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# LABELLING OF CARBOHYDRATE BY [$^{14}$C]GLYCEROL SUPPLIED TO SUSPENSION CULTURES OF SOYBEAN

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**Key Word Index**—*Glycine max*; Leguminosae, soybean; suspension cultures, [U-$^{14}$C]glycerol metabolism to starch, [U-$^{14}$C]sucrose metabolism

**Abstract**—Suspension cultures of *Glycine max* were incubated for 4, 12 and 24 hr in [U-$^{14}$C]glycerol in 0.2 M potassium dihydrogen phosphate, in [U-$^{14}$C]glycerol in growth medium, and for 24 hr in [U-$^{14}$C]sucrose in growth medium. The detailed distribution of $^{14}$C was determined. Starch, other polysaccharides, sucrose, glucose and fructose were labelled by [U-$^{14}$C]glycerol. This labelling of starch was appreciable in comparison to that achieved by [$^{14}$C]sucrose. The results are held to be consistent with the view that, in non-photosynthetic cells, conversion of sucrose to starch involves breakdown of sucrose to triose phosphate, entry of the latter into the amyloplast and conversion therein to starch

## INTRODUCTION

Amyloplasts from suspension cultures of soybean contain the enzymes needed to convert triose phosphate to starch, viz: triosephosphate isomerase, aldolase, fructose bisphosphatase, glucosephosphate isomerase, phosphoglucomutase, ADPglucose pyrophosphorylase and starch synthase. The capacities of these enzymes in the amyloplasts exceed the rate at which the cells accumulate starch These observations, and the intracellular distribution of other enzymes of carbohydrate metabolism, are consistent with the view that, in non-photosynthetic cells of plants, at least some of the sucrose destined for starch is first metabolized in the cytosol to triose phosphate which then enters the amyloplast for conversion to starch [1]. Against this hypothesis might be set the view that gluconeogenesis and fructose bisphosphatase are not normally found in non-photosynthetic cells of higher plants unless these cells are converting fat or protein to carbohydrate However, an inability to carry out the latter conversions is likely to be due in the first instance to the absence of the glyoxylate cycle [2] and phosphoenolpyruvate carboxykinase [3]. A non-photosynthetic cell could lack both the glyoxylate cycle and the carboxykinase, and yet be capable of converting triose phosphate to starch, provided it had a fructose bisphosphatase. There is evidence that soybean cultures [1] and roots of pea and maize [4] contain this enzyme.

If non-photosynthetic, non-gluconeogenic cells of plants do make sucrose into starch via triose phosphate, then the addition of [$^{14}$C]glycerol to such cells would be expected to label triose phosphates and thence starch. The aim of the work reported in this paper was to see if such labelling of starch occurred in soybean cultures.

## RESULTS AND DISCUSSION

We used 7-day-old cultures because we knew their starch content (3.4 mg/g fr. wt) and the rate (4 nmol/min per g fr. wt) at which they accumulated starch [Leja, M and apRees, T., unpublished observations]. Comparable samples of cells were incubated for 4, 12 and 24 hr in [U-$^{14}$C]glycerol in 0.2 M potassium dihydrogen phosphate and in [U-$^{14}$C]glycerol in half-strength growth medium. We varied the feeding conditions to see if any labelling of starch depended upon the physiological state of the cells Cells in growth medium represent growing cells; those in glycerol for 4 hr, stationary cells; and those in glycerol for 24 hr, starving cells. To compare any labelling of starch by [$^{14}$C]glycerol with that from the more normal precursor, we incubated samples of cells in half-strength growth medium in which the sucrose was labelled.

