Plant Physiol. (1982) 69, 448–455
0032-0889/82/69/0448/08/$00.50/0

Adenylate Levels, Energy Charge, and Phosphorylation Potential during Dark-Light and Light-Dark Transition in Chloroplasts, Mitochondria, and Cytosol of Mesophyll Protoplasts from *Avena sativa* L.[1]

Received for publication April 3, 1981 and in revised form July 21, 1981

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ABSTRACT

The compartmentation of cellular energy relations during dark-light and light-dark transitions was studied by means of a newly developed technique to fractionate oat (*Avena sativa* L., var. Arnold) mesophyll protoplasts. Using an improved microgradient system with hydrophobic and hydrophilic layers of increasing density, a pure plastid pellet (up to 90% of total chloroplasts) could be separated from an interphase of only slightly contaminated mitochondria (70 to 80% of total mitochondria), and a cytoplasmic supernatant could be obtained within 60 seconds. Appropriate controls indicate that, under the conditions employed, metabolic interconversions of adenylates can be kept to a minimum and, thus, be determined and corrected for. Cross contamination of the fractions, as well as liberation of organelles to the supernatant, was assessed by specific markers, and the metabolite levels recorded were corrected accordingly. Using this technique, we found that, during dark-light transition, the chloroplastic and cytosolic ATP exhibits a rapid increase, while the mitochondrial ATP level decreases. In all compartments, ADP levels mirror alterations of the ATP pool in the opposite way, at least to some extent. To compensate fully for the rise in ATP, chloroplastic and mitochondrial AMP levels change accordingly, indicating that, due to the more or less unchanged level of total adenylates, there is no net flux of adenylates between the compartments. In contrast to the organelles, no AMP could be detected within the cytosol. When the light is turned off, a decrease of ATP coincides between chloroplast stroma and the cytosol for only about 30 seconds. Under prolonged dark treatment, cytosolic ATP rises again, while stroma ATP levels exhibit a further decrease. After about 60 seconds of darkness, the cytosolic ATP level is back to its initial value. This obviously is due to the immediate rise in mitochondrial ATP upon darkening, which cumulates after about 60 seconds; then, caused by an ATP/ADP exchange with the cytosol, it levels off again at the state before changing the conditions, as soon as the cytosolic ATP is also back to its original level. All of these events are closely mirrored by the change in the ATP/ADP ratio and the energy charge within the compartments. While the values for chloroplasts exhibit considerable differences between dark and light, those calculated for mitochondria and the cytosol exhibit only transient changes. These are limited to about 60 seconds of undershoot or overshoot, with respect to the cytosol, and then return to nearly the levels observed before changing the conditions. Adenylate kinase was found to be exclusively associated with chloroplasts (90% of total activity level) and mitochondria. Isotonic liberation of vacuoles did not point toward a significant association of adenylates with this compartment.

The results are discussed with respect to an effective collaboration between photosynthetic and oxidative phosphorylation in order to keep the cytosolic energy state at a constant, preset value.

[1] Supported by a grant from the Deutsche Forschungsgemeinschaft (Ha 970/5).

Energy production in heterotrophic cells of higher organisms takes place mainly in mitochondria. The ATP built up in these organelles is exported to ATP consuming sites of the cell and a back flow of ADP maintains phosphorylation (18, 20).

In cells of eucaryotic green plants, a similar situation probably exists in the dark. Under illumination, however, the production of ATP by chloroplasts was shown to exceed considerably that by mitochondria, both organelles yielding about 90% of the ATP which is generated by the cell (30). Because many energy-requiring reactions proceed outside of these organelles, *e.g.* synthesis of protein, fatty acids, and sucrose, transfer and regulation of phosphorylation between organelles and cytosol is a prerequisite.

Investigations on adenine nucleotide transfer demonstrated the existence of specific adenylate carriers on the transport-limiting membranes of both mitochondria and chloroplasts (15, 18, 19).

While such a carrier is also highly active in plant mitochondria (similar to animal mitochondria), the exchange of phosphorylation power across the chloroplast envelope is primarily of indirect nature via a dihydroxyacetone-P/glycerate-3-P shuttle (15, 18).

Interactions between mitochondria, chloroplasts, and cytosol have not been studied in detail up to now. Of particular interest in this respect is the manner in which photosynthetic events interact with mitochondrial respiration. On the basis of physiological evidence, *e.g.* specific radioactivity of $^{14}CO_2$ evolved in the light or O_2 effect on CO_2 compensation point, many investigators favor the view that dark respiration is inhibited in the light either partially or totally.

The most likely mediators of the interaction between photosynthesis, respiration, and cytosolic metabolism are the adenylates, Pi, and nicotinamide adenine dinucleotides. These cofactors are known to control many respiratory enzymes (10).

Recently, we have developed a microgradient method that meets the requirements for a rapid separation of mitochondria and plastids from protoplast homogenates with acceptably low levels of cross contamination, as shown by the use of specific markers (11). Meanwhile, we have further improved this technique and used it to determine pool sizes of adenylates and inorganic phosphate.

The results given in this paper on events during dark → light and light → dark transition for the first time clearly demonstrate the close interaction of all compartments in keeping the cytosolic energy charge constant.

