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Compartmentation in *Vicia faba* Leaves

I. KINETICS OF ^{14}C IN THE TISSUES FOLLOWING PULSE LABELING[1, 2]

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ABSTRACT

Leaflets of *Vicia faba* were pulse-labeled with $^{14}CO_2$ to follow the subsequent movement of photosynthate between leaf tissues. Samples were taken during a $^{12}CO_2$ chase, quick frozen, freeze-substituted, and embedded in methacrylate. Paradermal sections provided tissue samples consisting only of upper epidermis, palisade parenchyma, spongy parenchyma and veins, spongy parenchyma, or lower epidermis. Most CO_2 fixation occurred in the palisade parenchyma, but its ^{14}C content declined rapidly after labeling. Concomitant with the decline of activity in the palisade parenchyma, there was an increase in activity in the spongy parenchyma and upper epidermis and a slow increase in the lower epidermis. Activity in the palisade parenchyma and spongy parenchyma eventually reached similar levels and remained constant. Tissue samples containing veins were consistently the most radioactive, and activity in those samples showed a decline. Very little change occurred in the insoluble fraction from any tissue. The results support previous assumptions regarding the pathway of assimilate transport to the veins, and demonstrate the rapidity of such transport. Sucrose is apparently the principal mobile compound.

The leaves of many plants, particularly those of mesomorphic dicots, are differentiated into upper epidermis, lower epidermis, palisade parenchyma, spongy parenchyma, and veins. Interpretations of physiological relationships among these tissues have been based substantially upon their supposed role in photosynthesis and the subsequent movement of assimilates to the veins. Because of its large surface area, many chloroplasts and, because of the proximity to the upper surface of the leaf, the palisade parenchyma is presumed to be the major site of CO_2 fixation, while the spongy parenchyma has been thought to act as an intermediate conducting tissue for the movement of photosynthate to the veins as well as a photosynthetic tissue. These assumptions have been given considerable support by comparative studies of leaf anatomy. Haberlandt (5) emphasized the apparent adaptation of leaf tissue arrangement to the efficient removal of photosynthate (his "principle of expeditious translocation"). Wylie (11–14) and Philpott (7, 8) demonstrated complementary relationships between interveinal distances, laterally oriented tissue (epidermis and spongy parenchyma), and palisade parenchyma. Nevertheless, the difficulty of localizing water-soluble compounds and tracing their movement within such small dimensions have left even those reasonable assumptions without experimental verification. In addition, related questions, such as the identity of the transported compound(s), the accessibility of the epidermal layers to exchange of organic compounds with the mesophyll, and the degree of metabolic differentiation between the spongy and palisade parenchyma are not amenable to techniques of comparative anatomy. We have attempted to provide answers to these functional questions of leaf organization by directly sampling the contents of the different tissues in *Vicia faba* leaflets.

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 (400–700 nm) was 170 μeinsteins m^{-2} sec^{-1} (about 900 ft-c) provided by a mixture of incandescent and fluorescent bulbs.

Two hours prior to an experiment, the shoot above the youngest fully expanded leaf was excised. The stem below the leaf was sealed into an opening in the bottom of a Plexiglas chamber. The chamber top was added just before labeling. The labeling chamber was connected in series with a circulating pump with sufficient capacity to give the chamber air a turnover time of 10 to 15 sec. Illumination was provided by four fluorescent lights, giving 170 μeinsteins m^{-2} sec^{-1} of photosynthetically active radiation at leaf level. The leaflets were pulse-labeled with 0.15 to 0.30 mCi of $^{14}CO_2$ generated by adding 10% perchloric acid to $Ba^{14}CO_3$. The initial CO_2 concentration was about 600 μl/l and did not change significantly during the course of the experiment.

^{14}C Kinetics in Individual Leaf Tissues. After pulse labeling, leaf punches were taken, quick frozen, freeze-substituted in propylene oxide at −80 C, and embedded in methacrylate to retain water-soluble compounds (2). Monitoring of the methacrylate mixtures used for infiltration showed that virtually no loss of water-soluble ^{14}C occurred during those steps (the results were essentially similar to those reported for Epon infiltration [2]). Furthermore, in processing other tissues, whenever they contained anthocyanins, the anthocyanins were always retained and were limited to the vacuole (see ref. 3 for observations on anthocyanins). Paradermal sections 2 to 4 μm thick were cut with a dry glass knife on an ultramicrotome.

