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Purification of Enzymatically Isolated Mesophyll Protoplasts from C_3, C_4, and Crassulacean Acid Metabolism Plants Using an Aqueous Dextran-Polyethylene Glycol Two-Phase System[1]

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ABSTRACT

Enzymatic digestion of leaf segments with 2% cellulase, in combination with a pectinase in some species, yields intact protoplasts mixed with epidermal tissue, vascular tissue, broken protoplasts, and chloroplasts. Epidermal and vascular tissue are removed with sieves of various porosity. Intact protoplasts in the filtrate are separated from other components by an aqueous two-phase system which consists of dextran-polyethylene glycol, with sorbitol and sodium phosphate. Intact protoplasts partition at the interphase, while chloroplasts and broken protoplasts partition in the lower phase when the separation is facilitated by low speed centrifugation. The optimum conditions for purification of maize mesophyll protoplasts with high yields are centrifugation of the two-phase system at 300g for 6 minutes at 2 C with a mixture including 0.46 M sorbitol, 10 mM sodium phosphate, 5.5% polyethylene glycol 6000, and 10% dextran of average molecular weight of 20,000 to 40,000. The collection of protoplasts at the interphase was proportional to the amount of chlorophyll added over a wide range of concentrations regardless of the initial contamination of the preparation by other cellular debris. The two-phase system is applicable for protoplast purification from a wide variety of species, including C_3, C_4, and Crassulacean acid metabolism plants, regardless of protoplast size.

Over the past 12 years there has been a rapid development in the enzymatic isolation and utilization of plant protoplasts for various research purposes (4, 6). Factors which need to be considered in the isolation of protoplasts include: (*a*) use of appropriate cell wall-degrading enzymes which do not disturb the integrity of the protoplast activity (the type and optimum concentration may vary with the plant tissue); (*b*) use of an osmoticum such as sorbitol or sucrose at levels in which the protoplasts are stable; and (*c*) a method for the efficient purification of protoplasts from the mixture of undigested tissue, broken protoplasts, chloroplasts, and other organelles. Recently, we have isolated mesophyll protoplasts and bundle sheath strands from leaves of many C_4 plants[4] for comparative biochemical studies on the role of the two cell types in C_4 photosynthesis (13, 14). It was necessary to purify the isolated protoplasts as well as to obtain suitable yields for metabolic studies. Purification of mesophyll protoplasts of tobacco and cereal leaves, tomato fruit, and onion roots by floating the protoplasts on sucrose solutions has been reported (9, 11, 17, 18), although in most studies yield data have not been given.

Attempts to collect the protoplasts of maize, a C_4 plant, by floating on sucrose has not provided adequate quantitative preparations for our investigations. This led to the development of another purification method with a new principle. We have found that an aqueous dextran-polyethylene glycol two-phase system is applicable for purifying leaf protoplasts from a wide variety of plants including C_3, C_4, and CAM plants. Details of the characteristics of the two-phase system for protoplast purification are described in this report. The viability of the protoplasts was discussed in relation to their photosynthetic capacity.

MATERIALS AND METHODS

Plants were grown in a growth chamber under 16 hr of light and 8 hr of dark with a day temperature of 30 C and a night temperature of 20 C. Light was provided by a combination of incandescent and fluorescent lamps giving a quantum flux density of 40 nE/cm^2·sec between 400 and 700 nm.

For protoplast isolation, 1 to 2 g of young leaves (C_3 and C_4 plants 2–4 weeks old; CAM plants, 4–8 weeks old) were cut vertically across the leaf to give segments less than 1 mm in width. Leaf segments of C_3 and C_4 plants were vacuum infiltrated in 20 ml of an enzymatic digestion medium and incubated in a reciprocal shaker at room temperature as previously described (13, 14). The enzyme medium contained 0.6 M sorbitol, 5 mM $MgCl_2$, 20 mM MES buffer (pH 5.5), and 2% cellulase. With some species 0.1% pectinase was added in the digestion medium to promote protoplast liberation. The incubation medium was changed each 2 hr, and the chlorophyllous material released from the segments was collected at

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[4] Abbreviations: Evans Blue: 6,6′-[(3,3′-dimethyl-4,4′-biphenylene)bis(azo)]-bis(4-amino-5-hydroxy-1,3-naphthalenedisulfonic acid), tetrasodium salt); PEG: polyethylene glycol; C_4 plants: plants having the C_4-dicarboxylic acid pathway and the reductive pentose phosphate pathway of photosynthesis; C_3 plants: plants having only the reductive pentose phosphate pathway of photosynthesis; CAM plants: plants having Crassulacean acid metabolism.

