Plant Physiol. (1980) 66, 400–403
0032-0889/80/66/0400/04/$00.50/0

Isolation of Cytoplasmic Enzymes from Pollen[1]

Received for publication December 3, 1979 and in revised form April 7, 1980

NORMAN F. WEEDEN AND LESLIE D. GOTTLIEB
Department of Genetics, University of California, Davis, California 95616

ABSTRACT

The cytoplasmic isozyme of many cytoplasmic-organelle isozyme pairs, as well as other cytoplasmic enzymes in plants, can be readily obtained from pollen by soaking it in an appropriate buffer for 4 hours. Enzymes localized in subcellular organelles appear not to be released during the soaking period, although they are released if the pollen is crushed. The technique is a useful initial step in studies of subcellular localization of enzymes or for obtaining small quantities of cytoplasmic enzymes free of organellar contaminants.

The intracellular localization of enzymes has provided considerable information regarding the compartmentation of metabolic pathways. Techniques are now available for the isolation of various organelles from plant tissues (see ref. 19 for recent review). However, the cytoplasm and its complement of enzymes has been difficult to obtain free from contamination from organelle enzymes because techniques used to disrupt the plant cell also disrupt a certain proportion of the organelles. Cytoplasmic enzymes have been obtained following protoplast formation (14), but this procedure requires care and practice for optimal results. We report that many cytoplasmic enzymes can be obtained from pollen of many plant species by a simple and rapid method.

MATERIALS AND METHODS

Fresh pollen was collected from *Pisum sativum* L. cv. Alaska and *Spinacia oleracea* L. cv. Early Hybrid No. 7 because these species have been favorite materials for enzyme localization studies. In addition, we examined enzymes released from pollen of *Zea mays* L., *Clarkia unguiculata* Lindley, *Cedrus atlantica* Manetti, *Oenothera biennis* L., *Lycopersicon esculentum* Mill., and *Stephanomeria exigua* ssp. *coronaria* Gottlieb. The pollen was usually placed directly in buffer, although dry storage for 24 h at room temperature did not seriously impair the analysis. The buffer was generally 50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 14 mM ME[2]. Both 100 mM K-phosphate, pH 7.0, and 50 mM EDTA, pH 8.6, were also used. Approximately 5 mg pollen were placed in 0.5 ml chilled buffer and allowed to soak for 1 to 12 h. The extract then was used for starch gel electrophoresis. Crushed pollen extracts were obtained by grinding the soaked pollen suspension by hand in a chilled mortar for 1 to 2 min, which resulted in approximately 70% cell breakage. The slurry was centrifuged for 2 min at 7,000*g*. The supernatant fraction was removed by pipette and the pellet was discarded. Extracts from green leaf tissue were obtained by grinding 0.5 g tissue in 0.5 ml chilled 50 mM K-phosphate, pH 8.2, containing 2 mM EDTA and 14 mM ME.

Starch Gel Electrophoresis. Starch gels were prepared and electrophoresis was conducted as described previously (7). Assays were as follows: PGI (23); PGM (modified from (4) to contain 3 mM disodium α-D-glucose-1-P and 0.9 μM dipotassium α-D-glucose-1,6-diP); AAT (7); ADH (21); ALDO (modified from ref. 7 by using 100 mM Tris-HCl, pH 8.5, as the buffer and 0.3 mM MTT in place of NBT); NAD- and NADP-specific GA3PDH (with 0.13 mM NADP in place of the NAD when assaying for the NADP-specific form) (4); catalase (23); TPI (modified from ref. 28 by using 100 mM Tris-HCl, pH 8.2, as the buffer and including 0.3 mM MTT and 100 μM PMS); NAD-specific MDH (100 mM malate-Tris, pH 7.2, containing 0.4 mM MTT, 100 μM PMS, and 0.45 mM NAD); SKDH (100 mM Tris-HCl, pH 8.5, containing 1.5 mM shikimic acid, 0.3 mM MTT, and 100 μM PMS).

