Carbohydrate Metabolism of Avocado. II. Formation of Sugars During Short Periods of Photosynthesis [1]

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Although mannoheptulose and perseitol have been known as major constituents of avocado sugars for many years (14) no mechanism for the synthesis or accumulation of these seven carbon sugars has been demonstrated. Recently mannoheptulose has been found to be more widely distributed than previously believed, having been found in leaves of figs (4) and alfalfa (15). Other heptoses and heptuloses (5, 7, 8, 10, 18) as well as octuloses, octitols, and a nonulose (5, 16, 17) have also been isolated from biological materials. Thus, the synthesis of these less common sugars has become of somewhat wider interest in metabolic investigations.

Tracer experiments by Nordal and Benson (14) with photosynthesizing avocado leaves established that a phosphate ester of mannoheptulose was formed during early photosynthesis. This was confirmed in this laboratory (3) with the additional demonstration that mannoheptulose appears concurrently with sucrose during photosynthesis in avocado leaves. Perseitol, in these experiments, seemed to be a secondary product, derived from mannoheptulose. Rendig and McComb (13, 15) fed various sugars to alfalfa in attempts to elucidate the mechanism of mannoheptulose synthesis. They apparently did not find significant increases in mannoheptulose but some sugars gave rise to new heptuloses, presumably by transketolase and transaldolase reactions.

Since mannoheptulose appears to be a major photosynthetic product in avocado leaves, a kinetic study of the labeled intermediates in short period photosynthesis with $C^{14}O_2$ might aid in elucidating the mechanism of synthesis. This paper reports the results of studies in avocado leaves using photosynthetic exposure periods of 10 seconds to 16 minutes.

Material & Methods

As in previous work (3), freshly picked leaves from avocado trees (*Persea gratissima* Gaertn., var. Bacon) were used in all experiments. Disks, 1.1 cm in diameter, were cut from the leaves under water for use in the photosynthetic chambers.

An apparatus similar to that in figure 1 (differing mainly in having four rather than six leaf chambers) was constructed for simultaneous exposure of several sample disks to identical atmos-

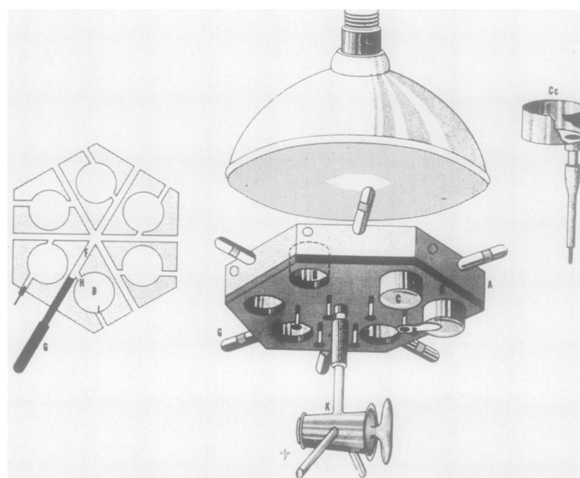

FIG. 1. Photosynthetic apparatus. The main body of the photosynthetic apparatus (A) was made from plexiglass sheet, drilled for flow passages and the sample chamber areas (B). The sample chamber caps (C) were plastic vial caps with a wire platform to hold the leaf disk sample. Not used in the experiments reported here were the modified caps (Cc) for continuous flow. The caps, containing the samples were firmly held in place with the adjustable spring clips (D) against the rubber gasket (E). The entrance passages (F) were opened by pulling the plungers out so that the ring seal gaskets were past the chamber inlets (H). The chamber vents (J) were unplugged so that a gentle flow of air could be passed through all the chambers during the pre-illuminations period by applying a slight vacuum at the 3-way stopcock (K). $C^{14}O_2$ was generated in an evacuated tube attached to the other arm of K. After 40 minutes pre-illumination, the chamber vents were re-plugged and a vacuum (half an atmosphere) drawn upon the chambers momentarily. The stopcock (K) was turned to the $C^{14}O_2$ tube and then a second stopcock on the generating system was opened to the atmosphere to allow air to sweep the $C^{14}O_2$ into the exposure chambers. After the proper exposure times (minimum of 5 sec) each sample in turn was removed by closing the valve for a given chamber (ring gaskets on each side of chamber entry) and the cap, with sample, simply pulled away from under the clip and the sample dropped quickly into boiling alcohol. The closing of each chamber before sample removal allowed different times of exposure for each sample without disturbing the remaining samples. The lamp was normally maintained at 12 inches from the chambers. A heat absorbing unit, consisting of thermal glass immersed in a circulating water bath was used between the lamp and the chambers.