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Except for those sections which contained both spongy parenchyma and veins, paradermal sections of a *Vicia* leaflet provided "tissue samples" consisting almost entirely of a single tissue type (*i.e.* epidermis, palisade parenchyma, or spongy parenchyma). Contaminating cell types (except in the case of veinal tissue) and plastic without tissue were dissected away. Data for the veins were obtained by collecting sections of spongy parenchyma which contained veins. These data are expressed for the veins alone, a calculation made by assuming that the spongy parenchyma is a relatively homogenous tissue (which we recognize to be only a rough approximation). Water-soluble compounds were extracted from the sections by floating them for 9 min on a drop of water at room temperature, briefly immersing them in the water and repeating the extraction in another drop.

Two-dimensional paper chromatography (23:12:15; l-butanol-propionic acid-water in the first direction and 80% aqueous phenol in the second direction) was used to compare the water-soluble extract from methacrylate sections of palisade and spongy parenchyma to the water-soluble fraction from an 80% ethanolic extract from a similar freeze-substituted, but unembedded sample in one experiment (Fig. 1). In a control experiment, a comparison between the distribution of ^{14}C in water-soluble compounds in an embedded leaflet and an unembedded, but freeze-substituted, leaflet showed that all the labeled water-soluble compounds were quantitatively extracted. Additionally, the ^{14}C remaining in the sections after extraction was always consistent with the activity of insoluble compounds from the unembedded sample. The small amounts of activity in extracts from the palisade parenchyma and spongy parenchyma samples made it necessary to minimize the size of the origin on the chromatograms. For this purpose, the extract was evaporated to less than 25 μl and pulled into a 25-μl pipette which had been previously coated with Siliclad (Clay Adams). A 0.75 mm by 1.5 mm piece of chromatography paper was inserted into the end of the pipette which was then placed in a desiccator. All of the radioactivity was deposited on the filter paper as the extract was pulled onto the paper by capillarity during evaporation from its exposed surface. The paper was then inserted into a pinhole at the origin of the chromatogram and was teased and meshed into the chromatography paper. After development, the chromatograms were autoradiographed.

Sample size was determined by dissolving a water-soluble fluor (1% [w/v] BBOT[5]) in the methacrylate monomer prior to embedding. The monomer was centrifuged briefly to remove insoluble particles. After embedding and polymerization, the volume of plastic in the samples (several paradermal sections) was determined by dissolving the sections in 1 to 2 ml of toluene and measuring the fluorescence of BBOT at 475 nm (exciting wavelength, 338 nm). Two precautions were necessary in using this method to determine sample size. First, since there was some variation from embedment to embedment, the amount of fluorescence produced per weight of plastic had to be determined for each block. Several sections from each block were weighed on a quartz fiber fishpole balance (constructed and calibrated with quinine bromide as described by Lowry and Passonneau [6]), dissolved in toluene and the fluorescence measured. Second, the ratio of fluorescence to plastic weight was highly variable in the outer 0.5 mm or so of the block. For this reason, a small piece of cheesecloth was put in the bottom of each capsule before polymerization to keep the tissue away from the block surface. With these two precautions, BBOT fluorescence proved to be a reliable measure of sample weight and never varied more than 3%. This fact was verified by making both weight and fluorescence measurements on some samples. Also, with a few small samples, the volume was calculated by measuring their area under a microscope and multiplying by thickness as determined by interference microscopy. The actual and predicted fluorescence values were in consistent agreement.

In its final form, the data are expressed as the amount of activity per mm^2 of leaf area. To calculate these values from the raw data (as dpm/sample weight), corrections were made for methacrylate density (1.08 g cm^{-3}) and the leaf thickness occupied by each tissue (determined from cross-sections of the same leaf: representative values are given in Table I).