Time-Course Studies and Spectrophotometric Assays. Approximately 10 mg *Clarkia* pollen, weighed to the nearest 0.1 mg, was placed in an Eppendorf microcentrifuge tube containing 0.5 ml of 50 mM K-phosphate, pH 8.5, with 1 mM EDTA and 14 mM ME. The mixture was gently shaken by hand and allowed to stand at room temperature. Periodically, 0.025-ml aliquots were taken for enzyme assays at 22 C on a Zeiss PM6 recording spectrophotometer using a 0.5-ml cuvette. Catalase activity was measured using the method of Lück (15); fumarase, by the method of Racker (20). The assay mixtures for PGI, NAD- and NADP-specific GA3PDH, ALDO, ADH, and MDH were the same as those given above except that MTT (or NBT) and PMS were omitted. For these enzymes, the activity was determined by measuring the production of reduced dinucleotides at 340 nm. Blanks lacking substrate were run to allow correction for interfering reactions.

Chloroplast Isolation. Plants used for chloroplast extractions were kept in the dark for 16 h prior to harvest in order to reduce chloroplast starch content. Pea chloroplasts were extracted in a buffer modified (from ref. 17) to contain 0.33 M sorbitol, 100 mM Tricine (pH 7.5), 10 mM KCl, 1.5% (w/v) Ficoll, 0.1% (w/v) BSA, and 1 mM isoascorbic acid. Five g leaf tissue were sectioned into 1- to 2-cm^2 pieces and homogenized in 30 ml chilled extraction buffer by five 1-s bursts on a Sorvall Omni-Mixer. The slurry was filtered through four layers of Miracloth, with glass wool inserted between layers, and then centrifuged for 60 s at 1000*g*. The supernatant was discarded, and the pellet was resuspended in 20 ml extraction buffer and centrifuged again for 60 s at 1000*g*. Spinach chloroplasts were extracted in the buffer described in reference 3 except that 25 mM Hepes, pH 7.5, was used instead of Mes, and sodium pyrophosphate was not added. The initial homogenization and centrifugation steps were similar to those used for the pea preparation.

The chloroplast pellets of both species were further purified in

[1] The research for this paper was supported by National Research Service Award Grant 5 T32 GM07467 (to N. F. W.) and National Science Foundation Grant DEB 77-08448 (to L. D. G.).

[2] Abbreviations: ME, 2-mercaptoethanol; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; AAT, aspartate aminotransferase; ALDO, fructose-1,6-diphosphate aldolase; GA3PDH, glyceraldehyde-3-phosphate dehydrogenase; TPI, triose phosphate isomerase; MDH, malate dehydrogenase; SKDH, shikimic dehydrogenase; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; NBT, nitro blue tetrazolium; PMS, phenazine methosulfate.

the following manner. Each was resuspended in 6 ml of the extraction buffer initially used for each species and placed on top of a 20 to 55% continuous sucrose gradient. This was centrifuged for 5 min at 4000 rpm and then for 5 min at 7000 rpm in a Sorvall HS-4 swinging bucket rotor. Two discrete Chl-containing bands were present after centrifugation. The lower band, containing the intact chloroplasts, was collected and divided into two aliquots. One of these was used for electron microscopy, and the other was diluted with an equal volume of 50 mM phosphate buffer (pH 8.2), containing 2 mM EDTA and 14 mM ME, and centrifuged for 2 min at 7000*g*. The supernatant fraction was used as the chloroplast extract in the electrophoretic studies.

