Plant Physiol. (1975) 55, 704-711

Compartmentation in *Vicia faba* Leaves

II. KINETICS OF ^{14}C-SUCROSE REDISTRIBUTION AMONG INDIVIDUAL TISSUES FOLLOWING PULSE LABELING[1, 2]

Received for publication June 4, 1974 and in revised form October 31, 1974

WILLIAM H. OUTLAW, JR.,[3, 4] DONALD B. FISHER, AND A. LAWRENCE CHRISTY[5]
Department of Botany, University of Georgia, Athens, Georgia 30602

ABSTRACT

Leaflets of *Vicia faba* L. were pulse labeled with $^{14}CO_2$ and the kinetics of ^{14}C-sucrose redistribution among individual tissues was followed. Sucrose specific activity in the whole leaf peaked about 15 minutes after labeling and declined with a half-time of about 80 minutes. In one experiment, leaflet discs taken at various times during the $^{12}CO_2$ chase were quick frozen, freeze-substituted, and embedded in plastic. The tissue was sectioned paradermally and sections of palisade parenchyma, of spongy parenchyma, and of spongy parenchyma that contained veins were collected. Water extracts from these sections were assayed for sucrose specific activity. Sucrose specific activity in the palisade parenchyma was higher than that of the spongy parenchyma and reached a maximum in both tissues 9 to 15 minutes after labeling. Sucrose specific activity initially declined rapidly in the palisade parenchyma followed by a period during which little or no loss occurred. Sucrose specific activity in sections containing veins peaked at 15 minutes with a maximum value substantially higher than either mesophyll tissue, indicating that recently synthesized sucrose was preferentially exported from the mesophyll. Decline of activity in these sections containing veins continued for the remainder of the experiment. Sucrose specific activity in lower epidermal peels peaked several minutes after that of the whole leaflet and remained lower. Sucrose specific activity in upper epidermal peels was variable (probably due to contamination), but the limited data suggest that the sucrose specific activity there reached somewhat higher values than those of the lower epidermis. The experiments indicate that each leaf tissue contains a kinetically identifiable sucrose pool (which we refer to as "histological compartmentation"), and that further compartmentation may occur at the intracellular level. A simulation of leaf sucrose compartmentation is presented.

The possibility that leaf sucrose might be compartmentalized has been suggested on several grounds, but evidence for multiple pools has been indirect. In itself, the differentiation of a leaf into photosynthetic (the mesophyll), nonphotosynthetic (the epidermises), and conducting tissues implies some degree of metabolite compartmentation. The kinetics of ^{14}C-sucrose movement in a source leaf has been of particular interest to translocation physiologists, for it is there that the translocation profile, apparently, is generated (1, 5, 6, 13). Because there is a lag between the time of maximum sucrose specific radioactivity in the leaf (5 min in soybean [2]) and the time of the maximum export rate of ^{14}C-sucrose from the leaf (20 to 40 min, [2, 13]), there must be compartmentation of leaf sucrose. Additionally, Shiroya *et al.* (11) found limited evidence that suggested a storage pool of sucrose in pine needles: a larger percentage of recently assimilated ^{14}C was translocated from needles in a seedling that was placed in an atmosphere of compensation point CO_2 concentration than in a seedling that was placed in an atmosphere containing 400 μl/l of CO_2. More recently, Outlaw and Fisher (8) showed a marked difference in the kinetics of ^{14}C redistribution among the tissues, which was attributed to sucrose movement.

Our present experiments demonstrate, by direct determination of sucrose specific activity in different tissues, that there is significant compartmentation of sucrose in a leaf. Additionally, our results imply compartmentation at the intracellular level in the palisade and spongy parenchyma.

MATERIALS AND METHODS

Growth and Labeling of Plants. *Vicia faba* L. seeds were planted in a vermiculite-soil mixture and grown under greenhouse conditions for 3 to 5 weeks. At least 5 days prior to an experiment, the plants were transferred to a growth chamber under a 14-hr photoperiod at 21 C during the day and 15 C during the night. Photosynthetically active radiation at the plant level was 170 μeinsteins m^{-2} sec^{-1} (about 900 ft-c) provided by a mixture of incandescent and fluorescent lamps.

Two hours prior to an experiment, the shoot above the youngest fully expanded leaf was excised. The leaflet to be labeled was placed in an open Plexiglas chamber by sealing the stem immediately below the leaflet into an opening in the bottom of the chamber. Neither the leaflet nor the shoot below the leaflet were excised during these manipulations. The chamber was closed just before labeling. The chamber was connected in series with a circulating pump with sufficient capacity to give the chamber air a turnover time of 1 to 2 sec in the experiment reported in Figure 1, and 10 to 15 sec in the experiments reported in Figures 3 and 4. Illumination was provided by two incandescent lamps (250 w, General Electric cool beam clear flood lamps) in the first experiment and by four fluorescent lamps (30 w, Westinghouse daylight) in the others. Photosynthetically active radiation was 170 μeinsteins

[1] This work was supported by Grant GB-33905X1 from the National Science Foundation to D. B. F.

[2] This work was presented in part at the 1973 meetings of the American Society of Plant Physiologists.

[3] Supported in part by a National Defense Education Act IV Predoctoral Fellowship.

[4] Present Address: Department of Biochemistry, Michigan State University, East Lansing, Mich. 48824.

