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Enzymic Assay of 10^{-7} to 10^{-14} Moles of Sucrose in Plant Tissues[1]

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ABSTRACT

Procedures are described for measuring sucrose in plant extracts or freeze-dried tissue in the range between 10^{-7} and 10^{-14} moles. The method is based on the destruction of pre-existing glucose and fructose, followed by the hydrolysis of sucrose and reduction of $NADP^+$ by a series of coupled enzymic reactions. Depending on the sensitivity required, the NADPH is determined directly with a spectrophotometer or a fluorometer, or is amplified as much as 30,000 times before fluorometric assay. The procedures suggested for the macro level are simpler than current methods, and those suggested for microanalysis are several orders of magnitude more sensitive.

With this technique, single palisade parenchyma cells and single spongy parenchyma cells of *Vicia faba* leaflets were each found to contain about 2.2 pmoles of sucrose.

Sucrose is an important plant metabolite. The concentration of sucrose in plant tissues can regulate such diverse functions as the rate of carbon export from leaves (7), differentiation, enzyme activities, and hormonal action (see 17). It is also the principal compound transported in the phloem of most plants.

The content of sucrose in plant cells is remarkably variable, reaching levels as high as 80% of the dry weight in some storage tissue and there is evidence to suggest that sucrose is compartmentalized in such cells (see 6). Furthermore, 11 kinetically different intercellular and intracellular pools of sucrose have been postulated to exist in leaflets of *Vicia faba* (14). Indeed, the most widely accepted theory of phloem transport requires that adjacent cells have very different sucrose concentrations (*e.g.* 5). Thus, there is clearly need for an assay suitable to measure sucrose content of plant tissues at the cellular or even subcellular level.

In this paper we describe such a method. It is a flexible method, capable of measuring sucrose over a 10 million-fold range down to the amount present in $^1/_{200}$ of a single mesophyl cell. The principle used to achieve this sensitivity can be extended to other analytical problems of plant metabolism.

MATERIALS

Biological Material. *V. faba* L. plants were grown in a soil-sand mixture in pots. Sunlight was supplemented for 16 hr each day from "Gro-lux" fluorescent tubes and also for 8 hr each day from 150 w incandescent "Horticulture" lamps (Sears). Radiation from the incandescent lamps passed through a water filter 1 cm deep. Plants were watered weekly with Hoagland solution. A constant temperature of 24 C was maintained.

Whole leaf extracts used for the kinetic studies were made with $HClO_4$. Individual palisade and spongy parenchyma cells were dissected from leaflets after freeze-drying according to the following procedure. Several mature leaflets were harvested from various parts of the canopy and immediately frozen in liquid N_2 previously cooled to its melting point by boiling off part of the liquid under vacuum. (This accelerates freezing by prevention of gas bubble formation on contact.) To facilitate drying, the leaflets were broken into fragments of 1 to 3 mm on a side in powdered CO_2. The fragments were freeze-dried at −35 C under a vacuum of less than 10 μm of mercury for 96 hr. Except when samples were taken for dissection, the tissue was stored at −20 C under vacuum.

Biochemicals. All enzymes except invertase were from Boehringer Corporation. Enzymes which were supplied as $(NH_4)_2SO_4$ suspensions were freed of most of the salt by centrifuging. (Sulfate inhibits most of the enzymes used.) The precipitate was dissolved in a convenient volume of 25 mM tris-HCl (pH 8.1) which contained 0.02% (w/v) BSA. Crystalline invertase of high specific activity (400 units/mg) from Sigma Chemical Co. was dissolved in the same buffer. Glutamate dehydrogenase was supplied in a glycerol solution and was used without further treatment. Enzyme solutions were stored at 4 C and were prepared fresh weekly. Low fluorescence imidazole was from Sigma.

The optical density of NADPH was measured at 340 nm in a Zeiss PMQ II spectrophotometer. For fluorometric measurement, a Farrand filter-type fluorometer was used. The primary filter was Corning No. 7-37 (peak transmission, about 360 nm) and the seconary filters were Corning No. 4-72 and 3-72 (peak transmission, about 485 nm).