Buffer Concentration Study. To test the effect of increasing the osmotic potential of the soaking buffer, approximately 10 mg *Clarkia* pollen was placed in 0.5 ml of each of three solutions: (*a*) 50 mM K-phosphate, pH 8.2, containing 2 mM EDTA and 14 mM ME; (*b*) the above solution supplemented with 1.0 M mannitol; (*c*) the chloroplast extraction buffer (without the isoascorbic acid but including 14 mM ME). The mixtures were shaken gently and allowed to stand at room temperature. After 30, 60, 120, and 270 min, 0.025-ml aliquots were taken from each solution, and the PGI activity was measured on the spectrophotometer. In a parallel study, 2.5 mg pea pollen was placed in 0.5 ml solution (*a*) above, and a second 2.5 mg was placed in 0.5 ml of the same solution containing 1% (w/v) NaCl since this has been reported to inhibit germination and prevent the pollen membrane from bursting (16). Aliquots of 0.025 ml were taken at 30 and 120 min for PGI assays.

RESULTS

The electrophoretic patterns of the various enzyme systems were examined in extracts of whole leaf, soaked pollen, and whole chloroplast preparations. The purities of the spinach and pea chloroplast preparations were verified by electron microscopy.

PGI, TPI, and PGM were examined by electrophoresis in both spinach and pea. For each system, the leaf extracts of both species contained two isozymes (Table I). The spinach chloroplast extract contained only the more anodal isozymes of PGI, TPI, and PGM. These results confirm previous observations that the more anodal isozymes of PGI (22) and PGM (18) are plastid-specific. The spinach soaked-pollen extract contained only the less anodal isozyme of these three systems. The pea chloroplast extract contained only the more anodal PGI and the less anodal TPI and PGM isozymes. The pea soaked-pollen extract contained only the less anodal PGI and the more anodal TPI and PGM (Fig. 1). For both species, the chloroplast isozyme of each system was present within pollen and could be obtained as an additional isozyme by soaking the pollen for a prolonged time or by mechanical crushing (Fig. 2). Thus, the plastid isozymes are present but retained within the pollen, whereas the cytoplasmic isozymes are readily released.

Two AAT isozymes could be clearly resolved by electrophoresis in pea leaf extracts. A third AAT isozyme with intermediate mobility was occasionally observed. The least anodal isozyme was present only in soaked-pollen extracts, and the most anodal one was present only in the chloroplast extract (Table I).

The most anodal MDH isozyme of pea, previously reported to be in the cytoplasm (29), was found only in the soaked-pollen extracts. None of the MDHs was found in the chloroplast extracts. Their compartmentation was not studied further. Electrophoresis revealed only a single enzyme for each of the other systems examined in pea. The plastid marker, NADP-specific GA3PDH, was present in extracts from whole leaves and the chloroplasts, but not in soaked pollen. However, the enzyme was present in the crushed-pollen extracts. Similar results were obtained for ALDO and SKDH (Table I).

The peroxisomal marker catalase exhibited low activity in soaked pollen extracts, suggesting that it is not entirely confined to extracytoplasmic compartments in pollen. Indeed, cytoplasmic catalase has previously been reported in castor bean (6). Catalase activity was 20-fold higher in the crushed-pollen extracts.

The mitochondrial marker fumarase was not resolved by electrophoresis. However, spectrophotometric analysis indicated it was present in the crushed-pollen extracts but absent from soaked pollen.

The cytoplasmic marker NAD-specific GA3PDH was present in extracts from soaked pollen. Thus, all of these results are consistent with the model that only cytoplasmic enzymes are readily extracted from pollen.

A number of enzymes were also examined in soaked-pollen extracts of *C. unguiculata* in order to test the generality of this finding. The soaked pollen extracts contained the cytoplasmic enzymes NAD-specific GA3PDH, ADH, and MDH but did not contain the organelle markers NADP-specific GA3PDH and fumarase. The cytoplasmic isozyme of PGI was present in the pollen extract but not the more anodal chloroplast PGI. We also examined PGI in *O. biennis*, *L. esculentum*, and *S. exigua* ssp. *coronaria*. In all three species, two regions of PGI activity were observed after electrophoresis of green leaf extracts, the more anodal being localized in the chloroplast fraction (unpublished data). Soaked-pollen extracts from each species contained only the more cathodal PGI isozyme.

