METABOLISM OF APPLES

Conversion of Glucose to Sucrose in Apple Tissue

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When radioactive glucose is infiltrated into apple disks, radioactive sucrose, but not free fructose, is formed. The process is inhibited by the exclusion of oxygen. Although both moieties of the sucrose molecule are labeled, most of the activity is found in the glucose portion. In addition to the sucrose labeling, some activity appears in the amino acids, notably alanine, γ-aminobutyric acid, glutamine, and asparagine.

THE CONVERSION OF GLUCOSE TO SUCROSE in isolated apple tissue was undertaken as part of a broad study of the metabolic processes which persist in fruit between the time of harvesting and processing. For the present investigation the technique of vacuum infiltration with radioactive glucose was employed. The formation of sucrose from glucose in isolated plant tissue had been shown as early as 1933 by Nelson and Auchincloss (*8*) in the case of the potato. Virtanen and Nordlund (*16*), McCready and Hassid (*5*), and Hartt (*1*) extended these observations to include various green plants and parts thereof. It became clear from these studies that glucose and fructose, among other substances, could serve as precursors of sucrose independently of photosynthesis and that oxygen was required. More recently Hassid and Putman (*2, 10*) have demonstrated, using glucose-C^{14}, that the sucrose formed in *Canna indica* leaf disks was labeled in both moieties, although the free fructose was relatively unlabeled. Fructose-C^{14} gave rise to sucrose labeled in both component sugars, but formed virtually no free glucose. Vittorio, Krotkov, and Reid (*17*) have obtained similar results with isolated tobacco leaves.

Experimental Work

Material Newton Pippin apples, grown in the Watsonville area of California and stored for approximately 7 months at 5° C., were used in this experiment. Apples of the 1952 and 1953 harvests were used with qualitatively similar results, but because more detailed information is available for the work with the 1953 apples, only this work is described here.

Infiltration A plug of apple tissue, 14 mm. in diameter, taken approximately midway between the core and peel of the fruit with the aid of a cork borer, was sectioned transversely to yield disks weighing approximately 0.24 gram each. These disks were placed two at a time in a 1.25% solution of uniformly labeled glucose (approximately 0.5 μc. per mg.) in the smallest practicable container, and submerged with a glass stopper. The beaker was placed in a small bell jar which was evacuated with a water aspirator. When bubbles ceased to appear from the sections, the vacuum was slowly released. The disks were now translucent and no longer floated. Three disks were quickly transferred to a moist filter paper in a covered Petri dish. Three other disks were placed in a Thunberg tube on a crumbled piece of moist filter paper. The air in the tube was replaced with nitrogen by repeated evacuation and flushing.

Extraction of Carbohydrate And Amino Acids After the disks had been held for 22 hours at 25° in the dark they were ground with 10 ml. of hot 80% ethanol in a Potter-Elvehjem homogenizer (*9*), which was immersed in hot water at the boiling point of alcohol. The residues were washed several times with 3-ml. portions of alcohol. The alcoholic extracts were taken to dryness at room temperature, dissolved in 5 ml. of water, passed through a column of Dowex 50 (acid form, 200-mesh, 5 mm. in diameter × 50 mm. long) to remove the amino acids and then eluted from the column with 5% ammonium hydroxide.

Chromatography of Carbohydrates In order to determine the relative amounts of radioactivity in glucose, fructose, and sucrose, small portions of the extracts which were concentrated to approximately 0.5 ml. at room temperature were spotted on Whatman No. 1 filter paper for chromatographing, the remaining portions being chromatographed as bands, using two 22-inch-wide sheets for each lot. Development was carried out in butanol-ethanol-water (10:1:2, by volume) in the descending direction; 96 hours was allowed for the spots and 150 hours for the bands. The sugars were identified by running known sugars simultaneously. In addition to glucose, fructose, and sucrose, a trace of xylose was present. The aniline dipping reagent (*7*) permitted the detection of fructose and sucrose as well as glucose and xylose when the chromatogram was viewed in ultraviolet light. These sugars were also located with the naphthoresorcinol spray (*3*). Fructose and sucrose were detected with the resorcinol dipping reagent (*6*) as well. Radioactive areas were found by radioautography.

Chromatography of Amino Acids The ammonium hydroxide eluates were concentrated to a small volume, spotted on 5 × 5 inch sheets of S. and S. No. 589 blue label filter paper, and chromatographed in two dimensions as described by Underwood and Rockland (*15*), with ammoniacal phenol in the first direction and butanol–formic acid in the second direction. Approximately half of the total yield of the amino acids was used because of their very low radioactivity. Radioautographs of the chromatograms were prepared with Eastman No-Screen x-ray film, at exposure times ranging from 24 to 788 hours. The time required for the appearance of darkened areas was noted as a rough indication of the relative amount of labeling in the various amino acids. The amino acids were located with the ninhydrin-acetone dipping reagent of Smith (*12*). Five per cent collidine was added to the reagent since, as Levy and Chung (*4*) have shown, this base causes many of the amino acids to give specific "off-shades" with ninhydrin on moderate heating, thus aiding greatly in their tentative identification.

