A Flux Model of Glycolysis and the Oxidative Pentosephosphate Pathway in Developing *Brassica napus* Embryos*

Received for publication, April 2, 2003

Published, JBC Papers in Press, May 20, 2003, DOI 10.1074/jbc.M303432200

**Jörg Schwender**, John B. Ohlrogge, and Yair Shachar-Hill

From the Department of Plant Biology, Michigan State University, East Lansing, Michigan 48824

Developing oilseeds synthesize large quantities of triacylglycerol from sucrose and hexose. To understand the fluxes involved in this conversion, a quantitative metabolic flux model was developed and tested for the reaction network of glycolysis and the oxidative pentose phosphate pathway (OPPP). Developing *Brassica napus* embryos were cultured with [U-13C6]glucose, [1,13C]glucose, [6,13C]glucose, [U-13C12]sucrose, and/or [1,2-13C2]glucose, and the labeling patterns in amino acids, lipids, sucrose, and starch were measured by gas chromatography/mass spectrometry and NMR. Data were used to verify a reaction network of central carbon metabolism distributed between the cytosol and plastid. Computer simulation of the steady state distribution of isotopomers in intermediates of the glycolysis/OPPP network was used to fit metabolic flux parameters to the experimental data. The observed distribution of label in cytosolic and plastidic metabolites indicated that key intermediates of glycolysis and OPPP have similar labeling in these two compartments, suggesting rapid exchange of metabolites between these compartments compared with net fluxes into end products. Cycling between hexose phosphate and triose phosphate and reversible transketolase velocity were similar to net glycolytic flux, whereas reversible transaldolase velocity was minimal. Flux parameters were overdetermined by analyzing labeling in different metabolites and by using data from different labeling experiments, which increased the reliability of the findings. Net flux of glucose through the OPPP accounts for close to 10% of the total hexose influx into the embryo. Therefore, the reductant produced by the OPPP accounts for at most 44% of the NADPH and 22% of total reductant needed for fatty acid synthesis.

*Brassica napus* (rapeseed, canola) is one of the world’s major oilseed crops and is also a well studied model for oilseed metabolism (1–21). The main storage compounds in seeds are derived from sugars and amino acids taken up from the surrounding endosperm liquor (11, 12, 22, 23). Because of its high oil content and ease of genetic transformation, *B. napus* has also been a target for metabolic engineering of oil metabolism. However, some attempts to engineer plant oils have had limited success (for a review, see Ref. 24). In order to make advances in improving oil yield and quality, a more detailed understanding of metabolism during seed development is needed. In particular, a number of fundamental metabolic issues remain unresolved. These include the source(s) of reductant and ATP for fatty acid synthesis; the degree to which cytosolic, plastidial, and mitochondrial metabolic fluxes are integrated; and the chief metabolic and transport route(s) by which carbon flows from maternal sources to seed storage products.

Fatty acid synthesis has a high demand for reductant, and in other systems there is evidence that the supply of reductant can limit lipid accumulation (25, 26). Thus, determining the source of reductant for fatty acid synthesis in developing oil seeds is important, and in particular the contribution of NADPH made by the oxidative pentose phosphate pathway (OPPP)3 to fatty acid synthesis is not known. Of the two reducing steps of fatty acid synthesis, *in vitro* data indicate that the first (3-ketoacyl-ACP reductase; EC 1.1.1.100) requires NADPH (27), whereas the second (enoyl-ACP reductase, EC 1.3.1.9) requires NADH (28). NADH can be provided by the pyruvate dehydrogenase reaction in plastids, whereas it has long been thought that NADPH for reductive syntheses in nonphotosynthetic plastids is produced by the OPPP (29). However, reductant could also be provided by steps in glycolysis (e.g. GAP-dehydrogenase; EC 1.2.1.13), by photosystems of green seeds, or by the import into the plastid of reducing equivalents generated in the mitochondria or cytosol. Thus, the OPPP represents one of several possible sources of reductant for oil synthesis in seeds, and the *in vivo* contribution of these alternatives has not been established.

