Rapid Fractionation of Wheat Leaf Protoplasts Using Membrane Filtration

THE DETERMINATION OF METABOLITE LEVELS IN THE CHLOROPLASTS, CYTOSOL, AND MITOCHONDRIA

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ABSTRACT

A technique is presented for measuring the in vivo metabolite levels in the chloroplast stroma, the cytosol, and the mitochondrial matrix of wheat (Triticum aestivum, var 'Timmo') leaf protoplasts, in which membrane filtration is used to prepare fractions enriched in the different subcellular fractions within 0.1 seconds after disruption of the protoplasts. By closing a syringe, protoplasts are forced through a net and disrupted, diluting the cytosol into the medium and also releasing intact chloroplasts and mitochondria which can then be immediately removed on membrane filters. By varying the membrane filters, different filtrates are obtained corresponding to (a) mainly cytosol, or (b) cytosol and mitochondria with only low levels of chloroplasts; alternatively, (c) the entire protoplast contents are obtained by omitting the filters. The filtrates are immediately split, half flowing into HClO4, where they are immediately quenched for subsequent metabolite analyses; the other half flows into detergent and is used to monitor the exact distribution of marker enzymes in each individual fractionation. Using the measured distributions of metabolite and of marker enzymes in the three filtrates, the subcellular distribution of the metabolite can be algebraically calculated. The method is presented using ATP as an example.

The quench time (0.1 second) made possible by membrane filtration is considerably faster than has been possible in the previously developed techniques using silicone oil centrifugation for chloroplasts (1 second) or mitochondria (1 minute). This rapid quench makes it possible to investigate subcellular pools which have a rapid turnover, like the adenine nucleotides. The latter technique achieves separation of the chloroplasts and a quench of their metabolism within about 1 s. Silicone oil centrifugation has also been used to separate mitochondria from rapidly ruptured protoplasts (4). In this procedure, however, the time elapsing between rupture of protoplasts and quenching of metabolism of the separated mitochondria was about 1 min. This method therefore appears to be limited to the assay of those mitochondrial substances which have a relatively slow turnover. The measurement of the mitochondrial adenine nucleotide pools, which have a turnover time in the order of 1 s, requires alternative methods.

The rate of separation of organelles by centrifugation depends partly on their mass. Due to their relatively low mass, mitochondria sediment much more slowly than chloroplasts. On the other hand, small particles can be separated almost instantaneously by filtration techniques. Membrane filtration has been previously used for rapid removal of isolated mitochondria from the incubation medium (8). The present publication describes a new technique, also based on membrane filtration, for fractionating protoplasts and quenching metabolism in the separated fractions within about 0.1 s, which allows metabolite levels to be measured in the chloroplast, cytosol, and mitochondria. The application of this technique for the determination of subcellular adenine nucleotide levels in wheat leaf protoplasts under illumination and darkness will be described in a subsequent publication (12).

MATERIALS AND METHODS

Plant Material. Wheat (Triticum aestivum, var 'Timmo'; Rothwell Plant Breeders, Lincoln, U.K.) was grown in hydroponic culture using the medium described by Randall and Bouma (9) in a glasshouse with supplementary artificial lighting. Plants were grown for 8 to 11 d and harvested in the morning.

Protoplast Preparation. Protoplasts were prepared by a method modified from Edwards et al. (3). Both the cut leaf segments and the protoplasts were well washed, removing phenols which are at high levels in wheat and can impair yield and photosynthetic activity. The washing of protoplasts was improved by using a raffinose/sorbitol flotation gradient with a large volume. Calcium was excluded from the final stages of the protoplast preparation as it impairs the activity of some enzymes and the filtration of protoplast extracts.

Leaves (10 g) were cut into transverse sections 0.5 to 1 mm wide, immediately washed twice in 100 ml medium 1 which contained 0.5 mM sorbitol, 5 mM Mes (pH 6.0), 1 mM CaCl2, 0.05% BSA, and 0.05% PVP, drained and placed in a crystallizing dish with 40 ml of medium 1 adjusted to pH 5.5, and containing additionally 2.5% Celulase (Onozuka SS) and 0.5% Macerase R-10 (enzymes purchased from Welding and Co., Hamburg, Germany). The material was incubated for 3.5 h in an illuminated glasshouse.