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pheres with provision for removing one sample at a time rapidly without disturbing the remaining samples. Samples were placed in the four chambers and pre-exposed to the light during a period of 40 minutes with a stream of air flowing through the apparatus. Experiments using the Liston-Becker Infra-Red CO_2 Analyzer, Model 15, to measure photosynthetic rates established that 25 to 30 minutes of illumination was required to attain maximal photosynthesis and steady state. A General Electric Projector Flood lamp (150 w) provided illumination at a level of 2500 to 4000 ft-c. Thermal glass immersed in a circulating water bath was used to absorb the infra-red radiation.

Following the exposures to the radioactive atmosphere for periods of 10 seconds to 16 minutes, the samples were killed in boiling alcohol and extracted as previously described (1, 3). Counting procedures and general chromatographic procedures have also been described (1, 2, 3).

The organic moieties of phosphate esters were identified by elution of the chromatographic spots and treatment with alkaline phosphatase followed by rechromatography. In some cases, the ester spots were treated directly with phosphatase on the paper and then eluted directly onto the second chromatographic sheet by chromatographic transfer.

Rechromatography was required to obtain complete resolution of some of the original components. Glucose, mannoheptulose, and glycine were coincident on the original two-dimensional chromatograms. These were resolved by elution onto a second filter paper strip (chromatographic transfer) and developing with ethyl acetate-pyridine-water (8:2:1). This moved the mannoheptulose and glucose away from the glycine, which remained at the origin in this system. The glycine spot was cut off and the glucose in the glucose-mannoheptulose area was oxidized to gluconic acid by treating the paper with fungal glucose oxidase. Development once more with the ethyl acetate-pyridine-water solvent separated the gluconate and mannoheptulose.

Most of the confirmatory identification procedures for various sugars (in addition to the chromatographic positions) have been presented in previous papers (1, 3). Oxidation by fungal glucose oxidase was used as the criterion for glucose identification. Decarboxylation with ninhydrin was used for amino acid confirmation (2). Since organic acids were characterized only by chromatographic positions in the usual solvents (water-saturated phenol, butanol-acetic acid-water) and by rechromatography in pentanol-5 M formic acid (1 : 1), their specific identifications are only tentative.

Results

The incorporation of C^{14}-activity into gross fractions of the avocado leaf disk is represented in figure 2. The total quantities of activity fixed by the leaves varied as much as fivefold in the various experiments but the general pattern of uptake remained very much alike in all five experiments.

A schematic diagram of the positions of the major labeled components in the extracts as they appeared on the chromatograms and radioautograms is given in figure 3. Despite a careful search for labeled components which might give qualitative evidence for new synthetic pathways, very few clues could be found as to the intermediates in mannoheptulose formation. Mannoheptulose was positively identified as a phosphate ester overlapping with glucose monophosphate. No evidence could be obtained for labeling of a perseitol phosphate, free perseitol, or other polyols in these short period experiments. Nor was it possible to detect activity in significant amounts in pentose phosphates other than ribose and ribulose although a search was specifically made for arabinose-5-phosphate which might be a potential acceptor in a *cis-* condensation of an abnormal transketolase reaction. No other sugar phosphates were discovered which might logically be construed to act as intermediates in novel paths leading to the unique configurations of the avocado sugars.

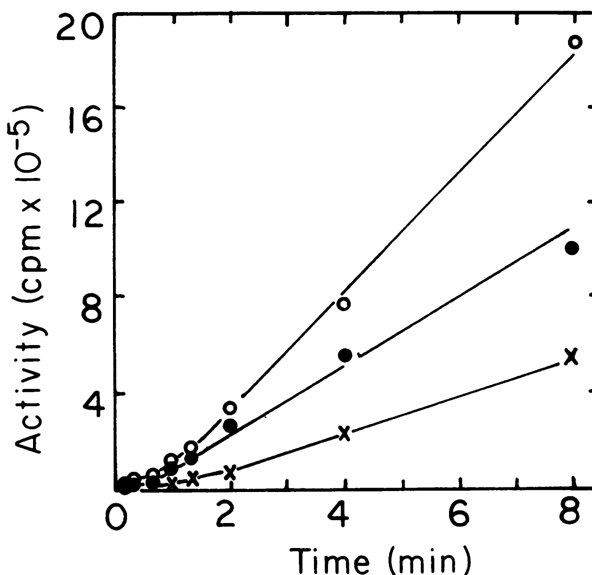

FIG. 2. Assimilation of $C^{14}O_2$ in avocado leaf disks during photosynthesis. ○, Total activity; ●, alcohol-soluble activity; X, alcohol-insoluble activity.

