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The Pentose Phosphate Pathway in Relation to Fat Synthesis in the Developing Castor Oil Seed[1]

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ABSTRACT

Slices of 25- to 28-day-old developing castor bean endosperm were incubated with various ^{14}C- and ^{3}H-labeled substrates to determine the amount of glucose dissimilated in the pentose phosphate pathway and to determine the use of the reduced nucleotides so produced in fatty acid synthesis. Ten to 12% of the metabolized glucose traversed the pentose phosphate pathway, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) production would be sufficient to supply 51 to 68% of the reducing equivalents required for fat synthesis. However, using ^{3}H-NADPH produced from 3-^{3}H-glucose as a tracer, it was found that only 40% of the NADPH produced in the pentose phosphate pathway was used in fat synthesis. Thus the actual contribution of the reducing equivalents generated from the pentose phosphate pathway to fat synthesis was 20 to 27% of that required. Because of the methods and assumptions, this value represents a minimal estimate of NADPH used in fat synthesis, and the actual contribution may be somewhat higher. However, tritium from ^{3}H-NADH generated from 1-^{3}H-ethanol was incorporated into fatty acids, and it is contended that NADH may supply a large proportion of the reducing equivalents necessary for fat synthesis in this tissue.

Fatty acid synthesis *in vitro* from acetyl-CoA or malonyl-CoA requires NADPH (5, 10, 18, 23) or NADPH and NADH (7, 9, 20, 25) as a source of reducing power. Direct proof for the *in vivo* reduced nucleotide requirement is difficult to obtain, but the problem can be studied by relating the amount of NADPH generated in the pentose phosphate pathway to its utilization in the cell with reference to fat synthesis. Using this method, Katz *et al.* (11) observed with rat adipose tissue that the NADPH produced in the PPP[3] was sufficient to supply 59 to 88% of the required reducing equivalents for fat synthesis. Kikuta and Erickson (15) showed that NADPH produced via the PPP was one-half of the reducing power necessary in avocado while Agrawal and Canvin (3) demonstrated that NADPH produced via the PPP would supply 50 to 72% of the reducing power required for fat synthesis in the developing castor bean endosperm. While such studies show the amount of NADPH that is available for fat synthesis, they provide no evidence to show that the NADPH produced in the PPP is indeed used for fat synthesis. Since the reductive hydrogen is retained on the fatty acid (6, 19), it should be possible to obtain direct evidence on the source of reducing hydrogen by generating intracellularly tritiated NADPH or NADH. With this approach it has been observed that ^{3}H-NADPH generated from 1-^{3}H-glucose was a better source of reducing power for fat synthesis than ^{3}H-NADH generated from 2-^{3}H-glycerol or 1-^{3}H-ethanol (8, 21). Katz and Rognstad (12) showed that 75% of the hydrogen equivalents were provided by NADPH and 25% by NADH. They also demonstrated that there was a considerable isotope discrimination against a tritium transfer from 1-^{3}H-glucose to ^{3}H-NADPH and showed that it was superior to use 3-^{3}H-glucose to measure ^{3}H-NADPH production in the PPP. In this report the production and use of reduced nucleotides in the developing castor bean endosperm were studied by generating tritiated NADPH and NADH intracellularly from specifically labeled tritium substrates and following their incorporation into fatty acids.

MATERIALS AND METHODS

Plant Material. Plants were grown as described by Agrawal and Canvin (2).

Radioactive Material. 1-^{14}C-Glucose, 6-^{14}C-glucose, U-^{14}C-glucose, 1-^{3}H-glucose, and 6-^{3}H-glucose were purchased from Radiochemical Centre, Amersham, England. 1-^{3}H-Ethanol and 1-^{14}C-ethanol were purchased from New England Nuclear Corporation, Waltham, Massachusetts. 3-^{3}H-Glucose was a generous gift from Dr. Joseph Katz, Cedars-Sinai Medical Center, Los Angeles, California.