In contrast to the rapid release of cytoplasmic enzymes from the pollen of *Clarkia*, the soaked-pollen extracts of maize and *Cedrus* were unusual in that they contained very little activity of PGI or ADH (other enzymes were not tested). Freezing or thawing their pollen before soaking yielded higher activity, although this treatment also resulted in release of small amounts of the plastid isozymes.

The time-course experiment indicated that PGI, NAD-specific GA3PDH, MDH, and ADH all diffused from *Clarkia* pollen shortly after it was immersed in the buffer. The activities of these enzymes increased more or less at a linear rate for the first 90 min and were essentially completed after 4 h soaking (Fig. 3). Evidence that a significant portion of the cytoplasmic PGI was not released into the buffer, even after 24 h soaking, was obtained by transferring soaked pollen into fresh buffer and crushing the pollen. Both the plastid and the cytoplasmic forms of PGI were obtained after crushing. Since both isozymes were present in this extract, their relative activity could not be determined spectrophotometrically.

The effect of increasing the osmotic potential upon the release of PGI was also tested using *Clarkia* pollen. The presence of 0.33 M sorbitol or 1 M mannitol in the extraction buffer did not affect the amount or rate of enzyme release, evaluated as per cent of the activity released in the crushed-pollen extract (Table II). The total PGI activity in the crushed pollen was similar in the control and in both test buffers (9.5 ± 2.2 units/mg pollen; one unit is defined as the amount of enzyme that causes an increase of 0.1 *A* units/min under the above conditions.

In a parallel experiment, the influence of 1% NaCl upon PGI release from pea pollen was also tested. No difference was noted in the PGI activities in the two buffers (Table II). The total PGI activity in the crushed pea pollen was 4.7 ± 0.3 units/mg pollen; units are defined as above.

DISCUSSION

Nearly a century ago, Green (8) mentioned that moistened pollen grains released a substance, presumably amylase, that liquified starch paste. Our data indicate that only cytoplasmic enzymes are released from pollen during the first 12 h of soaking in buffer, whereas organelle-bound enzymes are not. Consequently, pollen provides a convenient source of these enzymes. That enzymes are readily released from pollen is well known and has been demonstrated in *Iris* (10), *Phalaris* (12), *Ambrosia* and *Gladiolus* (13), and *Salsola*, *Pyrus*, and *Plantago* (25), in addition to the species studied by us. Stanley and Linskens (24) present a

Table I. *Isozymes Resolved by Electrophoresis of Leaf, Chloroplast, and Pollen Extracts*

Numbers indicate mobility of isozyme relative to the borate front.

Enzyme	Species	Isozymes			Intracellular Location	Reference
		Leaf	Chloroplast	Pollen		
PGI	Pea	0.51	0.51		Chloroplast	27
		0.39		0.39	Cytoplasm	27
	Spinach	0.47	0.47		Chloroplast	22
		0.28		0.28	Cytoplasm	22
TPI	Pea	0.54		0.54	Cytoplasm	1
		0.48	0.48		Chloroplast	1
	Spinach	0.54	0.54		Chloroplast	This paper
		0.44		0.44	Cytoplasm	This paper
PGM	Pea	0.66		0.66	Cytoplasm	27
		0.58	0.58		Chloroplast	27
	Spinach	0.56	0.56		Chloroplast	18
		0.42		0.42	Cytoplasm	18
AAT	Pea	0.37	0.37		Chloroplast	27
		0.27		0.27	Cytoplasm	27
MDH	Pea	0.48		0.48	Cytoplasm	29
		0.45			Microbody	29
		0.20			Mitochondria	29
Aldolase	Pea	0.36	0.36		Chloroplast	2
GA3PD(NAD)	Pea	0.25		+[a]	Cytoplasm	19
GA3PD(NADP)	Pea	0.18	0.18		Chloroplast	19
Catalase	Pea	0.15	ND[b]	faint	Peroxisome	19
SKDH	Pea	0.33	0.33		Chloroplast	5

[a] GA3PDH activity in soaked pollen extracts from pea was too low to produce a clear stain on starch gels but could be measured using the spectrophotometric assay.