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water bath at 28°C (two 100-w incandescent lamps at 40 cm distance, 2 cm thick water filter). The enzyme-containing medium was carefully drained from the leaf sections which were then agitated gently in 20 ml medium 1. This and all subsequent operations were at 2° to 4°C. The medium, containing released protoplasts, was filtered sequentially through nylon mesh with 1 mm and then with 100 μm apertures. The leaf sections were rewashed twice, and the pooled filtrates centrifuged at 100g for 3 min (swing-out rotor), the supernatant removed, and the pellet resuspended in 20 ml medium 2 (0.3 M sorbitol, 0.2 M raffinose, 50 mM MES, 1 mM MgCl₂, pH 6.0). This suspension was subdivided among four 50-ml glass centrifuge tubes, and overlaid with 20 ml medium 3 (0.32 M sorbitol, 0.18 M raffinose, 50 mM MES, 1 mM MgCl₂, pH 6.0) and 5 ml medium 4 (0.5 M sorbitol, 50 mM MES, 1 mM MgCl₂, pH 6.0). After centrifugation (15 min, 100g), protoplasts were removed from the upper interphase with a Pasteur pipet. The pooled protoplast suspension was diluted with 3 volumes of medium 2, overlaid with 3 ml medium 4 and recentrifuged as before. The protoplasts were collected from the interface. The final protoplast suspension contained 0.5 to 1.8 mg Chl in a concentration range 100 to 300 μg Chl/ml. The lower yields and concentrations occurred in mid winter. For maximal yield and photosynthetic activity, it was essential to use young (7–10 d old) plants and to avoid taking too much plant material.

Medium.  Protoplasts (25 μg Chl/ml) were suspended in 0.32 M sorbitol, 0.1 M raffinose, 50 mM Hepes, 10 mM KCl, 0.5 mM MgCl₂, 1.0 mM NaHCO₃, 0.2% (w/v) PVP, and 0.06% BSA adjusted to pH 6.8 with KOH at 20°C. This medium was isosmotic with respect to the protoplasts, avoiding the need for constant stirring to maintain the protoplasts in suspension.

Membrane Filtration.  Protoplast suspension (2–2.5 ml) was slowly drawn into a transparent plastic syringe (2 ml, Braun Melsungen AG) modified by inserting an 0.85 mm diameter polyethylene tube into its outlet. The syringe was attached directly to a 25 mm diameter membrane filter holder (Sartorius GmbH, Göttingen, Germany), and the assembly was held vertically with the membrane filter holder uppermost (Fig. 1). The compartment of the filter holder on the exit side was filled with glass beads to reduce the dead volume to 200 μl. A capillary T-piece attached to the outlet led to two 1.5-ml microcentrifuge tubes. One contained 50 μl 70% (v/v) HClO₄, to quench metabolism, yielding an extract for metabolite measurements. The other, for measurement of marker enzyme distribution, contained 5 μl detergent (2.4% Ami-

noxide WS-35; Theo Goldschmidt, AG, Munich) and was immediately snap-frozen in 50-μl aliquots at −85°C. This procedure allowed the exact cross-contamination in each individual fractionation to be determined.

The filter holder contained a 17-μm mesh nylon net (Scrynel, Beutelcheaufabric AG, Rischlikon, Switzerland) on the inlet side to rupture the protoplasts. Membrane filters (Sartorius) of pore size and sequence specified below, were placed behind this net for separation of the released organelles. In all cases, polyester mem-

brane separators (Sartorius) were inserted between filters and between the nylon net and a filter. The following combinations were used. (a) Nylon net alone: filtrate (F₁₉₉) containing the entire disrupted protoplast suspension (cytosolic, mitochondrial, and chloroplast material). (b) Nylon net and 8 μm membrane filter (cellulose nitrate): filtrate (F₂) containing mainly cytosolic and mitochondrial material. (c) Nylon net, 8-μm (cellulose nitrate), and 0.45-μm (cellulose acetate) membrane filters: filtrate (F₃) containing mainly cytosolic material.