MATERIALS AND METHODS

Preparation of Protoplasts. Primary leaves from 7-day-old oat seedlings (*Avena sativa* L., var. Arnold) were sliced into segments of 0.5- to 1-mm width and macerated by incubation in 2% Cellu-

lysin (Calbiochem), 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, essentially as described previously (12). The resulting protoplast suspension was purified on a sucrose-sorbitol step 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 in a 50-μl volume in 0.38 M sorbitol, 20 mM Hepes-KOH (pH 7.6), 7.5 mM $CaCl_2$, and 10 mM $NaHCO_3$ were placed above a 20-μm nylon mesh, fixed in a 400-μl microfuge tube by a 6-mm length of tubing (11, 31). Additionally, the tubes contained (starting from the tip) 20 μl 0.6 M sucrose or 15% (v/v) $HClO_4$, 30 μl silicone oil (density at 25°C, 1.070; Wacker Chemie, Munich, Federal Republic of Germany), 50 μl 0.4 M sucrose without or fortified with 10 mM EDTA, 5 μg ml^{-1} oligomycin, 1 μM antimycin A, 50 μl silicone oil (density at 25°C, 1.030), and air space of about 70 μl. The tubes, four parallels each time, were centrifuged for 60 s in a Beckman microfuge (11). If experiments under different light regimes were performed, illumination (150 w m^{-2}) of the tubes containing protoplasts was carried out with the tubes already fixed in the microfuge head and continued during centrifugation.

In parallel experiments, intact protoplasts or isolated vacuoles (see below) and medium were separated by directly pipetting the suspension over 50 μl silicone oil (density at 25°C, 1.028) and centrifugating as above for 20 s into 50 μl 0.6 M sucrose or 15% (v/v) $HClO_4$.

In all experiments, the tubes were kept at temperatures between 18 and 20°C before insertion into the microfuge, which was brought to 4 to 8°C before starting the experiment. If illumination was carried out with the tubes already positioned in the microfuge head, the light source kept the surface of the tubes at the required temperature. At the end of a centrifugation (60 s), the surface of the tubes did not show a temperature lower than 16 to 18°C. This was due to a warming of the air inside the centrifuge during an experiment. If the centrifuge was not precooled, sample temperatures reached about 30°C after 60 s of centrifugation. Under these special precautions, temperatures during incubation and fractionation could be kept fairly constant. Thus, a disturbance of the protoplast metabolism by a larger transient of temperature could be avoided.

Critical Assessment of the Method and the Data Obtained. The experimental approach used to fractionate protoplasts offers some uncertainty about the data given for the mitochondrial fraction. As the agents used for halting mitochondrial metabolism require about 30 s for full inhibition (Fig. 1), changes of mitochondrial levels of metabolites are to be expected and are actually shown in Table II. Therefore, with respect to the ATP/ADP ratio, the values reported for mitochondria should be lower compared to those for organelles contained in intact protoplasts before fractionation.

Preparation of Vacuoles. Vacuoles were prepared isoosmotically from protoplasts by short-time addition of DEAE-dextran (Pharmacia), according to the method of Dürr *et al.* (8) as modified by Schmidt and Poole (29), and purified by centrifugation (100,000*g*, 30 min; SW 27, Beckman L-5/50) on a three-step discontinuous Ficoll-400 gradient (Pharmacia) (25). The vacuoles were carefully removed from the gradient with a 1,000-μl Eppendorf pipette, diluted 10 times with Ficoll-free medium, and pelleted again (100*g*, 5 min) to reduce the amount of Ficoll. Aliquots were used to determine, in parallel metabolites, osmotic space (tritiated water − [^{14}C]sorbitol space) and contamination by other cellular constituents.

Correction for Cross-Contamination. Cross-contamination of the different compartments was routinely monitored in samples of unquenched gradients (0.6 M sucrose instead of $HClO_4$; 0.4 M sucrose without inhibitors), which were run in parallel and verified by the determination of specific markers (RuBP[2] carboxylase, NADP-dependent triose-P dehydrogenase, fumarase, Cyt *c* oxidase, catalase, PEP carboxylase, and acid phosphatase [for assays, see Ref. 11; compare, also, Table I]). Thus, all the metabolite concentrations given in this presentation have been corrected for those in the medium and for cross-contamination, as described earlier (11). Adenylate kinase activity was determined by an optical method (28).

Determination of Metabolites. To determine adenylate levels, gradients containing $HClO_4$ and inhibitors were used for protoplast fractionation, and the amount of metabolic interconversion during fractionation was checked by control experiments (see "Results" and Ref. 11). Immediately after centrifugation, the tubes were frozen in degassed liquid N_2, cut into different segments (11), 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 aliquots of the supernatants were brought to pH 7.8 by adding 1 M bicine in 5 M KOH. After 15 min on ice, the precipitated $KClO_4$ was pelleted (8,600*g*, 2 min) and the adenylates determined in triplicate by the luciferin-luciferase method after enzymic interconversion and internal standardization (31). The resulting luminescence (Lumit, Abimed, Federal Republic of Germany) was integrated over 10 s (Biolumat, Labor Prof. Berthold, Wildbad, Federal Republic of Germany).

Pi was measured spectrophotometrically by a two-step method which increases sensitivity and decreases nonextract blanks deriving from biochemicals and enzymes, as described by Wirtz *et al.* (31). [^{14}C]sorbitol and ^3H_2O spaces of protoplasts, vacuoles, chloroplasts, and mitochondria were measured and calculated, as described by Heldt and Sauer (21). Protein was determined according to Bradford (5) using the Bio-Rad protein assay; Chl was determined by the method of Arnon (1).