At the end of each incubation the sample was checked for microbial contamination, washed, killed and analysed. All data are from samples that showed no sign of contamination after 3 weeks incubation on two different media. We conclude that our results reflect solely the metabolism of the soybean cells. We analysed the washings from the 24 hr samples from each type of medium At least 85% of the label in the washings was recovered as the compound initially supplied, and no significant accumulation of label was found in any other compound We argue that washing the samples did not remove significant amounts of labelled metabolites The washed samples were divided into ethanol-soluble and -insoluble material Volatile constituents and lipids (ether-soluble fraction) were removed from the former which was then divided into its basic (mostly amino acids), acidic (mostly organic acids) and neutral (mostly sugars) components. Starch was removed from the ethanol-insoluble material by dispersal in boiling water followed by treatment with amyloglucosidase and α-amylase. Glucose was isolated from this hydrolysate and its labelling is taken as a measure of the labelling of starch We investigated the reliability of this procedure by subjecting the dispersion to a second digestion with the hydrolases We did this for each type of sample. In no instance did the second digestion increase

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the amount of [$^{14}$C]glucose recovered The complete ethanol-insoluble fraction was also hydrolysed and the products were analysed Label recovered in the neutral components of the hydrolysate is attributed to sugars derived from both storage and structural polysaccharides The difference between the labelling of this neutral fraction and that of starch almost certainly represented incorporation into cell-wall polysaccharides The basic components of the hydrolysate are held to represent protein In order to compare different treatments and different samples, the $^{14}$C recovered in each fraction of a sample is expressed as a percentage of the total $^{14}$C metabolized by that sample This total is obtained for each sample by summing the label in $^{14}CO_2$, the ethanol-soluble and the ethanol-insoluble fractions The $^{14}$C recovered in the ultimate fractions of our analyses amounted to 85–95% of that metabolized, thus, our data are not seriously affected by losses during analysis

The growing cultures readily absorbed [$^{14}$C]sucrose and metabolized it in the expected way (Table 1) The label recovered as $^{14}CO_2$ and in the fractions that contained lipids, organic acids, amino acids and proteins represents that portion of the [$^{14}$C]sucrose that entered the respiratory pathways, and amounted to 47% of that metabolized There was appreciable labelling of starch but it was not more than 15% of the total metabolized and was well below the 24% of label recovered in the neutral components of the hydrolysate of the total ethanol-insoluble material This suggests that sucrose was as readily converted to structural polysaccharides as to starch

When [$^{14}$C]glycerol was supplied alone it was steadily absorbed and metabolized After 4 hr 39% of that supplied had been metabolized and the detailed distribution of label (not shown) was similar to that at 12 hr except that there was less in carbon dioxide (14%) and more in the ethanol-soluble material (79%) The absorbed [$^{14}$C]glycerol was rapidly and extensively metabolized As expected there was appreciable incorporation into the fraction that contained the lipids Labelling of carbon dioxide and the fractions that contained the organic acids, amino acids and proteins was extensive, 57% of the metabolized label This provides compelling evidence that much of the [$^{14}$C]glycerol was converted to dihydroxyacetone phosphate which then entered the respiratory pathways The crucial feature of [U-$^{14}$C]glycerol metabolism is that not only was starch labelled but there was also label in other polysaccharides and in sugars The percentage of metabolized label in each of the above was small but the amounts of $^{14}$C were appreciable and readily measurable The total $^{14}$C recovered in sugars isolated from the neutral fraction of the hydrolysate, and in free sugars was 5 5, 7 0 and 5 9, respectively, for cultures incubated for 4, 12 and 24 hr The most noteworthy

Table 1 Distribution of label from [U-$^{14}$C]glycerol and [U-$^{14}$C]sucrose supplied to suspension cultures of soybean