Syringe Closure.  For a syringe containing 2.3 ml of protoplast suspension, the optimal closure time was 0.8 s. Satisfactory syringe closure was made by hand. A mechanical device (Fig. 2) permitted a more accurate closure time and enabled the three filtrations to be made simultaneously. This device was driven by an electronically variable electric hand drill, which provided reproducible, load-independent speeds. A rotating threaded shaft (a) drove a nut upwards (b) which was itself prevented from rotating by the solenoid (c). By a support (d), the upward moving nut was connected to a plunger with a circular base (e). The resulting upward movement of the plunger closed three syringes (f) (of which in Fig. 2 only two are to be seen) at the same time. In order to attain a constant motor speed when driving the syringe pistons, the syringes were initially fitted about 3 mm above the plunger. The empyting of the syringes was stopped abruptly when the microswich (g) reached the adjustable stop (h). The power to the drill was then turned off, and the solenoid (c) was simultaneously retracted, releasing the nut (b). The ball bearing (i) then enabled free rotation of the nut. This stopped the flow of protoplast suspension through the filter instantly by disconnecting the motor drive from the plunger movement.

During the entire procedure, the syringes were kept in a thermostated waterbath (20°C). When required the syringes and the filters were illuminated with white light from tungsten quartz-iodine light sources of approximately 180 w/m².

Extracts of Total Unfractionated Protoplasts. In parallel with the preparation of the various filtrates from disrupted protoplasts forced through the filter assembly, extracts were also prepared for assay of enzymes and substrates from directly frozen or quenched protoplasts, respectively. For enzymes, protoplasts were pipetted directly (from the prevailing illumination conditions) into a microcentrifuge tube in an aluminum block at −85°C. For substrates, protoplasts (300 μl) were injected rapidly from a plastic syringe (outlet, 0.85 mm diameter) into an illuminated (or darkened) microcentrifuge tube containing 50 μl 70% (w/v) HClO₄.

Separation of Protoplasts and Medium.  Intact protoplasts were filtered from the medium by passage through the F₂ filter system, but using a syringe with an outlet 2.8 mm in diameter and a closure time of 90 s. Because of the very slow flow rate, the protoplasts remained intact. This filtrate was designated Fmed.

Assay of Marker Enzymes.  The following marker enzymes were used: NADP-GAPDH dehydrogenase (EC 1.2.1.13) for the chloro-

plast stroma, fumarase (EC 4.2.1.2) for the mitochondrial matrix, and PEP carboxylase (EC 4.1.1.31) for the cytosol. The 50-μl aliquots, stored at −85°C, were thawed immediately before assay for these enzymes. A dual wavelength spectrophotometer was used for measurement of GAP dehydrogenase (340 versus 400 nm) (14) and fumarase (240 versus 320 nm) (1). For PEP carboxylase (14), six replicate assays from at least three separately frozen aliquots were made, and for GAP dehydrogenase at least two determinations from separately frozen aliquots.

Substrate Determinations.  For measurement of adenine nucleo-
tides, the HClO₄ extracts (stored at −85°C) were neutralized (adding 125 μl 5 M KOH, 1 M triethanolamine), diluted with 25 mM Hepes (pH 7.75), 4 mM MgSO₄, 3 mM KCl, and 0.2 mM PEP pyruvate.

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3 Abbreviations: GAP, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate.
RAPID SUBCELLULAR FRACTIONATION BY MEMBRANE FILTRATION

**RESULTS AND DISCUSSION**

The Fractionation Procedure. To illustrate the principle of the procedure, the marker enzyme distributions obtained in a typical

### Table 1. Distribution of Marker Enzymes in Filtrates Collected with Different Membrane Filter Combination

<table>
<thead>
<tr>
<th>Filter Construction</th>
<th>Syringe Closure Time</th>
<th>Total Activity Recovered in Filtrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GAP dehydrogenase</td>
<td>PEP carboxylase</td>
</tr>
<tr>
<td></td>
<td>ms</td>
<td>%</td>
</tr>
<tr>
<td>Nylon net (17 μm)</td>
<td>Membrane filter</td>
<td></td>
</tr>
<tr>
<td>8 μm + 0.45 μm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F_{total}</td>
<td>0.7–1.0</td>
<td>100</td>
</tr>
<tr>
<td>F_{1}</td>
<td>0.7–1.0</td>
<td>17.3</td>
</tr>
<tr>
<td>F_{2}</td>
<td>0.7–1.0</td>
<td>13.4</td>
</tr>
<tr>
<td>F_{medium}</td>
<td>0.7–1.0</td>
<td>90</td>
</tr>
</tbody>
</table>