2, 4, and 6 hr. Washing the segments with 0.6 M sorbitol immediately after removal of the incubation medium at 2, 4, and 6 hr resulted in further release of protoplasts. For C_4 plants the extract was filtered successively through a 35 mesh screen, 80-μm, and 44-μm nylon net which removed undigested leaf segments and epidermal tissue on the 35 mesh screen, and vascular strands on the nylon nets. For C_3 plants the extract was filtered through a 35 mesh screen and 80-μm nylon net. The filtrates were centrifuged at 400g for 3 min. The precipitates were resuspended in 0.6 ml of 0.6 M sorbitol containing 0.05 M Tricine-KOH buffer (pH 8.0), and 5 mM $MgCl_2$. This gave crude preparations of mesophyll protoplasts from C_3 and C_4 plants.

For CAM plants the epidermis was removed from at least one side of the leaf with forceps before cutting segments approximately 1 mm thick with a sharp razor. Certain precautions were taken to avoid breakage of CAM protoplasts. The leaf segments were incubated with the enzyme medium for 1 hr on a reciprocal shaker, and thereafter they were not shaken except gently swirling by hand just before collecting the protoplasts at 2, 4, and 8 hr. The extracts were filtered through a 12 mesh screen and centrifuged at 150g for 1 min. The precipitate was resuspended in 0.6 ml of 0.6 M sorbitol containing 0.05 M Tricine-KOH buffer (pH 8.0), and 5 mM $MgCl_2$ to give crude preparations of CAM protoplasts.

An aqueous liquid-liquid two-phase method as developed for the partition of protoplasts consists of dextran and PEG containing sorbitol and salts. In this two-phase system, intact protoplasts collected at the interphase of the two liquid phases, while chloroplasts and broken protoplasts suspended in the lower phase. After optimizing the variables in the system, a standard mixture chosen for the purification of maize protoplasts is shown in Table I. The two-phase solution is thoroughly mixed in a 13 × 100 mm test tube by inversion prior to adding 0.6 ml of the crude protoplast preparation. After adding the protoplast suspension, the solution is again thoroughly mixed by inversion and then centrifuged at 300g for 6 min in a swinging bucket centrifuge. A layer of green material is collected at the interphase which consists of spherical protoplasts having a tendency to stick to each other. The protoplasts at the interphase can be sucked off easily with a disposable Pasteur pipette, transferred directly to a second interphase solution with the same composition, mixed, and the centrifugation repeated. The protoplasts collected at the second interphase are suspended in 0.6 M sorbitol containing 0.05 M Tricine-KOH buffer (pH 8.0) and 5 mM $MgCl_2$; centrifuged at 400g for 90 sec; and resuspended in a small amount of the same medium. The entire procedure for the two-phase separation is run at 2 C. Running the two-phase system at room temperature will result in some contamination of the protoplast preparation with other chlorophyllous material. The procedure described above is designated as "standard procedure."

The purity of the protoplast preparation was routinely verified by light microscopy. To examine for intact protoplasts, an equal volume of 2.5% Evans Blue in 0.6 M sorbitol was mixed with the protoplast preparation on a microscope slide and examined under a light microscope for the exclusion of the dye by the intact protoplasts. Chlorophyll was determined in 96% ethanol by the method of Wintermans and De Mots (21).

Cellulase (Onozuka R-10) and pectinase (Macerozyme R-10) were obtained from All Japan Biochemical Co., Ltd., Nishinomiya, Japan. Dextran was obtained from Pharmacia Fine Chemicals; T_{20}, T_{40}, and T_{70}, represent approximate average molecular weight of the dextran, *i.e.*, 20,000, 40,000, 70,000. The source of dextran may be important for reproducible results as noted in Table I. PEG 6000 (average mol wt of 6000–7500) was obtained from J. T. Baker Chemical Co.

Table I. *Composition of Dextran-Polyethylene Glycol System Used for the Standard Procedure to Purify Maize Mesophyll Protoplasts*

Stock Solution	ml Added	Final Concentration
Polyethylene glycol 6000 30% (w/w)	1.1	5.5%
Dextran T_{40} (Pharmacia)[1] 20% (w/w)	3.0	10.0%
Na_2HPO_4-NaH_2PO_4 0.2 M, pH 7.5	0.3	10 mM
Sorbitol 2.4 M	1.0	460 mM
Cell suspension containing	0.6	
$MgCl_2$ 5 mM		0.5 mM
Tricine 50 mM, pH 8.0		5.0 mM
Sorbitol 0.6 M		

[1] The source of dextran may be important, as dextran having an average molecular weight of 40,000 from Sigma Chemical Co. did not provide similar results.