Measurement of Radioactivity In cases where the carbohydrates were chromatographed as spots, the areas bearing the various sugars were excised and counted in a Tracerlab windowless Geiger counter. Where an area was too extensive to count in one piece, it was counted in several sections. An effort was always made to remove the center of the spot in one segment, so that the bulk of the radioactive sugar could be centered in the

Table I. Incorporation of Radioactivity into Sucrose in Apple Tissue Infiltrated with Glucose-C^{14}

(Activity, counts per minute)

N_2 Incubation			Air Incubation		
		Sucrose			Sucrose
Glucose	Sucrose	Glucose + sucrose	Glucose	Sucrose	Glucose + sucrose
221	4	0.0178	326	27	0.0765
415	6	0.0143	328	15	0.0437
630	11	0.0172	638	45	0.0659
1,976	32	0.0159	673	47	0.0653
2,396	46	0.0188	1026	83	0.0748
6,999	123	0.0171	4415	336	0.0707
Total 12,637	222	0.0173	7406	553	0.0695
Activity (% of total)	1.73			6.95	

counter to minimize geometrical inequalities. The peripheral areas, in general, carried such a small portion of the total activity that deviations from optimum geometry were not serious. In all cases the radioactive paper was on a stainless steel disk mounted on a brass cylinder which brought the paper as close as possible to the upper portion of the counter. In any event, trials with symmetrically and asymmetrically placed pieces of radioactive paper showed relatively small differences in counting rates. The windowless counter proved to be approximately three times as sensitive as a thin-window mica counter (Tracerlab TGC-2, 1.6 mg. per sq. cm.) positioned directly over the paper.

In most cases, counting was continued long enough to keep the standard counting error below 5%. Net values below 20 counts per minute are based on at least 60 minutes of counting with a background rate of approximately 17 counts per minute.

The relative specific radioactivities of sucrose and glucose obtained by elution of the band chromatograms were determined by evaporating suitable aliquots of the sugar on stainless steel disks. The sucrose and glucose contents of these solutions were determined by the methods of Roe (*11*) and Somogyi (*13*), respectively.

Results

The actual distribution of counts in the glucose and sucrose present after incubation of the apple tissue is shown in Table I. The results obtained by direct counting of the paper for a number of chromatographic separations performed on various-sized applications of extracts are tabulated individually to indicate the experimental variation encountered. It is clear from these values that glucose is indeed incorporated into sucrose by the apple tissue under the conditions studied and that the extent of its conversion into sucrose on the basis of the total activity present is greatly reduced by exclusion of oxygen. None of the glucose is converted to free fructose, which is in agreement with the observations of Putman and Hassid (*10*) and Vittorio *et al.* (*17*) for leaf tissue.

The relative specific activities of the sucrose were determined by direct counting of aliquots of the sucrose eluted from the band chromatograms and dried on stainless steel planchets. The activities were 1.27×10^3 and 0.68×10^3 counts per minute per micromole of hexose in the air and nitrogen experiments, respectively.

The relative specific activity of the glucose, determined under similar counting conditions, was 29.4×10^3 counts per minute per micromole. It is thus clear that total equilibration between glucose and sucrose had hardly been approached.

The distribution of radioactivity between the two halves of the sucrose molecule was determined by inversion of the sucrose with invertase, chromatographic separation of the glucose and fructose, and direct counting of the appropriate areas. As shown in Table II, the glucose moiety was more highly labeled than fructose in both the air and nitrogen experiments.

Relatively little activity was incorporated in the amino acids. Judging from the exposure time required for producing discernible darkening on a radioautograph in the nitrogen experiment, alanine and γ-aminobutyric acid were most highly labeled; glutamine was next and asparagine least. These results were apparent after 90 hours' exposure. After the longest exposure, 788 hours, many darkened areas appeared, but they were too diffuse to permit any conclusions as to which amino acids they represented. Similar results were noted in the air experiment, except that the γ-aminobutyric acid area was barely perceptible until after the maximum exposure.

Discussion

It thus appears that the conversion of glucose-C^{14} to radioactive sucrose goes on in apple tissue in much the same way as originally discovered by Hassid and Putman in *Canna* leaves and shown by Vittorio *et al.* (*17*) in tobacco leaves. In all three tissues the presence of oxygen promotes the conversion to sucrose. Vittorio and coworkers obtained satisfactory sucrose synthesis with the leaf in nitrogen, but only in the presence of light. Light could have stimulated the formation of oxygen by permitting photosynthesis to occur. This appears to have been the case, as sucrose formation was considerably retarded when both oxygen and light were excluded.