The protoplasts were fractionated and filtered as described in "Materials and Methods." The results of a typical fractionation are expressed as percentage of total activity in unbroken protoplasts. The total enzymic activities were as follows: GAP dehydrogenase, 1100; PEP carboxylase, 2; fumarase, 15 (μmol/mg Chl-h).
fractionation are shown in Table I. The marker enzyme activities in the $F_{\text{total}}$ filtrate do not differ significantly from those in intact protoplasts. The $F_1$ filtrate contains predominantly the cytosolic marker PEPC carboxylase (80–95%) and the mitochondrial marker fumarase (65–80%), but only 5% to 15% of the chloroplast marker GAP dehydrogenase, showing that most of the chloroplasts are retained on the 8-$\mu m$ membrane filter. In the $F_2$ filtrate, the activities of PEPC carboxylase and GAP dehydrogenase resemble those in $F_1$, but only 2% to 10% of the fumarase activity are found in there. Obviously, the 0.45-$\mu m$ membrane filter is effective in retaining the mitochondria. The somewhat lower yield of PEPC carboxylase in the presence of the membrane combinations is due to a small proportion of the protoplasts being filtered off intact within the filter apparatus (see below). The very small proportion of enzyme marker activities in the medium ($F_{\text{med}}$) indicates that protoplast breakage in the reaction mixture was low. The separation of protoplasts from the medium as obtained in this way was even more satisfactory (results not shown) than by the previously described silicone oil centrifugation procedure (14). It may be noted that the activity of PEPC carboxylase in the medium is higher than that of GAP dehydrogenase and fumarase. Broken protoplasts in the suspension will have released many of their organelles intact, and any such free intact organelles will be retained on the filter.

**Calculation of Subcellular Metabolite Levels.** The levels of metabolites, e.g. ATP, actually present in the compartments of the cytosol (C), the mitochondrial matrix (M), and the chloroplast stroma (S), can be calculated from those found in the filtrates $F_{\text{total}}$, $F_1$, and $F_2$. The data used in the calculation ($A_1$, $A_2$, $A_3$) represent the amount of metabolite in these filtrates after subtraction of the corresponding amounts measured in the protoplast medium ($F_{\text{med}}$). Similarly, the enzyme activity in the medium is first subtracted from the enzyme activities in the various filtrates, before using these values to correct for cross-contamination. The proportion of the total marker enzyme activity from intact protoplasts recovered in the filtrates $F_1$ and $F_2$ is represented by the following symbols. Activity in ($F_1 - F_{\text{med}}$)/($F_{\text{total}} - F_{\text{med}}$): fumarase, $u$; GAP dehydrogenase, $v$; PEPC carboxylase, $w$; Activity in ($F_2 - F_{\text{med}}$)/($F_{\text{total}} - F_{\text{med}}$): fumarase, $x$; GAP dehydrogenase, $y$; PEPC carboxylase, $z$. For the distribution of metabolites and marker enzymes among the three compartments, the following three equations can be written.

$$A_{\text{total}} = C + S + M$$
$$A_1 = wC + vS + uM$$
$$A_2 = zC + yS + xM$$

**Table II. Calculation of Subcellular ATP Contents from the Distribution of Marker Enzymes and ATP in the Filtrates**

The same experiment as in Table I. The calculation of subcellular ATP levels using the marker enzyme activities shown in Table I is described in the text. In order to show the cross-correction involved, the source of ATP is also given for $F_1$ and $F_2$.