In recent years, it has become clear that measuring fluxes through the OPPP presents technical challenges and requires careful experimental design and interpretation. The effects of cycling among hexose, triose, and pentose pools via reversible reactions leads to label redistributions that must be quantitatively considered if one is to understand the sources of carbon and reductant (30). Understanding flux through metabolic networks that involve reversible, branching, and parallel pathways has been greatly aided by the development of steady state labeling methods using stable isotopes and isotopomer analysis (31–34). Analysis of isotopomer distributions in intermediates

---

*This work was supported by Department of Energy Grant DE-FG02-87ER13729, National Science Foundation Grant MCB 0224655, and United States Department of Agriculture Grant 83786. This work was also supported by the Michigan Agricultural Experiment Station. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Plant Biology, Michigan State University, Wilson Dr., East Lansing, MI 48824-1312. Tel.: 517-355-5237; Fax: 517-353-1926; E-mail: Schwind2@msu.edu.

3 The abbreviations used are: OPPP, oxidative pentose phosphate pathway; C18, octadecanonic acid; C18:1, octadecenonic acid; C22, eicosenonic acid; C22:1, docosenic acid; DHAP, dihydroxyacetone phosphate; Fru-6-P, fructose 6-phosphate; GAP, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; GC, gas chromatography; MS, mass spectrometry; PEP, phosphoenol pyruvate; Rib-5-P, ribose 5-phosphate; Ru-5-P, ribulose 5-phosphate; Suc, sucrose; TAG, triacylglycerol; TA, transaldolase; TK, transketolase; Xa-5-P, xylulose 5-phosphate; PDH, pyruvate dehydrogenase complex.
Flux Model in Developing Plant Embryos

and end-products of metabolism can provide information on the relative fluxes through alternative pathways and on flux ratios at branch points between pathways (see, for example, Refs. 35–37). With in vivo labeling, this approach yields quantitative information on systems unperturbed by cell disruption, mutations, or transgenic manipulation. The results of this approach can therefore distinguish the relative contributions of competing pathways and help guide rational engineering of metabolism.

To take advantage of such methods, we have recently established culture conditions for developing B. napus embryos that mimic in planta growth and allow steady state labeling during storage product accumulation (22). After feeding 13C-labeled carbon sources, the labeling pattern of various intermediates of central carbon metabolism are “imprinted” on seed oil and on the amino acids of seed protein; these can be measured by gas chromatography/mass spectrometry (GC/MS) and by NMR spectroscopy. Using these techniques, we deduced that the pyruvate that provides acetyl-CoA units for fatty acid is derived from Glc almost entirely by glycolytic cleavage (Embden-Meyerhof pathway) and that glycolysis rather than the OPPP accounts for most embryo hexose catabolism. Based on a preliminary analysis of labeling in fatty acids, we estimated that the net flux of Glc-6-P into OPPP is in the range of 5–10% of total influx of Glc-6-P. However, this preliminary estimate was based on making key assumptions about the reversibilities of transketolase (TK; EC 2.2.1.1) and transaldolase (TA; EC 2.2.1.2). In the present study, we have developed a quantitative model of glycolysis and OPPP and tested its ability to account for isotopomer labeling patterns and to yield reliable flux parameters in developing B. napus seeds.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—[U-13C6]Glc, [1,2-13C2]Glc, [1-13C]Glc, [6-13C]Glc, and [2,3-13C2]Glc (all 99% 13C abundance) were purchased from Isotec (Miamisburg, OH) and Omicron (South Bend, IN). Methoxyamine hydrochloride, α-amyloglucosidase (EC 3.2.1.1) and Aspergillus niger α-amylglucosidase (EC 3.2.1.3) were purchased from Sigma.