RESULTS

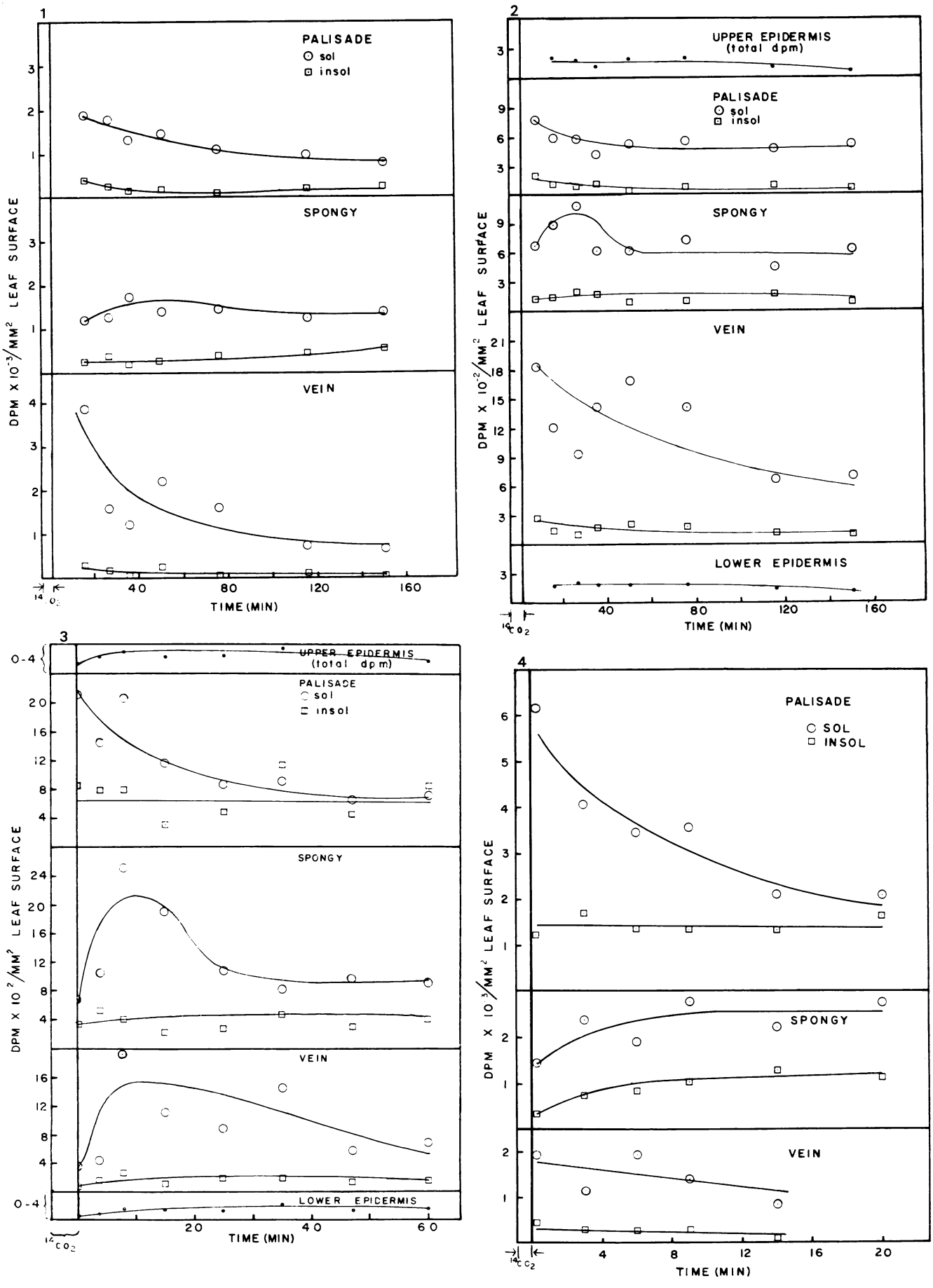
Figures 1 to 4 show the kinetic behavior, in four experiments, of soluble and insoluble ^{14}C in leaf tissues after pulse labeling with $^{14}CO_2$. In three of the experiments (Figs. 1 to 3), the labeling time was 4.5 min and the kinetics of ^{14}C were followed for 2.5 hr (two experiments) or for 1 hr (one experiment). In the fourth experiment (Fig. 4), labeling was for 1 min and the kinetics of ^{14}C were followed for only 20 min.

Several consistent patterns of ^{14}C distribution are readily recognizable. The rate of CO_2 fixation, as reflected by the initial ^{14}C distribution, was several times higher in the palisade parenchyma than in the spongy parenchyma (Figs. 1–4). Soluble ^{14}C in the palisade parenchyma declined roughly exponentially, with a half-time of about 10 min, to a level similar to that of the spongy parenchyma (Figs. 3 and 4). Because the tissue volumes of the palisade parenchyma and spongy parenchyma are similar (Table I), the total counts in these tissues give a good indication of relative counts per tissue volume. Soluble ^{14}C in the spongy mesophyll increased after the $^{14}CO_2$ pulse, and reached a final level similar to that of the palisade parenchyma. In two experiments (Figs. 2 and 3) there were definite peaks of activity in the spongy parenchyma, in one case around 12 min, and in the other around 25 min. This feature was not striking in Figure 1. At the end of the experiment shown by Figure 4, activity in the spongy mesophyll had not begun to decline. Insoluble activity remained fairly constant in all tissues, with the exception of the spongy parenchyma, where there was a brief increase just after labeling (Fig. 4).