[5] Present Address: Agricultural Research Department, Monsanto Company, St. Louis, Mo. 63166.

m^{-2} sec^{-1} (about 900 ft-c) in all experiments. The leaves were pulse-labeled with 0.05 to 0.1 mCi of $^{14}CO_2$ per leaflet, generated by adding 10% perchloric acid to $Ba^{14}CO_3$ (50 mCi $mmole^{-1}$).

Comparison of Specific Activities in the Palisade Parenchyma, Spongy Parenchyma, and Veins. Following a 1-min pulse of $^{14}CO_2$, samples were taken with a paper punch at various times during the air chase. Samples were quick frozen and freeze-substituted in propylene oxide and embedded in methacrylate as described by Fisher (3). Solvents and resin monomers used in the procedure were previously dried over molecular sieves. Paradermal sections of the embedded samples were cut at a setting of 4 μm with an ultramicrotome. Sections containing only palisade parenchyma, or spongy parenchyma, or spongy parenchyma and veins were extracted in 100 μl of H_2O.

The amounts of sucrose and glucose in the extracts were assayed by the enzymatically catalyzed amplification procedure of Lowry and Passonneau (7, p. 175; "Cycling Assay"). A 10-μl aliquot of the sample was diluted to a concentration in the range of the standards (1 to 10×10^{-12} moles/5 μl). Sucrose in a 5-μl aliquot of the diluted sample was hydrolyzed with highly purified invertase and the resulting glucose was assayed. A 10-μl aliquot was assayed for glucose without hydrolysis with invertase to correct for glucose in the extract. Three replicates of each aliquot were run, with the results agreeing to $\pm 5\%$. A set of sucrose and glucose standards were included each time the assay was run and further controls, such as NADPH and 6-P-gluconate were included to monitor the cycling procedure. Preliminary studies in which sucrose and glucose standards were added to extracts indicated no interference from materials in the extract.

Because the amounts of radioactivity present in the extracts from sections were so small that an unusually long exposure time would have been required for localization of sucrose by autoradiography, the following procedure was used. Seventy-five μl of the extract were chromatographed on Whatman No. 4 paper (l-butanol–propionic acid–water; [23:12:15; v/v/v]) with 50 μg of sucrose. Sucrose was located by spraying the chromatograms with invertase and, after drying, the chromatograms were sprayed with Glucostat reagent (Worthington Biochemical). This resulted in the production of a reddish spot in the area containing sucrose. The spot was eluted in 1 ml of H_2O in a vial for 30 min; 10 ml of aqueous cocktail were added, and the sample was assayed for radioactivity with a liquid scintillation counter. Trials run with larger amounts of ^{14}C-sucrose (*i.e.* localizable by autoradiography) showed that there was no loss of radioactivity associated with this procedure. All activities were converted to dpm using an efficiency obtained by including an internal ^{14}C-toluene standard in several samples. Data are expressed as dpm/μg of sucrose.

For determination of sucrose pool sizes, paradermal methacrylate sections containing palisade parenchyma, the lower layer of spongy parenchyma, or spongy parenchyma with veins were extracted in H_2O. Sucrose was assayed by Lowry and Passonneau's cycling assay (7). The volume of the sections (and therefore of the tissue extracted) was determined by measurement of a fluor incorporated into the plastic (8). Taken together, these measurements provided the amount of sucrose per tissue volume. The total amount of sucrose in each tissue could then be calculated from the total thickness of each tissue in the leaf, as determined from cross sections. In order to estimate the pool size of the veins alone, we assumed that the sucrose concentration (*i.e.* μg sucrose/μm^3 [cell volume]) of the upper layer of spongy parenchyma was equal to the sucrose concentration in the lower layer of spongy parenchyma. Justification for this assumption comes from the fact that the measured sucrose concentrations in the palisade parenchyma and the lower layer of spongy parenchyma were similar. Because the cell volume of the upper layer of spongy parenchyma (if the bundle sheath is included) is approximately 1.5 times the cell volume of the lower layer of spongy parenchyma, the sucrose pool size of the former tissue was assumed to be 1.5 times that in the latter tissue.

Comparison of Average Leaf Sucrose Specific Activity to Sucrose Specific Activity in the Lower Epidermis. Sucrose-specific activity in the lower epidermis was determined in epidermal peels taken by excising a leaflet at various times after pulse labeling, peeling off the lower epidermis, and washing the peel briefly in 10 mM $CaCl_2$ to remove the contents of damaged cells. Areas of a peel containing green color were discarded. From the same leaflet, an area of intact (*i.e.*, unpeeled) tissue was taken for determination of average sucrose specific activity. Samples were extracted twice in boiling 80% aqueous ethanol, the extract was dried, and the lipids were removed by extraction with propylene oxide. Water-soluble compounds were separated by two-dimensional ascending chromatography on Whatman No. 1 paper, using 80% aqueous phenol and l-butanol–propionic acid–water (23:12:15; v/v/v). Sucrose was located by autoradiography, eluted with water, hydrolyzed with invertase, and assayed by the Glucostat procedure. Samples were run in triplicate and, with a few exceptions, all agreed to $\pm 5\%$, and most agreed to $\pm 2.5\%$.

Radioactivity in water-soluble compounds was counted by a gas-flow counter. Insoluble activity was determined by digesting the sample in Soluene (Packard), adding a scintillation cocktail, and counting in a liquid scintillation counter.

To estimate the sucrose pool size in the lower epidermis, the volume of some of the epidermal peels was estimated. Their areas were measured and multiplied by the thickness of the epidermal layer as determined from cross sections of methacrylate-embedded leaflets. Because a large fraction of epidermal cells are ruptured during peeling, an estimate of the fraction of viable cells to be expected in the samples was made by vital staining similar peels with 0.01% neutral red at pH 7.4. Neutral red uptake occurred only in cells showing good cytological organization and cytoplasmic streaming. Twenty per cent of the epidermal cells and all of the guard cells were viable by this criterion.

