positions indicated (broken lines). In order to quench metabolic reactions, 0.6 M sucrose is replaced by 15% $HClO_4$, and the middle layer fortified with antimycin A and oligomycin in addition to EDTA. Reactions within the supernatant are minimized by closing the tubes (after the addition of protoplasts) with punctured caps, containing a droplet of HCl in their cavity. *S* supernatant; *M* middle layer; *P* pellet; *WE* whole extract. Silicone oils: (a)=AR-Type; (b)=CR-Type. For further details see Methods

Assay of Markers. RuBP carboxylase (EC 4.1.1.39) activity was measured after activation, as described by Lorimer et al. (1977), in a total volume of 0.5 ml at 30° C.

NADP-dependent triose-P dehydrogenase (EC 1.2.1.9) activity was determined by the decrease in absorbance at 340 nm due to the oxidation of NADPH by PGA, following the method of Wolosiuk and Buchanan (1976).

Fumarase (EC 4.2.1.2) was measured at 240 nm according to the method of Racker (1950), but using 50 mM tricine buffer, pH 8.4; the reaction was initiated by the addition of L-malate (final concentration 10 mM).

Cyt c oxidase (EC 1.9.3.1) was determined by following the decrease of reduced Cyt c at 550 nm as described earlier (Hampp 1979). The reaction was started by the addition of extracts that had been preincubated in 0.2% (W/v Triton X-100 for 5 min at 20° C.

NADH-dependent Cyt c reductase (EC 1.6.99.3) was assayed according to Lord et al. (1973) in a medium containing 50 mM phosphate buffer (pH 7.2), 20 µM oxidized Cyt c, 80 µM NADH, and 1 mM KCN. The reaction was initiated by adding the particle suspension (preincubated in 0.1% Triton X-100 for 5 min), and the reduction of Cyt c followed at 550 nm. Antimycin A was added to give a final concentration of 1 µM.

Catalase (EC 1.11.1.6) was measured at 20° C by following the rate of O_2 evolution with a Clark-type O_2 electrode. The assay mixture (1 ml) contained 50 mM HEPES-KOH (pH 7.6) and 1.2 mM H_2O_2, and the reaction was started by the addition of 10 µl of the respective fraction.

PEP carboxylase (EC 4.1.1.31) activity was assayed by the incorporation of labeled bicarbonate. According to Robinson and Walker (1979), the reaction mixture contained 50 mM glycylglycine (pH 7.9), 10 mM $MgCl_2$, 2 mM PEP, 0.4 mM NADH, 2.1 mM [^{14}C]$NaHCO_3$ ($1.2 \cdot 10^5$ Bq µmol^{-1}), 2.5 µg malate dehydrogenase (0.17 µmol·s^{-1}), and aliquots of the respective fractions in a total volume of 0.5 ml. The reaction was started with the extract and terminated 5, 10, and 15 min later by injecting 150 µl aliquots into 200 µl ethanol/acetic acid (95/5). After evaporation at 40° C the incorporation of label into acid stable fixation products was determined by liquid scintillation counting.

Acid phosphatase (EC 3.1.3.2) activity was determined with p-nitrophenyl phosphate (2.5 mM) as the substrate and in acetate buffer (0.3 M, pH 5.6) at 37° C (Bergmeyer 1970). The reaction was stopped after 5, 10, and 15 min by rapidly mixing of 1 ml assay with 0.5 ml KOH in ethanol [2 M; 90% (V/V)], and the change in absorbance at 450 nm was compared to a control to which KOH/ethanol was added at time zero. If not otherwise stated the assays were performed at 25° C and in a total volume of 2 ml.

Protein was determined according to Lowry et al. (1951), cell number by haemocytometer counting (Fuchs-Rosenthal).

Determination of Metabolites. In order to determine metabolite levels a modified gradient was used for protoplast fractionation. In this case the lowermost layer consisted of 15% (V/V) $HClO_4$ and the middle layer was fortified by oligomycin (5 µg ml^{-1}) and antimycin A (1 µM). Additionally, a lid containing 10 µl of 0.15 M HCl in the well was fitted over the microfuge tube as described by Wirtz et al. (1980). A fine pore in the bottom of the lid allowed for the penetration of the HCl into the supernatant above the upper silicone layer (Fig. 1) during the centrifugation step, resulting in a pH of about 2.8. Immediately after centrifugation the tubes were frozen in liquid nitrogen, cut into different segments as shown in Fig. 1, and thawed in concentrated $HClO_4$ to give a final concentration of 15% $HClO_4$. The combined and cooled samples were centrifuged at 8,600 *g* for 2 min and the supernatants brought to pH 7.8 by adding 1 M bicine in 5 M KOH. After 15 min on ice, the precipitated $KClO_4$ was removed (8,600 *g*; 2 min) and the metabolites determined.