RESULTS

Purification of Maize Protoplasts. The crude protoplast preparation from the maize leaf segments contained a mixture of intact protoplasts, chloroplasts, and broken protoplasts. Besides being spherical in shape the intact protoplasts were easily recognized by using the Evans Blue stain, which has been used to identify live cells in tissue slices (10). Intact protoplasts excluded Evans Blue, while the pigment was strongly absorbed by broken protoplasts as shown by light microscopy with maize leaf extracts (Fig. 1A).

In a preliminary experiment, crude protoplast preparations from leaf segments of maize and wheat were suspended in solutions of 0.4, 0.6, or 0.8 M sucrose and attempts to collect the spherical protoplasts by floating, were made by either standing for 30 min at room temperature or centrifuging at 200g for 5 min, as previously reported for tomato fruit, tobacco, and cereal leaf protoplasts (9, 11, 18). No protoplasts floated to the top of the sucrose solution by standing at room temperature for 30 min. With the low speed centrifugation, there was a tendency for some protoplasts of both maize and wheat to move to the upper surface of the 0.8 M sucrose, but there was an incomplete separation of the spherical protoplasts from chloroplasts and broken protoplasts as observed with Evans Blue staining. Many intact protoplasts were found in the precipitate. In comparison, with low speed centrifugation in sorbitol, both broken protoplasts and intact protoplasts were in the precipitate.

In considering other methods for purification of protoplasts, it was found that a dextran-PEG system could be useful for separating intact protoplasts from broken protoplasts and chloroplasts. Figure 1B shows a preparation of maize mesophyll protoplasts suspended in 1.25% Evans Blue after purification with the two-phase method. The absence of broken protoplasts and chloroplasts from the preparation and the exclusion of the dye by the protoplasts was apparent. When necessary, further purification can be obtained by repeating the two-phase separation. The protoplasts are stable for at least 2 days at 4 C, and they can be easily handled by pipetting and low speed centrifugation without breakage.

Factors Affecting the Two-Phase Method. A number of variables in the two-phase system were examined using crude preparations of maize mesophyll protoplasts. The yield of purified protoplasts collected after the second interphase separation was proportional to the amount of crude chlorophyll added except at the low levels of chlorophyll. When less than 0.05 mg of chlorophyll was added to the two-phase solution the amount of protoplasts was so low at the interphase that