RESULTS

Purity and Yield of Fractions. The separation of cellular compartments from each other can be monitored by the determination of specific markers. In a previous report (11), it was shown that, by a technique of integrated homogenation of protoplasts and centrifugal filtration of the homogenate on a gradient of silicone oils, a pure plastid pellet and an only slightly contaminated mitochondrial fraction can be separated from the rest of the protoplast within 60 s. This was indicated by the distribution of a series of specific markers across the microgradient used. In Table I, results are given which show, using some selected markers, that the efficiency of separation can still be improved when a Tris medium is injected into the supernatant during centrifugation to give a final concentration of 50 mM Tris (pH 7.6), 100 mM KCl, and 5 mM $MgCl_2$. This is achieved by covering the microtube by a punctured cap in the cavity of which an aliquot of the medium is pipetted before starting the centrifugation. A comparison shows that, possibly due to the increased ion concentration (which is suggested to reduce adhesion of cellular organelles to each other [29]), nearly 90% of the chloroplast marker NADPH-dependent glyceraldehyde phosphate dehydrogenase is recovered in the pellet, whereas only small amounts are left in the middle layer and in the supernatant. On the other hand, the recovery of fumarase, a mitochondrial marker, under Tris treatment is considerably higher in the middle fraction (up to 80% of the total mitochondrial activity) than it is in the absence of high ion concentrations. There is, however, a slight increase in contamination of the plastid fraction by fumarase, which is still far below 10% of the total level of activity. In contrast, markers for cytosol (PEP carboxylase), vacuolar constituents (acid phosphatase), or microbodies (catalase)

[2] Abbreviations: PEP, phosphoenolpyruvate; RuBP, ribulose 1,5-bisphosphate.

Table I. *Distribution of Markers following Fractionation of Avena Mesophyll Protoplasts in the Presence and Absence of High Ion Concentrations*

Protoplasts were layered on top of the nylon net, and the tube was covered by a punctured cap. In the cavity of the cap were pipetted either 5 µl bidistilled H_2O (−) or 5 µl of a solution containing 0.5 M Tris (pH 7.6), 1 M KCl, 50 mM $MgCl_2$ (+; compare 'Materials and Methods"). The values represent the rates of activity for whole protoplast extracts and the three fractions (P + M + S). Means of at least four independent experiments. Average SD, ±3%.

Marker	Rates of Activity (Pellet + Middle Fraction + Supernatant)							
	Whole extract		Pellet		Middle fraction		Supernatant	
	−	+	−	+	−	+	−	+
	%							
NADP triose-P-dehydrogenase	100.6	102.4	74.5	87.3	16.2	6.9	9.3	5.8
Fumarase	103.2	112.0	0.3	6.5	47.2	76.3	52.7	17.2
Acid phosphatase	103.8	110.9	0.9	1.5	1.8	1.6	97.3	96.9
Catalase	103.2	101.0	1.0	2.3	13.5	11.7	85.5	86.0
Adenylate kinase	ND[a]	86.0	ND	77.5	ND	17.2	ND	5.3

[a] ND, Not determined.

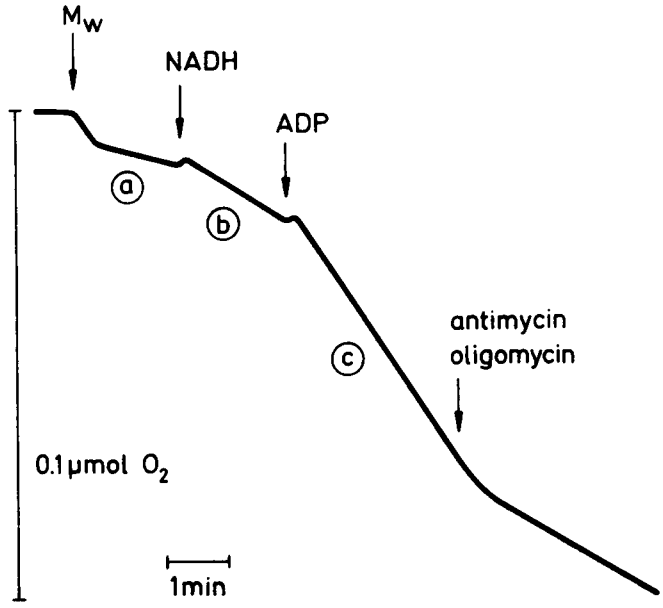

FIG. 1. Oxygen electrode tracing of respiration by mitochondria isolated from *Avena* leaf tissue by differential centrifugation (1,000*g*, 5 min; 10,000*g*, 10 min). The 1-ml reaction medium contained 0.35 M mannitol, 25 mM Hepes (pH 7.2), 5 mM $MgSO_4$, 5 mM KH_2PO_4, 0.1% BSA, and about 0.3 mg mitochondrial protein. Additions were: (a) none; (b) 0.6 µmol NADH; and (c) 100 nmol ADP, 1 nmol antimycin, and 5 µg oligomycin. State 3 respiration (c) refers to the rate of O_2 uptake in the presence of ADP, and state 4 refers to the rate upon depletion of ADP, *i.e.* when all ADP has been phosphorylated, and was identical with trace (b; no ADP added). M_w, washed mitochondria; temperature, 20°C.

under both conditions contribute negligibly to the plastid, as well as to the mitochondria-enriched, fractions. As seen by the recovery of the total marker activity, addition of Tris to the supernatant did not affect the levels of marker enzyme activities, nor could a changed degree of leakage of adenylates from plastids or mitochondria be observed in comparison to fractionations without Tris medium. Thus, the results given in Table I demonstrate an improved separation of plastids, mitochondria, and cytosolic fractions from each other within 60 s and in the presence of high ion concentrations, but only during the homogenization step (*i.e.* for a few seconds, until the organelles have passed the upper oil layer).

Table II. *The Ratio of ATP/ADP within Conventionally Isolated Mitochondria under Different Conditions of Respiratory State and Metabolic Quenching*

Washed mitochondria were incubated in an O_2 electrode, as shown in Figure 1, and samples were taken at the metabolic states indicated. They were either pipetted into ice-cold $HClO_4$ (final concentration, 10%) or immediately subjected to silicone oil filtration under the conditions described for protoplast fractionation. The silicone oil was underlayered by 50 µl of the respective solution. Respiratory control was 2.8. Means of two to three independent experiments.

	ATP/ADP			
		After filtration across silicone		
	$HClO_4$, without filtration	$HClO_4$	Antimycin/oligomycin/EDTA in 0.4 M sucrose	Sucrose, 0.4 M
	ratio			
No substrate added (a)[a]	0.94	0.92	0.87	0.72
State 4 (b)	1.50	1.45	1.23	1.04
State 3 (c)	1.70	1.63	1.0	0.60

[a] (a), (b), (c), Respective traces in Figure 1.