PRINCIPLE

Any free glucose or fructose present is first destroyed by alkali and heat. Sucrose, which is stable in alkali, is then acted upon by four enzymes which carry out the following reactions in a single analytical step, see top of page 380.

The NADPH is then measured in one of our ways, depending on the amount present: (A) by its absorption ($5\text{-}70 \times 10^{-9}$ mol); (B) by its native fluorescence ($0.1\text{-}5 \times 10^{-9}$ mol); (C) by the greater fluorescence when converted back to $NADP^+$ with H_2O_2 and then treated with strong NaOH ($0.01\text{-}1 \times 10^{-9}$ mol); or (D) by amplification with enzymic cycling (10^{-12}–10^{-14} mol).

The capacity to use the greater sensitivity of (C) and (D) depends on the fact that the excess $NADP^+$ is easily destroyed by weak alkali without detectable loss of NADPH.

To achieve the highest sensitivity requires not only enzymic cycling (described below) but also accurate submicroliter constriction pipettes plus the "oil well" technique to avoid evaporation. Details of the oil well technique and the construction of the necessary micropipettes have been described (9).

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$$\text{sucrose} \xrightarrow{\text{invertase}} \text{glucose} + \text{fructose} \quad (1)$$

$$\text{glucose} + \text{fructose} + 2\ \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-P} + \text{fructose-6-P} + 2\ \text{ADP} \quad (2)$$

$$\text{fructose-6-P} \xrightarrow{\text{P-glucoisomerase}} \text{glucose-6-P} \quad (3)$$

$$2\ \text{glucose-6-P} + 2\ \text{NADP}^+ \xrightarrow{\text{glucose-6-P dehydrogenase}} 2\ \text{6-P-gluconate} + 2\ \text{NADPH} \quad (4)$$

COMMENTS ON THE GENERAL PROCEDURE

Destruction of Preformed Glucose and Fructose. This preliminary procedure has been used in an earlier sucrose method (16) and in a method for glycogen (10). The initial heating in alkali destroys not only free glucose and fructose, but enzymes from the tissue which might interfere (*e.g.* NADPH oxidase). If such enzymes are not present, and the levels of glucose and fructose are no greater than that of sucrose, this step could be eliminated in the direct methods (A and B). In this case, invertase is omitted from the specific reagent and added instead after the free glucose and fructose have reacted (less than 10 min).

Specific Enzyme Reaction Sequence. For simplicity, the reactions shown above are carried out in a single step. The enzymes have different pH optima. At pH 6.9, which is a compromise, relatively large amounts of invertase are required (pH optimum, approximately 4.5). The activity of different invertase preparations varies; therefore, with a new lot of invertase, it is recommended that the time course of the reaction be followed with standard sucrose. The invertase concentration is then adjusted to give a half-time for the over-all reaction of not more than 5 min so that the reaction will be at least 98% complete by 30 min.

If increasing the invertase does not reduce the half-time to 5 min, one of the other enzymes may be at fault. This can be tested by adding standard amounts of glucose-6-P, fructose-6-P, and glucose in succession until the weak link is located.

A 10 × stock solution may be stored at −70 C. Repeated freezing and thawing does not affect the stability of this reagent. Storage of the complete stock solution at −20 C is not satisfactory because the enzymes, especially P-glucoisomerase, are not stable.

INDIVIDUAL PROTOCOLS

A. SPECTROPHOTOMETRIC METHOD (5–70 × 10^{-9} MOL)

Step. 1. The tissue extract (at least 25 μM in sucrose) is made 0.02 N in NaOH and heated 30 min at 95 C.

Step 2. An aliquot of 200 μl or less, depending on the concentration, is added to 1 ml of reagent composed of imidazole buffer (pH 6.9) (40 mM imidazole base, 60 mM imidazole-HCl), 0.4 mM $NADP^+$, 1 mM ATP, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.02% (w/v) BSA, 20 μg/ml yeast invertase (EC 3.2.1.26), 2 μg/ml yeast hexokinase (EC 2.7.1.1), 1 μg/ml yeast P-glucoisomerase (EC 5.3.1.9), and 1 μg/ml yeast glucose-6-P dehydrogenase (EC 1.1.1.49).