| | $^{14}$C per fraction as % total $^{14}$C metabolized | | | | |
|---|---|---|---|---|---|
| | [$^{14}$C]Sucrose in culture medium for | [$^{14}$C]Glycerol in $KH_2PO_4$ for | | [$^{14}$C]Glycerol in culture medium for | |
| Cell fraction | 24 hr | 12 hr | 24 hr | 4 hr | 24 hr |
| $CO_2$ | 20 3 | 24 0 | 35 8 | 10 3 | 13 9 |
| Starch | 14 5 | 1 6 | 0 5 | 5 8 | 6 3 |
| Ethanol-insoluble material | 36 8 | 10 7 | 13 8 | 12 8 | 15 4 |
| Basic components | 6 5 | 5 8 | 0 1 | 3 1 | 3 1 |
| Acidic components | 2 2 | 0 6 | 0 9 | 6 3 | 1 2 |
| Neutral components | 23 9 | 3 3 | 2 8 | 7 4 | 7 1 |
| Galactose | 2 0 | 0 7 | 0 4 | 0 5 | 0 5 |
| Glucose | 11 9 | 1 5 | 0 5 | 4 4 | 4 2 |
| Mannose | 1 7 | 0 7 | 0 6 | 0 4 | 0 4 |
| Arabinose | 0 3 | 0 2 | 0 1 | 0 1 | 0 1 |
| Xylose | 1 1 | 0 3 | 0 3 | 0 3 | 0 3 |
| Ethanol-soluble material | 42 9 | 66 0 | 50 7 | 77 4 | 71 0 |
| Volatile constitutents | 16 6 | 22 5 | 9 6 | 27 9 | 18 6 |
| Ether-soluble constituents | 4 9 | 6 6 | 9 1 | 24 2 | 32 8 |
| Water-soluble constituents | 21 2 | 36 9 | 30 2 | 25 3 | 19 6 |
| Basic components | 8 9 | 12 1 | 13 2 | 8.8 | 5 4 |
| Acidic components | 6 1 | 15 3 | 9 0 | 10 9 | 5 0 |
| Neutral components | 8 6 | 5 2 | 5 2 | 2 7 | 3 3 |
| Sucrose | 6.9 | 2 9 | 2 4 | 1 0 | 0 8 |
| Glucose | 0.3 | 0 4 | 0 8 | 0 2 | 0 1 |
| Fructose | 0.3 | 0 3 | 0 8 | 0 2 | 0 2 |
| Glycerol | 0 3 | 0 6 | 0 3 | 0 6 | 0 3 |
| Sum of $^{14}$C recovered in fractions as % of $^{14}$C metabolized | 90 0 | 93 5 | 84 1 | 87 5 | 86 7 |
| $^{14}$C metabolized as dpm × $10^{-4}$ | 584 | 206 | 278 | 267 | 304 |
| as % $^{14}$C supplied | 35 | 53 | 72 3 | 70 2 | 80 2 |

differences between the patterns at 12 and 24 hr are that at 24 hr there had been a decline in the labelling of starch and an increase in that of carbon dioxide. These changes probably reflect consumption of starch and the increasing use of the supplied glycerol by the respiratory pathways in the starving cells.

The presence of the growth medium increased the rate at which the added [$^{14}$C]glycerol was metabolized. The distribution at 12 hr was intermediate between those at 4 and 24 hr, and is not shown. The patterns observed are qualitatively similar to those for [$^{14}$C]glycerol alone but differ quantitatively in some respects. More label was recovered in the lipid fraction and less in those that contained the respiratory products. This suggests that in the growing cells more of the [$^{14}$C]glycerol was used for lipid synthesis and, thus, less was available for conversion to dihydroxyacetone phosphate. More importantly, there was a 4–6-fold increase in the proportion of metabolized label recovered in starch. After 24 hr this proportion was over 40% of that found for sucrose under similar conditions (Table 1). In contrast to [$^{14}$C]sucrose, when [$^{14}$C]glycerol was fed to growing cells most of the label in the neutral fraction of the hydrolysate could be accounted for as starch. This strongly suggests that, whilst [$^{14}$C]sucrose labels starch and structural polysaccharides more or less equally readily, [$^{14}$C]glycerol preferentially labels starch.

Regardless of the length of the incubation and irrespective of whether the cells were growing, stationary, or starving, starch was always labelled by [$^{14}$C]glycerol. In the presence of growth medium the proportion of [$^{14}$C]glycerol converted to starch was almost half that found for the usual precursor of starch. Bearing in mind that these samples used nearly a third of the absorbed [$^{14}$C]glycerol for lipid synthesis, and that the remainder entered the respiratory pathways below the points at which the respiration of sucrose is controlled, then the conversion of [$^{14}$C]glycerol to starch must be regarded as comparable to that found for sucrose. This indicates that there is a route from triose phosphate to sucrose in these cells. Further support for this view is provided by the fact that [$^{14}$C]glycerol labelled other polysaccharides and also free sugars. The preferential labelling of starch, as opposed to structural polysaccharides, by [$^{14}$C]glycerol, and the lack of this preference by [$^{14}$C]sucrose might be expected if sucrose is converted to starch via triose phosphate. Indirect support for the view that non-photosynthetic non-gluconeogenic cells of plants can convert glycerol to polysaccharide is provided by the evidence that glycerol can support some degree of growth [5, 6] and differentiation [7] in certain tissue cultures.