**Growth in the Presence of 13C-Labeled Sugars**—Oilseed rape plants (B. napus L., cv. Reston) were grown as described before (22). Siliques were harvested 20 days after flowering, and embryos were immediately dissected under aseptic conditions and transferred into culture medium (22). In order to obtain fully labeled TAG and seed protein for analysis by GC/MS, five embryos were isolated at the early stage of oil accumulation (0.5–1 mg fresh weight) and were grown for 14 days, in each 5 ml of growth medium under low light conditions (continuous light, 50 μmol m−2 s−1) at 25 °C under a stream of nitrogen, this approach yields quantitative

**Measurement of Lipid Labeling**—For analysis by GC/MS, Glc was derivatized to Glc methoxime penta-acetate. 1 ml of methoxyamine hydrochloride in pyridine (20 mg/ml) was added to 50–100 μg of Glc and heated to 50 °C for 1 h. After cooling to room temperature, 1 ml of acetic anhydride was added, and the sample was again heated to 50 °C for 1 h. Finally, the derivative was extracted with toluene after adding 1 ml of H2O to the reaction. The isomers m/z 398, m/z 288, and m/z 99 (C15H22O9N; Glc(1-13C2), C16H22O10N; Glc(1-13C, 6-13C2), and C16H22O11N; Glc(1-13C 2), respectively) were monitored by GC/MS.

**Measurement of Label in Amino Acids of Storage Proteins**—Proteins were hydrolyzed in 6 N HCl for 24 h at 100 °C. HCl was evaporated at 50 °C under a stream of nitrogen. Amino acids were dissolved in 0.1 N HCl and loaded on an H+ exchange column (AG 50W-X4; Bio-Rad). After washing with 5 volumes of H2O, amino acids were eluted with 2 N NH4OH. After most of the NH4OH was removed under a stream of nitrogen, the sample was lyophilized and then derivatized to their N-tert-butyldimethylsilyl derivatives by adding 100 μl of N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetic acid (1:1) to 100 μg of amino acids and heating at 120 °C for 1 h (36, 42). The identities of the tert-butyldimethylsilyl fragments of the N-tert-butyldimethylsilyl amino acid derivatives in mass spectra were derived from the literature (36, 42).

**GC Conditions**—One microliter of each derivatized sample (100–500 ng) was analyzed with a HP 5890 II (Hewlett-Packard) gas chromatograph/mass spectrometer (HP 5972 quadrupole MS). Carrier gas was helium at 1 ml/min. For fatty acid methyl esters, a DB23 column (0.25 mm I.D. × 30 m, 0.25 μm film; J&W Scientific, Rockwood, CA) was used. For N-tert-butyldimethylsilyl derivatives of amino acids, Glc methoxime penta-acetate, and glycerol trifluoroacetate, a 30 m × 0.25-mm DB1 column was used (J&W Scientific). The GC conditions for fatty acid methyl esters and N,O-succinimidyldimethylsilyl derivatives of amino acids were as previously described (22). For glycerol trifluoroacetate, the injector temperature was 250 °C. Initial temperature was 80 °C for