RESULTS

Comparison of Sucrose Specific Activity in the Palisade Parenchyma, Spongy Parenchyma, and Veins. Sucrose specific activities in the palisade parenchyma, spongy parenchyma, and veins from a pulse-labeled *Vicia* leaflet are reported in Figure 1. In this experiment, to avoid the variability in sucrose specific activity that occurred from leaflet to leaflet (Fig. 4), samples were taken from a single leaflet. Sucrose specific radioactivity in the palisade parenchyma reached a maximum of 375 dpm μg^{-1} about 9 to 15 min after labeling and then declined rapidly for the next 10 min. After 30 min, there was little change. Sucrose specific activity in the lower layer of spongy parenchyma reached a maximum of 225 dpm μg^{-1} and, qualitatively, the kinetics in this tissue did not show any marked difference from those in the palisade parenchyma.

Values for ^{14}C-sucrose in sections containing veins were substantially different from the mesophyll. Sucrose specific activity in these samples showed a sharp peak at 15 min and reached a value (>550 dpm μg^{-1}) that was substantially higher than that of the palisade parenchyma and more than twice that of the spongy parenchyma. Specific activity in the veins more than doubled between 9 and 15 min whereas, by contrast, the specific activity in the mesophyll did not change appreciably

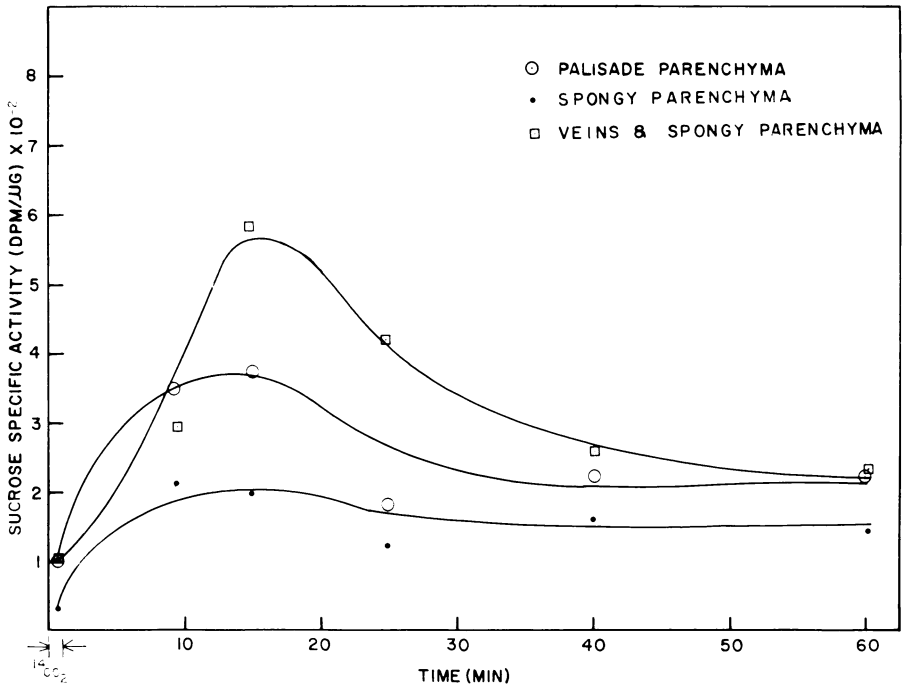

FIG. 1. Kinetics of ^{14}C-sucrose specific activity in the palisade parenchyma, the lower layer of spongy parenchyma, and the upper layer of spongy parenchyma which contained veins. The leaf was pulse-labeled for 1 min.

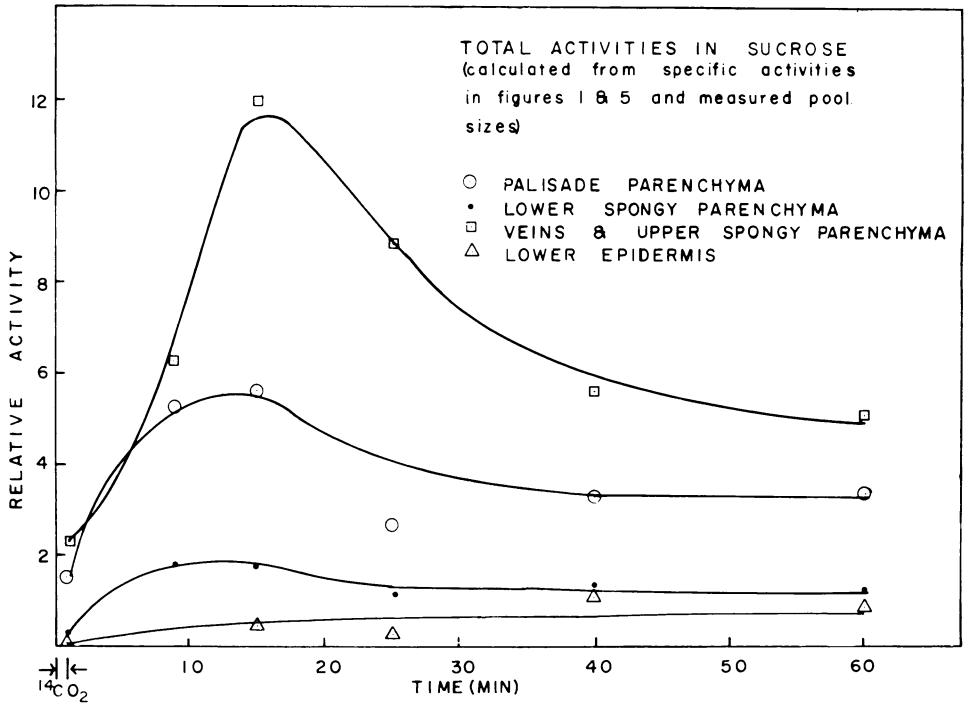

FIG. 2. Total ^{14}C-sucrose in individual tissues after pulse labeling. The pool size of each tissue was measured and multiplied by the appropriate specific activity shown in Figs. 1 and 5.