[b] ND, not determined.

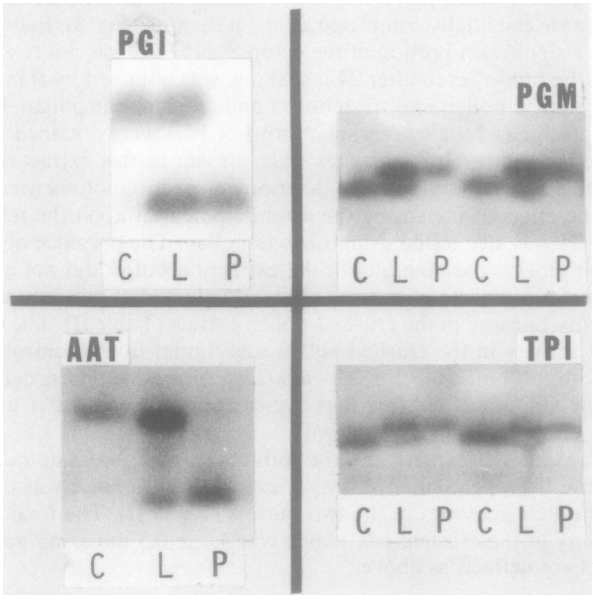

FIG. 1. Electrophoretic patterns of isozymes of PGM, PGI, AAT, and TPI on starch gels. Comparison of chloroplast (C), leaf (L), and soaked-pollen (P) extracts from pea. Anode is at the top of the figure.

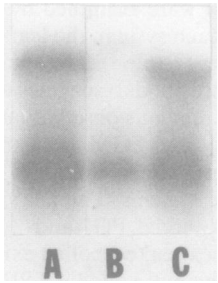

FIG. 2. Electrophoretic patterns of PGI isozymes in spinach extracts. A, crushed-pollen extract; B, soaked-pollen extract; C, leaf extract. Anode is at the top of the figure.

list of approximately 80 enzymes that have been detected in pollen extracts.

The absence of enzyme activity in a soaked-pollen extract does not necessarily indicate that the enzyme is localized within an organelle. The enzyme could be attached to the ER or other membrane, may not be expressed in pollen, or may simply not be leachable [*e.g.* maize and also pine and palm (25)]. The technique is a worthwhile initial step in studies of subcellular localization and for obtaining small quantities of cytoplasmic enzymes free from organellar contaminants. Its simplicity recommends it.

Our attempts to determine the total activity of specific enzymes per mg pollen proved difficult because the amount of activity released from the pollen appears to be affected by its age, the age of the plant, and environmental conditions. In addition, the amount of enzyme may be species-specific; thus, *Clarkia* and pea pollen extracts had different amounts of PGI activity.

Previous studies (26) of the genetic basis for the cytoplasmic PGI in *Clarkia* demonstrate that this enzyme, at least, is synthesized by the pollen and not by surrounding tapetal cells. This follows because the intragenic hybrid enzyme characteristic of individuals heterozygous for the coding gene locus is not observed in soaked pollen extracts. If the PGI were of tapetal origin, all three forms of the enzyme (*i.e.* both homodimers and the heterodimer) would be expected in the pollen of such plants. Other studies (9, 10) showed that acid phosphatase, ribonuclease, and esterase that leach from pollen are also synthesized therein.