Incubation of Tissue with Radioactive Substrates. Seeds were harvested 25 to 28 days after pollination. Slices were prepared and tissues were incubated with radioactive substrates at 25 C for 3 hr according to the method described by Agrawal and Canvin (3). Four isotopic combinations were obtained by mixing the ^{14}C- and ^{3}H-labeled substrates: 1-^{14}C-1-^{3}H-ethanol, 1-^{14}C-1-^{3}H-glucose, U-^{14}C-3-^{3}H-glucose, and 6-^{14}C-6-^{3}H-glucose. The activity used per sample of tissue was 2 to 3 μc for ^{14}C-glucose; 0.5 μc ^{14}C-ethanol; 10 to 15 μc 1-^{3}H-glucose, 6-^{3}H-glucose, or 1-^{3}H-ethanol; and 7 μc 3-^{3}H-glucose.

Tritiated substrates were tested for nonenzymatic exchange with water and found to contain the following amount of exchangeable tritium: 1-^{3}H-glucose, 0.28%; 3-^{3}H-glucose, 0.62%; and 6-^{3}H-glucose, 0.30%. All the reported values for tritium content in water have been corrected for this exchange.

All experiments were performed twice and both results are presented.

Extraction of Tissues. Tissues and incubation medium were frozen separately, after the termination of experiments, in a Dry Ice-acetone bath. Both were lyophilized separately to trap ^{3}H-water formed during the experiment. The tissues were then

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[3] Abbreviation: PPP: pentose phosphate pathway.

extracted with boiling ethanol (3). Fractionation of lipids and water-soluble materials and analysis of the incubation medium were done as described by Agrawal and Canvin (3).

Assay of Radioactivity. Radioactivity was measured in a Nuclear-Chicago liquid scintillation counter. Water-soluble materials were counted with dioxane scintillator; ether-soluble and $^{14}CO_2$ were counted with toluene scintillator (2, 3). Full details of the separate counting of ^{3}H and ^{14}C in the same sample were presented by Agrawal (1).

RESULTS AND DISCUSSION

1-^{14}C-Ethanol was rapidly metabolized by castor bean endosperm, and 18 to 30% of the ^{14}C was incorporated into fatty acids (Table I). Presumably this occurred via its conversion to acetate, and thus 1-^{3}H-1-^{14}C-ethanol could be used to generate ^{3}H-NADH, since both the alcohol and aldehyde dehydrogenases are NAD-specific (16). 1-^{14}C-1-^{3}H-Glucose and U-^{14}C-3-^{3}H-glucose were used to generate ^{3}H-NADPH (12) and to compare the efficiency of hydrogen transfer from these labeled compounds to fatty acids. 6-^{14}C-6-^{3}H-Glucose was used to calculate the incorporation of carbon-bound tritium into fatty acids from 1-^{14}C-1-^{3}H-glucose (12).

The incorporation of ^{3}H and ^{14}C from the above substrates into various fractions was determined (Table I), and the specific yields were calculated (Table II). The specific yield is the ratio of the radioactivity found in a product to the total radioactive substrate metabolized. The major labeled product derived from tritium in all cases was water, the yield ranging from 74 to 94% (Table II). However, the tritium content in water from 1-^{3}H-ethanol (Table II) may not represent the true value because tritium in the water was determined by a freeze-drying method and some 1-^{3}H-ethanol may appear in the condensed water.

The recovery of utilized tritium to that of ^{14}C was in close agreement except for 3-^{3}H-glucose (Table I). It should be emphasized here that with 3-^{3}H-glucose uniformly labeled ^{14}C-glucose was present. All the tritium from 3-^{3}H-glucose entering the PPP would be removed, but, for every three molecules of U-^{14}C-glucose metabolized via the PPP, two-thirds would be regenerated and would mix with the common hexose pool (14). Thus ^{3}H recovery in the products would be greater than ^{14}C and a discrepancy in the balance sheet would appear.