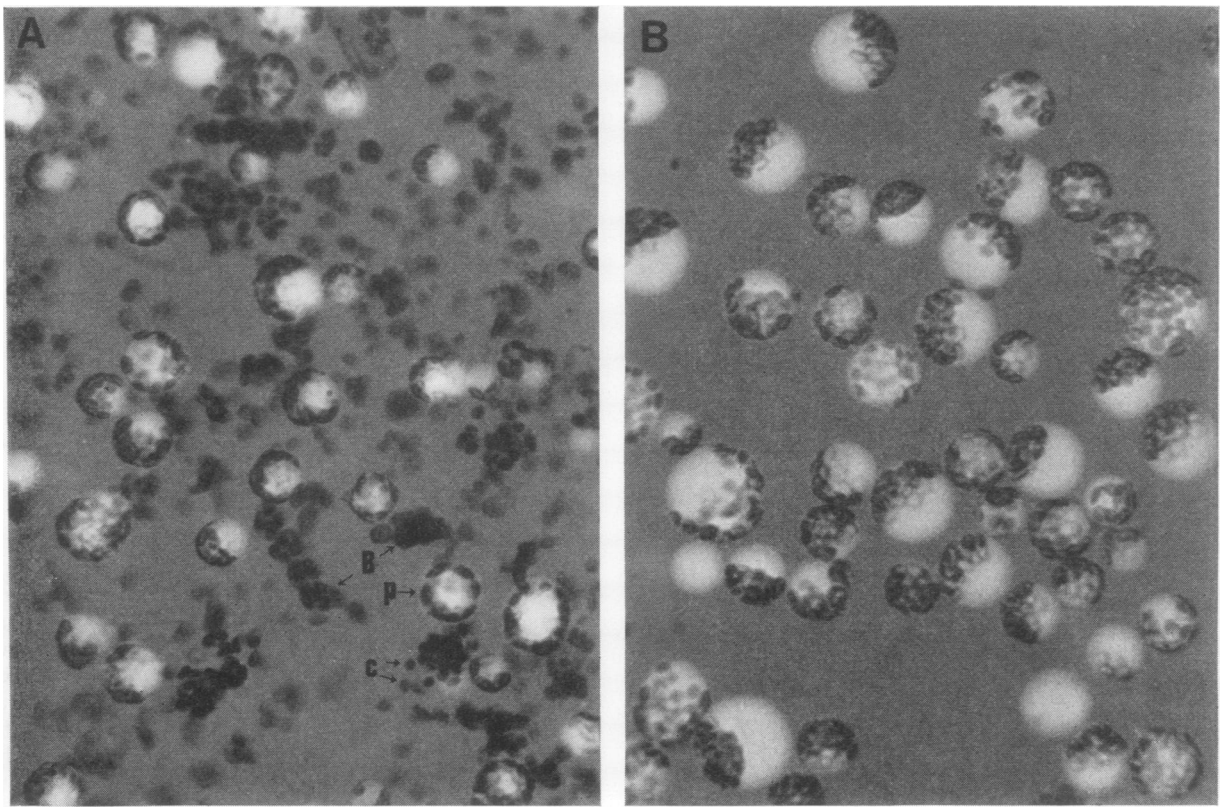

FIG. 1. A: Light micrograph of a field of intact protoplasts, broken protoplasts, and chloroplasts from the enzymatic leaf extract of maize prior to purification, ("crude protoplast preparation") 340 ×. Preparation is in 1.25% Evans blue. P indicates intact protoplast by exclusion of Evans blue, B indicates broken protoplast, and C indicates chloroplast. B: Light micrograph of intact mesophyll protoplasts of maize after purification with the two-phase system, 340 ×. Protoplasts are in 1.25% Evans blue. Lighter background of protoplasts is due to exclusion of blue pigment.

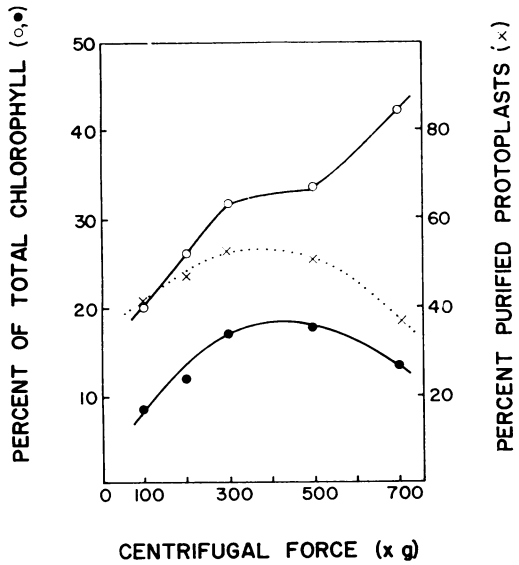

FIG. 2. Effect of centrifugal force on the purification of maize mesophyll protoplasts by the two-phase method. ○: amount of green particles collected at the interphase at variable centrifugal forces; ●: amount of purified protoplasts obtained by using standard two-phase procedure (300*g*, 6 min) on the green particles collected at varying centrifugal forces; ×: percentage of purified protoplasts calculated as ratio of purified protoplasts to the crude preparation collected at the first interphase $\left(\frac{\text{value of } \bullet}{\text{value of } \circ} \times 100\right)$. Data are average of three experiments.

quantitative collection by pipetting was difficult. A point of saturation of protoplasts at the interphase was not reached with the range of chlorophyll concentrations used (up to 0.22 mg of chlorophyll in one experiment). All other experiments were run within the linear range of chlorophyll concentrations.

The effect of centrifugal force on the purification of protoplasts by the two-phase method is shown in Figure 2. Of the crude protoplast preparation from maize leaves 0.6 ml was mixed with 5.4 ml of the two-phase system according to the standard procedure. However, a series of phase solutions were centrifuged at varying forces for 6 min, and the total green material was collected from the interphase and expressed as a percentage of the total chlorophyll added to each tube. There was a plateau between 300 and 500*g* with a further increase at 700*g* of the percentage of total chlorophyll collected at the interphase. A duplicate set of two-phase solutions was centrifuged at varying forces and the green particles collected from the interphase were added directly to a second interphase solution, mixed, and centrifuged for 6 min at 300*g* (standard procedure). The protoplasts were collected from the interphase, the purity confirmed by light microscopy, and the yield of protoplasts determined by chlorophyll measurements (Fig. 2, closed circles). The results show that the yield of protoplasts was highest when the centrifugal force was between 300 and 500*g* for the initial two-phase centrifugations. Below 300*g* only part of the protoplasts were coming to the interphase (light microscopy showed some protoplasts remaining in the lower phase), while above 500*g* chloroplasts and broken protoplasts were also coming to the interphase. Apparently at 700*g* some intact protoplasts were broken during

centrifugation which resulted in a lower yield of purified protoplasts. Ruesink (20) has noted that low centrifugation is generally required to avoid protoplast breakage.