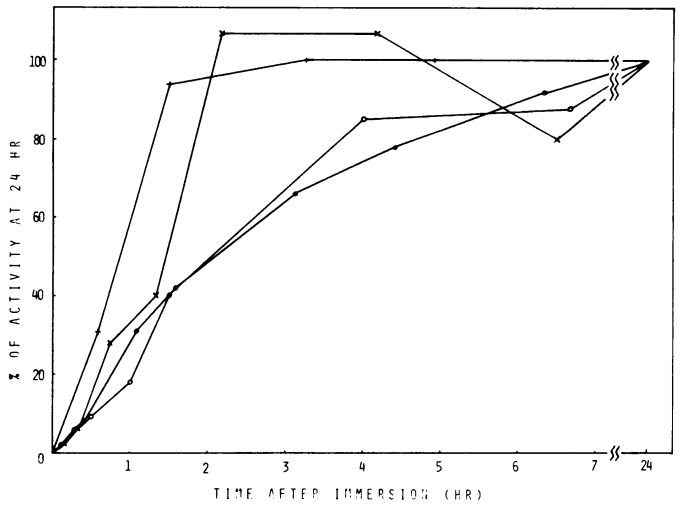

FIG. 3. Results of time-course study for PGI (●——●), MDH (○——○), ADH (×——×), and NAD-specific GA3PDH (+——+) from *Clarkia* pollen. Values on ordinate are percentage of enzyme activity in extract relative to that found after 24 h soaking. The PGI data points are the average of three independent experiments. Data points for MDH, ADH, and NAD-specific GA3PDH are the average of two pollen extracts examined during a single experiment. Standard errors for the data points are similar to those given in Table II.

Table II. *Effect of Buffer Solute Concentration on Leaching Process*

PGI activity in soaked pollen extracts as a percentage of activity in the crushed pollen extract. Values are the means ± SE of three independent extractions with two replicates each for *Clarkia* pollen in 0.05 M phosphate, and one experiment with two replicates for the sorbitol test and three replicates for the mannitol tests. The test in pea was a single experiment with two replications.

Time after Immersion	*Clarkia* PGI			Pea PGI	
	0.05 M Phosphate	0.33 M Sorbitol	1.0 M Mannitol	0.05 M Phosphate	1% NaCl
min			%		
30	6.0 ± 1.2	9.0 ± 5.0	10 ± 0.8	15 ± 1.0	13 ± 1.0
60	16 ± 2.6	26 ± 2.0	25 ± 9.0		
120	42 ± 6.6	45 ± 1.5	48 ± 7.4	23 ± 0.2	20 ± 0.3
270	60 ± 12	53	53 ± 7.8		

The mechanism of enzyme release from the pollen appears to be the same for all of the enzymes we assayed since they followed a similar time course. Their release even in buffers having high osmotic potential may mean that they are located in the intine layer as proposed for other enzymes (11, 12). Alternatively, the pollen plasmalemma may be more "porous" than the membranes surrounding the organelles. Regardless of the actual mechanism of enzyme release, pollen is likely to serve as a useful source of cytoplasmic enzymes.

Acknowledgment—We thank Lisa Morrison for examining the spinach and pea chloroplast preparations by electron microscopy.