during the same interval. This rapid increase in specific activity was followed by a decline that was evident for the remainder of the experiment. The decline of sucrose specific activity in the upper layer of spongy parenchyma and veins showed a half-time of about 20 min.

Totals of ^{14}C-sucrose in the palisade parenchyma, lower spongy parenchyma, upper spongy parenchyma and veins, and in the lower epidermis (Fig. 2) were calculated from measured pool sizes. These data demonstrate more clearly the role of each tissue and allow a more direct comparison to the compartmental model (see "A Compartmental Analysis").

Comparison of Average Leaf Sucrose Specific Activity to Specific Activity in the Epidermis. The variability of the sucrose specific activities from leaflet to leaflet, in both the peeled lower epidermis and in the whole leaf, was too great for direct comparison of the data from different leaflets. However, the ratio

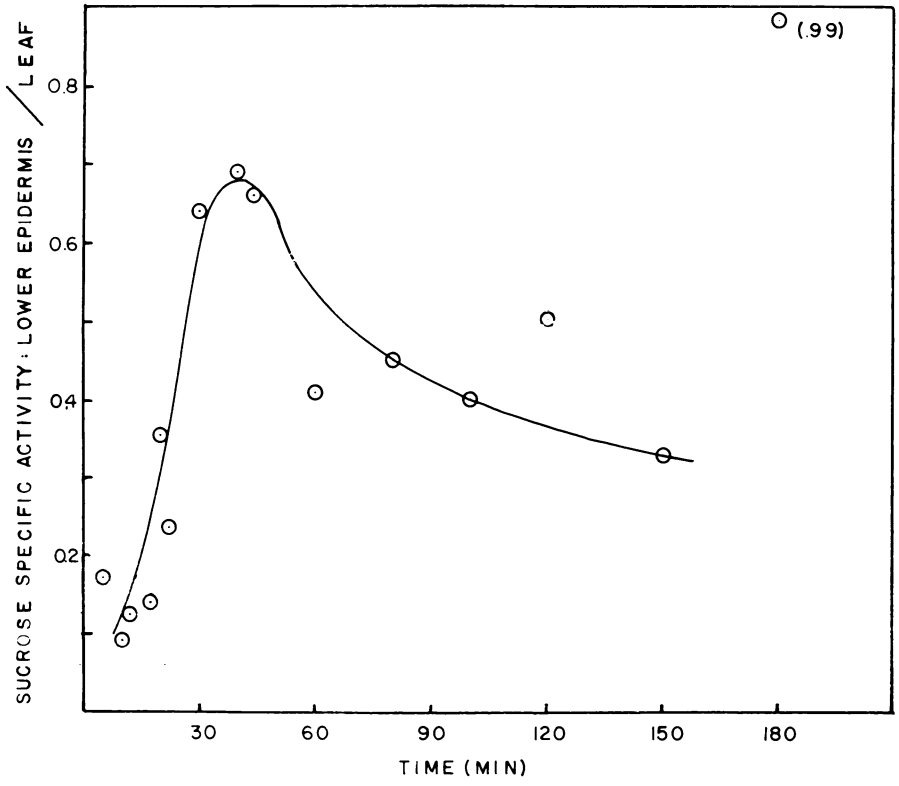

FIG. 3. Ratio of the sucrose specific activity from lower epidermal peels to the sucrose specific activity from an intact area of the same leaflet. The leaflets were pulse-labeled with $^{14}CO_2$. Each point represents a different leaflet.

of sucrose specific activity in the lower epidermis to that in the whole leaflet followed a regular kinetic sequence even in comparing different experiments. The kinetics of this ratio are plotted in Figure 3. The data suggest that there was little or no sucrose synthesis from $^{14}CO_2$ in the lower epidermis and demonstrate that sucrose in the lower epidermis had not equilibrated with the remainder of the leaf sucrose even several hours after labeling.

Two similar experiments were conducted to follow the kinetics of sucrose specific activity in the upper epidermis. However, even the ratios of epidermal to average leaf sucrose specific activity were erratic. Because of this erraticism and the fact that there was substantial contamination of the peels with intact palisade cells, the data are not presented in their entirety. Nevertheless, they indicated compartmentation between the upper epidermis and the remainder of the leaf. Although the average of 13 ratios was close to unity (1.05), most were different. The ratios ranged from 0.5 to 1.8. The maximum ratio of 1.4 in the first experiment was reached at 32 min, whereas there was no apparent relationship of ratio to sampling time in the second experiment.