The effect of the molecular weight of dextran on the protoplast purification is shown in Figure 3. The two-phase solution was the same as in Table I, except various molecular weights of dextran were tested at a final concentration of 10% (w/w). After centrifugation for 6 min at 300*g*, the highest percentage of the total chlorophyllous material (73%) was obtained at the interphase using dextran having an average molecular weight of 117,000 (T_{125}). Examination of the interphases by light microscopy indicated, however, that dextran T_{125} gave more contamination by broken protoplasts than T_{20}, T_{40}, and T_{70}. From another set of two-phase solutions with dextrans of various molecular weights the green material collected at the interphase was transferred to a second set of the standard two-phase solutions (as in Table I), and the protoplasts were purified by the standard procedure. As seen from Figure 3, the highest yields of purified protoplasts were obtained using dextran T_{40} and T_{70}. Although the yield was slightly lower, the highest purity of protoplasts was obtained with dextran T_{20} in the one step treatment of the two-phase system.

In considering the sorbitol concentration in the two-phase system, the green particles partitioning at the interphase of tubes after centrifugation with various levels of sorbitol were purified by the standard two-phase mixture (Fig. 4). The highest yield of purified protoplasts was collected from 0.38 to 0.46 M sorbitol in the initial two-phase solutions. Concentrations of sorbitol below 0.38 M apparently caused breakage of many of the protoplasts due to osmotic shock while concentrations above 0.46 M retained some of the intact protoplasts in the lower phase. Therefore, the highest percent purity of protoplasts was obtained in a narrow range of sorbitol concentrations (0.38–0.46 M in final concentration). A concentration of 0.46 M sorbitol has been used in the standard two-phase mixture. Experiments with various concentrations of sucrose instead of sorbitol gave similar results for the purification of maize protoplasts.

The species of salt and their concentration in the two-phase solution have profound effect on the partition of protoplasts from the crude protoplast preparation. With maize there is a linear increase in the amount of chlorophyllous material collecting at the interphase with increasing concentrations of sodium phosphate, at pH 7.5, up to 25 mM (Fig. 5). The material collected from the interphase using various concentrations of sodium phosphate was purified with the standard two-phase solution. The maximum yield of protoplasts was obtained at 10 mM sodium phosphate with maize; there is an increasing amount of chloroplasts and broken protoplasts collecting at the interphase with sodium phosphate concentrations higher than 10 mM. With crude protoplast preparations from sugarcane leaves, the amount of chlorophyllous material collecting at the interphase increased more rapidly than with maize preparations by increasing concentrations of phosphate (Fig. 5). To avoid contamination of the protoplasts at the interphase with chloroplasts and broken protoplasts 5 mM sodium phosphate was the optimum concentration with sugarcane. This concentration was used as a standard to purify the sugarcane protoplasts collected at the interphase with varying concentrations of sodium phosphate. Under these conditions the yield of purified protoplasts from sugarcane was similar to maize. It should be noted that without sodium phosphate in the two-phase solutions, the protoplasts of maize or sugarcane did not form a distinct coagulated layer at the interphase. The use of phosphate apparently causes an attraction between the protoplasts at the interphase which makes the protoplasts easy to collect. In considering the effect of variable pH of sodium phosphate

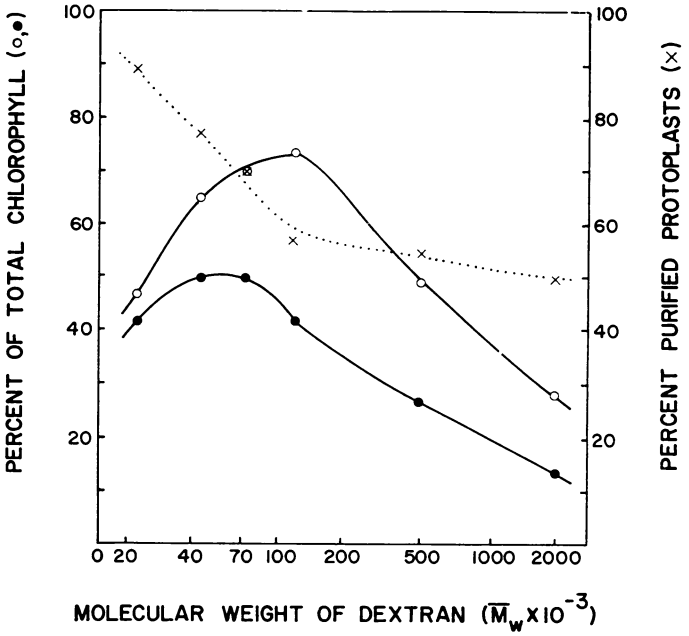

FIG. 3. Effect of the molecular weight of dextran on the purification of maize mesophyll protoplasts by the two-phase method. ○: amount of green particles collected at the interphase with various average molecular weights of dextran; ●: amount of purified protoplasts obtained by using standard two-phase procedure on the green particles collected with various molecular weights of dextran; ×: percentage of purified protoplasts $\left(\frac{\text{value of } \bullet}{\text{value of } \circ} \times 100\right)$.