RuBP. As a further marker for chloroplasts and less developed stages of etiochloroplasts, the distribution of RuBP across the different fractions was assayed according to Latzko and Gibbs (1972) at 25° C in a medium (1 ml) consisting of neutralized extract, 10 mM $MgCl_2$, 10 mM [^{14}C]$NaHCO_4$ ($3 \cdot 10^5$ Bq µmol^{-1}), 500 µg RuBP carboxylase (0.17 nmol s^{-1}), and bicine-KOH (0.1 M, pH 7.8). The reaction was terminated after 1 h (no more change in the amount of label incorporated between 30 and 60 min) by the addition of 0.5 ml 1 M HCl and the samples dried (60° C) and counted by liquid scintillation. In a parallel assay the reaction was calibrated by the addition of 5 to 50 nmol RuBP.

ATP was measured by the luciferin-luciferase method with the samples acidified and neutralized as described for the determination of RuBP. Aliquots of 10 to 25 µl were mixed with 100 µl of 10 mM $MgSO_4$ in 50 mM glycylglycine (pH 7.75) and automatically reacted with 100 µl Lumit (Abimed, Düsseldorf, FRG). The resulting luminescence was integrated over 10 s (Biolumat, Labor Prof. Berthold, Wildbad, FRG). Each sample was measured in duplicate and internally standardized by the addition of 10 pmol ATP.

Results and Discussion

A satisfactory resolution of plastids (different developmental stages between etioplasts and chloroplasts), mitochondria, and other cellular constituents from each other can be identified by examining the location of enzymes specific to distinct cellular compartments. In Table 1, such a distribution of markers and of protein, following a 60-s fractionation of protoplasts from etiolated (3 h, 6 h, 24 h illuminated) and light-grown primary leaves of *Avena* by the method described in Fig. 1, is shown. Usually, more than 70% (see Table 2) of the two plastid stromal enzymes NADP-dependent triose-P dehydrogenase and RuBP carboxylase were recovered in the pellet fraction (P, Fig. 1). This finding shows that under optimum conditions (time of centrifugation, type of silicone oil) the majority of plastids, independent of their developmental stage, remain intact during the passage through the nylon net, as only intact organelles can penetrate the oil layers (Hampp 1978).

This finding is supported by the similar distribution of RuBP, which is a stromal constituent with only a very limited envelope permeability.

In contrast, of the marker enzymes for mitochondria (fumarase, Cyt c oxidase), microsomes (catalase), and soluble cytoplasmic or vacuolar constituents (PEP carboxylase, acid phosphatase), a maximum of 1% of the total cellular activity is able to pass the lower silicone layer.

Compared to the pellet there is a distinct difference in the distribution in levels of activity of mitochondrial and plastid marker enzymes in the middle fraction (M; Fig. 1). Within this fraction the dominant cellular constituents are mitochondria which, according to the percentage of activity of fumarase and Cyt c oxidase, yield up to 50% of the total amount. In addition, an average of about 18% of the plastid markers can be found within this fraction, as far as the enzymes are concerned. The relative amount of

Table 1. Distribution of markers and of protein following fractionation of *Avena* protoplasts. Protoplasts were isolated from 7 d old etiolated primary leaves of *Avena* without, or after 3, 6, or 24 h of illumination (8.7 Wm^{-2}) and compared to protoplasts from 7 d old light-grown (lg) leaves. After centrifugation for 60 s the fractions of microtubes (see Fig. 1) were combined and the enzyme activities (µmol product formed or substrate consumed h^{-1}), and the protein content (mg) in each fraction determined. RuBP levels (nmol) were measured in quenched gradients (see Methods). For reasons of better comparability all values are referred to P+M+S=1 mg protein. Density of silicone oils (25° C): (a) 1.030 for all stages; (b) 1.066 (0 h); 1.068 (3–6 h); 1.070 (24 h and light-grown)