To get a general idea of the absolute behavior of sucrose specific activity in the whole leaf and, from the ratios in Figure 3, of sucrose specific activity in the lower epidermis, sucrose specific activities in six separate leaflets were followed by taking leaf punches after pulse labeling with $^{14}CO_2$. The results of these experiments are illustrated in Figure 4. Although the kinetics of sucrose specific activity followed a smooth time course within any individual leaflet, there were appreciable differences in the absolute values between leaflets. The average data from all six experiments are plotted in Figure 5 (upper curve), which is taken to be representative of the sucrose specific activity kinetics in an "average" *Vicia* leaflet. Sucrose specific activity in this combined plot reaches a maximum at about 15 min after labeling and then declines with a half-time of about 80 min. The absolute behavior of sucrose-specific activity in the lower epidermis is also plotted in Figure 5 (lower curve). These values were obtained by multiplying the values for whole leaf sucrose-specific activities (upper curve) by the ratios in Figure 3. Sucrose-specific activity in the lower epidermis reached a maximum later than for the leaf as a whole, and did not show as much of a decline afterwards.

DISCUSSION

The occurrence of sucrose compartmentation in *Vicia* leaves is demonstrated directly by the existence of histologically separate pools having substantially different specific activities. Sucrose specific activity in the lower epidermis not only followed quite different kinetics from the leaf as a whole, but the two still had not equilibrated even several hours after pulse labeling. Although the layers of mesophyll tissues showed qualitatively similar kinetics, the sucrose specific activity in the lower layer of spongy parenchyma was always lower than that in the palisade parenchyma. This difference results, at least in part, from the lower rate of CO_2 fixation in the spongy parenchyma (9). The low specific activity of sucrose in the lower epidermis apparently reflects the low specific activity of the spongy parenchyma pool with which it exchanges. The level of sucrose specific activity in samples containing veins was particularly striking in that it attained even higher values than did sucrose in either the spongy or palisade parenchyma which supply ^{14}C-sucrose to the veins (8). The data therefore not only demonstrate histologically separate pools of sucrose (which we refer to as "histological compartmentation") but also strongly imply the presence of sucrose compartmentation at the intracellular level. Newly synthesized sucrose apparently is transported preferentially to the veins from the sites of $^{14}CO_2$

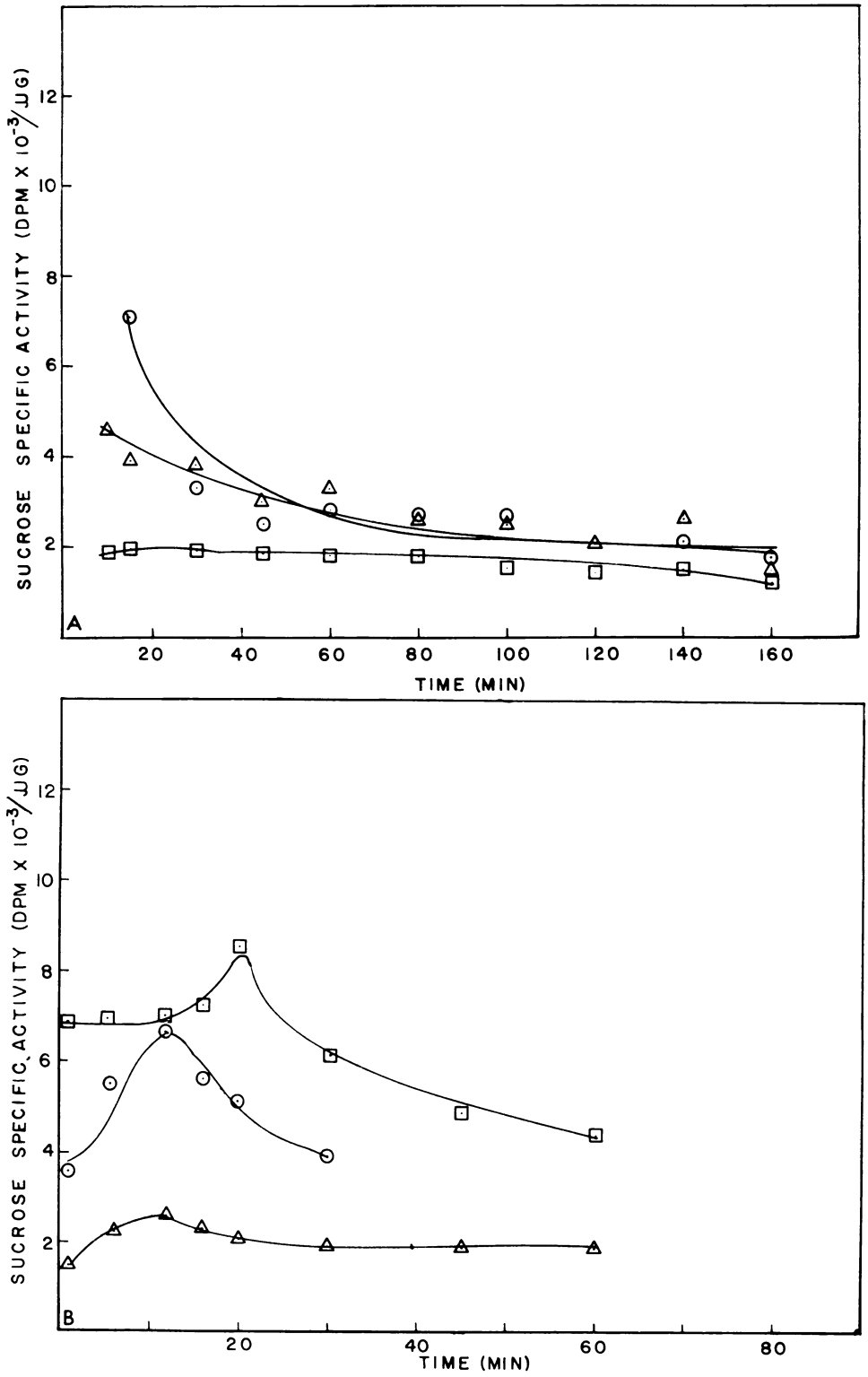

FIG. 4. A and B: Kinetics of sucrose specific activity in six pulse-labeled leaflets. Each point was obtained by taking a punch from a leaflet at various times. Each curve represents the kinetics in an individual leaflet.