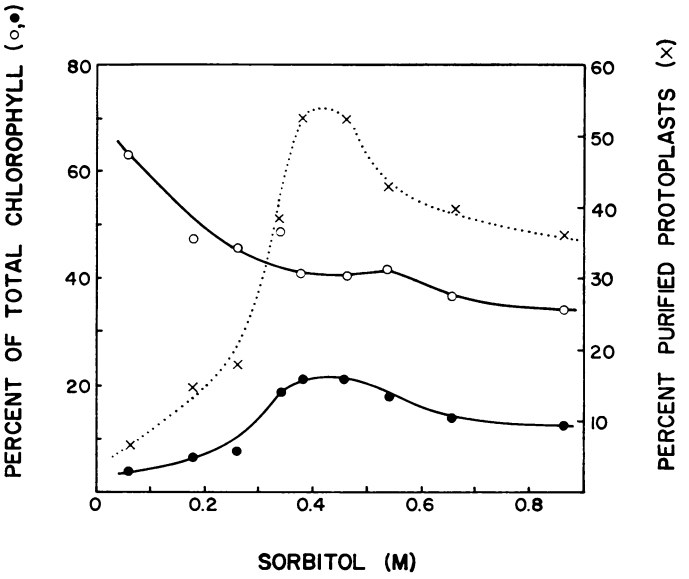

FIG. 4. Effect of sorbitol concentration on the purification of maize mesophyll protoplasts by the two-phase method. ○: amount of green particles collected at the interphase with various sorbitol concentrations; ●: amount of purified protoplasts obtained by using standard two-phase procedure on the green particles collected at various sorbitol concentrations; ×: percentage of purified protoplasts $\left(\frac{\text{value of } \bullet}{\text{value of } \circ} \times 100\right)$. Data are average of two experiments.

on the protoplast purification, a broad optimum around pH 7.5 was found. When NaCl or KCl was substituted for sodium phosphate with maize preparations there was much less effect on the partitioning of the particles at the interphase. The particles collected from the interphase of two-phase solutions with

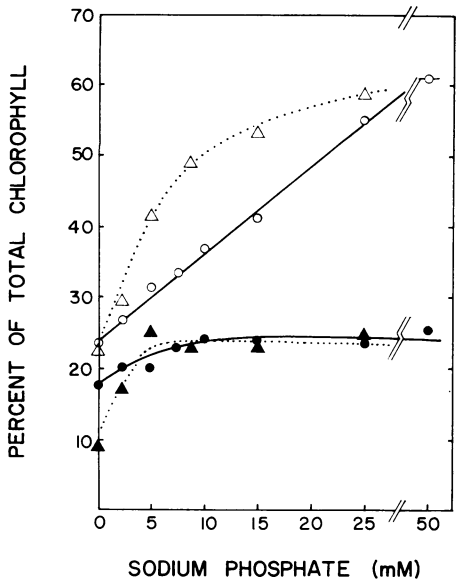

FIG. 5. Effect of phosphate (pH 7.5) concentration on the purification of maize and sugarcane mesophyll protoplasts by the two-phase method. ○: amount of green particles from maize crude preparation collected at the interphase with various Na phosphate concentrations; ●: amount of purified maize protoplasts obtained by using standard two-phase procedure on the green particles collected at various Na phosphate concentrations; △: amount of green particles from sugarcane crude preparation collected at the interphase with various Na phosphate concentrations; ▲: amount of purified sugarcane protoplasts obtained by using standard two-phase procedure on the green particles collected at various Na phosphate concentrations. Data are average of three experiments.

various concentrations of NaCl and KCl were purified by the standard two-phase mixture. In contrast to sodium phosphate there was an initial decrease in the yield of protoplasts with the addition of 2.5 mM NaCl or KCl with a constant yield between 2.5 and 50 mM. Therefore, with maize and sugarcane, the effects of sodium phosphate were more desirable for protoplast purification than NaCl or KCl.