Some unusual components did appear among the products in these experiments. In the phosphate ester area, component eight (fig 3) was found to contain glucose but it does not correspond chromatographically with the known phosphate esters, nucleotides, or cyclic phosphates of glucose. This compound was not found in all experiments, however. It is also notable that glucose, rather than fructose, ribulose, and sedoheptulose, seemed to be the major labeled component of the diphosphate area although the others were present.

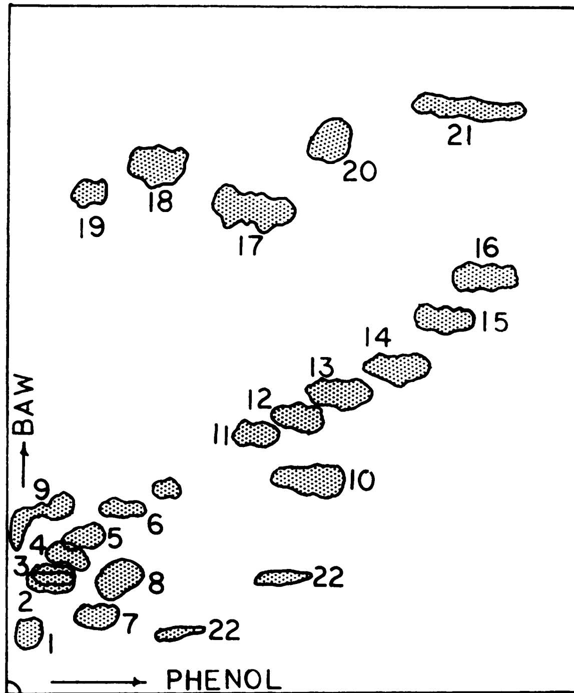

FIG. 3. Diagram of chromatographic positions of labeled components in avocado photosynthesis in $C^{14}O_2$. Chromatograms were on Schleicher and Schull No. 589 White Ribbon filter paper developed first in water-saturated phenol and then twice in butanol-acetic acid-water (52:13:33). 1, diphosphates, mainly glucose with fructose, ribulose, and sedoheptulose; 2, mannoheptulose (mono)phosphate; 3, glucose monophosphate; 4, sedoheptulose and glucose monophosphates; 5, fructose and glucose monophosphates; 6, pentose phosphate; 7, glucose and galactose phosphates (probably nucleotides); 8, glucose containing phosphate appearing only in some sample series; 9, phosphoglyceric acid; 10, sucrose; 11, serine and perseitol; 12, glucose, mannoheptulose and glycine; 13, sedoheptulose; 14, fructose; 15, alanine; 16, neutral, possibly ribulose; 17, glyceric acid; 18, malic acid; 19, citric acid; 20, succinic acid; 21, lactic acid; 22, unidentified neutral compounds.

A number of neutral components were found which did not coincide with compounds usually labeled during photosynthesis in other plants. Spot 16 (fig 3) appears to coincide with free ribulose. Free sedoheptulose was definitely identified as a product of early photosynthesis. A number of slower moving neutral components, in low concentration and activity, remain unidentified but may correspond either with longer carbon chain ketoses or oligosaccharides.

An analysis of the incorporation of activity into the various free sugar components during a typical time sequence is presented in figure 4. A very rapid labeling of free sugars, particularly ketoses, is found. The labeling of the phosphate esters in a similar experiment is given in figure 5. These results show that the ratio of activity in free sugars of avocado leaf to activity in phosphate esters is rather high in comparison with the proportions found in other leaves during short periods of photosynthesis. This is emphasized in table I which gives a comparison of total sugar activity and phosphate ester activity for bean leaf disks and for avocado leaf disks after 10 or 20 seconds photosynthesis in $C^{14}O_2$. Although conditions were identical in these experiments, the

Table I

Differences between Bean Leaves & Avocado Leaves in Activity Distributions after Short Periods of Photosynthesis in $C^{14}O_2$

Leaf disks were exposed and treated as described in the text. Activities represent the totals for all sugar or phosphate ester areas by direct count on chromatograms of one tenth of entire extracts.