<table>
<thead>
<tr>
<th>Filtrate</th>
<th>Amount of ATP Measured in filtrate</th>
<th>Calculated subcellular source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg Chl</td>
<td>Stroma</td>
</tr>
<tr>
<td>$F_{\text{total}}$</td>
<td>63.3</td>
<td></td>
</tr>
<tr>
<td>$F_1$</td>
<td>42.6</td>
<td>2.2</td>
</tr>
<tr>
<td>$F_2$</td>
<td>38.6</td>
<td>1.5</td>
</tr>
<tr>
<td>$F_{\text{med}}$</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>Subcellular content</td>
<td>18.3</td>
<td>37.1</td>
</tr>
</tbody>
</table>

Solved for $C$:

$$C = \frac{x A_{\text{total}} - A_2}{y - x} + \frac{A_1 - uA_{\text{total}}}{v - u}$$

Solved for $S$ and $M$:

$$S = C \cdot \frac{u - w}{v - u} + \frac{A_1 - uA_{\text{total}}}{v - u}$$

$$M = A_{\text{total}} - S - C$$

The results of a typical calculation for ATP are shown in Table II. In addition to the final figures calculated for the subcellular ATP distribution for whole protoplasts, the amounts present in each filtrate are also shown.

**Factors Affecting the Filtration.** To remove an organelle by filtration, the pore size of the filter has to be small enough to retain the organelle, but organelles break if the pores are too small. For example, chloroplasts are almost totally disrupted on filters with a pore size of less than 1.2 $\mu m$ (data not shown). It seems that the pores must be large enough to allow organelles to penetrate into the matrix of the filter, where they can be retained without blocking the filter. Once the pores are blocked, the organelles are destroyed due to the increasing pressure difference across the membrane. This explains why polycarbonate surface filters were unsuitable either allowing whole organelles to pass through or disrupting the organelles totally (data not shown). For this reason, in the $F_2$ filter combination, the 8-$\mu m$ membrane filter is included in order to remove the chloroplasts first, before removing the mitochondria by the 0.45-$\mu m$ filter.

Preliminary experiments showed that the separation was very dependent on the speed of the filtration. Therefore, a mechanical apparatus was built to close the syringes (see "Materials and Methods"). This machine allowed a preselected speed to be maintained constantly throughout the closure of the syringes. Under our conditions (2.3 ml, 20 $\mu g$ Chl ml$^{-1}$), the optimal closure time was 0.8 to 0.9 s. Faster closure led to increased disruption of chloroplasts on the filter. Slower closure increased the retention of protoplasts in the filter apparatus, because more protoplasts were retained on the nylon net, or passed through without being ruptured and were then retained on the filters (data not shown). Protoplast breakage could be aided by a narrow syringe aperture. However, when the exit was smaller than 0.8 mm, more chloroplasts broke during the filtration. The separation was also improved by using a medium which, by addition of raffinose, was made isopycnic with the protoplasts. The Chl concentration and
the volume passed through the filter are also critical; in standard conditions, 1.5 to 1.8 ml of suspension (20–25 μg Chl ml⁻¹) can pass through the membrane filters before these saturate and organelle breakage increases. The speed of closure, syringe design, protoplast concentration, and volume all interact to determine the efficiency of the separation.

The composition of the medium was chosen to prevent nonspecific binding of organelles, substrates, or enzymes onto the negatively charged membrane filter material. PVP was essential to prevent binding of PEP carboxylase or of mitochondria. Divalent cations at higher concentrations increased binding of mitochondria onto the 8-μm filter. Where possible, cellulose acetate filters were used instead of cellulose nitrate because the latter bound more material. BSA was included not only to prevent nonspecific binding but also to protect the marker enzymes against inactivation by proteases and phenolic compounds. It might be noted that binding is much stronger on filters with a smaller pore size, presumably due to the larger internal surface area.

Control experiments showed that under our conditions there was no binding of adenine nucleotides when they were present at levels resembling those in a disrupted protoplast suspension (data not shown). When a protoplast extract was prepared by passing protoplasts through a nylon net, identical activities of PEP carboxylase were found before and after passage through a 17-μm filter.

