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Subcellular Localization of Hexokinase in Pea Leaves

EVIDENCE FOR THE PREDOMINANCE OF A MITOCHONDRIALLY BOUND FORM*

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Hexokinase (ATP : D-hexose-6-phosphotransferase, EC 2.7.1.1) activity was determined in subcellular fractions prepared from pea (*Pisum sativum*) leaf homogenates. About 60% of the total detectable activity of hexokinase was found associated with a particulate fraction consisting essentially of mitochondria and chloroplasts and free of cytosol contamination. The hexokinase specific activity of the particulate fraction was 2-fold higher than that of the homogenate and about 4-fold higher than that of the cytosol.

Using a specially designed isokinetic-isopycnic sucrose density gradient centrifugation method, the distribution of hexokinase activity correlated with that of the mitochondrial marker (cytochrome oxidase) and not with that of the chloroplast membrane marker (chlorophyll) or that of the cytosol marker (phosphoenolpyruvate carboxylase). Thus, the hexokinase/mitochondria ratio was close to 1.0 along the entire gradient, while the hexokinase/chloroplast ratio varied over a 10-fold range.

The results strongly suggest that hexokinase is predominantly bound to mitochondria of pea leaves, and that pea leaf chloroplasts are essentially devoid of any specifically associated hexokinase activity. This work provides the first demonstration of a specific association of hexokinase with mitochondria from photosynthetic tissues of higher plants.

The subcellular localization of hexokinase in plant cells has been a matter of neglect or of controversy (1-7). Particulate hexokinase activity in plants was originally reported almost 3 decades ago (8, 9) but, to date, little is known about its precise subcellular localization in nonphotosynthetic and photosynthetic plant tissues, or of the physiological role of particulate hexokinase in the plant cell. The subcellular localization of hexokinase in animal cells has also been a matter of controversy until recently. Despite the fact that in 1945 Utter *et al.* (10) observed that hexokinase activity found in homogenates of rat brain is markedly reduced upon centrifugation, and that in 1953 Crane and Sols (11) partially characterized particulate hexokinase, it was not until 1967 that Rose and Warms (12) firmly established that the particulate behavior of hexokinase was due to an association of this enzyme with the outer surface of the external mitochondrial membrane. Mitochondrial hexokinase in animal cells is now known to fall within the category of "ambiquitous" enzymes as proposed by Wilson (13, 14). Thus, mitochondrial hexokinase can remain bound to mitochondria or become detached according to the relative concentrations of specific metabolites such as ATP, Mg^{2+}, or Glc-6-P[1] (12, 14-16). Significantly, mitochondrial hexokinase has been shown to be primarily responsible for the high aerobic glycolytic rate characteristic of many animal tumor cells (15, 17-20). The regulation of this key enzyme appears to be critical in shifting the cell from a low to a high glycolytic state or vice versa (15, 17-20).

The role of hexokinase in the compartmentalization of function in the photosynthetic plant cell has been obscured by the lack of experimental information on its precise subcellular localization. There seems to be a possible association of hexokinase with mitochondria in nonphotosynthetic cells according to recent reports which describe hexokinase activity in particulate fractions prepared from homogenates of *Allium sativum* bulbs (21) and lentil roots (22). With respect to hexokinase activity associated with particulate fractions of photosynthetic tissues such as leaves, a recent report by Stitt *et al.* (4) showed very conclusively that hexokinase was absent from the stroma of pea chloroplasts, and suggested that it might be associated with the external surface of the chloroplast envelope.