fixation before it mixes completely with larger pools of lower specific activity in the mesophyll. We presume that the latter pool is contained in the vacuole and that the smaller mobile pool of newly photosynthesized sucrose is located in the cytoplasm (or in both the cytoplasm and cell wall, since there is nothing to indicate whether transport is symplastic or apoplastic).

The following picture of sucrose compartmentation is inferred from experiments with *Vicia* leaves (including the data in ref. 8). Newly synthesized sucrose is transported rapidly from the mesophyll to the veins without extensive exchange with vacuolar sucrose. We presume that most veinal sucrose is contained in phloem parenchyma cells that are engaged in sieve tube loading (*i.e.* virtually all veinal sucrose is in a

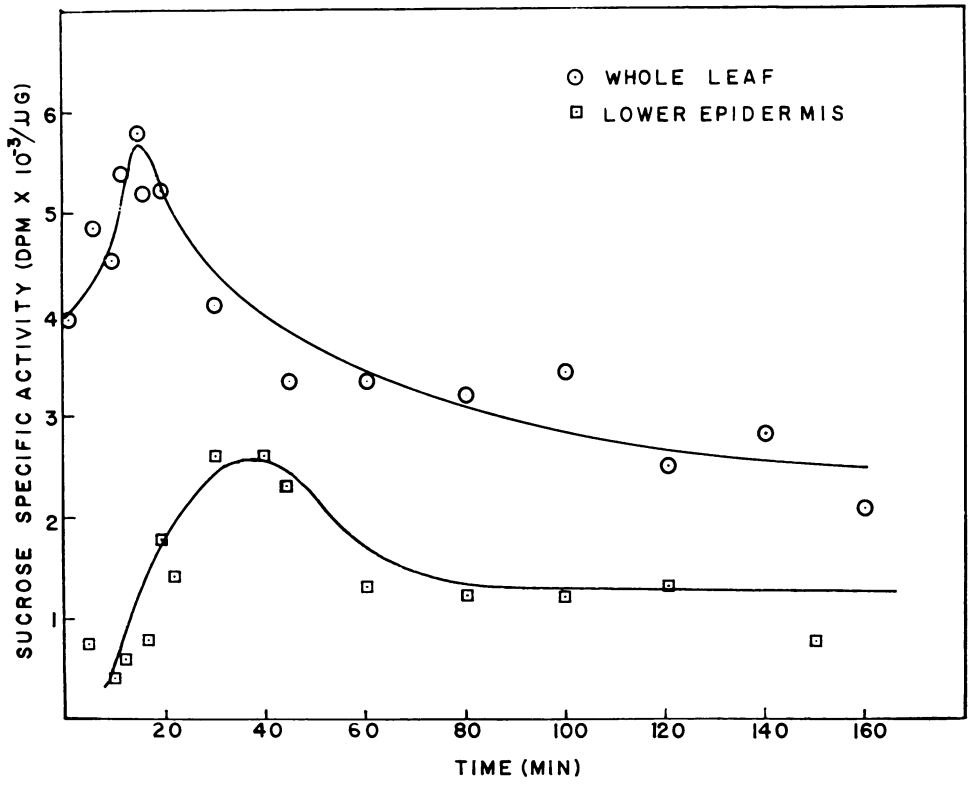

FIG. 5. Average kinetic behavior of whole leaf sucrose specific activity (from Fig. 4) and the sucrose specific activity in the lower epidermis (plotted from the ratios in Fig. 3).

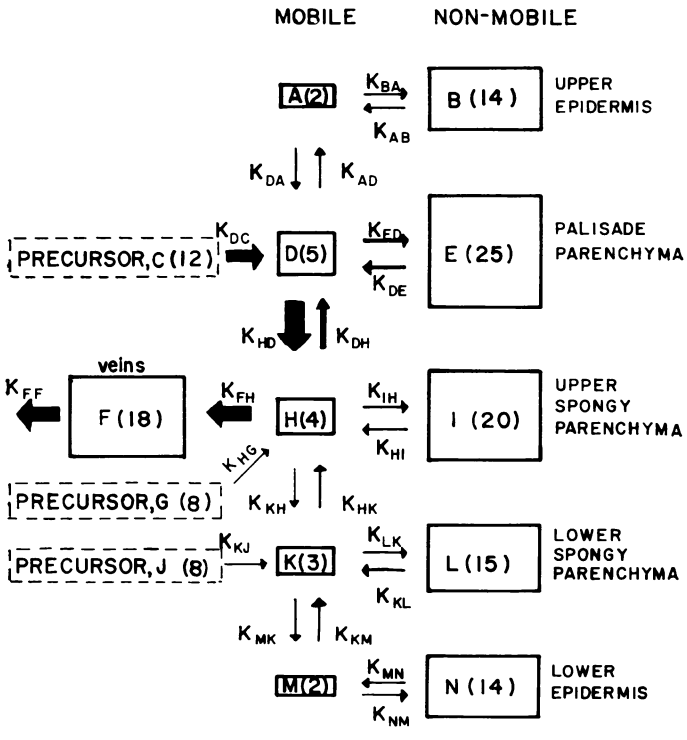

FIG. 6. Block diagram for compartmental analysis of sucrose in a *Vicia* leaf. The letter inside each block identifies the compartment and the number indicates the relative pool size used in the stimulation. If the latter figures were in micrograms they would approximate the sucrose pool sizes in a leaf area of 1.2 cm². The size of the block indicates the pool size and the width of arrows indicates the transport rates (see Table I for exact values of the latter).