**Table IV. Recovery of Adenine Nucleotides Added to the Medium before Disruption and Filtration of Protoplasts**

Protoplasts were incubated in the light for 5 min before addition of small amounts of adenine nucleotides (see below) followed by fractionation after 7 min in the light. Two 300-μl aliquots (with and without added adenylates) were quenched by direct injection into 1 ml HClO₄, and the difference in their adenylate content was taken as representing the amount of each adenylate present in the medium at the moment of protoplast disruption (= 100%). Simultaneously, three samples of protoplasts from each of these suspensions (with and without added adenylates) were subjected to filtration giving filtrates F₁, F₂, and F₃ (without addition) and F₄, F₅, and F₆ (with added adenylates). The difference between the adenylate content in a given filtrate from protoplast suspensions with and without added adenylates was then divided by the amount of added adenine nucleotides, giving the recovery of added adenylate in the various filtrates (mean ± se of four experiments).

<table>
<thead>
<tr>
<th>Amount Added</th>
<th>Recovery in</th>
<th>F total</th>
<th>F₁</th>
<th>F₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol/mg Chl</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>92 ± 15</td>
<td>109 ± 9</td>
<td>105 ± 9</td>
<td>103 ± 5</td>
</tr>
<tr>
<td>ADP</td>
<td>33 ± 6</td>
<td>114 ± 14</td>
<td>95 ± 11</td>
<td>106 ± 12</td>
</tr>
<tr>
<td>AMP</td>
<td>14 ± 1</td>
<td>108 ± 21</td>
<td>88 ± 7</td>
<td>75 ± 12</td>
</tr>
</tbody>
</table>

**Table V. Comparison of Adenylate Levels in a Filtrate Containing Intact and Broken Chloroplasts**

The filtrate containing intact chloroplast was obtained by forcing protoplasts through a filter holder containing only a 17-μm nylon net (see "Materials and Methods"). To obtain a filtrate with broken chloroplast, a 0.6-μm polycarbonate surface filter (Nuclepore, Sydney, Australia) was included immediately after the 17-μm nylon net. The protoplasts were illuminated for 7 min before the fractionation. The enzyme activities are expressed as a percentage of those in the unfractinated protoplast suspension.

<table>
<thead>
<tr>
<th>Chloroplasts</th>
<th>Activity in Filtrate</th>
<th>Amount in Filtrate</th>
<th>nmol/mg Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEPCX GAPDH Fumarase ATP ADP AMP ΣADN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>%</td>
<td>100 96 102 64 13 12 89</td>
<td></td>
</tr>
<tr>
<td>Broken</td>
<td>%</td>
<td>97 98 88 61 15 10 87</td>
<td></td>
</tr>
</tbody>
</table>

combination (data not shown). After disruption of the organelles with 0.12% Amonoxide detergent before passage through a F₂ filter combination, there was no retention of GAP dehydrogenase or of fumarase on the filters.

**On the Method.** The present technique was designed to allow a rapid quench of metabolism after rupturing the protoplasts. At a flow rate of more than 2 ml/s and a total dead volume in the filtration apparatus between the nylon net and the outlet of about 200 μl, the transit time for the disrupted cell material between the nylon net and the outlet into concentrated HClO₄ was less than 0.1 s. This is more than 10 times faster than can be achieved by centrifugal silicone oil filtering techniques for separation of chloroplasts and cytosol (10, 14), and 300 to 600 times faster than for separation of mitochondria (4). No evidence for substantial changes in the adenine nucleotide levels during the fractionation procedure could be found. The total amounts of ATP, ADP, and AMP in whole protoplasts, as quenched by direct injection into concentrated HClO₄, were compared with the summed levels from the chloroplasts, cytosol, and mitochondria, which had been determined from the filtration procedure (Table III). For both illuminated and darkened protoplasts, the recovery of the total adenine nucleotide pool was complete, and only very small (5%) changes in the levels of individual adenine nucleotides were found, apart from AMP in illuminated protoplasts. Accurate measurement of AMP using the luciferase method is difficult if the ATP and ADP are much higher than AMP. This is because the AMP value is obtained by subtracting the total (ATP + ADP) from the sum of the adenine nucleotides (see "Materials and Methods" and also Ref. 12). In another series of experiments, the recovery of small amounts of ATP, ADP, and AMP, added individually to the medium in which the protoplasts were suspended, was determined (Table IV). No gross changes in the added adenine nucleotides during filtration using the F₂ total, F₁, or F₂ systems occurred, showing that there was no significant metabolism of adenine nucleotides released to the medium during filtration.