In general, a higher proportion of tritium was incorporated into fatty acids from glucose than from ethanol (Table II). Of the tritium from 6-^{3}H-glucose 5.1% was found in the fatty acids (Table II, experiment 1), and this should all be incorporated as carbon-bound tritium. The $^{3}H/^{14}C$ ratio from 6-^{14}C-6-^{3}H-glucose was 0.20 (Table III), a value lower than expected if the tritium was only labilized from acetate during its conversion to fatty acids. The expected value of the $^{3}H/^{14}C$ ratio from 6-^{14}C-6-^{3}H-glucose in fatty acids depends upon chain length and for a $C_{18:0}$ fatty acid it can be calculated as follows:

$$\frac{1 + 8 \times 0.33}{9} = 0.41$$

Thus a ratio of 0.41 would be expected if no other loss of ^{3}H occurred. However, the observed ratio of 0.20 (Table III) indicated that approximately 50% of the tritium was labilized prior to its conversion to acetate. $^{3}H/^{14}C$ ratios of 0.15 in fatty acids were also observed by Katz and Rognstad (12) from the incorporation of 6-^{14}C-6-^{3}H-glucose into fatty acids by rat adipose tissue. These authors ascribed their low yield of tritium to the cycling of pyruvate through oxalacetate via a carboxylation and decarboxylation. However, the $^{3}H/^{14}C$ ratios in fatty acids and their $^{3}H/^{14}C$ ratios of 0.35 in lactic acid would necessitate that all the pyruvate is recycled. This would seem unlikely. Although there seems to be no evidence to support the idea, a loss of 50% of the ^{3}H during the con-

Table I. *Recovery of ^{3}H and ^{14}C in Products*

Castor bean endosperm slices (500 mg) from 25- to 28-day-old seeds were incubated for 3 hr in a total volume of 1 ml containing 0.5 ml of 0.2 M potassium phosphate buffer, pH 7.0; 7 to 15 μc of ^{3}H-glucose and 2 to 3 μc of ^{14}C-glucose (30 μmole); or 10 μc of 1-^{3}H-ethanol and 0.5 μc of 1-^{14}C-ethanol (3 μmole). In experiment 2 only 5 μc of 3-^{3}H-glucose were present.

Experiment	Substrate	$^{3}H_2O$	$^{14}CO_2$	Fatty Acid	Glycerol	Organic Acid	Amino Acid	Total
		μmoles substrate per 500 mg tissue per 3 hr						*μmoles*
1	Ethanol-1-^{3}H	2.02	...	0.07	...	0.21	0.02	2.32
	Glucose-1-^{3}H	4.10	...	0.39	0.10	0.48	0.22	5.29
	Glucose-3-^{3}H	5.00	...	0.63	0.07	0.35	0.16	6.21
	Glucose-6-^{3}H	4.23	...	0.28	0.09	0.65	0.27	5.52
	Ethanol-1-^{14}C	...	1.47	0.47	...	0.46	0.26	2.66
	Glucose-1-^{14}C	...	1.83	1.05	0.09	1.49	0.81	5.27
	Glucose-6-^{14}C	...	0.72	1.58	0.09	1.66	0.90	5.05
	Glucose-U-^{14}C	...	1.84	0.97	0.13	1.50	0.61	5.05
2	Ethanol-1-^{3}H	1.68	...	0.06	...	0.05	...	1.79
	Glucose-1-^{3}H	4.53	...	0.58	0.13	0.41	0.18	5.83
	Glucose-3-^{3}H	5.36	...	0.87	...	0.05	...	6.28
	Glucose-6-^{3}H	4.10	...	0.42	0.15	0.71	0.20	5.58
	Ethanol-1-^{14}C	...	0.57	0.33	...	0.14	0.06	1.10
	Glucose-1-^{14}C	...	2.57	1.58	0.11	1.20	0.54	6.00
	Glucose-6-^{14}C	...	1.05	1.99	0.14	1.72	0.83	5.73
	Glucose-U-^{14}C	...	2.49	1.26	0.10	1.19	0.68	5.72

Table II. *Specific Yield of ^{3}H and ^{14}C in Products from ^{3}H- and ^{14}C- Labeled Substrates*

Specific yield is the ratio of the radioactivity found in a product to the total radioactive substrate metabolized.