Application of Two-Phase Methods to Various Plant Protoplasts. The isolation of mesophyll protoplasts and the use of the two-phase system for purification of mesophyll protoplasts has been tested and successfully used with many plants including various species from three photosynthetically different groups of plants; C_3 plants, C_4 plants, and CAM plants (Table II). There was a wide range in the size of the mesophyll protoplasts between species from an average diameter of 17.4 μm with *Panicum capillare* to 118.8 μm with *Sedum rubrotinctum*. There was also considerable variability in size of the protoplasts within a species, particularly with *Triticum aestivum* and the CAM species. The trend among the plants examined is that C_4 mesophyll protoplasts were smaller than C_3 mesophyll protoplasts, while C_3 mesophyll protoplasts were smaller than CAM mesophyll protoplasts.

In testing the effectiveness of the two-phase system for protoplast purification with the various species, dextran T_{20} was compared to dextran T_{40} with other conditions the same as in Table I. The purification of protoplasts was successful with a variety of C_4 plant preparations by the standard procedure with dextran T_{40}. However, dextran T_{20} gave better preparations than dextran T_{40} by a single interphase separation, particularly with the C_3 and CAM plants. The percentages of dextran and PEG used are close to the critical point (point below which only one phase is formed) with dextran T_{20} (3); therefore, the concentrations must be accurately measured and solutions maintained at lower temperatures (4 C) for two-phase formation. Dextran T_{20} seems widely applicable for the purpose of protoplast purification. However, the choice between dextran T_{20} and T_{40}, as well as salt species and their concentration, should be made with the particular plant species being studied.

DISCUSSION

Progress towards understanding the function of differentiated cells in plant tissues is partially dependent on obtaining pure cellular preparations. This is true for elucidation of the photosynthetic mechanism in C_4 plants; the leaves of which consist of two types of chlorophyllous cells, mesophyll and bundle sheath cells. Chloroplasts of maize leaves have been isolated with a functional reductive pentose phosphate pathway (16) but not with a functional C_4 pathway. It should be an advantage for biochemical studies to isolate mesophyll protoplasts or cells which maintain their C_4 carboxylating potential.

Table II. *List of Plant Species from Which Mesophyll Protoplasts Have Been Isolated and Purified*

C_3 and C_4 protoplasts were suspended in 0.6 M sorbitol, whereas CAM protoplasts were suspended in 0.8 M sorbitol. Average size represents the average diameter from 100 measurements per species by light microscopy.

Plants	Enzyme[1] Mixture	Range in Diameter	Average Diameter
		μm	
C_4 Species			
Panicum capillare L.	A	10.0–21.5	17.4
Panicum maximum Jacq	B	11.4–38.6	20.5
Chloris gayana Kunth	B	12.9–30.0	20.4
Eleusine indica L.	A	14.3–31.5	20.7
Sorghum bicolor L. (var. Atlas sorgo)	B	12.9–25.7	20.8
Digitaria sanguinalis L.	A	10.6–28.3	21.1
Setaria lutescens Weigel	A	5.1–31.5	22.4
Paspalum notatum Flugge	A	14.3–35.8	24.2
Zea mays L. (var. Sugar King)	A	14.3–45.8	25.9
Saccharum officinarum L.	B	15.7–48.6	27.6
C_3 Species			
Nicotiana tabacum L.	B	17.2–42.9	28.6
Phaseolus vulgaris L. (var. Sanilac)	B	22.9–47.2	31.3
Triticum aestivum L. (var. Timwin)	B	11.4–71.5	33.7
Hordeum vulgare L. (var. Trophy)	B	22.9–60.1	36.6
CAM species			
Kalanchoe fedtschenkoi Hamet and Perrier	B	28.6–91.5	60.4
Kalanchoe pinnata Pers.	B	28.6–103.3	64.8
Kalanchoe daigremontiana Hamet and Perrier	B	28.6–260.3	73.0
Sedum telephium L.	B	22.9–180.2	109.1
Sedum rubrotinctum Clausen	B	51.5–200.2	118.8

[1] A: isolation medium contained 2% cellulase; B: isolation medium contained 2% cellulase and 0.1% pectinase.

In order to study the comparative biochemistry of photosynthetic metabolism in various plants we have concentrated on methods for isolating leaf mesophyll protoplasts.