The main objective of the work presented here was to ascertain the precise subcellular localization of hexokinase in pea leaf homogenates. This objective has been accomplished via the use of an isokinetic-isopycnic sucrose density gradient centrifugation technique to partition the homogenate into regions where the mitochondria/chloroplasts ratios vary markedly. Through the use of appropriate marker enzymes, the results presented in this paper provide strong evidence for a predominant association of hexokinase with the mitochondria of pea leaves.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

DISCUSSION

Results presented in this paper provide the first demonstration of an association of hexokinase with the mitochondria

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[1] The abbreviations used are: Glc-6-P, glucose 6-phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

[2] Portions of this paper (including "Experimental Procedures," "Results," and Figs. 1-5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-0559, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE I

Subcellular distribution of organelle markers in pea leaf homogenates

A homogenate was prepared from pea leaves and fractionated by differential centrifugation as described under "Experimental Procedures." Enzyme assays were carried out spectrophotometrically under conditions specified under "Experimental Procedures." The results reported represent means ($n = 6$) ± S.D.

Subcellular fraction	Cytochrome oxidase[a]	Chlorophyll[b]	P-enolpyruvate carboxylase[c]	Protein[d]
		%		
Homogenate	100	100	100	100
Particulate	75 ± 8	83 ± 4	2 ± 1	31 ± 2
Cytosol	25 ± 5	5 ± 1	92 ± 1	63 ± 4
Average recovery	100	88	94	94

[a] Cytochrome oxidase specific activity of the homogenate was 163 nmol of cytochrome *c* oxidized per min × mg.

[b] Chlorophyll concentration in the homogenate was 0.4 mg/ml.

[c] P-enolpyruvate carboxylase specific activity of the homogenate was 48 nmol of oxalacetate formed per min × mg.

[d] Protein concentration in the homogenate was 5.6 mg/ml.

TABLE II

Variation of the mitochondria/chloroplasts ratio in sucrose density gradients

Data presented in this table were taken from experiments shown in Fig. 3*A*. Thus, experimental conditions were specified in the legend to Fig. 3 and details concerning the sucrose gradients are described in "Experimental Procedures." Values are reported as means ($n = 10$) ± S.D.

Fraction number	Cytochrome oxidase	Chlorophyll	Per cent cytochrome oxidase / Per cent chlorophyll
	%		
1	19.0 ± 2.2	3.9 ± 1.0	4.9
2	13.4 ± 2.3	5.8 ± 0.6	2.3
3	5.2 ± 0.8	10.0 ± 2.7	0.5
4	5.6 ± 0.8	6.9 ± 1.2	0.8
5	9.0 ± 1.7	6.5 ± 1.3	1.4
6	15.6 ± 3.0	18.7 ± 4.5	0.8
7	14.8 ± 4.0	30.0 ± 6.0	0.5

from photosynthetic organs of plants. Several lines of experimental evidence point toward the predominance of a mitochondrially bound form of hexokinase in the pea leaf. (i) About 60% of the hexokinase activity detectable in pea leaf homogenates was found associated with a particulate fraction. (ii) The hexokinase specific activity of the particulate fraction is 2-fold higher than that of the homogenate and about 4-fold higher than that of the cytosol. (iii) The particulate fraction was found to be enriched in both mitochondria and chloroplasts and devoid of cytosol markers. (iv) The mitochondria/chloroplast ratio was found to vary markedly when the pea leaf homogenate was centrifuged in isokinetic-isopycnic sucrose density gradients. (v) The hexokinase/chloroplast ratio was found to vary dramatically over a 10-fold range. (vi) The hexokinase/mitochondria ratio was found to be constant along the entire gradient.

The specific association of hexokinase with the outer surface of the external mitochondrial membrane is a firmly established phenomenon in many animal cells (10–17). Mitochondrial hexokinase can exist in the animal cell either bound to the mitochondria or released from them (12, 13, 16) (the so-called ambiquitous behavior proposed by Wilson (14)). The bound hexokinase form and the soluble form differ in their apparent affinities for their substrate MgATP (16). Importantly, such a bound to soluble shift can be effected by specific metabolites such as ATP, Glc-6-P, or Mg^{2+} (12, 13, 16). It is interesting to note that hexokinase, which is the first enzyme in the glycolytic pathway, is actually bound to the outer membrane of the organelle responsible for oxidative production of most of the ATP in the animal cell (15, 17). In fact, it is now known that mitochondrially bound hexokinase is actually capable of phosphorylating glucose present in the cytosol at the expense of mitochondrially synthesized ATP (15, 32). Thus, mitochondrially bound hexokinase seems to be more efficient in phosphorylation of glucose than the soluble form since (i) it has a higher apparent affinity for MgATP, and (ii) it is located adjacent to the major ATP source of the cell, thus gaining increased accessibility to this substrate.