Sample	Photosynthesis period	Activities	
		Free sugars	Phosphate esters
	seconds	cpm	cpm
Bean leaf	10	150	8810
Avocado leaf	10	1140	3420
Bean leaf	20	400	14900
Avocado leaf	20	3440	4450

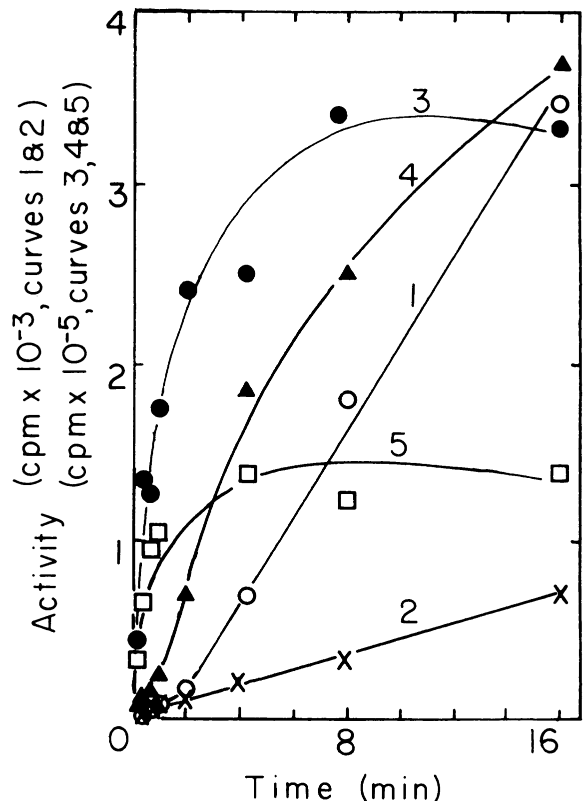

FIG. 4. Assimilation of $C^{14}O_2$ into free sugars of the avocado during short period photosynthesis. ○, sucrose (curve 1); X, mannoheptulose (curve 2); ●, fructose (curve 3); ▲, glucose (curve 4); □, sedoheptulose (curve 5).

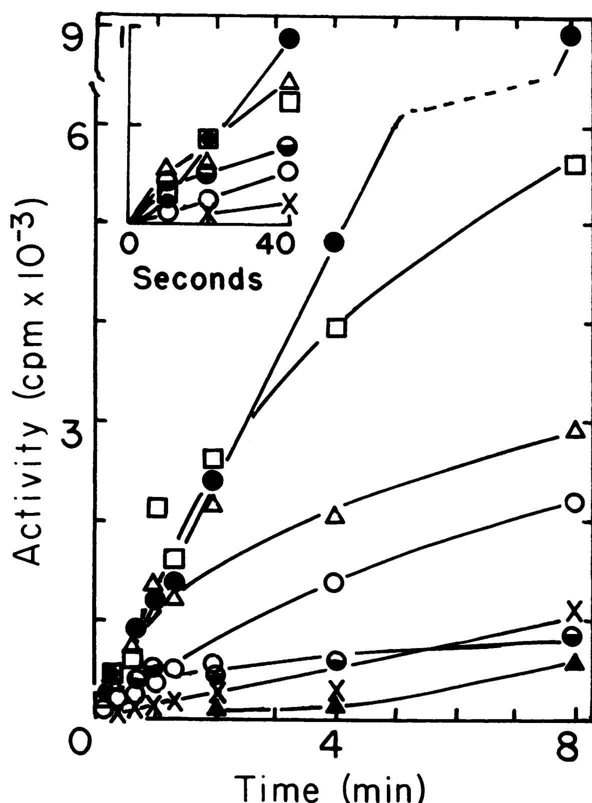

FIG. 5. Assimilation of $C^{14}O_2$ into the phosphate esters of avocado leaves during short period photosynthesis. △, phosphoglyceric acid; ○, diphosphates (mainly glucose); ●, glucose monophosphates; □, sedoheptulose monophosphate; X, mannoheptulose monophosphate; ○, fructose monophosphate; ▲, nucleotides.

sugar activity in beans amounts only to about 2% of the phosphate ester activity while the free sugar activity in avocados amounts to more than a third that of the phosphates.