Table I. *Relative Rates of Transfer of Sucrose Between Compartments*

K_{YZ} = amount transported per min from compartment Z to compartment Y.

$K_{DC} = 1.25$	$K_{IH} = 0.30$
$K_{HG} = 0.125$	$K_{HI} = 0.30$
$K_{KJ} = 0.125$	
	$K_{LK} = 0.22$
$K_{BA} = 0.25$	$K_{KL} = 0.22$
$K_{AB} = 0.25$	
	$K_{NM} = 0.20$
$K_{ED} = 0.375$	$K_{MN} = 0.20$
$K_{DE} = 0.375$	
	$K_{HK} = 0.20$
$K_{KM} = 0.25$	$K_{KH} = 0.075$
$K_{MK} = 0.25$	
	$K_{HD} = 2.00$
$K_{DA} = 0.25$	$K_{DH} = 0.75$
$K_{AD} = 0.25$	
	$K_{FF} = 1.50$
$K_{FH} = 1.50$	

mobile pool). This is inferred from autoradiographic observations in the minor veins of sugar beet (4, 12) and from similar observations on soybean (D. B. Fisher, unpublished findings). By analogy with the type of intracellular compartmentation implied in the mesophyll tissues, the epidermal layers probably also contain mobile and nonmobile sucrose pools. The former

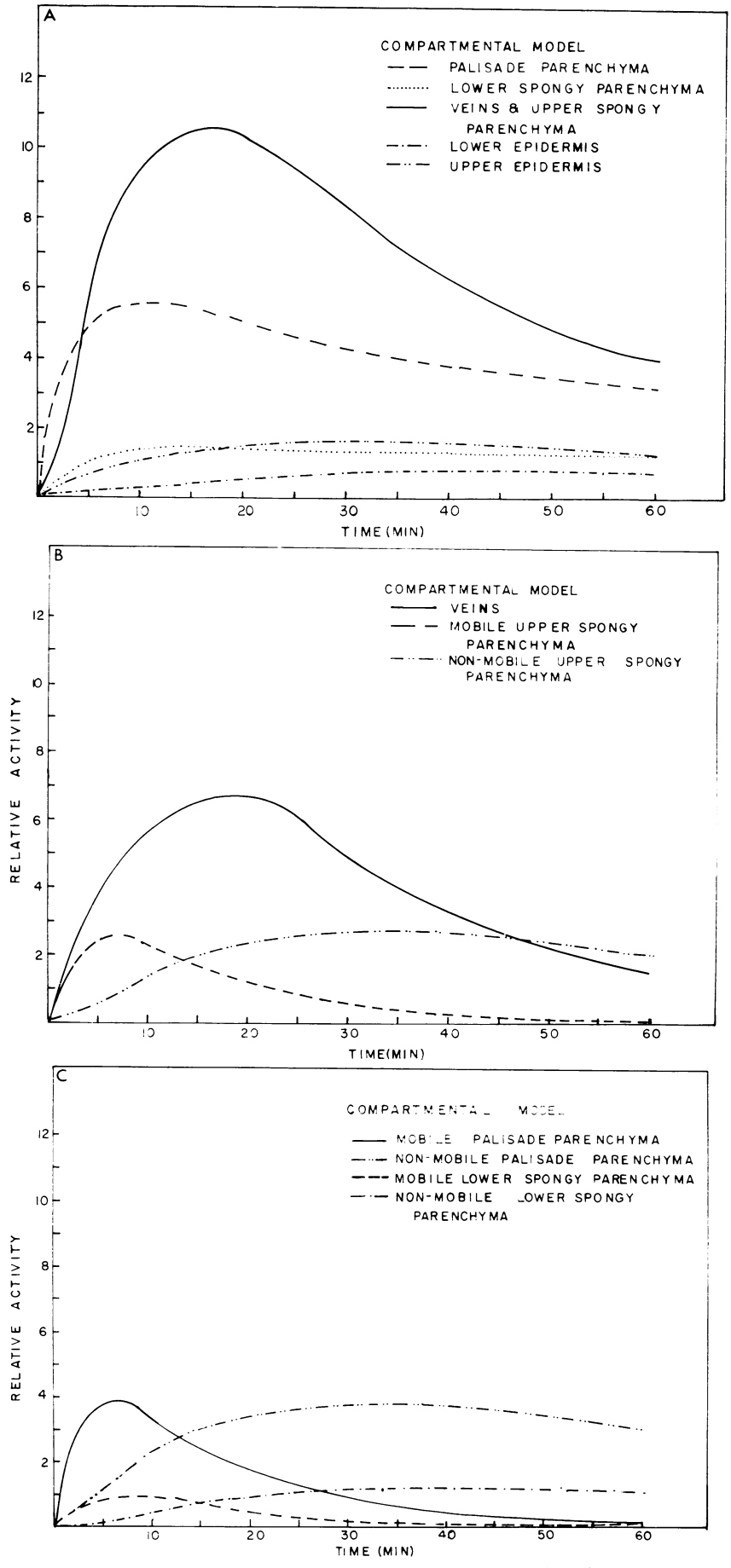

FIG. 7. Response of the compartmental model to a pulse of $^{14}CO_2$, using the transport rates listed in Table I and the pool sizes shown in Fig. 6. A: Kinetics of total ^{14}C-sucrose in the palisade parenchyma, the lower layer of spongy parenchyma, the veins and upper layer of spongy parenchyma, the lower epidermis, and the upper epidermis (compare with Fig. 2). B: Kinetics of total ^{14}C-sucrose in each of the compartments contained in the veins and upper spongy parenchyma. C: Kinetics of total ^{14}C-sucrose in the intracellular compartments in the palisade parenchyma and in the lower layer of spongy parenchyma.