Marker	Period of illumination (h)	Amount of protein, RuBP, or level of enzyme activity				
		P	M	S	P+M+S	Whole extract
NADPH triose-P dehydrogenase	0	11.0	2.2	0.98	14.2	15.4
	3	15.0	2.5	2.00	19.5	20.5
	6	18.3	7.6	2.80	28.7	27.6
	24	40.5	6.9	6.70	54.1	53.6
	lg	55.2	9.1	4.90	69.2	65.0
RuBP carboxylase	0	1.33	0.37	0.09	1.8	1.76
	3	2.53	0.56	0.40	3.5	3.60
	6	2.91	1.17	0.82	4.9	5.20
	24	4.50	0.82	0.88	6.2	6.30
	lg	7.68	1.36	0.36	9.4	10.2
RuBP	0	0.82	0.04	0.24	1.1	1.2
	3	0.97	0.13	0.30	1.4	1.6
	6	0.98	0.08	0.44	1.5	1.4
	24	1.73	0.25	0.42	2.4	2.7
	lg	2.75	0.09	0.36	3.2	2.9
Cyt c oxidase	0	0	1.8	1.8	3.6	3.2
	3	0	1.8	1.7	3.5	3.9
	6	0	1.7	2.1	3.8	3.7
	24	0	1.7	1.5	3.2	2.8
	lg	0	1.1	1.7	2.8	2.6
Fumarase	0	0	0.29	0.23	0.52	0.53
	3	0	0.37	0.33	0.70	0.76
	6	0	0.17	0.26	0.43	0.42
	24	0	0.19	0.18	0.37	0.40
	lg	0	0.15	0.27	0.42	0.42
Catalase	0	0	112.9	460.1	573	550
	3	5.8	134.9	443.3	584	602
	6	3.3	74.7	752.0	830	921
	24	6.9	34.9	530.2	572	595
	lg	19.3	78.8	705.9	804	820
PEP carboxylase	0	0.003	0.004	0.172	0.18	0.18
	3	0.001	0.003	0.156	0.16	0.15
	6	0.003	0.003	0.153	0.16	0.15
	24	0	0.004	0.116	0.12	0.13
	lg	0.001	0.004	0.145	0.15	0.16
Acid phosphatase	0	0.101	0.101	16.7	16.9	17.9
	3	0.057	0.257	14.0	14.3	15.0
	6	0.098	0.236	12.1	12.4	12.2
	24	0.022	0.335	10.4	10.8	11.7
	lg	0.102	0.179	12.5	12.8	13.1
NADH Cyt c reductase	0	0.225	0.225	0.751	1.20	1.34
	3	0.279	0.354	0.717	1.35	1.43
	6	0.241	0.223	0.956	1.42	1.39
	24	0.184	0.184	0.773	1.14	1.20
	lg	0.216	0.216	0.868	1.30	1.33
Protein	0	0.41	0.22	0.37	1.0	0.87
	3	0.40	0.31	0.29	1.0	0.94
	6	0.45	0.28	0.27	1.0	0.78
	24	0.41	0.34	0.25	1.0	1.09
	lg	0.54	0.16	0.30	1.0	1.05

P = pellet; M = middle fraction; S = supernatant

Table 2. Distribution of markers following fractionation of *Avena* protoplasts. The values represent the rates and levels given in Table 1 for whole protoplast extracts and the three fractions respectively as per cent of the summed fractions (P+M+S). Means of all developmental stages investigated ± standard error ($n=9$)

Marker	Percentage of (pellet + middle fraction + supernatant)			
	Whole extract	Pellet	Middle fraction	Supernatant
NADPH triose-P dehydrogenase	100.6±2.8	74.5±2.8	16.2±2.6	9.3±1.0
RuBP carboxylase	103.8±1.9	72.0±3.6	17.6±1.9	10.4±2.5
RuBP	101.0±1.6	66.8±3.9	8.2±3.2	25.0±4.8
Cyt c oxidase	95.4±2.2	0	47.0±4.2	53.0±3.7
Fumarase	103.2±1.9	0.3±0.3	47.2±3.9	52.7±4.0
Catalase	103.2±2.4	1.0±0.4	13.5±3.3	85.5±3.2
PEP carboxylase	101.0±3.0	1.0±0.4	2.5±0.3	96.5±0.3
Acid phosphatase	103.8±1.7	0.9±0.2	1.8±0.4	97.3±0.4
Cyt c reductase	104.6±2.3	17.8±0.8	18.7±1.9	63.5±2.7

RuBP, however, is considerably lower in the middle fraction but higher in the supernatant when compared to the plastid marker enzymes (see also Table 2). Therefore, it can be suggested that slightly damaged plastids already loose their RuBP in the supernatant before they enter the first oil layer, whereas the slower diffusing stromal enzymes are carried to a higher extend into the middle fraction.