However, extrapolation to plant cells of the important regulatory role exerted by hexokinase on energy metabolism in animal cells is not automatic. Photosynthetic cells contain chloroplasts in addition to mitochondria. Moreover, chloroplasts are organelles that are known to catalyze ATP synthesis driven by dissipation of the electrochemical potential generated mainly by the light reactions of photosynthesis (33). Therefore, if hexokinase were associated with the chloroplast membrane, it would also gain preferential access to chloroplast-synthesized ATP; conceivably, such a membrane-bound hexokinase form could have a kinetic advantage as well.

Consequently, a primary step in the understanding of the regulatory aspects of the interaction between the glycolytic and oxidative pathways of plant energy metabolism seemed to ascertain the precise subcellular location of hexokinase in photosynthetic tissues. Results presented in this paper provide strong experimental support for the predominance of a mitochondrially bound form of hexokinase in the pea leaf. In addition, our results do not indicate a specific association of hexokinase with chloroplasts of pea leaves. Thus, it could be conceived that mitochondrial hexokinase might play a role in the regulation of energy metabolism in the pea leaf perhaps similar to that described for animal tissues.

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SUPPLEMENTAL MATERIAL TO

SUBCELLULAR LOCALIZATION OF HEXOKINASE IN PEA LEAVES:
Evidence for the Predominance of a Mitochondrially-Bound Form

Eric Cosio and Ernesto Bustamante

EXPERIMENTAL PROCEDURES

Materials

The following reagents were purchased from Sigma Chemical Co.: cytochrome C type III, D-mannitol, Hepes , defatted bovine serum albumin (BSA), NAD^+, ATP, Coomassie brilliant blue G-250, NADH, phosphoenolpyruvate (PEP), sucrose, Glc-6-P dehydrogenase (from Leuconostoc mesenteroides, which can use either NAD^+ or $NADP^+$ as cofactor), and malate dehydrogenase. The following were obtained from New England Nuclear Corp.: Triton X-100, 2,5-diphenyloxazole (PPO), and p-bis(2,5-phenyloxazolyl)-benzene (POPOP). Toluene was Packard scintillation grade. [^{14}C]D-glucose was purchased from Amersham Radiochemical Centre. All other reagents were of the highest purity commercially available. Pea (Pisum sativum, cultivar Alderman) seeds were generously provided by the Department of Horticulture of the Universidad Nacional Agraria of Peru.

Methods

Cultivation of Plants and Homogenization of Leaves. Growth was initiated by soaking pea seeds in running water for six hours. Soaked seeds were subsequently planted in wet vermiculite and kept in a growth chamber at 22° and illuminated with a 16 hour photoperiod. Plants were watered daily with a 33% (w/v) Hoagland's solution. Plants were harvested 15 to 20 days after seeding. Approximately 4 g of totally expanded young leaves were homogenized at 4° using a porcelain mortar in 10 ml of HMB buffer. HMB buffer consisted of 50 mM Hepes, 330 mM mannitol, 5 mM $MgCl_2$, 0.1% (w/v) BSA, pH 7.5. The resulting suspension was filtered through four layers of cheesecloth and centrifuged at 120 x g for one min to sediment unbroken cells and multicellular aggregates. The resulting supernatant is referred to as the homogenate.