[2] Carbon atoms in different molecules are denoted as subscripts. For example, Glc(1-13C2) refers to the part of the molecule comprising carbons 1, 2, and 3 of Glc, and pyruvate(1-13C2) refers to carbons 1 and 2 of pyruvate.
Measurements of Fractional Labeling by Mass Spectrometry—In mass spectra of labeled compounds, selected molecular fragments were monitored. Single ion monitoring was generally used with >20-ms acquisition time for each ion. The mass spectra of each ion were integrated over the entire chromatographic peak to avoid the influence of possible isotope fractionation during GC separation. Background correction was performed with mass spectra taken just before each chromatographic peak. Reproducibility of isotope ratios was checked with unlabeled reference substances over a concentration range of 2 orders of magnitude. The ion clusters were corrected for natural isotope abundance in reference substances over a concentration range of 2 orders of magnitude. The ion clusters were corrected for natural isotope abundance in reference substances over a concentration range of 2 orders of magnitude. The molar abundances of molecule fragments containing i labeled carbons were referred to as $m_i$. The identity of ions was checked by comparison of the measured mass distribution of a fragment of unlabeled compounds with the theoretical distribution, as derived from the elemental composition and natural isotope abundances (43). Only fragments that were in good agreement with the theoretical mass distribution were used for measurements. In the case of TBDMS-amino acids and Glc methoxime penta-acetate, the ion purity was also verified by derivatization of $^{13}$C-labeled amino acids (hydrolysis of $^{13}$C-labeled protein, 99% $^{13}$C, Isotoc and Glc [11.0 $^{13}$C][Glc], [6.0 $^{13}$C][Glc], [1.2, $^{13}$C][Glc], and [1.0, 0 $^{13}$C][Glc], respectively, which leads to mass shifts of the isotopomer clusters defined by the presence of one or more $^{13}$C-labeled carbon atoms in the monitored fragment. The fragmentation of glycerol trifluoroacetate during MS analysis was established by analogy to glycerol triacetate (44). The fragment $m/z$ 158 contains glycerol, 1-$^{13}$C. In the mass spectra of saturated fatty acid methyl esters, the ion $m/z$ 74 can be used to measured labeling in C18(1-$^{13}$C) (22). Since in the extracted TAG, C18(1-$^{13}$C) dominated over C18, and fatty acids were derivatized before GC/MS analysis, the measured C18(1-$^{13}$C) represents mainly C18(1-$^{13}$C). Comparison of Measured and Simulated Labeling in Glucose by Least Squares Fitting—Embryos were labeled for 14 days with [U-$^{13}$C]2 Suc/[U-$^{13}$C]6 Glc (each diluted 1:10 with unlabeled sugar). Labeling in the glycosyl units of starch was measured by GC/MS. The fractional $^{13}$C enrichment was measured in the fragments Glc-1-$^{13}$C, Glc-3-$^{13}$C, and Glc-6-$^{13}$C. The measured mass isotopomers $m_i$ and $m_2$ of Glc-1-$^{13}$C, Glc-2-$^{13}$C, Glc-3-$^{13}$C, and Glc-6-$^{13}$C were compared with values predicted by the computer model. For each mass isotopomer $i$, the difference between measurement and prediction ($A_i$) was calculated. The sum of squared differences ($\sum A_i^2$) was calculated as a measure for the similarity between measured and predicted mass isotopomers. By variation of the model parameters $X$, $V_{TPC}$, $V_{PC}$, and $V_{TPC}$, minima for $\sum A_i^2$ were determined as shown in Figs. 3 and 4.

NMR Analysis—NMR analyses of aqueous extracts (containing predominantly Suc) of Glc (isolated from starch) and of storage lipids (mainly triacylglycerols) were performed with a Varian VXR 500 MHz spectrometer equipped with a 5-mm $^{13}$C-1H switchable probe. $^1$H and $^13$C NMR spectra were measured with a 90° pulse angle, 1$^1$H waltz decoupling during acquisition only (for $^1$H spectra), and full relaxation (recycle times = 60 s). Data processing included zero filling and multiplication of the free induction decays by an exponential function to improve the signal-to-noise ratio. NMR peak assignment for Glc, Suc, and TAG was performed using literature values (45) and by comparison with pure reference substances. The absolute $^{129}$C enrichment in Suc and Glc was determined by $^1$H NMR of Suc glucosyl C-1 and $\alpha$-C-1 of Glc, respectively. In addition, absolute $^{13}$C enrichment was determined by GC/MS of methoxamine penta-acetates of Glc.

Flux Model in Developing Plant Embryos

The crucial first stage in building quantitative models of metabolic flux is constructing a map of the metabolic network to be modeled. Such a scheme for the principal flows of carbon into protein, starch, and oil in developing B. napus embryos is shown in Fig. 1. This map is the basis for the quantitative modeling of fluxes described below, and it is based on the biochemical literature including enzyme localization in vivo and in vitro labeling studies. Since Brassica and Arabidopsis

---

Footnote:

1 The molar abundances of molecule fragments containing i labeled carbons are referred to as $m_i$. Sugar Catabolism—During oil accumulation, B. napus embryos use Suc, as well as Glc and fructose, as carbon sources for fatty acid synthesis (12, 22, 23). Suc is mostly cleaved by Suc synthase (EC 2.4.1.13) (12, 23). The cleavage products are metabolized through glycolysis, the enzyme activities of which are present in both cytosol and plastid (3, 9, 14). Resynthesis of Fru-6-P from triose phosphate is possible by plastidic fructose-1,6-biphosphatase (EC 3.1.3.11) or cytosolic pyrophosphatase-dependent fructose-6-phosphate-1-phosphotransferase (EC 2.7.1.90) (9). Exchange of intermediates between cytosol and plastids can occur by the transport of Glc-6-P, triose phosphate, PEP, pentose phosphate, and pyruvate (3, 9, 14, 46).