All samples and blanks are brought to the same total volume with 0.02 N NaOH.

Step 3. After 30 min at 20 to 25 C, the absorption is measured at 340 nm.

Comment. Although we regard the direct fluorometric method below as usually more convenient, the spectrophotometric method is necessary if the need to standardize sucrose solutions arises.

If the samples to be analyzed have significant absorption at 340 nm, this needs to be evaluated. The simplest approach is to omit glucose-6-P dehydrogenase from the reagent. After 30 min (to allow conversion of glucose and fructose to glucose-6-P), the absorption is read and the reaction is completed quickly (1–2 min) by adding 10 μl of 1 mg/ml glucose-6-P dehydrogenase.

B. DIRECT FLUOROMETRIC METHOD (0.1–5 × 10^{-9} MOL)

This assay is conducted as described for the spectrophotometric method except that step 2 is carried out in a 3-ml fluorometer tube (10 × 75 mm) and the step 2 reagent is altered: imidazole is reduced to 50 mM, ATP to 0.2 mM, and $NADP^+$ to 0.1 mM (0.03 mM for the 0.1–0.5 × 10^{-9} mol range to keep the fluorescence blank to a minimum).

Sucrose standards covering the expected ranges, as well as blanks, are required.

Comment. It should seldom be necessary to use more than 50 μl of sample (50 μl of 2 μM sucrose is equal to 0.1 × 10^{-9} mol). All samples can be brought to the same total volume or any small differences in volume can be taken into account in the calculations.

The upper limit of the useful range is 5 μM (5 × 10^{-9} mol in 1 ml) because with more than 10 μM NADPH, proportionality of fluorescence is lost. The lower limit is set by the fluorescence blank of the sample or reagent. With care in preparing glassware and in selection and storage of reagents, the fluorescence blank of the reagent can be kept to the equivalent of less than 0.5 μM sucrose (0.5 × 10^{-9} mol in 1 ml).

For samples in the lowest part of the range, as well as for samples with significant fluorescence, the procedure is altered (as suggested for the spectrophotometric method) by omitting glucose-6-P dehydrogenase from the specific reagent. Readings are then taken before and 5 or 10 min after adding 10 μl of 0.2 mg/ml glucose-6-P dehydrogenase. The difference in fluorescence due to NADPH can be assessed more accurately than by comparison between sample and blank tubes. Blanks are still required, however, and any small difference in their readings when the dehydrogenase is added is taken into account.

C. ASSAY WITH FLUORESCENCE ENHANCEMENT (0.01–1 × 10^{-9} MOL)

Step 1. This step is unchanged although to take advantage of the high sensitivity, the volume at this step has to be small. This presents a problem in regard to heating without evaporation. Volumes of 10 μl or more could be heated in small sealed tubes; for smaller volumes, heating under oil in oil wells is suggested (see method D).

Step 2. (The reagent is the same as for method B.) Ten μl or less of sample are added to 50 μl of the reagent in a 3-ml fluorometer tube.

Step 3. After 30 min at 20 to 25 C, 50 μl of phosphate buffer (0.25 M Na_3PO_4, 0.25 M K_2HPO_4) are added and mixed with care not to spatter the step 2 reagent up on the walls before mixing with alkali.

Step 4. After heating 15 min in a 60 C water bath, 1 ml of 6 N NaOH containing 10 mM H_2O_2 and 10 mM imidazole (8) is added with immediate and thorough mixing. The tubes are heated to 60 C for 15 min.

Step 5. The tubes are cooled exactly to room temperature, wiped clean, and the fluorescence is read. (The fluorescence is somewhat light-sensitive, therefore, samples should not be left in strong light before reading. Otherwise the fluorescence is almost indefinitely stable.)

Comment. Thorough mixing at step 3 is essential because any $NADP^+$ which escapes destruction at this step will be converted to a highly fluorescent product at step 4.