Assessment of Metabolic Alterations of the *in Vivo* Metabolite Pools during Fractionation. For the determination of metabolite pools by this technique, the lowerest layer (0.6 M sucrose) is replaced by 15% (v/v) $HClO_4$. This results in rapid quenching of metabolic interconversions as soon as chloroplasts enter this layer and was established by comparing the ATP/ADP ratios of chloroplasts which were isolated from protoplasts and, after illumination, injected into $HClO_4$ either directly or after microgradient centrifugation under continued light treatment. Both trials gave identical values. In addition, the middle layer (0.4 M sucrose) was fortified with oligomycin and antimycin A (inhibition of oxidative phosphorylation), together with EDTA (inhibition of liberated adenylate kinase). For comparison, experiments were also performed in which, similar to the technique of Tris injection, the supernatant was mixed with a droplet of HCl (11, 31) to quench metabolic interconversion of adenylates in the cytosolic fraction. Between both treatments, no significant differences in adenylate recovery (ATP + ADP), as well as in the ATP/ADP ratio, could be observed; this was possibly due to the lack of adenylate kinase outside chloroplasts and mitochondria (see Table VI). Therefore, for reasons of better comparability, all gradients (quenched and sucrose only) were run by injecting Tris medium during centrifugation. Whereas the quenching of stroma and supernatant metabolism does not create any obvious problems, we tried to assess to what extent changes in the metabolite pool contained within mitochondria do occur during fractionation. For this purpose, we conventionally isolated mitochondria from oat leaves by differential centrifugation. Immediately after recording of their respiration rates in the O_2 electrode (Fig. 1), aliquots were taken and either injected directly into $HClO_4$ (final concentration, 10%) or pipetted on the microgradient used for protoplast fractionation but containing 0.4 M sucrose, 0.4 M sucrose + antimycin A + oligomycin + EDTA, or 10% $HClO_4$, respectively. In each case, the microtubes were centrifuged for 60 s, and the samples were analyzed for ATP and ADP. As presented in Table II, there is

almost no difference in the ATP/ADP ratio between $HClO_4$-quenched organelles and those centrifuged across the gradient for 60 s into a layer of $HClO_4$ or sucrose plus inhibitors when no substrate for respiration is added (Fig. 1A); without any inhibition, the ATP/ADP ratio is slightly decreased. On addition of NADH, mitochondria exhibit state 4 respiration with rates of about 0.18 μmol O_2 consumed/min·mg protein. In this case, the quenching of metabolic interconversions as compared to $HClO_4$ extracts is less effective but still yields about 85% of the ATP/ADP ratio found under direct $HClO_4$ precipitation. However, after inducing a state 3 respiration by the addition of ADP (Fig. 1C), the ATP/ADP ratio in the presence of inhibitors decreases to about 60% of that calculated for $HClO_4$ extracts and is further decreased in the presence of sucrose only (35%). If *in vivo* respiration is between states 4 and 3, as has been suggested for animal cells (6, 7), then a maximal underestimation of the recovered ATP/ADP ratio between 15 and 40% has to be regarded. Thus, the values for energy charge calculated for mitochondria (see below) are possibly to some degree smaller then those existing *in vivo*, *i.e.* before fractionation of protoplasts.

Molarities of Adenylates and Inorganic Phosphate within Different Cellular Compartments. In parallel to metabolite determinations, the volumes of different cellular spaces were measured. For this purpose, protoplasts were incubated in ^3H_2O and [^{14}C]-sorbitol and, after about 2 to 5 min of incubation (constant labeling), filtered through silicone oil, as described in "Materials and Methods." To obtain the osmotic space of chloroplasts and mitochondria, protoplast homogenates were treated as above and subsequently filtered on a microgradient, omitting the sucrose interphase between oil layers. Isotonically liberated vacuoles were treated as shown for intact protoplasts. After correction for cross-contamination and adhering medium, the osmotic spaces (organelles, vacuoles, protoplasts) were obtained. The results of all these measurements are summarized in Table III. According to these values, the osmotic space of chloroplasts is about 26 μl mg^{-1} Chl or about 10% of the total osmotic space of oat protoplasts, while mitochondria occupy only about 4% of the cell. The osmotic space of vacuoles was derived from microscopic analysis and osmotic space determinations of isotonically liberated vacuoles and found to be between 70 and 80% of the protoplast volume (*i.e.* about 18 μl 10^{-6} protoplasts). However, with respect to this compartment, errors can be considerably higher than those for organelles, due to possible osmotic stresses during preparation, although care was taken to keep the osmotic environment as constant as possible. Based on these spaces, molarities of adenylates and of Pi in the different compartments of darkened protoplasts are given in Table III.

With respect to the adenylate concentrations, plastids and mitochondria show comparable levels, while the extra organellar space exhibits a much lower overall concentration (cytoplasm + vacuoles). In separate experiments, we analyzed the amount of adenylates associated with isolated vacuoles and corrected it as far as possible for cytosolic and organellar contamination due to the amount of specific markers contained in oil-filtered vacuoles (percentages of total protoplast activity: fumarase, 28; NADP-dependent triose-P dehydrogenase, 13; and PEP carboxylase, 31; for assays, see Ref. 11). These determinations indicated no considerable (<5% of total adenylates) amount of adenine nucleotides contained in the vacuolar compartment. The molarities of Pi, calculated in the same way, are obviously rather identical in all spaces (darkened chloroplasts, vacuoles) except mitochondria; these organelles exhibit a matrix concentration of Pi that is about 5 times that measured for total protoplasts on an average.