Cell Fractionation. All operations were carried out at 4°. The homogenate was centrifuged at 280 x g for 3 min in the Sorvall SM-24 rotor to sediment nuclei, which were discarded. The resulting supernatant was centrifuged at 13,000 x g for 15 min in the Sorvall SM-24 rotor. The supernatant was saved, and the sediment was resuspended in HMB buffer and centrifuged at 13,000 x g for 15 min in the Sorvall SM-24 rotor. The final suspended sediment will be referred to as the particulate fraction. The supernatants were pooled and centrifuged at 160,000 x g for 60 min in the Beckman 75Ti rotor. The resulting final supernatant will be referred to as the cytosol fraction.

Isokinetic-Isopycnic Sucrose Density Gradient Centrifugation. A modification of the isokinetic-isopycnic method originally described by Miflin and Beevers (23) was used. All operations were performed at 4°. A discontinuous sucrose density gradient was made manually in 5 cc Beckman nitrocellulose tubes as follows, starting from the bottom of the tube: 0.5 ml of 60% sucrose [bottom], 0.6 ml of a 57% to 42% sucrose gradient (in 0.1 ml steps and with a difference of 3% sucrose concentration between each 0.1 ml step and the next), 0.6 ml of 42% sucrose, 1.2 ml of a 39% to 30% sucrose gradient (in 0.3 ml steps and with a difference of 3% sucrose concentration between each 0.3 ml step and the next), 0.4 ml of 30% sucrose [top]. All sucrose concentrations described above were made in 50 mM Hepes, pH 7.5 and their concentrations are expressed as percent (w/w) sucrose as determined by refractometry at 20°.

A 0.5 ml aliquot of the homogenate [the 330 mM mannitol in the HMB buffer was replaced with 30% sucrose] was layered on top of the gradient and centrifuged in the Beckman SW65 rotor at 5,000 rpm (1,800 x g) for 5 min. Immediately thereafter the rotor speed was accelerated to 12,000 rpm (10,300 x g) for 10 min. Brake was applied to the rotor only after it had decelerated to 5,000 rpm in order to minimize disturbances on the gradient. Fractions (0.3 ml) were collected from the top of the tubes using an ISCO gradient fractionator model 640. Usually three identical tubes were centrifuged simultaneously and the equivalent fractions were pooled making up a volume of 0.9 ml per fraction.

Enzyme Assays. Hexokinase activity was determined according to two methods:
a) Spectrophotometric Procedure. Hexokinase activity was assayed at 28° and pH 7.7 in a final volume of one ml containing 30 mM Hepes, 20 mM glucose, 3 mM ATP, 10 mM $MgCl_2$, 3 mM NAD^+, and one unit of Glc-6-P dehydrogenase, by following the formation of NADH at 340 nm.
b) Radiometric Procedure. Hexokinase activity was assayed by a modification of the method of Radojkovic et al. (24) at 25° and pH 7.7 in a medium containing 40 mM Hepes, 6 mM ATP, 10 mM $MgCl_2$, and 0.5 mM [^{14}C]D-glucose (1 mCi/mMol). After a period ranging between 10 and 20 min, the reaction was stopped with one ml of a solution containing 0.1 N formic acid - 0.1 M glucose. The mixture was passed through a 0.8 x 1 cm Dowex 1X8 anion exchange column equilibrated with formate ions, so that labeled Glc-6-P could bind the resin. The column was washed 10 times with 4 ml each of 0.1 N formic acid to eliminate free labeled glucose. Labeled Glc-6-P was eluted with 2.5 ml of 0.4 M ammonium formate pH 5.3. Aliquots were mixed with a Triton X-100 based toluene scintillation cocktail and radioactivity was measured with a Packard scintillation spectrometer.

Cytochrome oxidase (EC 1.9.3.1) activity was used as a marker for mitochondria. It was assayed according to the procedure of Fritz and Beevers (25) at 28° and pH 7.7 in a 2-ml final volume containing 30 mM sodium phosphate, 0.02 mM reduced cytochrome C [reduction was effected using sodium dithionite], and 0.2% (v/v) Triton X-100. Initial rates of the decrease in absorbance at 550 nm were taken as representative of zero-order reaction velocities.