Owing to the difficulty of getting samples, data were taken for the epidermal layers in only two experiments (Figs. 2 and 3). Aside from their thinness, these layers sectioned poorly, necessitating a disproportionate effort to get a useable count rate. Only total activity was determined. Apparently neither epidermis fixed much ^{14}C directly, but gained most of it from the mesophyll after the labeling period (Fig. 3). Radioactivity increased more rapidly in the upper epidermis than in the lower.

Data for the veins were erratic, but indicated a consistent decrease in their ^{14}C content as time progressed. Other than this general result, the kinetic data for the veins must be regarded with caution. Most of the radioactivity in this sample would be in the phloem (4), which is only a few μm in diameter. Since the minor veins occurred at somewhat different levels in the leaflets, reproducibility of this sample would be expected to be poor. The figures for these samples are instructive largely because of the amount of activity associated with the veins and the decline in activity. They were consistently the most radioactive samples counted. This result can be appreciated from the fact that although the palisade parenchyma and spongy parenchyma each occupied about 30% of the cellular volume in the leaf, the veins occupied only about 10% yet

[5] Abbreviation: BBOT: 2,5-bis-(5-*tert*-butylbenzoxazolyl)-thiophene.



FIGS. 1–4. Histological distribution of ^{14}C in *V. faba* leaflets after pulse labeling a leaflet with 0.15 to 0.30 mCi of $^{14}CO_2$.

Table I. *Thickness of Tissues in Leaflets used for Kinetics Experiments*

	Upper Epidermis	Palisade Parenchyma[1]	Spongy Parenchyma[2]	Lower Epidermis
		μm		
Fig. 2	26	97	169	26
Fig. 3	20	127	187	18
Fig. 4		116	155	

[1] 34% of total volume is cell volume.
[2] 26% of total volume is cell volume.

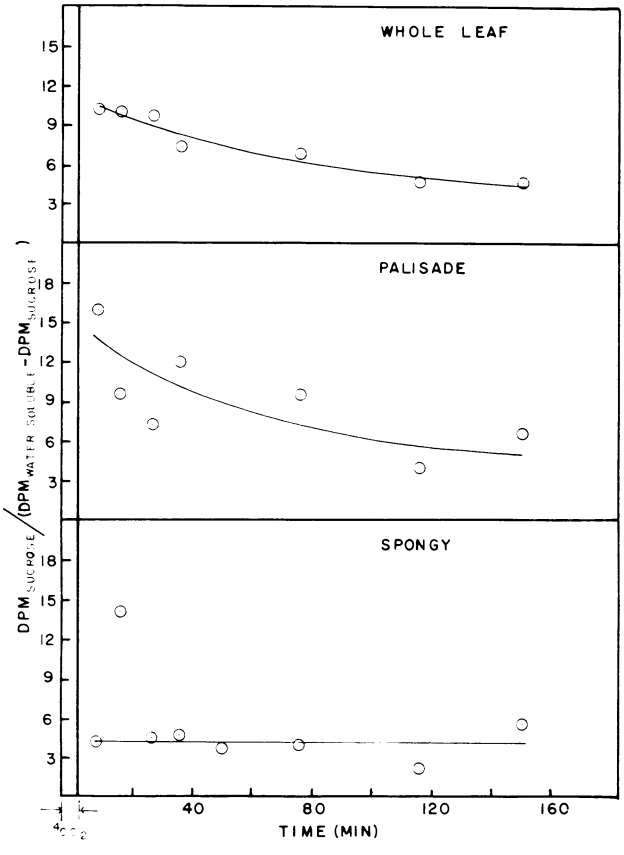


FIG. 5. Relative amounts of ^{14}C represented by sucrose in the spongy parenchyma, palisade parenchyma, and whole leaflet following pulse labeling. (Kinetics of total ^{14}C are shown in Fig. 1.)

usually contained an amount of ^{14}C comparable to either mesophyll layer. The fraction of insoluble activity in the veins was generally less than in mesophyll samples.

Figure 5 describes the kinetic behavior of the relative amount of ^{14}C-sucrose in water-soluble compounds in the palisade mesophyll and in the spongy mesophyll. These data come from the experiment reported in Figure 1. A large proportion of soluble ^{14}C was in the form of sucrose. The proportion of ^{14}C-sucrose was lowest in the spongy parenchyma and showed no change. In the palisade parenchyma, the proportion of ^{14}C-sucrose was relatively high at first and declined to almost the same value as in the spongy parenchyma. Average values for the leaf were between those for the two mesophyll layers. The probable role of sucrose in intercellular transport may be inferred by comparing these data with Figure 1. Half of the soluble ^{14}C was lost from the palisade mesophyll during the postlabeling period (Fig. 1). Because the ratio of sucrose to nonsucrose compounds declined by a similar fraction, apparently most or all of the ^{14}C lost from the palisade parenchyma was sucrose.