Adenylate Levels and Energy Charge during Dark-Light and Light-Dark Transition. Figure 2 (A–C) summarizes the compartmented fluctuations of adenylates during the first 5 min of illumination of protoplasts kept in the dark for at least 20 min (all measurements are corrected for metabolites in the medium deriving from contaminating free organelles and cross-contamination of the fractions). The response of chloroplast associated adenylates is shown in Figure 2A. Upon illumination, ATP levels increase up to 240% of the dark values, reaching a maximum after about 30 s. This increase is followed by a slight decrease under continued illumination, leveling off at around 190% of the dark value after 5 min in the light. Inasmuch as ADP is the natural phosphate acceptor in photophosphorylation, the response of this adenylate is the reverse of that of ATP. However, the change in ADP levels is not equivalent to that for ATP. This is due obviously to a considerable drop in AMP during the first 30 s of illumination. In parallel to the reduction in ATP level both, ATP and AMP exhibit an increase during continued light treatment. All of these changes markedly influence the energy charge ([ATP] + ½[ADP]/[ATP] + [ADP] + [AMP]) of the chloroplast stroma which rises upon illumination from 0.55 to 0.81 and reaches a steady state at 0.71 (Table IV). If the amounts of ATP, ADP, and AMP are added, a more or less constant figure is obtained (Fig. 2, A–C), which may indicate that there is no net flux of total adenylates between the compartments investigated.

In Figure 2B, the corresponding adenylate levels associated with mitochondria are illustrated. The results clearly demonstrate an immediate but transient reduction in ATP upon illumination, which is closely mirrored by an increase in AMP, whereas the ADP level stays fairly constant as does the sum of all adenylates. In parallel, there is a drop in mitochondrial energy charge from 0.68 to 0.54, which recovers again to the dark level after about 5 min of illumination.

In contrast to the organellar adenylate pools, no significant amounts of AMP could be detected in cytosolic fractions, corrected for contamination by mitochondria and chloroplasts (Fig. 2C). As a response to photophosphorylation, there is a concomitant rise in the cytosolic ATP level, which accumulates about 30 s later, compared to that in chloroplasts, obviously due to a somewhat retarded energy transfer between chloroplast stroma and cytosol. Again, under prolonged illumination, the ATP level drops to

Table III. *Molarities of Adenylates and of Pi within Different Cellular Compartments of Protoplasts in the Dark*

The volumes represent osmotic spaces ([^{14}C]sorbitol impermeable) of protoplasts, chloroplasts, mitochondria, and vacuoles. Before starting the experiment, protoplasts were kept in the dark for 20 min at 20°C ± SD (n = 4–7).

Compartment	Volume	ATP	ADP	AMP	Total ADN	Pi
	μl 10^{-6} protoplasts			*mM*		
Chloroplasts	2.5[a] ± 0.2	0.32	0.60	0.20	1.12	6.4 ± 1.6
Mitochondria	1.05 ± 0.2	0.81	0.53	0.23	1.57	32.6 ± 9.6
Vacuoles + cytosol	19.5 ± 1.3	0.08	0.06	0	0.14	4.5 ± 1.8
Vacuoles	18.0 ± 2.8					4.4 ± 2.2
Protoplasts	23.05 ± 1.1	0.15	0.12	0.04	0.31	6.7 ± 1.1

[a] 26 μl mg chl·$^{-1}$.

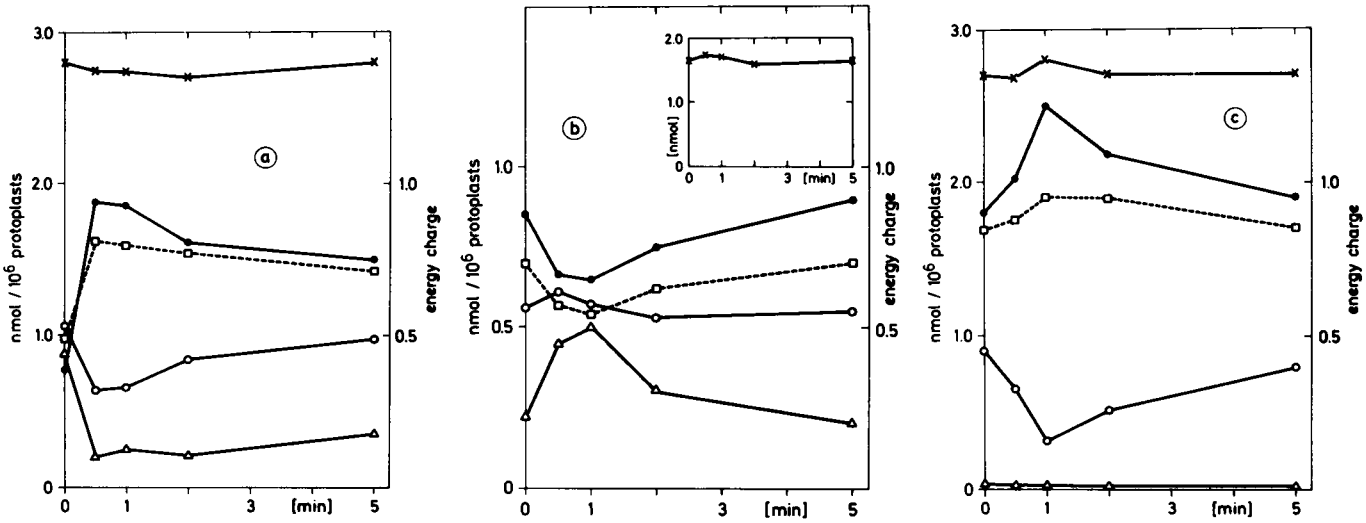

FIG. 2. Time course of changes in adenylate levels during transition from dark to light. Before starting the experiment, protoplasts were kept in the dark for 20 min (20°C). Illumination (150 $w \cdot m^{-2}$) was performed with the suspension of intact protoplasts above the nylon net, and centrifugation started at the times indicated. During centrifugation, illumination was continued. (●——●), ATP; (○——○), ADP; (△——△), AMP; (x——x), total adenylates; (□- - -□), energy charge. (a) Chloroplasts; (b) mitochondria; (c) cytosol.