Exposure of the NaOH (used in step 4) to sunlight for 2 days reduces the fluorescence blank. H_2O_2 is added to this reagent within 1 hr of use. Immediate and thorough mixing upon the addition of the viscous NaOH in step 4 is especially important, since in weak alkali, much of the $NADP^+$ (from NADPH oxidation) is destroyed without the production of a fluorescent product(s).

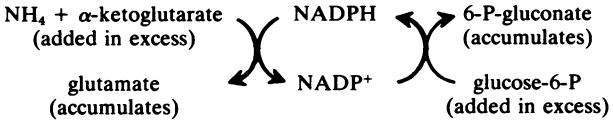
The emission and excitation peak of the fluorescent product is identical to that of NADPH.

D. CYCLING METHODS

Because cycling can increase sensitivity many thousandfold, a wide range of methods is possible, limited only by the ability to work with small initial volumes. Examples are given in the 10^{-12} mol, 10^{-13} mol, and 10^{-14} mol range. All are designed for use with freeze-dried tissues but could be adapted to other purposes. The initial steps in all three methods are carried out in oil wells. However, for samples down to about 10^{-12} mol, the methods could easily be adapted to small test tubes (see suggestion after the DI method).

All of the methods in this section depend upon the selective destruction of the excess $NADP^+$ after the specific step and the subsequent enzymic amplification of the NADPH.

The scheme used to amplify NADPH is shown below.



NADPH is alternately oxidized and reduced by high levels of glutamic dehydrogenase and glucose-6-P dehydrogenase and their respective substrates. One of the accumulated products (6-P-gluconate) is then measured (fluorometrically) by the NADPH produced with a new excess of $NADP^+$ plus 6-P gluconate dehydrogenase.

Analysis at the 10^{-12} to 10^{-14} mol level can be carried out routinely in 1 day. However, if desired, the procedure can be interrupted for at least as long as 72 hr after the heating following either the specific step or cycling.

D1. METHOD FOR 1 TO 10 × 10^{-12} MOL

Step 1. A freeze-dried tissue sample is pushed into a 1-μl droplet of 0.02 N NaOH which is under oil to prevent evaporation (see "oil well technique" in ref. 9). The oil well rack is heated to 95 C for 30 min. Standards consist of 1 μl of 3 and 10 μM sucrose in 0.02 N NaOH.

Step 2. One μl of specific reagent is added to the droplet and incubated at 20 to 25 C for 30 min. The reagent is the same as for method B except that the buffer is 80 mM imidazole base, 20 mM imidazole-HCl and all other components are double strength.

Step 3. Ten μl of 0.06 N NaOH are added to the droplet in the oil well and the oil well rack is heated in an oven at 80 C for 20 min.

Step 4. Two μl of the oil well droplet are transferred to a 3-ml tube which contains 100 μl of cycling reagent (100 mM tris-HCl [pH 8] 5 mM α-ketoglutarate, 1 mM glucose-6-P, 10 mM ammonium acetate, 100 μM 5-ADP, 50 μg/ml beef liver glutamate dehydrogenase [EC 1.4.1.3], and 4 μg/ml glucose-6-P dehydrogenase). The cycling reagent and tubes are kept on ice until all transfers are made. The test tubes are covered with foil and transferred to a 38 C water bath for 1 hr and then to a 100 C water bath for 4 min.

Step 5. The 6-P-gluconate which accumulated during the cycling step is assayed enzymically by adding 1 ml of indicator reagent (50 mM imidazole-acetate [pH 7] 0.04 mM $NADP^+$, 0.1 mM EDTA, 30 mM ammonium acetate, 5 mM $MgCl_2$, 5 μg/ml 6-P-gluconate dehydrogenase) to each tube. After the reaction is complete (30 min at 20–25 C), the fluorescence of NADPH is read.

Comment. The initial steps in this method could easily be adapted to small test tubes (*e.g.* commercial 6 × 50 mm o.d. serological tubes) by increasing all volumes in steps 1 to 3 by a factor of 5. Steps 4 and 5 would be unchanged, except that with samples below 5 × 10^{-12} mol, it would be advantageous to increase the aliquot for cycling to 10 μl.