Experiment	Substrate	$^{3}H_2O$	$^{14}CO_2$	Fatty Acid	Glycerol	Organic Acid	Amino Acid
1	Ethanol-1-^{3}H	0.843	...	0.038	...	0.108	0.011
	Glucose-1-^{3}H	0.775	...	0.074	0.019	0.091	0.042
	Glucose-3-^{3}H	0.805	...	0.101	0.011	0.056	0.026
	Glucose-6-^{3}H	0.766	...	0.051	0.016	0.118	0.049
	Ethanol-1-^{14}C	...	0.553	0.177	...	0.172	0.098
	Glucose-1-^{14}C	...	0.347	0.199	0.017	0.283	0.154
	Glucose-6-^{14}C	...	0.142	0.313	0.018	0.329	0.198
	Glucose-U-^{14}C	...	0.365	0.192	0.026	0.297	0.120
2	Ethanol-1-^{3}H	0.942	...	0.031	...	0.027	...
	Glucose-1-^{3}H	0.778	...	0.099	0.022	0.070	0.031
	Glucose-3-^{3}H	0.853	...	0.139	...	0.008	...
	Glucose-6-^{3}H	0.735	...	0.075	0.027	0.127	0.036
	Ethanol-1-^{14}C	...	0.520	0.300	...	0.127	0.054
	Glucose-1-^{14}C	...	0.430	0.264	0.018	0.200	0.089
	Glucose-6-^{14}C	...	0.183	0.348	0.025	0.300	0.145
	Glucose-U-^{14}C	...	0.435	0.220	0.018	0.208	0.119

Table III. *$^{3}H/^{14}C$ Ratios in Fatty Acids and Glycerol*

Labeled Substrate	Fatty Acid	Glycerol
Ethanol-1-^{3}H-1-^{14}C	0.17	...
Glucose-1-^{3}H-1-^{14}C	0.37	1.15
Glucose-3-^{3}H-U-^{14}C	0.67	0.27
Glucose-6-^{3}H-6-^{14}C	0.20	1.40

version of 2-phosphoglyceric acid to pyruvate would also explain the final $^3H/^{14}C$ ratios. Thus the $^3H/^{14}C$ ratio of pyruvate and acetate would be 0.5 and the ratio expected in fatty acids by the method previously outlined would be 0.2.

Since carbon 1 and 6 of glucose metabolized in the Embden-Meyerhof-Parnas (EMP) pathway are considered to be equivalent at the triose phosphate level, the $^3H/^{14}C$ ratios 6-^{14}C-6-^{3}H-glucose can be used to calculate the amount of ^3H incorporated into fatty acids in association with carbon 1 of glucose. Correction for this nonreductive incorporation of ^3H can be done from the incorporation of ^{14}C from glucose-1-^{14}C into fatty acids (Table II, experiment 1, 19.9%) and the values for the $^3H/^{14}C$ ratios observed with 6-^{14}C-6-^{3}H-glucose (Table III, 0.20). Hence, the incorporation of ^3H attached to glucose carbon 1 was: $19.9 \times 0.20 = 3.9\%$. Thus, only 3.5% (7.4 − 3.9 = 3.5%) of the tritium from 1-^{3}H-glucose was incorporated into fatty acids via ^3H-NADPH.

Incorporation of ^3H from 3-^{3}H-glucose into fatty acids could occur only via ^3H-NADPH arising during the 6-phosphogluconate dehydrogenase reaction (13). Incorporation of ^3H from 3-^{3}H-glucose into fatty acids via ^3H-NADPH was three times that derived by the same mechanism from 1-^{3}H-glucose (Table II). This observed difference corroborates the earlier results of Katz, Rognstad, and Kemp (13) which were explained as being due to glucose 6-phosphate dehydrogenase discrimination against 1-^{3}H-glucose and preferential utilization of unlabeled glucose. If there had been no istotope discrimination effect, then ^3H incorporation via ^3H-NADPH from both glucose molecules would have been the same and the corrected $^3H/^{14}C$ ratios in the product would also have been the same (Table III). In cases of isotope discrimination, the isotope concentration in one of the products would be lower and in the other higher than the substrate. This is observed in this experiment, where the corrected $^3H/^{14}C$ ratio in fatty acid from 1-^{14}C-1-^{3}H-glucose was 0.17 (for ^3H incorporation attached to carbon it is the same as the $^3H/^{14}C$ ratio from 6-^{3}H-6-^{14}C-glucose) and the ratio in glycerol was 1.15 (Table III).