Table IV. *Energy Charge and ATP/ADP Ratio during Dark-Light and Light-Dark Transition in Chloroplasts, Mitochondria, and Cytosol of Oat Protoplasts*

The calculations are based on the values for adenylates given in Figures 2 and 3. Energy charge (EC) is defined as [ATP] + ½[ADP]/[ATP] + [ADP] + [AMP].

Treatment	Chloroplasts		Mitochondria		Cytosol	
	EC	ATP/ ADP	EC	ATP/ ADP	EC	ATP/ ADP
		ratio		*ratio*		*ratio*
Dark	0.55	0.53	0.68	1.50	0.84	1.45
Light, 30 s	0.81	2.92	0.57	1.07	0.88	3.12
Light, 60 s	0.79	2.71	0.54	1.11	0.95	8.33
Light, 120 s	0.77	1.94	0.62	1.42	0.94	4.40
Light, 300 s	0.71	1.58	0.70	1.64	0.85	2.38
Light, 60 s; dark, 30 s	0.63	1.21	0.58	1.09	0.72	0.77
Light, 60 s; dark, 60 s	0.55	0.73	0.77	2.83	0.81	1.70
Light, 60 s; dark, 120 s	0.54	0.64	0.62	1.03	0.85	3.51
Light, 60 s; dark, 300 s	0.56	0.83	0.58	0.75	0.89	3.66

somewhat above the dark values after 5 min of light. In this case, because of the lack of AMP, cytosolic ADP shows exactly the opposite behavior, compared to ATP. As a consequence, cytosolic energy charge rises transiently from 0.84 to 0.95.

If protoplasts are illuminated for 60 s and fractionated immediately afterwards or after up to 5 min in the dark, a completely opposite behavior emerges. This is illustrated by Figure 3 (A–C). In this case, chloroplasts (Fig. 3A) exhibit an instant fall in ATP, while ADP, and to a lesser extent AMP, show a concomitant rise. All three adenylates again tend to reach a steady state ratio between 2 and 5 min of dark treatment, which is also reflected by the constant energy charge level (about 0.55 after 1 min of darkness, compared to 0.75 after 1 min of illumination). As before, mitochondria exhibit an exactly opposite response; their ATP level increases immediately upon turning off the light, and this response is most pronounced between 30 and 60 s of darkness. After about 60 s, mitochondrial ATP accumulates, and, after a 5-min dark treatment, it is back at the initial level. Again, both ADP and AMP change their levels in a way opposite to ATP. All these fluctuations express their effect on the mitochondrial energy charge, which rises from 0.49 to 0.77 and comes back again to 0.58 after 5 min of continuous darkness.

During the first 30 s of darkness, cytosolic ATP decreases in parallel to stroma ATP (Fig. 3, A and C). But then, possibly due to mitochondrial oxidative phosphorylation and ATP export, there is a subsequent rise in cytosolic ATP which comes close to the initial ATP level, while stroma ATP still decreases. The cytosolic ADP level exactly mirrors the change in ATP in the opposite direction. Because of these alterations, there is only a very short-lived transient fall in cytosolic energy charge from 0.84 to 0.72 between 0 and 30 s of darkness.

As observed under the dark-light regime, no considerable net transfer of total adenylates from one compartment to another occurs, as can be derived from the addition of the analytical data for ATP, ADP, and AMP, also given in Figure 3 (A–C).

In Table IV, ATP/ADP ratios under the transients used are given, in addition to energy charge values. Here, interesting differences between the compartments emerge. Whereas the values for energy charge in plastids and mitochondria exhibit a relatively similar range from 0.5 to 0.8, depending on the metabolic state, that of the cytosol is considerably higher. The same is true for the ratios of ATP/ADP.

Computation of the ratios $[ATP][AMP]/[ADP]^2$ shows that the fluctuations in adenylate levels within each compartment do not simply reflect changes in the equilibrium concentrations, induced by altered AMP levels and catalyzed by adenylate kinase (Table V). It, thus, appeared important to investigate the compartmentation and levels of activity of adenylate kinase. From the percentage of adenylate kinase distribution across the three microgradient fractions (Table I), the amount of enzyme activity associated with each compartment was calculated after correcting for cross-contamination of the fractions by means of the distribution of specific markers. The results clearly indicate that adenylate kinase is exclusively associated with chloroplasts and mitochondria, the former showing 90% of the total cellular level of activity. These values were highly reproducible, and there never could be found any activity outside the organelles. The levels of activity ranged from 0.3 (chloroplasts) to 0.03 (mitochondria) μmol ADP formed from ATP and AMP per 10^6 protoplasts·min (Table VI). If organellar protein is used as a reference, then the specific activity of the chloroplast-associated kinase is twice of that of the mitochondrial enzyme. From these observations, it appears that both