Starch Metabolism—In developing B. napus seeds, starch is accumulated inside the chloroplasts mainly before the main stage of oil accumulation but is still present at later stages and is continuously turned over (5). Therefore, the labeling in starch can be assumed to represent the plastidial hexose phosphate during maximal oil deposition. For B. napus embryos, it was concluded that hexose is mainly imported into the plastids in the form of Glc-6-P, whereas Glc-1-P was not used by isolated plastids (9). Import of the starch precursor ADP-Glc into the plastids can be excluded because of the subcellular localization of ADP-Glc pyrophosphorylase (EC 2.7.7.27) in B. napus embryos (5, 14, 47).

Incomplete Cytosolic OPPP—In developing B. napus seeds, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity is found in plastids and the cytosol (9, 14). The regeneration of Fru-6-P from pentose phosphate involves ribose-5-phosphate isomerase (EC 5.1.3.1), ribulose-5-phosphate epimerase (EC 5.3.1.6), TK, and TA. In Arabidopsis, there are most probably only plastidic isoforms of TK and TA (48). Similar results for spinach leaves (49) and other tissues (50) also point to an incomplete OPPP in the cytosol. Therefore, cytosolic regeneration of Fru-6-P from pentose-phosphate by TK and TA were not included in the network. Instead, it was assumed that pentose phosphate, if produced in the cytosol, can be transported into the plastid by a pentose phosphate-specific transporter (48).

Transport of Carbon into Plastids—Import of carbon into isolated plastids of developing B. napus embryos has been reported for many substrates including Glc-6-P, DHAP, malate, pyruvate, PEP, and free hexoses (9). Evidence for Glc-6-P, DHAP, and triose phosphate transporters also comes from transcriptional profiling of developing seeds of A. thaliana (46).

During maximal oil synthesis, it has been proposed that the main flux of carbon enters the chloroplasts as PEP or pyruvate with a minor influx of Glc-6-P (3, 46, 51). This is supported by isotopic tracer experiments with isolated plastids and by the change of plastidial activities of enzymes of glycolysis during embryo development (2, 3, 8, 9). Furthermore, in developing embryos of A. thaliana, the expression of the PEP translocator follows the pattern of enzymes involved in oil synthesis (plastidic pyruvate kinase (EC 2.7.1.40) and plastidic pyruvate dehydrogenase (EC 1.1.1.49)), peaking with maximal oil synthesis, whereas the expression of cytosolic pyruvate kinase decreases with the onset of oil synthesis (46). Therefore, Fig. 1 includes a major carbon influx into the plastid at the level of PEP, although the in vivo contribution of other transport processes cannot be ruled out.

Plastidic Fatty Acid Synthesis and Cytosolic Elongation—in plant systems, fatty acid synthesis is localized predominantly...
in plastids (52, 53). Plastidic fatty acid synthesis produces C16 and C18 fatty acids, whereas the elongation of C18:1 by a cytosolic fatty acid elongation system produces C20 and C22 fatty acids (54, 55). Thus, labeling in the carboxyl-terminal acetate units of C18 and C22 fatty acids represent plastidic and cytosolic acetyl-CoA pools, respectively (22).

The Source of Plastidic Acetyl-CoA—Plastidic acetyl-CoA is mainly produced from pyruvate (22, 40). In developing B. napus embryos, most of the pyruvate dehydrogenase activity resides in the plastids (9). Also, in developing embryos of A. thaliana, the expression of the plastidic pyruvate dehydrogenase complex correlates with the activity of fatty acid synthesis (46, 56). Also consistent with plastidic pyruvate being a precursor of acetyl-CoA is the observation that the activity of plastidic pyruvate kinase follows the activity of fatty acid synthesis in embryos of B. napus (57). On the other hand, cytosolic acetyl-CoA is derived from mitochondrial metabolism, probably involving citrate cleavage (22).