pools would exchange sucrose with the mobile pools in the adjacent mesophyll layers. A total of at least nine sucrose compartments is therefore inferred from the data.

From the point of view of translocation kinetics, the effect of a sucrose pool in the veins is to delay the maximum rate of tracer efflux from the leaf in comparison with the time at which average leaf sucrose specific activity reaches a maximum (Fig. 5). The effects of interposing a sucrose pool between the sites of photosynthesis and the translocation stream are more complex than the two-compartment model suggested earlier for soybean (2), principally because of the presence of mobile and nonmobile sucrose pools in the mesophyll.

A COMPARTMENTAL ANALYSIS

Since the data reflect a relatively complex compartmentation of sucrose, the over-all behavior of the system becomes difficult to analyze from the data alone. In this section, a model of leaf sucrose compartmentation is presented and the effects of changing various model parameters are described.

The most satisfactory form of the model is diagrammed in Figure 6, the transport rates for which are shown in Table I. Differential equations were derived for each compartment, assuming a chemical steady state and instantaneous mixing within each compartment (see 10 for theory). Particular solutions to the resulting set of simultaneous differential equations were obtained on a digital computer. The kinetic response of the model after pulse labeling the precursor pools is shown in Figure 7, A, B, and C. Initial amounts of ^{14}C in the precursor pools were set at values reflecting the relative rates of photosynthesis in the parenchyma layers under the conditions of these experiments.

Several of the assumptions made for the model deserve particular comment, because, for lack of data, they are necessarily somewhat arbitrary. Except for the sucrose precursor pools, the model ignores the interconversion of sucrose with other compounds. Observations in this and in an earlier paper (8) suggest that this lack of interconversion is a reasonable assumption. The turnover times for sucrose in the nonmobile pools in the spongy parenchyma and palisade parenchyma were assumed to be similar. The exchange rate between the mesophyll and upper epidermis was assumed to be the same as between the mesophyll and lower epidermis, for which there were more data. We recognize that the lack of data on some points lends some ambiguity to the model. Nevertheless, we regard it as a reasonable and informative approximation of sucrose compartmentation in a *Vicia* leaf. With some exceptions mentioned below, the curves generated from the model are not greatly changed by moderate changes in pool sizes and transport rates. This result is to be expected in a real leaf, where these values would be subject to local variations. Otherwise, the kinetic data would be very erratic. That is, the pattern of compartmentation (Fig. 2) is a far more important factor in determining the kinetics than are the exact values for pool sizes and transport rates.

Instead of the nine compartments suggested earlier, the compartmental model consists of 11 compartments. Further separation of the spongy parenchyma into two layers (it is, in fact, comprised of two layers) seemed necessary. Models with only one layer could not, in our opinion, be realistically adjusted to give both high activity in the veins and low activity in the spongy parenchyma.

Estimates of histological pool sizes were available directly (*e.g.* palisade parenchyma, lower spongy parenchyma) or indirectly (*e.g.* veins), except for the upper epidermal pool, which is assumed to be equal to the lower epidermal pool. At the intracellular level, the sucrose specific activity data (Fig. 1) implied that the nonmobile pool was larger than the mobile pool. The ratios of pool sizes (Fig. 6) roughly reflect the relative volumes of the vacuole and cytoplasm. Further decreasing the mobile pool size resulted in a more rapid turnover of sucrose in the leaf and lower values at later times for total ^{14}C-sucrose in the measurable combinations of compartments.

In addition to examining the effects of pool sizes, we also examined the effects of changing transport rates between compartments. Increasing the exchange rate between the nonmobile and mobile compartments resulted in higher maximum tracer values in the parenchyma and ultimately diminished the retention of tracer in the leaf. Decreasing the exchange rate between the mobile and nonmobile compartments caused a rapid depletion of tracer from the parenchyma, a higher peak in the veins, and diminished retention of tracer at 60 min. Reducing exchange between the mobile pools in the parenchyma caused the tracer in the palisade parenchyma to peak too soon and decline too rapidly to fit the observed data while tracer in the lower spongy parenchyma did not reach empirically determined levels (Fig. 2). Increasing exchange between the mobile parenchyma pools broadened the peak in the veins and allowed too much tracer to enter the lower spongy parenchyma.

To investigate the possibility that a sucrose precursor might be involved in intercellular transport, exchange among the precursor compartments was simulated. However, even low exchange rates substantially diminished the difference in specific activity between the palisade parenchyma and spongy parenchyma. Transport of these compounds apparently must be very limited.

Acknowledgments—The authors wish to thank Drs. A. Goldstein and J. O. Lampen of Rutgers University for the invertase used in the enzyme cycling assay for sucrose. We gratefully acknowledge Dr. J. R. Webster's assistance in programming the compartmental model for solution on a digital computer.

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