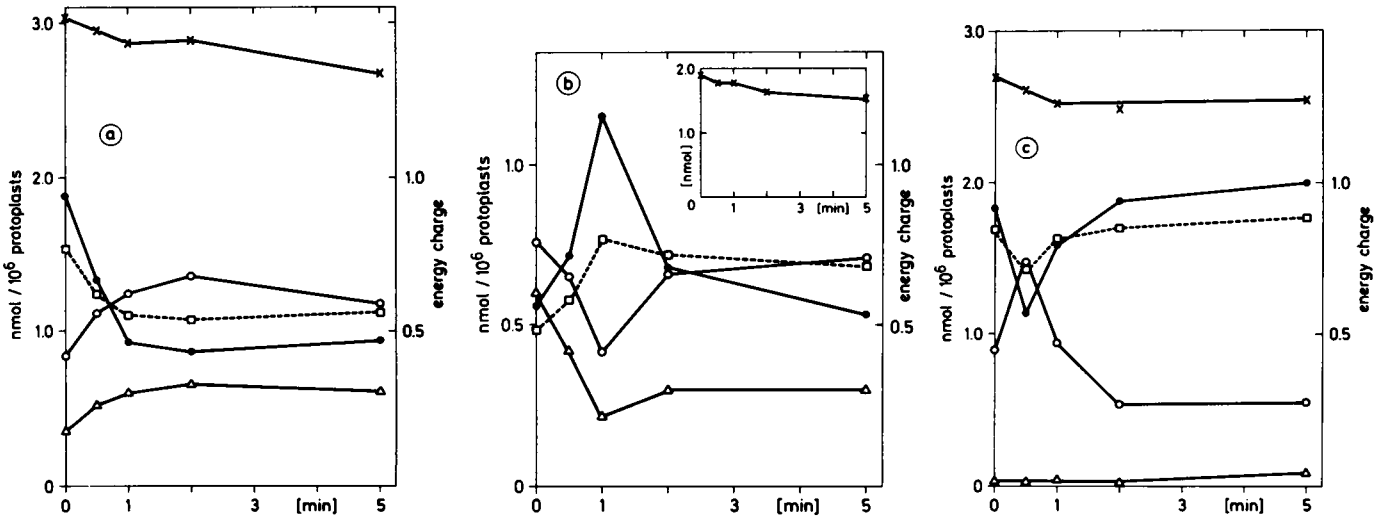

FIG. 3. Time course of changes in adenylate level during transition from light to dark. Before starting the experiment, protoplasts were kept in the dark for 20 min (20°C). Illumination (60 s, 150 $w \cdot m^{-2}$) was performed with the samples already positioned in the microfuge head and continued during centrifugation (0 min of darkness). When different periods of darkness were required, the samples were protected from light after 60 s of illumination, and centrifugation was started at the times indicated. Symbols are as in Figure 2.

Table V. *Mass Action Ratios [ATP][AMP]/[ADP]² in Chloroplasts and Mitochondria during Dark-Light and Light-Dark Transition*

Ratios are calculated from the values given in Figure 2 (A and B) (dark → light) and Figure 3 (A and B) (light → dark). For conditions, see legends to Figures 2 and 3.

Treatment	Chloroplasts	Mitochondria
	ratio	
Dark	0.50	0.66
Light, 30 s	0.90	0.78
Light, 60 s	0.98	1.00
Light, 120 s	0.46	0.80
Light, 300 s	0.58	0.60
Light, 60 s; dark, 30 s	0.57	0.71
Light, 60 s; dark, 60 s	0.48	1.43
Light, 60 s; dark, 120 s	0.31	0.46
Light, 60 s; dark, 300 s	0.45	0.53

mitochondria and chloroplasts contain sufficient adenylate kinase. However, under *in vivo* conditions (*i.e.* within the intact protoplast), initial changes in AMP levels are obviously too fast to be balanced immediately by this enzyme. The lack of adenylate kinase outside the osmotic spaces of chloroplasts and mitochondria is consistent with the absence of AMP.

The curves drawn in Figure 4A give the theoretical adenylate distribution at different energy charge values, as calculated under the assumption that adenylates are in the adenylate kinase equilibrium (equilibrium constant, 0.5; see Ref. 4; see, also, Table V, steady state conditions). They are compared to the distribution observed for chloroplasts, mitochondria (Fig. 4A), and cytosol (Fig. 4B) during dark-light and light-dark transition. In most cases, the measured adenylate distribution in mitochondria and chloroplasts fits the curves, indicating that at least close to steady state conditions (>2 min light or dark) the adenylates were in the adenylate kinase equilibrium. Larger deviations from the equilibrium occur only during the early transition stages. Due to the absence of AMP, however, the cytosolic ATP and ADP levels yield straight lines in relation to the energy charge (Fig. 4B).

DISCUSSION

First attempts to get information on the communication between chloroplastic and cytoplasmic adenylates under transient conditions were made by Heber and his coworkers. They designed experiments which allowed the independent analysis of chloroplasts and the nonchloroplast part of the cell by means of nonaqueous techniques. In these experiments, the ATP/ADP ratio in chloroplasts from intact leaves of *Spinacia*, *Beta*, and *Nicotiana* increased upon illumination from values below 1 up to about 3 (16, 17, 24, 28), and this behavior was mimicked by the extrachloroplastic part of the cell. Reverse changes were observed on darkening. Similar observations have recently been reported by Giersch *et al.* (9), using aqueously isolated spinach chloroplasts. As far as cytosol and chloroplast stroma are concerned, we get similar results with our protoplast system. However, as can be seen, the mitochondrial adenylate levels change independently from those in chloroplasts and cytoplasma.

As soon as chloroplasts start photophosphorylation, there is also a fast transfer of photosynthetic phosphorylation power to the cytosol; this considerably increases cytosolic energy charge. From investigations on animal mitochondria (6, 7), it was concluded that, under conditions which simulate those in the intact cell, the extramitochondrial ATP/ADP ratio determines the rate of entry of ADP into the mitochondria and, thus, the rate of respiration through coupled phosphorylation. Similar conclusions were drawn from manipulations with isolated plant mitochondria (26, 27). In this view, the immediate decrease of mitochondrial ATP in parallel to an increase of AMP upon illumination should be a consequence of an immediate reduction of respiration-coupled phosphorylation.