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) activity was used as a marker for cytosol. It was assayed according to the coupled assay system of Wong and Davies (26) following the formation of oxaloacetate via its reduction to malate. This assay was carried out at 28° in a 1-ml final volume containing 50 mM Tris, 5 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM $NaHCO_3$, 2.5 mM PEP, 0.2 mM NADH, and 2 units of malate dehydrogenase, by following the decrease in absorbance at 340 nm.

Determination of Chlorophyll. Chlorophyll was used as a marker of chloroplast membranes. It was determined by the method described by Arnon (27) by adding 0.1 ml of sample to 5 ml of 80% acetone. After vigorous mixing the extract was centrifuged at 1,000 x g for 5 min at 20° and absorbance readings at 645 nm and 663 nm were rapidly obtained in the resulting supernatant. Chlorophyll concentration was calculated from the following equation:

$$\mu g \text{ chlorophyll/ml} = 5 \times [202\ A_{645} + 80\ A_{663}]$$

Determination of Protein. Protein concentration was determined by a modification of the method described by Bradford (28) by adding samples smaller than 0.025 ml to 1 ml of an aqueous mixture containing 0.01% (w/v) Coomassie brilliant blue G-250, 10% (v/v) methanol, and 10% (v/v) H_3PO_4 [the mixture was filtered through Whatman #1 paper prior to use]. Absorbance was read at 595 nm against reagent blank. Protein concentration was calculated from a standard curve made with a 1.4 mg/ml BSA solution [calibrated using an $E_{1\%}$ value of 6.7 at 280 nm].

RESULTS

Hexokinase Activity in Pea Leaf Homogenates. Results shown in Fig. 1 indicate that hexokinase activity in homogenates from pea leaves, as assayed by the spectrophotometric method described in Experimental Procedures, is linear with respect to time over a period of several min (Fig. 1A). In addition, when rates of hexokinase activity are plotted against amount of homogenate protein added in the assay, a linear behavior is also observed (Fig. 1B). These results indicate that the rate of hexokinase activity in pea leaf homogenates, under our assay conditions, is proportional to total enzyme concentration; thus, the hexokinase reaction behaves in a zero-order fashion. When hexokinase activity was assayed according to the radiometric procedure [see Methods] linearity versus time and homogenate protein concentration were also observed (data not shown). Significantly, hexokinase activity in the homogenate was not increased in the presence of 0.1% Triton X-100 (data not shown) which indicates that the enzyme is not latent or encapsulated within a membrane barrier inaccesible to substrates.

It should be pointed out that our spectrophotometric procedure for assaying hexokinase will not overestimate hexokinase activity via putative 6-phosphogluconate dehydrogenase activity since the cofactor used for the auxilliary enzyme Glc-6-P dehydrogenase (see Materials) is NAD+ rather than NADP+. Consequently, hexokinase activity rates [expressed as nMol glucose phosphorylated per min] were obtained directly from data calculated as nMol NADH/min.

Hexokinase Activity in Pea Leaves is Particulate. Results presented in Fig. 2A show that about 60% of the total hexokinase activity present in pea leaves is associated with a 13,000 x g particulate fraction. Fig. 2B shows the same experimental data presented in the form of a de Duve plot (29). This type of graph emphasizes that the specific activity of the particulate fraction is about 2-fold higher than that of the homogenate and almost 4-fold higher than the specific activity of the cytosolic fraction. Thus, these results indicate that although only 60% of the hexokinase activity of homogenized pea leaves is found associated with a particulate fraction (Fig. 2A), this fraction is almost 4-fold more enriched in hexokinase activity than the cytosol (Fig. 2B).