Less than 15% of the peroxisomal marker, catalase, and about 2% of the marker enzymes for soluble cytoplasm were recovered in the middle fraction, suggesting that these cellular components are mainly kept back within the supernatant.

Tables 1 and 2 also indicate that for all levels of enzyme activity determined, the sum of the activities within the three fractions together (P+M+S) is very similar to that recovered in the protoplast homogenate not further fractionated (see Fig. 1).

In Fig. 2 the distribution of marker enzymes between the pellet, the middle, and the supernatant fraction is shown for protoplasts from etiolated and 24-h illuminated primary leaves of *Avena* after different times of integrated centrifugal fractionation and filtration. By optimizing the silicone oil layer (b) with respect to density and viscosity, similar patterns of labeling of the three fractions could be achieved for both etiolated (Fig. 2, a–c) and 24-h illuminated cells (Fig. 2, d–f). The time course for the distribution of the plastid markers (Fig. 2; a, d) shows that after 30 s already more than 60% of their total activity can be recovered in the pellet. In parallel, mitochondrial markers are enriched in the middle layer. With respect to the yield of organelles and because of the absence of any contamination of the pellet (as far as this can be expressed by the markers used, and except endoplasmic reticulum), as well as a relatively small amount of contamination of the middle layer by cellular compartments other than mitochondria,

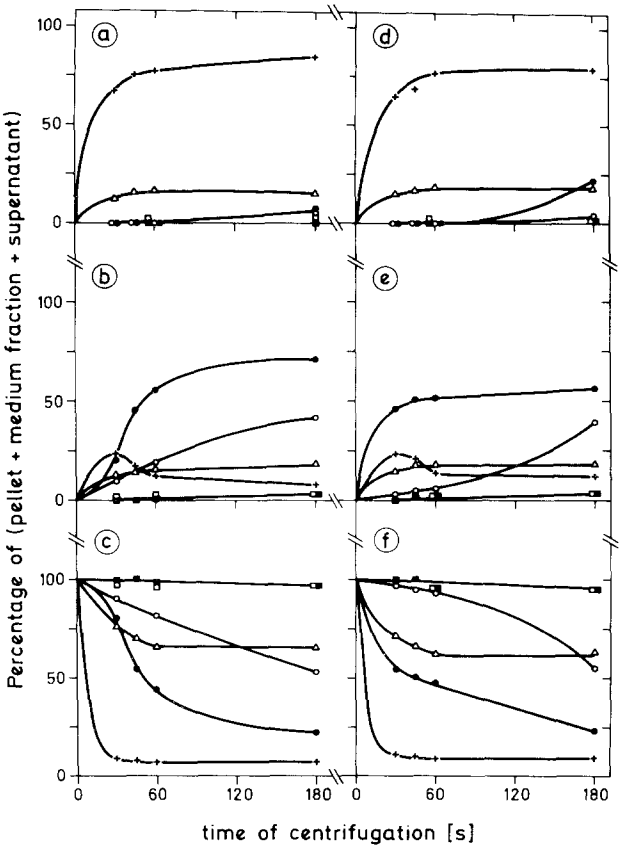

Fig. 2a–f. Distribution of marker enzymes after different times of centrifugal filtration (16,000 *g*, 8° C) between protoplast fractions as obtained by the methods illustrated by Fig. 1. The levels of activity are given as the percentage of the activity in the summed fractions (P+M+S), which could be recovered within the pellet (**a**; **d**), the middle fraction (**b**; **e**), and the supernatant (**c**; **f**). **a–c** protoplasts from etiolated leaf tissue (density of silicone oils at 25° C: **a**=1.030; **b**=1.066; **d–f** protoplasts from 24 h illuminated leaves (density of silicone oils at 25° C: **a**=1.030; **b**=1.070). +–+: RuBP carboxylase, NADPH-dependent triose-P dehydrogenase; △–△: NADH-dependent Cyt c reductase (antimycin A-insensitive); ●–●: fumarase, Cyt c oxidase; ○–○: catalase; □–□: acid phosphatase; ■–■: PEP carboxylase

a centrifugation time of between 45 and 60 s appeared most suitable.