D2. METHOD FOR 1 TO 10 × 10^{-13} MOL

Steps 1 and 2. are identical to procedure D1 except that volumes are reduced to 0.1 μl.

Step 3. The only change is to reduce the NaOH to 0.5 μl.

Step 4. Ten μl of cycling reagent (as in method D1 but with enzymes increased 4-fold) are added to the oil well droplets at regular intervals. An hr after the first addition, 2 μl of 0.5 N NaOH are added to each droplet in succession at the same time intervals to keep all incubation times the same within 1 min. The rack is heated 20 min at 80 C in an oven.

Step 5. Ten μl of each sample are added to 1 ml of indicator reagent and readings are made when the reaction is complete (as in method D1).

D3. METHOD FOR 1 TO 10 × 10^{-14} MOL

The method is identical to D2 except as follows. The volumes of 0.02 N NaOH, specific reagent, 0.06 N NaOH, and cycling reagent are all reduced by a factor of 5. Incubation time is increased to 2 hr and the NaOH added after cycling is 10 μl of 0.1 N solution. To increase precision, the fluorescence of each tube with indicator reagent is read before adding the 10-μl aliquot from the oil well.

RESULTS AND DISCUSSION

The fructose moiety of fructosides of the raffinose family (galactose$_{(n)}$-Glc-Fru) is hydrolyzed by the invertase used in this assay, although the rate of hydrolysis is slower with the larger oligosaccharides (Fig. 1). The differential in the rate of hydrolysis is a useful tool in examining the saccharide composition of raw plant extracts. The kinetics of the reaction with a $HClO_4$ extract of *V. faba* was identical to that of sucrose, indicating that neither raffinose nor stachyose was present in the extracts. In extracts of plants which contain other sugars of the raffinose family, analysis of sucrose may be made by deleting P-glucoisomerase from the specific step reagent and decreasing hexokinase (because it may contain a trace contamination of P-glucoisomerase). The yield of NADPH will be cut in half. If desired, after the glucose-6-P has reacted, the fructose moiety of all of

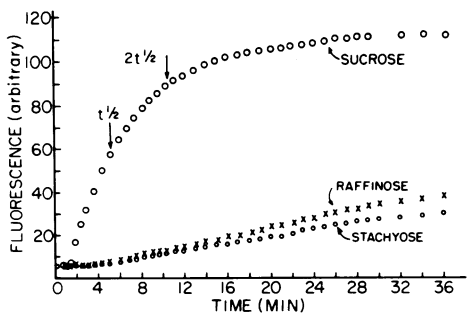


FIG. 1. Time course of specific step reaction with sucrose, raffinose, and stachyose. Time course of reaction with sucrose was indistinguishable from that of a $HClO_4$ extract of *V. faba* leaflet.

the invertase-susceptible sugars may be measured by adding P-glucoisomerase and taking another reading a few min later.

Other sugars which were tested and found not to interfere in the assay were trehalose, melezitose, cellobiose, melibiose, turanose, gentiobiose, and maltose.

NADPH in plant extracts introduces a small overestimate of sucrose in cases where sucrose content is very low. This would be quickly destroyed by the acid extracts and otherwise it is automatically subtracted out with other absorbing or fluorescing compounds when subtracting the blank in macro methods. Tissue blanks (samples without invertase) yielded values indistinguishable from 0.02 N NaOH blanks in an analysis of sucrose in single *V. faba* parenchyma cells. In special cases, the samples could be briefly acidified before analysis (NADPH destruction is 99% complete in 2 min at room temperature in 0.01 N HCl [9].)