If tritium incorporation into fatty acids from 1-^{3}H-glucose is compared to that from 3-^{3}H-glucose, it is readily apparent that a considerable underestimation of the PPP ^3H-NADPH production would result if only 1-^{3}H-glucose was used to estimate the value. This study shows that 3-^{3}H-glucose is preferred over 1-^{3}H-glucose as a substrate to study the production of NADPH in the PPP and further emphasizes that caution must be exercised in the interpretation of data from 1-^{3}H-glucose (13).

In order to assess the quantitative contribution of NADPH to fatty acid synthesis, using ^3H-NADPH generated in the PPP as a tracer, certain assumptions must be made. First, it must be assumed that there is no discrimination against ^3H-NADPH by the dehydrogenases of the fatty acid synthesis pathway. Discrimination (such as discussed above for the glucose 6-phosphate dehydrogenase) would result in a reduced amount of ^3H incorporated into fatty acids. Second, it must be assumed that ^3H from the β-stereo configuration of NADPH is incorporated into fatty acids. Stern and Vennesland (22) have shown that the hydrogen is transferred from either glucose 6-phosphate or 6-phosphogluconic acid to the β-position of NADPH. Matthes *et al.* (19) have shown that a fatty acid-synthesizing system from rat mammary glands incorporated tritium from the α-position of NADPH at three times the rate of tritium from the β-position. In the same paper, however, they have shown that a rat liver system incorporated ^3H into fatty acids equally well from either position. The specificity of the reductases in the castor bean are unknown, but any discrimination against the β-position would again reduce the amount of ^3H incorporated into fatty acids. Third, Lynen (17) has shown that the second reductase in the fatty acid-synthesizing system from yeast is FMN-specific, and it must be assumed that NADPH can reduce the moiety, if the castor bean system is the same. Tritium could also be incorporated from water via the FMN, but (if there is not specific localization in any treatment) this does not seem to be an interfering factor. Approximately the same amount of ^3H_2O was present in all treatments (Table I), but greatly different amounts of ^3H have been incorporated into the fatty acids (Table I). Because of the above difficulties and because one must also estimate the dilution, the incorporation of ^3H from ^3H-NADPH can only be used to provide a minimal estimate of the contribution of NADPH to fatty acid synthesis. Dilution from other NADPH-soluble dehydrogenases (15) cannot be estimated but dilution from the PPP can easily be estimated. ^3H-NADPH (0.63 μmole) from 3-^{3}H-glucose was used for fatty acid synthesis, and (assuming no discrimination) an equivalent amount of NADPH produced from carbon 1 of glucose could also be incorporated; *i.e.*, a total of 1.26 μmoles of NADPH was used in fatty acid synthesis. At this time 5.82 μmoles of carbon were incorporated into fatty acids from U-^{14}C-glucose, and this would require 5.2 μmoles of NADPH (3). Hence, the PPP supplied only 24% of the NADPH required ($1.26/5.20 \times 100$) for fatty acid synthesis. Similar calculations for experiment 2 (Table I) show that the PPP supplied only 26% of the NADPH required.

It can be calculated from the data of 1-^{14}C-glucose and 6-^{14}C-glucose incorporation (Table I and II) that the amount of ^3H-NADPH produced from 3-^{3}H-glucose should be of the same order of magnitude as that expected. The average PPP participation in glucose dissimilation calculated by the four methods of Katz *et al.* (11) is 11.6% for experiment 1 and 10.0% for experiment 2. ^3H-NADPH production in experiment 1 would be 1.76 μmoles and in experiment 2 1.72 μmoles. Since only 0.62 μmole and 0.87 μmole of ^3H-NADPH were observed in fatty acids, it means that only 35 to 50% of ^3H-NADPH produced was used in fatty acid synthesis. The remainder of the ^3H-NADPH must be used to reduce compounds other than fatty acids (*e.g.*, amino acids and organic acids) or is oxidized to water (Table I). These results differ from those of Katz and Rognstad (12), who showed that under conditions of rapid fat synthesis all of the NADPH produced in the PPP was used for fat synthesis.