DISCUSSION

Our findings generally confirm assumptions made on anatomical grounds concerning the relative roles of the mesophyll tissues in a dicot leaf. They thereby strengthen the supposed functional relationships suggested by earlier workers (5, 7, 8, 10–14). Under the conditions of these experiments, the palisade parenchyma was the most active tissue photosynthetically. Most of the photosynthate produced in the palisade parenchyma disappeared from that tissue. Because there was a concomitant increase and decline of ^{14}C in the spongy parenchyma, photosynthate was moving from the palisade parenchyma to the spongy parenchyma and from there to the veins. However, beyond supporting these more traditional assumptions, the data provide additional qualitative and quantitative insights into intercellular transport of photosynthate in leaves.

The major compound transported between tissues appears to be sucrose, although the data do not exclude a very small pool of some transportable compound(s) in rapid exchange with sucrose. The strongest evidence for the mobility of sucrose comes from the data illustrated in Figure 5, where all of the ^{14}C loss from the palisade parenchyma came from the sucrose pool.

The redistribution of photosynthate in the leaf was quite rapid. In fact, ^{14}C appeared in the veins so rapidly that in only one of the four experiments (Fig. 3) was an apparent initial increase in ^{14}C content observed in the veins. However, sampling may not have commenced quickly enough in two of the experiments to detect an increase (Figs. 1 and 2). The fourth experiment (Fig. 4) indicated that the ^{14}C content of the veins began to decline immediately after labeling. Because of the difficulty of obtaining comparable sections of veinal tissue, we feel that the data demonstrate reliably only that the veins contain a relatively large amount of ^{14}C and that this amount declined later. Although we realize that the data allow only an equivocal statement of this point, we presume that the tissues in the veinal samples do not photosynthesize much more rapidly than the palisade or spongy parenchyma. The rapid appearance of ^{14}C in the veins must therefore be due to its rapid transport from other mesophyll tissues, particularly from the palisade parenchyma. That an initial increase must occur in the ^{14}C content of the veins of pulse-labeled leaves is supported by kinetic data on ^{14}C translocation (1, 9, 10) which indicate that the maximum rate of ^{14}C export occurs about 20 to 40 min after pulse labeling. Better data for the initial kinetics in the veins might be obtained with the present approach if more samples were taken and if the specific radioactivity of ^{14}C sucrose were assayed.

Movement of assimilates in the leaf was not directed only toward the veins. Substantial amounts of radioactivity quickly appeared in the upper and lower epidermises. Within 10 min (Fig. 3), the ^{14}C in the upper epidermis amounted to about 20% of the radioactivity detected initially in the palisade parenchyma. The rate of ^{14}C movement into each epidermis reflected the level of ^{14}C in the adjacent mesophyll tissue (*i.e.* it was higher in the upper epidermis than in the lower epidermis).

The movement of photosynthate does not involve significant mobilization or deposition of insoluble ^{14}C. Some data (Figs. 1 and 2) indicated that insoluble ^{14}C decreased slightly in the palisade parenchyma but other experiments (Figs. 3 and 4) did not confirm this observation. Similarly, insoluble ^{14}C

increased slightly (Figs. 1 and 4) in the spongy parenchyma in some experiments, but not in others (Figs. 2 and 3). In any case, the amounts of insoluble ^{14}C possibly involved were small in comparison to the changes in soluble ^{14}C.

Several reasons (cited earlier) have been advanced to implicate the palisade parenchyma as the most active tissue photosynthetically. One of these, the fact that it is simply the uppermost layer of chlorenchyma in the leaf implies that shading might play a significant role in determining the relative rates of CO_2 fixation in the palisade and spongy parenchyma. An investigation of the respective rates of photosynthesis under different light intensities confirmed that possibility (Outlaw and Fisher, unpublished). In the present experiments, the leaves were illuminated at a light intensity which resulted in the maximum difference in photosynthesis between the tissues.

We have discounted the possibility of a CO_2 gradient within the leaf since epidermal stomatal densities are similar (3920 cm^{-2} in the upper epidermis and 4400 cm^{-2} in the lower epidermis) and the rate of CO_2 fixation was low.

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