The observed preponderance of radioactivity in the glucose moiety of the sucrose formed in the apple experiment must be regarded as significant. Putman and Hassid (*10*) made an elaborate time course study of the labeling in the two moieties of sucrose, involving a great many separate determinations. Their curves consistently showed a slightly higher activity in the glucose half when they used glucose-C^{14} and a higher activity in the fructose half when they used fructose-C^{14}. Vittorio *et al.* (*17*) also found that the small amount of sucrose formed in a leaf receiving glucose-C^{14} in the absence of air was labeled mainly in the glucose portion, although under favorable conditions for incorporation of glucose they found equal partition of activity between both moieties.

The pronounced asymmetry of labeling occurring in the authors' experiments in contrast to the smaller inequalities found by these two groups of workers may be a reflection of the relative sluggishness of the apple tissue compared to the leaf tissues in performing the interconversions. Whereas in the case of these leaf tissues both groups of investigators attained practically complete equilibration in a few hours or less between the monose precursor and sucrose, or what is virtually the same, complete

Table II. Distribution of Radioactivity of Sucrose Between Hexose Moieties

(Activity, counts per minute)

	N_2 Incubation		Air Incubation	
	Glucose	Fructose	Glucose	Fructose
	172	37	400	167
	51	8	98	32
Total	223	45	498	199
Activity (% of total in sucrose)	83.2	16.8	71.5	28.5

utilization of the monose, in the apple, on the contrary, the ratio of the specific activity of the sucrose to the glucose was 0.043 after 22 hours, a condition remote from complete equilibration.

Because of the inequality of the labeling, it must be concluded that both halves of the sucrose do not arise entirely from a common precursor. On the basis of the present information it would be premature to speculate on this point at length. Two obvious mechanisms by which an unequal distribution of label might arise are:

The conversion of glucose-C^{14} to the precursor of the glucose moiety may be more rapid than the interconversion of glucose to fructose (or fructose derivative), thus increasing the relative contribution of any endogenous precursor of the fructose moiety that might be present. Whether the endogenous precursor is simply free fructose (segregated in a pool distinct from the bulk of the fructose) or a fructose derivative is immaterial.

Transglycosidation could cause unequal labeling, although invertase has been reported absent from the apple (*14*).

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FEED DIGESTIBILITY

Use of Copper Derivatives of Chlorophylls in Ratio Method for Estimating Digestibility of Forages

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The "chromogen" ratio method was found unsatisfactory for use in determining digestion coefficients in studies with switch cane (*Arundinaria sp.*). A slight modification of the analytical procedure, treating samples with 0.1*M* copper chloride in 1*N* hydrochloric acid, overcame the difficulties. This proposed modification will be useful in cases where the forage under study is relatively low in chlorophyll and its derivatives.

THE "CHROMOGEN(S)" of Reid's ratio method for determining the digestibility of forages are not a single entity but a mixture of pigments, composed predominantly of chlorophylls and pheophytins and small amounts of carotenoids (*2, 5*). Because the procedure lacks specificity in the sense of involvement of either a single pigment or a fixed ratio of pigments, conceivably this method might fail to yield valid digestion coefficients when the test forage is low in chloroplast pigments and high in pigments that can be lost or changed during passage through the digestive tract (*1*). In view of the importance of indirect methods for determining digestibility in pasture research and the soundness of the basic idea underlying the chromogen(s) method, it seemed desirable to study possible modifications of Reid's method to increase its specificity and useful range.

Experimental Work

Previous experience (*5*) has shown that the chromogen ratio method may be applied successfully to the estimation of digestibility of grass and legume hay by rabbits; therefore, four tortoise Dutch rabbits were used as experimental animals in this study. The test forage used was the edible leaves of switch cane (*Arundinaria sp.*), a plant which is grazed the year around by sheep and cattle on the coastal plain of North Carolina. Winter leaves were used in the first trial and spring leaves, collected after active growth had started, in the second.

Each diet was composed of 100 parts of ground switch cane leaves, 25 parts of glucose (Cerelose), and 7 parts of refined cottonseed oil (Wesson oil). The trials consisted of a 7-day preliminary period and a 7-day collection period. The samples of feed and of feces from these digestion trials were used in comparing digestion coefficients obtained by three methods of analysis: conventional, Reid's chromogen, and copper derivatives of chlorophyll.

In the first trial in which the winter collection of switch cane leaves was used, the chlorophyll derivatives were determined as sodium copper chlorophyllins according to a method of the Cerophyll Laboratories (*3*). This procedure offered promise as a substitute for Reid's method (*4*) for some plant species, but as many extra time-consuming steps were introduced, one of the main advantages of the chromogen method was lost.

An attempt to simplify the procedure led to the development of the following method.

Two grams of feed and of fecal samples were allowed to stand 2 to 4 hours in 20 ml. (or enough to cover the sample) of approximately 0.1*M* cupric chloride in 1*N*