By the enzymatic maceration of plant leaves with cellulase, intact protoplasts were released together with strips of epidermal tissue, fragments of vascular strands, broken protoplasts, and cellular organelles. The epidermal tissue and undigested leaf fragments are easily removed by filtration through stainless steel sieves, and bundle sheath strands of C_4 plants were separated by filtration techniques with nylon sieves (13, 14). The filtrate contains chloroplasts and other organelles, broken protoplasts, and intact protoplasts. Some researchers have reported purification of intact protoplasts from some C_3 species by floating on solutions of sucrose (9, 11, 17, 18). We have found these methods unsuccessful in our studies, particularly with mesophyll protoplasts from plants having the C_4-dicarboxylic acid pathway of photosynthesis. Floating on sucrose solutions may be applicable with protoplasts which have large vacuoles and a low concentration of organelles. De Vries and Wessels (7) found that protoplasts from *Schizophyllum commune* floated if isolated in 0.5 M $MgCl_2$ but not in sucrose, sorbitol, mannitol, NaCl, or KCl. They attributed the floating to a swelling of the vacuole in the presence of $MgCl_2$.

In liquid-liquid two-phase systems on the other hand, particles are fractionated by a delicate difference in the surface properties as well as their size and density as noticed by Albertsson (3). Therefore, liquid polymer phase systems are considered useful in biological research to separate (*a*) macromolecules such as nucleic acids and viruses; (*b*) cell components such as cell walls, mitochondria, and chloroplasts (3, 15); and (*c*) various types of cells such as bacteria, algae, and animal cells (3). Also, countercurrent distribution has been used with two-phase solutions to separate various cells including bacteria, algae, and erythrocytes (2). We have found that plant protoplasts have a strong tendency to partition at the interphase of the dextran-PEG two-phase system, containing sodium phosphate, leaving chloroplasts and broken protoplasts at the lower phase. The separation is achieved by low speed centrifugation; therefore, intact protoplasts can readily be collected by this method without employing countercurrent distribution apparatus which would be a laborious and time consuming method.

Salts can dramatically change the partition of particles between the phases of two-phase solutions. Albertsson (1) found that NaCl was quite effective in shifting the partition of *Chlorella* species, erythrocytes, and *Escherichia coli* from the upper phase to the interphase in a dextran T_{500}-PEG sysem. In our studies, sodium phosphate shifted the adsorption of particles from the lower phase to the interphase, while NaCl and KCl had little effect. Furthermore, as shown in Figure 5, protoplasts from different plant origin may respond quite specifically to a small difference in sodium phosphate concentration. Apparently in this case, the phosphate anion is specific in causing the phase shift of the plant protoplasts. By choosing an optimum level of sodium phosphate or other salts the intact protoplasts of a wide variety of plant species can be collected at the interphase with the other particles left in the lower phase.

In conclusion, by the methods described in this paper, we have isolated and purified leaf mesophyll protoplasts from a number of C_3, C_4, and CAM species. Leaf cells have also been isolated from a limited number of plant species for photosynthetic studies (5, 8, 12, 19). Some merits of the technique of protoplast isolation for biochemical studies are: (*a*) it is applicable to a large number of plants, (*b*) very pure cellular preparations are obtained, (*c*) the possibility of protoplast damage is minimized during purification as the protoplasts collect at the interphase by low speed centrifugation of the two-phase system, (*d*) the viability of the purified protoplasts can easily be examined by osmotic response in size to varying concentrations of sorbitol between 0.5 to 1.0 M, or exclusion of Evans Blue dye (molecular weight 961) which indicates the ability to maintain membrane semipermeability.

As a further criterion of protoplast viability, photosynthetic activities are being measured using mesophyll protoplasts of some C_3, C_4, and CAM plants. Protoplasts of a C_3 plant, *Nicotiana tabacum* isolated by techniques in this paper fix CO_2 in the light at rates of 30 to 50 μmoles/mg chl·hr without adding any CO_2 acceptor, (dark fixation was 0.6 μmoles/mg chl·hr) which compares favorably with rates obtained with enzymatically isolated cells of *Nicotiana tabacum* (12). Intact mesophyll protoplasts of C_4 plants are found to have high enzymatic activities of phosphoenolpyruvate carboxylase, and NADP-malate dehydrogenase (13, 14), and a high capacity for the Hill reaction (400–600 μmoles/mg chl·hr with added oxidant, Ku and Edwards, unpublished). Preliminary experiments with protoplasts of some C_4 plants indicate a high CO_2 fixation capacity when 10 mM phosphopyruvate is supplied in the reaction medium (500–700 μmoles/mg chl·hr). The fixation by mesophyll protoplasts of C_4 plants with phosphopyruvate occurs in the dark as well as the light. However, the fixation is low (generally less than 10 μmoles/mg chl·hr) in C_4 mesophyll protoplasts without the addition of phosphopyruvate. Mesophyll protoplasts of a CAM species, *Sedum telephium*, showed a light-induced fixation of CO_2 of 30 to 40 μmoles/mg chl·hr and a dark fixation of 0.9 μmoles/mg chl·hr, without a primer, when isolated around midday.

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