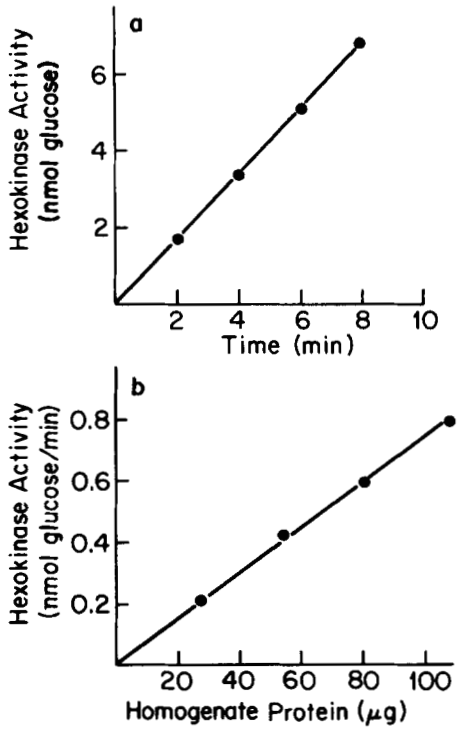

FIG. 1. Linearity of hexokinase activity versus time (A) and protein concentration (B). A homogenate was prepared from pea leaves and its hexokinase activity was assayed spectrophotometrically as described under Experimental Procedures. Results shown represent a typical experiment. Protein concentration of the homogenate was 2.7 mg/ml. The volume of homogenate used for the experiment shown in Panel A was 0.04 ml. The hexokinase specific activity of the homogenate was 7.9 nMol glucose/min x mg.

The Particulate Fraction of Pea Leaves is Enriched in Mitochondria and Chloroplasts. Organelle markers were used to further characterize the particulate fraction where an elevation of hexokinase activity was found (Fig. 2). Results shown in Table I clearly indicate that the particulate fraction contains about 80% of both the cytochrome oxidase activity and the chlorophyll of the homogenate. Moreover, the particulate fraction is also shown (Table I) to exhibit only 2% of the activity of the cytosolic marker PEP carboxylase found in the homogenate. Thus, the particulate fraction is highly enriched in both mitochondria and chloroplasts.

The Mitochondria/Chloroplast Ratio is Markedly Different upon Isokinetic-Isopycnic Centrifugation. Mitochondria and Chloroplasts are organelle populations difficult to separate from each other because their diameters and particle densities are dispersed over a wide and overlapping range (30,31). Therefore, methods of isokinetic centrifugation [which separate according to particle size] and of isopycnic centrifugation [which separate according to particle density] may be inadequate for a simultaneous separation of mitochondria and chloroplasts. However, quantitative separation of both organelle types was not really essential to answer our major question. Consequently, we employed a modified combined isokinetic-isopycnic centrifugation technique originally described by Miflin and Beevers (23) [see Methods for experimental details].

Fig. 3 depicts the distribution of organelle markers in sucrose density gradients loaded with an aliquot of pea leaf homogenate. Results presented in Fig. 3A indicate that the distribution of cytochrome oxidase overlaps that of chlorophyll. Nevertheless, it can be noticed (Fig. 3A) that there are some fractions richer in chlorophyll than in cytochrome oxidase (e.g. fractions 6 and 7) while others are richer in cytochrome oxidase than in chlorophyll (e.g. fractions 2 and 5). Thus, isokinetic-isopycnic centrifugation has partitioned the populations of mitochondria and chloroplasts differentially along the sucrose gradient.

Table II emphasizes this observation by tabulating the cytochrome oxidase/chlorophyll ratios in the several fractions collected from the gradients. Consequently, if these markers are taken as representative of the mitochondrial and chloroplast populations, it is evident that the mitochondria/chloroplast ratio is dramatically different along the sucrose density gradient, despite the fact that their distributions overlap.

Results presented in Fig. 3B show the distribution of the cytosolic marker PEP carboxylase and of protein concentration along the same fractions depicted in Fig. 3A. Significantly, about 65% of the homogenate protein did not enter the gradient. This value correlates well with the protein content of cytosol reported in Table I and with the fact that most of the PEP carboxylase activity was detectable only at the top of the gradient (Fig. 3B).