Discussion

There are a number of ways in which mannoheptulose might be formed during photosynthesis making use of variations of known reactions and common, or plausible intermediates. Among these would be: 1. an abnormal transaldolase or aldolase reaction forming the heptulose with a *cis-* rather than the normal *trans-* configuration at the point of condensation (9, 19, 20); 2. a *trans-* rather than normal *cis-* configuration resulting from a transketolase condensation; 3. direct epimerization of sedoheptulose or its phosphate ester, at C-4 as suggested by Venkataraman and Racker (20); 4. reduction of sedoheptulose at C-2 (giving the volemitol configuration) with re-oxidation at C-6 producing mannoheptulose with C-1 equivalent to the original C-7 of sedoheptulose. The original intent of this study was to determine whether a kinetic examination of the detectable, early products of photosynthesis might indicate a choice between these mechanisms. It was found that mannoheptulose phosphate, discovered in earlier experiments (3, 14), is formed very early in the photosynthetic sequence, although in low quantities. Extrapolation of the mannoheptulose phosphate labeling curve would indicate that it is formed either simultaneously or very shortly after the other ketose phosphates. No evidence could be found for isotope in any of the products which might be associated with the alternate reduction and oxidation of opposite ends of sedoheptulose so that the inversion pathway might be considered of very low probability. Although some anomolous phosphate esters were present in some experiments, these would not seem to have any apparent specific bearing upon the synthetic mechanism. No data were obtained which would enable choice between any of the other proposed mechanisms.

The kinetic data here indicate that Nordal and Benson (14) may have been prophetic in their proposal that mannoheptulose was formed from normal intermediates and might accumulate in avocados due to an active mannoheptulose phosphate phosphatase and/or a sluggish kinase. Mannoheptulose phosphate appears after only a few seconds of photosynthesis along with the normal phosphate esters. The ketoses, fructose, sedoheptulose, mannoheptulose, and probably ribulose, appear almost simultaneously with their corresponding phosphate esters. A number of factors suggest that this early labeling of the unphosphorylated ketoses may be normal within the avocado leaf and associated with the accumulation of mannoheptulose. It does not seem likely that the rapid hydrolysis of the phosphate esters could be simply an artifact of procedure. No similar hydrolysis of photosynthetically labeled phosphate esters was detected in leaves from a number of other plants (pinto bean, soy bean, sugar beet, barley) subjected to identical procedures of exposure, killing, extraction, and chromatography. Other experiments show (unpublished) the phosphate esters formed in avocado leaf disks from labeled sugars have little tendency to hydrolyze under similar conditions of processing making it unlikely that an unique condition of the avocado leaf is responsible for the ester hydrolysis. Instead, it would seem more likely that the rapid appearance of the unphosphorylated sugars may be due to the action of a phosphatase during photosynthesis. The high activity of the ketoses, relative to the activity in glucose, even where glucose phosphates may predominate, suggests that the phosphatase might be specific for ketoses.

The pools for sedoheptulose and for fructose appear to saturate rapidly (fig 4) while that for mannoheptulose continues to accumulate activity on a linear basis over a long period (3). This suggests that there may be a rather close equilibrium between the reactions for producing and re-utilizing the fructose and sedoheptulose while mannoheptulose is poorly re-utilized. A kinase, specific for the C-3, C-4 configuration of fructose and of sedoheptulose, lo-

cated in close proximity to the phosphatase could satisfactorily account for the observed behavior. Thus, the basis for mannoheptulose accumulation in avocado leaves might be the combined action of a phosphatase, giving rise to all ketoses rapidly, and a specific kinase which would return fructose and sedoheptulose, but not mannoheptulose, to active metabolic pools.

Previous studies (3) showed that fructose continued to accumulate activity at a slow rate during photosynthesis but dark experiments made it apparent that the fructose was being formed at the expense of labeled sucrose rather than from hydrolysis of photosynthetically formed fructose phosphate. Thus, it is clear that two distinct fructose pools are involved in the metabolism of the avocado leaf. One, a very small pool, rapidly labeled during photosynthesis, may be located within the chloroplast. The second pool, derived from the splitting of sucrose, would probably be in the cytoplasm or vacuole. The dark accumulation of activity in fructose was not observed in some other plants (3). Thus fructose in the latter pool may be subject to the same sluggish metabolism manifest for mannoheptulose. This is in sharp contrast with the apparent rapid re-utilization of fructose produced in the short periods of photosynthesis.

Summary

The assimilation of $C^{14}O_2$ into sugars and phosphate esters in avocado leaf disks during photosynthetic periods of 10 seconds to 16 minutes has been studied. The labeling of mannoheptulose phosphate after a short photosynthetic period was demonstrated. No evidence for perseitol phosphate was found.

Fructose, sedoheptulose, mannoheptulose, and possibly ribulose in their unphosphorylated form become labeled in avocado leaves very early but the pools of fructose and sedoheptulose appear to saturate rapidly while mannoheptulose and sucrose pools continue to accumulate activity at a linear rate over a long period of time.

A mechanism for the accumulation of mannoheptulose based on a phosphatase specific for ketose esters and a kinase specific for fructose and sedoheptulose configuration is suggested.

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