The time-dependent change in the levels of enzyme activity in the supernatant (Fig. 2; c, f) clearly mirrors the events in the other two fractions. If some protoplasts had retained their plasmalemma intact after passing the nylon net, this portion would have penetrated at least the upper oil layer (AR 150 is penetrated by intact protoplasts; see also Wirtz et al. 1980). In this case the levels of PEP carboxylase or acid phosphatase activity should be considerably higher in the middle fraction. Phase contrast microscopy of protoplast preparations forced through a 20 µm aperture nylon net by centrifugation revealed less than 2% of intact cells, as determined by haemocytometer counting.

As the rates given in Table 1 are always referred to as 1 mg of protein of the summed fractions (P+M+S), they also demonstrate developmental changes in enzyme activities during greening. For example, the rates of the plastid markers involved in photosynthetic carbon fixation greatly increase during greening. On the other hand, the mitochondrial enzymes, fumarase and Cyt c oxidase, exhibit a transient increase in activity. This is in accordance with recent reports on mitochondrial activity during chloroplast development, which showed that mitochondria increase their capacity for phosphorylation as well as their maximum rates of metabolite transport during the early stages of greening (Hampp 1979; Hampp and Wellburn 1980).

If the lowermost layer (Fig. 1) is replaced by 15% (V/V) $HClO_4$; the 0.4 M sucrose layer fortified with antimycin and oligomycin (inhibition of oxidative phosphorylation), in addition to EDTA (inhibition of liberated myokinase; Hampp 1979); and the supernatant acidified by a droplet of HCl during centrifugation (see Methods); metabolic reactions in all three fractions can be kept to a minimum. This is shown by the determination of ATP pool sizes.

ATP levels in protoplasts, killed immediately by the addition of perchloric acid to the suspension, were compared to the amounts of ATP recovered after fractionation into P, M, and S. Under the conditions employed, no significant differences were observed between acidified protoplasts and the summed fractions, as indicated by a percentage of recovery ranging from 92 to 107% (experiment shown in Table 3: 97% recovery). The efficiency of quenching of the fractions and of the assay technique for ATP was also shown by the fact that small amounts of ATP added to the different fractions before protoplast fractionation could quantitatively be regained (Tables 3 and 4), while there was a loss of up to 18% without any quenching. The results given in Table 3 also indicate that no ATP is taken from fractions of lower density to those of a higher one.

To determine a possible destructive effect on the organelle membranes by the addition of HCl to the supernatant during the centrifugation step, the distribution of plastid and mitochondria markers was measured in the absence and presence of 10 µl 0.15 M HCl. No significant changes were found, indicating that mitochondria and plastids penetrate the upper oil layer before any effect of the lowered pH (2.8 after mixing) can take place (see also Wirtz et al. 1980).

These findings formed the basis for the determina-

Table 3. Recovery of ATP. Determination of cellular ATP, and ATP added to the different gradient layers prior to the fractionation of protoplasts from etiolated tissue. To 4 microtubes were added 1.6 (P; 15% $HClO_4$); 4.8 (M; 0.4 M sucrose + oligomycin, antimycin, EDTA); 8.0 (S) nmol ATP. In the case of S a droplet containing the required amount of ATP was pipetted onto the surface of the upper oil layer before the nylon net was inserted. In parallel, protoplasts were fractionated on gradients with no ATP added. To measure a possible loss of ATP during the processing of the samples, intact protoplasts were killed with 1.5 volumes of 15% $HClO_4$, with or without 2 nmol of ATP. The same procedure was employed for the filtration of intact protoplasts (last column). Each value represents the average of three independent experiments

	Sample				Total protoplast homogenate (not fractionated)	Intact protoplasts after oil filtration	
	Pellet	M-Fraction	Supernatant	P+M+S		Supern.	Pellet
Sample ATP (nmol)	0.487	0.306	1.173	1.965 (97)[a]	2.020	0.242 (11.9)[a]	1.798 (89)[a]
+ATP (1.6; 4.8; 8; 2; 2; 2 nmol)	1.967	5.416	9.423	16.805	3.940	2.107	3.684
Total ATP–sample ATP (nmol)	1.480	5.110	8.250	14.84	1.920	1.865	1.886
Recovery of added ATP (%)	93	106	103	103	96	93	94

[a] Yield as percentage of the ATP concentration associated with unfractionated, $HClO_4$ quenched, protoplasts (100% = 2.020 nmol ATP)