The Distribution of Hexokinase in Sucrose Gradients is Identical to that of the Mitochondrial Marker. Results presented in Fig. 4A depict the relative distribution of hexokinase activity in fractions collected from sucrose density gradients subjected to isokinetic-isopycnic sedimentation. Significantly, most of the hexokinase activity entered the gradient. This is contrast with the behavior of the cytosolic marker (see Fig. 3B).

This is more noticeable when the distribution of hexokinase is expressed in terms of specific activity, as shown in Fig. 4B. The distribution of cytochrome oxidase activity (see Fig. 3A) and that of total hexokinase activity (see Fig. 4A) indicate a very close sedimentation behavior.

Fig. 5 depicts the hexokinase activity relative to the cytochrome oxidase activity and the chlorophyll concentration in fractions collected from the gradients described above. The hexokinase/cytochrome oxidase ratio is close to 1.0 in all fractions, a strong indication for a specific association of hexokinase with pea leaf mitochondria. On the other hand, the hexokinase/chlorophyll ratio (Fig. 5) varies 10 fold, making it unlikely that pea leaf hexokinase is specifically associated with chloroplasts.

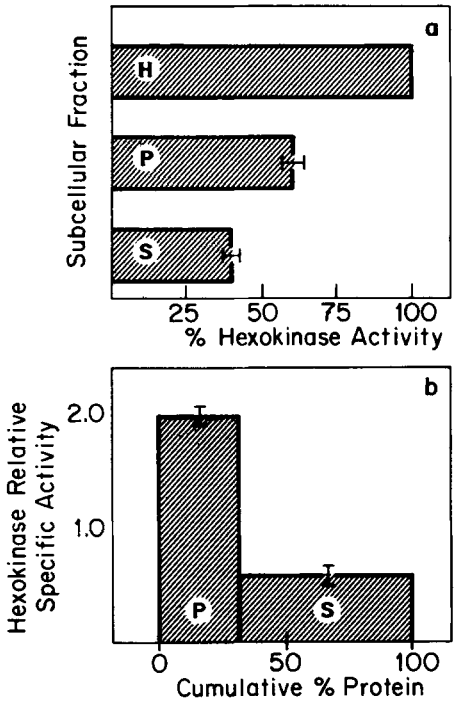

Fig. 2 Subcellular distribution of hexokinase activity in pea leaves. Pea leaves were homogenized and fractionated by the differential centrifugation procedure described under Experimental Procedures. The subcellular fractions were assayed for hexokinase activity by the spectrophotometric procedure described under Methods as well as for protein concentration. (A): Distribution of total hexokinase activity. (B): Distribution of relative specific activity. Absolute specific activities were: 8.7 mU/mg for the homogenate (H); 16.0 mU/mg for the particulate fraction (P); and 4.6 mU/mg for the cytosol fraction (S). In terms of chlorophyll contents, the hexokinase activity of the homogenate was 104 mU/mg chlorophyll. Results reported in this figure represent the average of 6 independent experiments and the bars indicate the SD.