Two very different sets of observations, the metabolism of [$^{14}$C]glycerol and the enzymic properties of amyloplasts concur in their support of the view that soybean cells can convert sucrose to starch via triose phosphate with the latter entering the amyloplast. The extent to which this is the sole route from sucrose to starch is not known. Further progress will depend largely upon the development of a means of isolating amyloplasts that retain their transport properties.

## EXPERIMENTAL

Cultures of soybean (*Glycine max* L. cv. Acme) derived from and grown as those in ref. [8] were harvested by filtration through glass-fibre paper and divided into samples of 0.7–0.9 g fr. wt. Each sample was incubated in 3 ml medium in a 100-ml flask fitted with a centre well that contained 10% KOH to absorb $CO_2$. The media were (a) the medium used to grow the cells but at half strength and containing [U-$^{14}$C]sucrose so that the concn. of labelled sucrose supplied to the cells was 41 mM and its sp. act. was 51.3 $\mu$Ci/mmol, (b) 2.5 mM [U-$^{14}$C]glycerol (0.33 Ci/mol) in 0.2 M $KH_2PO_4$ adjusted to pH 5.2 with KOH, (c) medium (b) plus half strength growth medium so that the concn. of sucrose was 41 mM and the sp. act. of [U-$^{14}$C]glycerol was 0.33 Ci/mol. The flasks were stoppered with cotton wool and shaken gently in the dark at 25°. At the end of the incubation small amounts of each sample were plated on half-strength growth medium in 1% (w/v) agar and on 2% (w/v) Bacto Difco nutrient agar and incubated at 25° and 37°, respectively, for 3 weeks.

At the end of the feeding period, cells were separated from the incubation medium by filtration, rinsed with 10 then 40 ml of unlabelled medium and killed with boiling 80% aq. EtOH. Each sample was then extracted × 3 with 10-ml vols. of 80% aq. EtOH to give the EtOH-insoluble and -soluble materials. The latter was evaporated to dryness at 20° *in vacuo*. The difference in $^{14}$C content between the residue obtained and the original EtOH soln. is attributed to volatile constituents. The residue was extracted with $Et_2O$ to give the $Et_2O$-soluble constituents and then with $H_2O$ to give the $H_2O$-soluble constituents.

The EtOH-insoluble material was dried at 70° for 20 min and homogenized in 5 ml $H_2O$. Total $^{14}$C was determined after treating a portion of this homogenate with NCS tissue solubilizer (BDH, Poole, U.K.). For measurement of label in starch, 2-ml samples of the suspension were autoclaved, made 0.1 M with NaOAc, pH 4.8, and then 2.7 units/ml amyloglucosidase (EC 3.2.1.13) and 0.2 units/ml pig pancreas α-amylase (EC 3.2.1.1) were added. This mixture was incubated for 12 hr at 37° and then centrifuged at 9000 *g* for 6 min. The pellet was washed with $H_2O$ by resuspension and centrifugation and the washings and supernatant were combined to give a soln. from which glucose was isolated by PC. Only material that co-chromatographed with glucose is ascribed to starch. For complete hydrolysis, 1 ml of the suspension of the EtOH-insoluble material was centrifuged. The pellet was incubated in 0.14 ml 26 M $H_2SO_4$ at 20° for 4 hr, then it was recombined with the supernatant and the whole suspension diluted to 0.33 M $H_2SO_4$ with $H_2O$, autoclaved and neutralized as in ref. [9].

The neutralized hydrolysate, the $H_2O$-soluble constituents and the washings were analysed by ion exchange chromatography as in ref. [10], which also describes how sugars were isolated by PC and $^{14}$C was assayed.

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