Table 4. Levels of ATP in acidified protoplasts from etiolated and greening primary leaves of *Avena*, and within quenched plastid, mitochondria and cytoplasmic fractions obtained therefrom by the method illustrated by Fig. 1. The terms "plastids", "mitochondria", "cytoplasm" represent 100% per cell of each respective compartment as calculated from the distribution of specific markers (for explanation see Results and Discussion). They are therefore different from the fractions P, M, and S obtained by gradient centrifugation; values ± standard deviation ($n=4$)

Compartment	Period of illumination (h)			
	0	2	24	Light-grown
Plastids				
nmol ATP per				
10^6 protoplasts	1.08 ± 0.04	3.5 ± 0.15	1.1 ± 0.12	0.8 ± 0.01
mg protein	0.8	1.3	0.7	0.5
mg protein 10^{-6} protoplasts	1.35	2.7	1.57	1.6
Mitochondria				
nmol ATP per				
10^6 protoplasts	1.31 ± 0.13	1.45 ± 0.14	1.12 ± 0.06	0.25 ± 0.01
mg protein	2.0	2.5	2.8	2.7
mg protein 10^{-6} protoplasts	0.66	0.60	0.4	0.09
Cytoplasm				
nmol ATP per				
10^6 protoplasts	5.27 ± 0.4	19.6 ± 0.8	3.6 ± 0.4	2.6 ± 0.2
mg protein	54	113.3	78	60
mg protein 10^{-6} protoplasts	0.1	0.17	0.05	0.04
Intact protoplasts				
nmol ATP per				
10^6 protoplasts	7.9 ± 1.3	24.1 ± 0.2	6.3 ± 0.6	3.8 ± 0.3
mg protein	3.9	7.6	2.9	2.5
mg protein 10^6 protoplasts	2.0	3.2	2.2	1.5

tion of ATP pool sizes within the three fractions obtained from protoplasts of different stages of greening (Table 4). In parallel to the measurement of ATP the distribution of markers was monitored. From the percentage of marker levels associated with each fraction, the total amount of plastids, and, by subtracting plastidic and cytoplasmic contaminations from the middle fraction, the total amount of mitochondria per cell together with the ATP levels associated with each compartment can also be calculated.

The ATP level within the cytoplasm was then obtained by subtracting the calculated ATP concentrations in total plastids and mitochondria per cell from the summed fractions (P + M + S). This was done after correcting the amount of ATP within the gradient supernatant for ATP measured in the supernatant which was obtained after silicone oil filtration of intact protoplasts (see Methods). This takes into consideration the level of free ATP in the protoplast suspension before homogenation, which is due to a certain amount of broken cells (about 7–12%; see Table 3: percentage of ATP in S of filtered intact protoplasts with respect to the total amount).

In Table 4 the results of the respective determinations and calculations are given as actual in vivo levels of ATP within the plastids, mitochondria, and the cytoplasm of 10^6 protoplasts or per mg protein, in relation to the developmental stage of the tissue. On a per cell basis as well as in relation to protein, there is a distinct increase in the ATP pool of plastids and of the cytoplasm after about 2 h illumination, when compared to the etiolated system. In contrast, the level of ATP associated with mitochondria is on a high level during the early stages of greening and then decreases with further development ($\geqq 24$ h) if expressed per 10^6 protoplasts. Referred to protein, however, ATP does not show significant changes during development. This suggests that mitochondria keep a constant ATP level, independent of their respiratory activity, by an appropriate adaption of the ATP/ADP exchange translocation (see Hampp and Wellburn 1980), and that the number of mitochondria per cell (i.e., protein) decreases significantly when plastids reach maturity.

While the ATP level of plastids (protoplast suspensions of all stages were kept in the dark for 15 min before fractionation) returns to that of the etiolated stage after prolonged times of illumination, that of

the cytoplasm rises up to 400% after 2 h illumination and comes down to about 50% in green tissue when compared to etiolated cells. Both the higher amount of mitochondrial protein and the significant increase in cytoplasmic as well as plastid ATP in the first stages of greening should be almost certainly due to a high demand for energy at a developmental stage, where no photophosphorylation can be performed but an intensive biosynthetic requirement exists (Wellburn and Hampp 1979).

It now appears of interest to investigate whether the increase in ATP is also true for other adenylates (ADP, AMP), i.e., to find out how the energy charge within the different compartments is altered during the transition from oxidative to photophosphorylation, if at all.

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