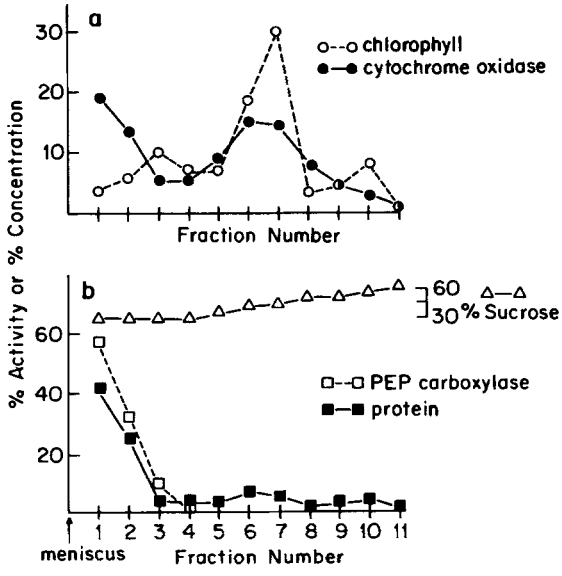

Fig. 3 Distribution of organelle markers in isokinetic-isopycnic sucrose density gradients. Panel (A): Distribution of cytochrome oxidase activity (●) and chlorophyll concentration (○). Panel (B): Distribution of PEP carboxylase activity (□) and protein concentration (■). 0.5 ml of a pea leaf homogenate prepared in a medium containing 50 mM Hepes, 30% (w/w) sucrose, 5 mM MgCl2, 0.1% (w/v) BSA, pH 7.5, as described under Methods was loaded onto a specially designed discontinous sucrose density gradient (see Methods). The gradient was centrifuged at 4° in the Beckman SW65 rotor as described under Experimental Procedures. 0.3 ml fractions were collected from the top of the tubes and assayed for the indicated markers under conditions specified in Experimental Procedures. Results shown represent averages of 10 independent experiments performed with plant groups grown separately. Average recoveries were: PEP carboxylase, 80%; cytochrome oxidase, 95%; chlorophyll, 78%; and protein, 95%. Sucrose concentration (Δ) was determined refractometrically at 20°.

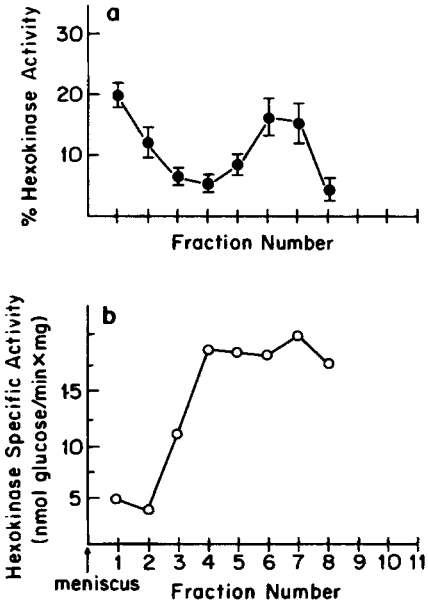

Fig.4 Distribution of hexokinase activity in isokinetic-isopycnic sucrose density gradients. (A) Distribution of hexokinase total activity (●). (B) Distribution of hexokinase specific activity (○). Pea leaf homogenates were processed essentially as described in the legend to Fig. 3. Hexokinase activity was assayed by the radiometric procedure described under Experimental Procedures. Results shown represent averages of 5 independent experiments (all five form part of the ten gradient experiments averaged in Fig. 3) performed with plant groups cultivated separately. Bars indicate SD.

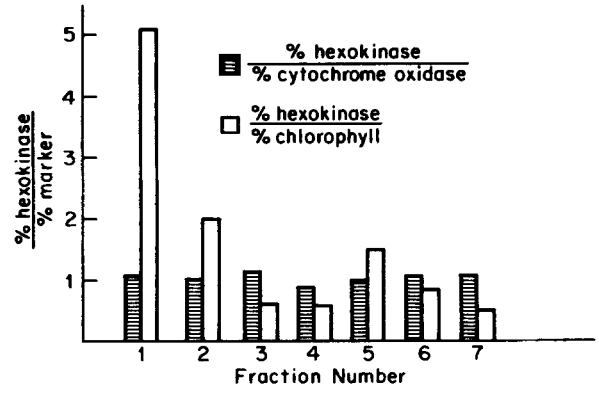

Fig. 5 Correlation of the distribution of hexokinase with that of organelle markers in fractions collected from isokinetic-isopycnic sucrose density gradients. Data were taken from results shown in Figs. 3 and 4. Thus, experimental conditions were as specified in the legends to Figs. 3 and 4.

Experimental details concerning the density gradient centrifugation method are described under Experimental Procedures. Values shown represent data averaged from 5 independent experiments.