LITERATURE CITED

1. ANDERSON LE 1971 Chloroplast and cytoplasmic enzymes. II. Pea leaf triose phosphate isomerases. Biochim Biophys Acta 235: 237–244
2. ANDERSON LE, I PACOLD 1972 Chloroplast and cytoplasmic enzymes. IV. Pea leaf fructose 1,6-diphosphate aldolases. Plant Physiol 49: 393–397
3. BAHR JT, RG JENSEN 1978 Activation of ribulose bisphosphate carboxylase in intact chloroplasts by CO_2 and light. Arch Biochem Biophys 185: 39–48
4. BREWER GJ 1970 An Introduction to Isozyme Techniques. Academic Press, New York, pp 62–137
5. FEIERABEND J, D BRASSEL 1977 Subcellular localization of shikimic dehydrogenase in higher plants. Z Pflanzenphysiol 82: 334–346
6. GONZALEZ E, H BEEVERS 1976 Role of the endoplasmic reticulum in glyoxysomal formation in castor bean endosperm. Plant Physiol 57: 406–409
7. GOTTLIEB LD 1973 Enzyme differentiation and phylogeny in *Clarkia franciscana*, *C. rubicunda*, and *C. amoena*. Evolution 27: 205–214
8. GREEN JR 1894 On the germination of the pollen-grain and the nutrition of the pollen-tube. Ann Bot 8: 225–228
9. HESLOP-HARRISON J, Y HESLOP-HARRISON, RB KNOX, B HOWLETT 1973 Pollen-wall proteins: 'gametophytic' and 'sporophytic' fractions in the pollen wall of the Malvaceae. Ann Bot 37: 403–412
10. KNOX RB 1971 Pollen-wall proteins: localization, enzymic and antigenic activity during development in *Gladiolus* (Iridaceae). J Cell Sci 9: 209–237
11. KNOX RB, J HESLOP-HARRISON 1971a Pollen-wall proteins: electronmicroscopic localization of acid phosphatase in the intine of *Crocus vernus*. J Cell Sci 8: 727–733
12. KNOX RB, J HESLOP-HARRISON 1971b Pollen-wall proteins: the fate of intine-held antigens of the stigma in compatible and incompatible pollinations of *Phalaris tuberosa* L. J Cell Sci 9: 239–251
13. KNOX RB, J HESLOP-HARRISON, C REED 1970 Localization of antigens associated with the pollen grain wall by immunofluorescence. Nature 225: 1066–1068
14. LARKIN PJ 1976 Purification and viability determinations of plant protoplasts. Planta 128: 213–216
15. LÜCK H 1965 Catalase. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis. Academic Press, New York, pp 885–894
16. MÄKINEN Y, JL BREWBAKER 1967 Isoenzyme polymorphism in flowering plants. I. Diffusion of enzymes out of intact pollen grains. Physiol Plant 20: 477–482
17. MIFLIN BJ, H BEEVERS 1974 Isolation of intact plastids from a range of plant tissues. Plant Physiol 53: 870–874
18. MÜHLBACH H, C SCHNARRENBERGER 1978 Properties and intercellular distribution of two phosphoglucomutases from spinach leaves. Planta 141: 65–70
19. QUAIL PH 1979 Plant cell fractionation. Annu Rev Plant Physiol 30: 425–484
20. RACKER E 1950 Spectrophotometric measurements of the enzymatic formation of fumaric and *cis* aconitic acids. Biochim Biophys Acta 4: 211–214
21. ROOSE ML, LD GOTTLIEB 1976 Genetic and biochemical consequences of polyploidy in *Tragopogon*. Evolution 30: 818–830
22. SCHNARRENBERGER C, A OESER 1974 Two isoenzymes of glucosephosphate isomerase from spinach leaves and their intracellular compartmentation. Eur J Biochem 45: 77–82
23. SHAW CR, R PRASAD 1970 Starch gel electrophoresis of enzymes—a compilation of recipes. Biochem Genet 4: 297–320
24. STANLEY RG, HF LINSKENS 1974 Pollen: Biology, Biochemistry, Management. Springer-Verlag, Berlin, pp 195–222
25. STANLEY RG, RW SEARCH 1971 Pollen protein diffusates. *In* J Heslop-Harrison, ed, Pollen: Development and Physiology. Butterworths, London, pp 174–176
26. WEEDEN NF, LD GOTTLIEB 1979 Distinguishing allozymes and isozymes of phosphoglucoisomerase by electrophoretic comparisons of pollen and somatic tissues. Biochem Genet 17: 287–296
27. WEEDEN NF, LD GOTTLIEB 1980 The genetics of chloroplast enzymes. J Hered 71: In press
28. WU R, E RACKER 1959 Regulatory mechanisms in carbohydrate metabolism. III. Limiting factors in glycolysis of ascites tumor cells. J Biol Chem 234: 1029–1035
29. ZSCHOCHE WC, IP TING 1973 Malate dehydrogenases of *Pisum sativum*. Tissue distribution and properties of the particulate forms. Plant Physiol 51: 1076–1081