Performance of Method in Macro Range (0.1-50 × 10^{-9} mol). Reproducibility and linearity with both the spectrophotometric and direct fluorometric procedures (methods A and B) are limited only by the precision of pipetting and stability of the spectrophotometer or fluorometer, and, in the case of fluorometry, recognition of some of the limitations. The first is that fluorescence has a negative temperature coefficient (about 1.5%/degree C), therefore, all samples and standards must be read at the same temperature. Second, fluorescence can only be linear up to the point where the absorption by the fluorescent compound begins to reduce significantly the intensity of the exciting wavelengths; this is at about 10 μM NADPH (from 5 μM sucrose). Finally, any significant absorption by the exciting wavelengths (340 nM region) or the emitted wavelengths (470 nM region) by other substances present will reduce the light which strikes the phototube. Because of the high sensitivity of the fluorometric procedure, extracts can be used at such high dilution that quenching is unlikely to occur. However, any doubt should be resolved by adding an internal sucrose standard to the suspected sample to see if the fluorescence increment is the same as that obtained in the absence of the sample.

Plant extracts for sucrose analysis in this range are conveniently made with $HClO_4$, although some caution must be exercised to prevent sucrose hydrolysis. Hydrolysis is strongly dependent upon temperature and $HClO_4$ concentration. In 0.3 M $HClO_4$, only 0.2% of sucrose was hydrolyzed in 1 hr at 0 C; however 12.5% was hydrolyzed at 23 C. In 1 M $HClO_4$, hydrolysis in 1 hr at 0 C was 2% but 43% at 23 C.

Most methods for sucrose analysis in this range are carried out by hydrolyzing sucrose enzymically in one step and subsequently analyzing glucose. The Glucostat procedure (Worthington Biochemical Corporation), perhaps the most commonly used of these methods, utilizes glucose oxidase to produce H_2O_2 which causes color development. Another additional step (H_2SO_4 treatment) is required for maximum sensitivity. The sucrose method of Bergmeyer and Bernt (1) and the glucose method of Finch *et al.* (4) are similar in principle to that proposed here. Chromatographic procedures (3, 11) also have been published. The proposed method is technically less difficult, more sensitive, and more flexible than any of these methods.

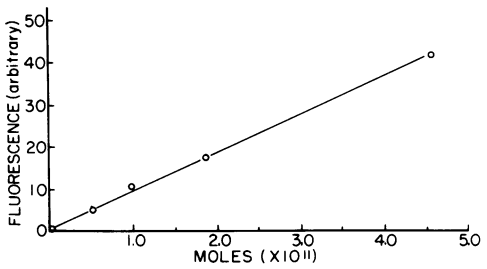


FIG. 2. Standard curve for sucrose in the 10^{-11} mole range. Method C was used. Standard deviation of triplicates was insignificant.

Performance in Intermediate Range (10^{-11} mol). A standard curve for sucrose is shown in Figure 2. Method C was used (final enhancement of fluorescence with strong NaOH). However, the use of cycling to provide the necessary increase in sensitivity is generally preferred for routine analyses in this range, because it can easily be extended to smaller samples. Nevertheless, for the investigator interested only occasionally in this sensitivity range, the fluorescence enhancement procedure offers the advantage of using simpler reagents.

A published method with similar sensitivity is based upon enzymic labeling of glucose followed by separation of the glucose-6-^{32}P and unreacted AT^{32}P (2).

Performance in Micro Range (10^{-12}-10^{-14} mol). A standard curve for authentic sucrose demonstrates that reproducibility and linearity are satisfactory down the range from 2 to 10 × 10^{-14} mol (Fig. 3). The range of sensitivity that is perhaps the most useful for investigation on quantitative histochemistry of plants is from 10^{-12} to 10^{-13} mol. A previous publication (14) reported the measurement of sucrose down to 10^{-12} mol and utilized the principle of enzymic hydrolysis of sucrose (1) followed by enzymic assay of glucose (1, 4, 9) and enzymic amplication (9). The present method requires fewer steps and reduces variability with tissue samples by the destruction of pre-existing reducing sugars.