The chloroplasts in the disrupted protoplast suspension (F total) were largely intact, while the GAP dehydrogenase in the filtrates obtained after passage through membrane filters (F₁, F₂) derived mostly from broken chloroplasts (data not shown). The cross correction for the contribution of chloroplast-derived adenine nucleotides to the filtrates F₁ and F₂ would be inaccurate if the adenine nucleotide levels contributed by intact and broken chloroplasts differed. Since in the light the stromal ATP has a half time of about 100 ms, it would be particularly susceptible to alterations. However, the levels of ATP, ADP, and AMP found in a normally prepared F total filtrate containing intact chloroplasts were very similar to the levels found in a protoplast filtrate in which the chloroplasts had been immediately disrupted (Table V). A complete disruption of the chloroplasts was achieved, when a 1-μm polycarbonate surface filter was placed behind the 17-μm nylon net. This experiment provides additional evidence that the adenine nucleotide levels in chloroplasts did not change greatly during the fractionation.

While these experiments make it unlikely that there were large alterations in the stromal and cytosolic adenine nucleotides during the fractionation, the evidence is not decisive for the mitochondria. Since only about 10% of the total adenine nucleotides occur in the mitochondria, these could alter without leading to large deviations in the total adenine nucleotide pool in the protoplasts. However, the rapid metabolic quench would limit the extent of any such changes. A typical respiratory rate of 0.15 μmol O₂ g⁻¹ Chl min⁻¹ (6) would support the synthesis of about 1 nmol ATP g⁻¹ Chl in 0.1 s. After protoplast disruption, the first effect on mitochondrial metabolism is likely to be a restriction of release of ATP in exchange for ADP via the adenine nucleotide translocator (5) due to the enormous dilution of the cytosolic ADP into the medium. In this case, a small rise in the mitochondrial ATP might
have occurred during the fractionation.

These considerations emphasize the importance of rapidly quenching mitochondrial metabolism, if the measured matrix adenine nucleotides are to resemble the *in vivo* levels. For this reason, the slower techniques using silicone oil centrifugation (4) seem basically unsuitable when the individual adenine nucleotides are to be assayed in the separated mitochondria. Even when isolated animal mitochondria are centrifuged through silicone oil directly into HClO4, some changes in the adenine nucleotide pool have been demonstrated (2). The likelihood of alterations when mitochondria are released from a cell into a diluted extract appear even greater than for isolated mitochondria, which at least remain in the same incubation medium during the centrifugation, especially as plant cells contain large amounts of malate, mostly in the vacuole (7), which becomes available to mitochondrial respiration in the time between cell disruption and the quench.

For the study of metabolism in the chloroplast stroma and cytosol, the faster separation by membrane filtration also provides advantages compared with the available silicone oil centrifugation techniques. This is especially so in the case of the cytosol, where a relatively uncontaminated fraction is obtained almost instantaneously. It should be noted that cytosol actually designates those compartments of the cell other than the chloroplasts and mitochondria and will include, for example, the vacuole. A measurement of cytosolic metabolic levels requires a correction to be made for the metabolites free in the medium, which otherwise would be included in the value for the cytosol (14). Under the conditions described here, the metabolites in the medium can be accurately evaluated after removing the protoplasts without significant breakage using a slow filtration. For chloroplasts, as of course also for mitochondria, the membrane filtration method has the disadvantage that the values are obtained by subtraction from the cytosol, so that the experiments require accurate analysis and repetition. However, in cases where significant alterations in metabolite levels could occur during a slower separation, it would be worthwhile sacrificing the purity of the individual fractions in order to quench these fractions faster.

Acknowledgments—R. McC Lilley was recipient of a Humboldt Fellowship. We thank Mr. Uwe Sader for constructing the apparatus used to fractionate the protoplasts.

**LITERATURE CITED**

Correction

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Page 968, column 2, the denominator of the equation under “Solved for C” should read:

\[
\frac{w - u}{v - u} + \frac{x - z}{y - x}.
\]