While under dark-light transition, a good correlation exists between stroma ATP levels and those recorded for the cytosol *per se*; there is a distinct difference after going from light to dark. In this case, our more detailed view on extrachloroplastic events does not fully agree with the observations reported by Heber and Santarius (17, 28), who, under comparable conditions, found an identical qualitative behavior of chloroplastic (stroma) and extrachloroplastic cellular space. There is some parallel between changes in stroma and cytosolic ATP and ADP only within the first 30 s after turning off the light (Fig. 3). But then, due to an immediately increasing ATP level in the mitochondrial matrix obviously combined with further ATP efflux, cytosolic ATP increased again while stroma ATP was still being reduced. This increase is most expressed between 30 and 60 s of darkness, and, as it tends to reach a steady state level (after about 2 min), mitochondrial ATP decreases again. This can be due both to

Table VI. *Compartmentation and Levels of Activity of Adenylate Kinase*

Association with specific compartments is calculated by correction for mutual contamination of the fractions by each other (see Table I and Ref. 11).

Compartment	Total Activity (Protoplast Homogenate)	ADP	Protein	ADP
	%	*μmol formed/min·10⁶ protoplasts*	*mg/10⁶ protoplasts*	*μmol formed/min·mg protein*
Chloroplasts	90	0.30	1.47	0.20
Mitochondria	10	0.032	0.31	0.11
Protoplasts − (chloroplasts + mitochondria)	0	0	0.02	0

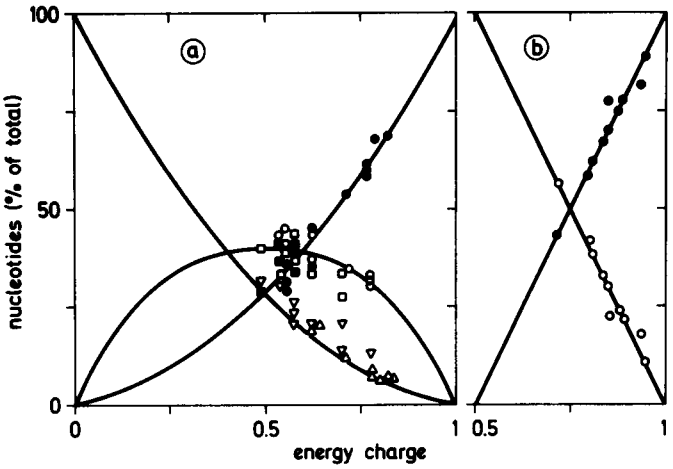

FIG. 4. Relative adenylate concentrations and energy charge in chloroplats, mitochondria (a), and cytosol (b) during transition from dark to light and vice versa. Drawn curves shown relative adenylate concentrations calculated under the assumption that adenylates are in the adenylate kinase equilibrium. The equilibrium constant [ATP] [AMP]/[ADP]² used for the calculation was 0.5 (4). (a) (●——●), ATP, chloroplasts; (■——■), ATP, mitochondria; (○——○), ADP, chloroplasts; (□——□), ADP, mitochondria; (△——△), AMP, chloroplasts; (▽——▽), AMP, mitochondria. (b) (●——●), ATP; (○——○), ADP.

enhanced ATP/ADP exchange by the mitochondrial translocase (23) and to restriction on mitochondrial phosphorylation as soon as the cytosolic energy charge is close to the level it was before the environmental conditions were changed.

We feel that the changes in compartmentalized adenylate levels strongly support the suggestion that there is, indeed, a very tight control of mitochondrial respiration by light via the cytosolic energy state (*i.e.* ATP/ADP ratio) to keep it constant.

As shown in this paper, the cytosol, corrected for chloroplastic and mitochondrial contamination, has a rather high energy charge of about 0.85. This high energy charge exhibits, only transiently, a slight over- (dark → light) or undershoot (light → dark) but regulates back to a required level, close to the value assumed by Atkinson (2) to be optimal for the interaction between energy-consuming and -producing metabolic reactions. In the present communication, this is shown to be due to chloroplastic and mitochondrial events. One of the reasons for the high cytosolic energy charge is the lack of AMP in this compartment. This finding gets additional support from the observation that the cytosol lacks adenylate kinase activity (Table VI).

In our experimental system, the kinase was exclusively associated with chloroplasts and mitochondria, showing a rather constant ratio between the two organelles of about 9:1, with respect to the total protoplast associated level of activity. This is a further observation that conflicts, to some extent, with that reported by Santarius and Heber (28). By determining the intracellular distribution of the enzyme between nonaqueous chloroplast and residue, they found that only 40% of the enzyme was located within the chloroplasts of leaf cells of spinach and *Beta*. This considerably lower value could, apart from the different plant material used, be due to inactivation of the enzyme by the nonaqueous isolation procedure, as suggested by the authors, or to problems inherent in the nonaqueous technique. It should be considered in this respect that some error may result from a higher degree of contamination of the fractions. For example, Bird *et al.* (3) reported that their purest chloroplast fraction was still considerably contaminated by nonchloroplast constituents of the cell. The absence of adenylate kinase from the cytosol could also be a reason for the higher ATP/ADP ratio in this compartment, as compared to the stroma and matrix space.

With respect to the calculated ATP/ADP ratios, the actual ratios could be higher, if one assumes that part of the measured adenylates is bound to membrane constituents. In relation to ATP, this can amount up to 40% of the total chloroplast pool present in the dark, as shown by Inoue *et al.* (22), and is in the range of ATP reported to be bound to the coupling factor (14). If, however, differential binding of adenylates to membranes was significant enough to decrease measured ATP/ADP ratios, as compared to the corresponding ratios of free adenylates within the different compartments, the calculated values would not fit the curves drawn in Figure 4 for the adenylate kinase equilibrium. Inasmuch as a rather close fit is observed in most cases, the conclusion drawn by Giersch *et al.* (9), that binding of adenylates is not considerable and can be neglected, is adopted.

Acknowledgments—Critical reading of the manuscript by Professor Heber, as well as experienced technical assistance by Mrs. H. Füllgraf, is gratefully acknowledged.

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