Tritium from ^3H-NADH generated from 1-^{3}H-ethanol was incorporated into fatty acids (Table II). In fact, approximately the same proportion of tritium was incorporated into fatty acids via ^3H-NADH as was incorporated via ^3H-NADPH from 1-^{3}H-glucose. If one does the appropriate calculations on Ragland and Hackett's data (21), the same results are found and in view of their extremely low ^3H incorporations into lipids (less than 0.01%) it is difficult to see the basis on which they concluded "that plant tissues use NADPH preferentially for the reductive synthesis of lipids." The dilution of ^3H-NADH must be more extensive than the dilution of ^3H-NADPH, since there are many known reactions in plants in which NADH is pro-

Table IV. *$^3H/^{14}C$ Ratios in Fatty Acids*

Labeled Substrate	Saturated	Oleic	Linoleic + Linolenic	Ricinoleic
Ethanol-1-^{3}H-1-^{14}C	0.15	0.12	0.13	0.14
Glucose-1-^{3}H-1-^{14}C	0.37	0.33	0.32	0.35
Glucose-3-^{3}H-U-^{14}C	0.83	0.59	0.54	0.64
Glucose-6-^{3}H-6-^{14}C	0.17	0.20	0.28	0.16

duced. Thus it is difficult to calculate the dilution of ^{3}H-NADH produced in the alcohol dehydrogenase and aldehyde dehydrogenase reactions and difficult to assess the exact contribution of NADH to fatty acid synthesis.

The fatty acids were separated into four groups as follows: saturated, oleic, linoleic + linolenic, and ricinoleic acids; and their ^{3}H/^{14}C ratios were calculated (Table IV). In general, within any ^{3}H/^{14}C combination the ratios were similar, indicating that NADPH or NADH was not selectively used to reduce any one group of fatty acids.

The appearance of ^{3}H in glycerol from 3-^{3}H-glucose (Table II) was not expected since all the ^{3}H should have been lost in the reversible isomerization of dihydroxyacetone-P to glyceraldehyde-3-P catalyzed by triosephosphate isomerase. However, if the rate of triose isomerization was slower than glycerol synthesis from dihydroxyacetone-P, then some ^{3}H could be found on carbon 1 of glycerol (12).

The amount of glucose metabolized through the PPP (10–12%) would be able to generate 51 to 68% of the reducing equivalents required for fat synthesis, a value in close agreement with that calculated earlier (3). However, since only 40% of this amount is actually incorporated, the over-all contribution of NADPH to fat synthesis is only 20 to 27% of the total reducing equivalents required. In determining the NADPH contribution to fat synthesis, we have ignored the possible production of NADPH by other soluble NADP-specific dehydrogenases. Kikuta and Erickson (15) observed that the PPP in avocado could supply only 50% of the NADPH required for fat synthesis and suggested that the remainder was supplied by the other soluble dehydrogenases.

Because the methods only estimate the minimal PPP contribution and ^{3}H incorporation, and because of possible NADPH production by other soluble NADP-specific dehydrogenases, it is tempting to conclude that NADPH is the preferred reductant for fatty acids and that the methods underestimate the total contribution. Other evidence, however, argues against this view and suggests that NADH may contribute the major portion (75%) of the reducing equivalents required. First, ^{3}H from ^{3}H-NADH was readily incorporated into fatty acids (Table II). Second, NADH is required at three times the concentration of NADPH for maximal conversion of malonyl-CoA to oleic aid in an *in vitro* enzyme system from castor beans (7). Third, a marked specificity for NADH was observed in the conversion of oleic acid to ricinoleic acid (9).

The use of NADH for fat synthesis in the developing castor bean provides an exception to the concept that NADPH is the primary nucleotide required for synthetic reactions in cells (21). It still leaves unanswered, however, the question as to what controls PPP activity in this tissue. Clearly, no correlation between PPP operation and rates of fat synthesis could be expected, and without knowledge of other NADPH-requiring reactions it is impossible to assess whether the rate of NADPH reoxidation controls the cycle (4).

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