It does not seem likely that there is need at present to measure less than 1 × 10^{-14} mol of sucrose. (A single leaf cell contains 200 times more sucrose than this.) However, this does not

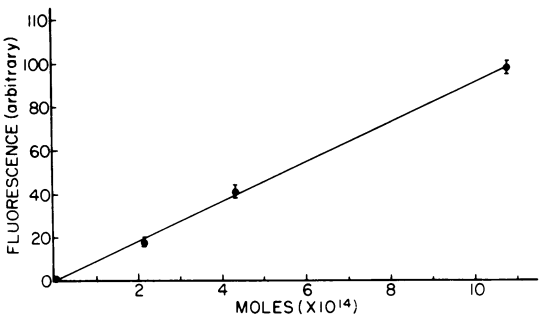


FIG. 3. Standard curve for sucrose in the 10^{-14} mole range. Method D3 was used. Bars represent standard deviations of five replicates.

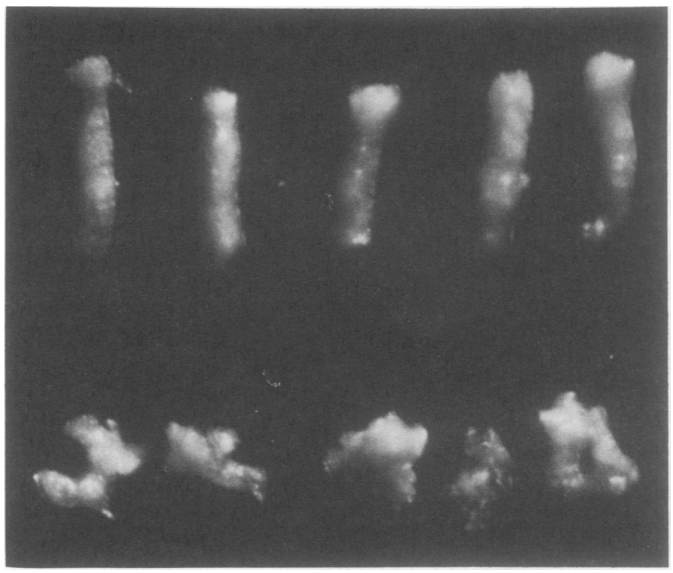


FIG. 4. Palisade parenchyma cells (top) and spongy parenchyma cells (bottom) dissected from a freeze-dried leaflet. Orientation of these samples demonstrates the ability to manipulate such small tissue samples manually.

Table I. Dry weight and sucrose content of individual leaf cells

Cells were randomly chosen from several leaflets which were under photosynthetic conditions prior to freeze drying.

	Single cell mass ng(dry) ± se	Sucrose content mmol/kg(dry) ± se
Palisade parenchyma	11.6 ± 0.4 (n=66)	185 ± 11 (n=55)
Spongy parenchyma	13.8 ± 1.1 (n=7)	160 ± 19 (n=12)

represent the limits of this analytical system. A standard curve between 1 and 10×10^{-15} mol was constructed by using the protocol for 10^{-14} mol and enhancing the fluorescence of the final NADPH with 6 N NaOH (*cf.* method C). At levels lower than 5×10^{-15} mol, it is probable that two cycling steps would be preferred. In this case, the only real limitation would be imposed by the need to keep initial volumes small enough not to introduce intolerably large blank values.

Application of the Method. Single palisade parenchyma cells and spongy parenchyma cells (Fig. 4) were weighed on a quartz fiber fishpole balance (9) and assayed for sucrose (Table I). The dry mass of the spongy parenchyma cells was 13.8 ng. This is slightly greater than the mass of the palisade cells. The sucrose concentration was about 175 mmol/kg(dry) and was not significantly different in the two cell types. The higher Chl to protein ratio in the palisade parenchyma (15) as well as higher level of $^{14}CO_2$ incorporation *in situ* at intermediate light levels (13) suggest that the palisade parenchyma would be the major photosynthetic tissue under the conditions of this experiment. Furthermore, kinetic studies of ^{14}C incorporation into sucrose in these tissues (12, 14) have shown that sucrose in the palisade parenchyma is in exchange with that in the spongy parenchyma. This evidence, along with anatomical observations, demonstrates that spongy parenchyma cells are intermediaries in the translocation of sucrose. However, the above results show that these cells do not concentrate this metabolite measurably.

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