The Absence of Fatty Acid Synthesis from Malate—It has been suggested that in B. napus embryos, malate produced by the sequential actions of cytosolic PEP carboxylase (EC 4.1.1.31) and malate dehydrogenase (EC 1.1.1.37) enters the plastids to supply fatty acid synthesis (20). Plastidic malate dehydrogenase and plastidic malic enzyme (EC 1.1.1.39) were proposed to supply NADPH and pyruvate to the plastidic biosynthesis of fatty acids (20). However, in isolated plastids of B. napus embryos, incorporation of label into fatty acids from malate is less than from Glc 6-phosphate, DHAP, or pyruvate (9). In addition, the results of isotope dilution experiments (22) show that oxaloacetate-derived metabolites do not significantly contribute to plastidic fatty acid synthesis. Therefore, in Fig. 1, the flux through plastidic malic enzyme into plastidic pyruvate and acetyl-CoA is considered to be minor compared with the flux from PEP to pyruvate to acetyl-CoA.

Amino Acid Biosynthesis—In steady state labeling experiments, the labeling of different amino acids gives information on the labeling of their respective precursors. Therefore, it is important to localize the biosynthesis of different amino acids in subcellular compartments. The biosyntheses of His, Val, Leu, and Ile are exclusively plastidic (58, 59). In the absence of photorespiration in B. napus embryos (22), serine is formed by the plastidic phosphorolysed serine biosynthetic pathway (60), in which serine is derived from 3-phosphoglyceric acid. Asparagine can be derived from oxaloacetate in different compartments by transamination (61, 62). Oxaloacetate in turn derives from cytosolic PEP carboxylase. Alanine is derived from pyruvate by different aminotransferases (63). Plants, alanine and pyruvate can be interconverted by cytosolic, mitochondrial, and peroxisomal transaminases (63, 64).

Modeling the Metabolic Network

In the network formed by glycolysis and OPPC, cyclic fluxes and reversible reactions cause the redistribution of label among different intermediates in ways that are not easily understood by inspection of labeling patterns (30, 65). Computer-aided modeling is needed if 13C label at multiple carbon positions is to be quantitatively interpreted (37, 66). In steady state flux models, flux rates are relative, and in the model presented here all fluxes are defined relative to the rate of uptake of hexose units by the developing embryo, which is assigned a value of 1. The intermediate pools in the model and the fluxes through them, including their mass balances, are shown in Fig. 2 and listed in Table I.

Metabolite Pools Considered in the Flux Model—The flux model is used to derive metabolic fluxes from labeling information. Therefore, only fluxes that influence labeling patterns in the metabolites that are analyzed can be usefully included, and fluxes between adjacent intermediates that lie between metabolic branch points are not resolved. Two pairs of metabolically adjacent intermediates, the hexose 6-phosphates and the GAP/DHAP pair of triose phosphates (shown in boxes in Fig. 2) have indistinguishable labeling patterns and are thus considered to be fully equilibrated (see “Results”). Hexose 6-phosphates and triose phosphates appear to have identical isotope patterns in the cytosol and plastid (see “Results”) and were thus considered to function as single pools (Fig. 2).
phosphates Xu-5-P, Ru-5-P, and Rib-5-P, which interconvert via ribulose-5-phosphate-3-epimerase (EC 5.3.1.6) and ribose-5-phosphate isomerase (EC 5.3.1.1), respectively, are also treated as one pool (PP, Fig. 2). A rapid exchange between Xu-5-P and Rib-5-P (via Ru-5-P), relative to the flux through oxidative decarboxylation of Glc-6-P, is supported by the observation of a TK signature in histidine (see “Results”).

Fig. 2. Illustrations of the flux model for carbon flux in the glycolysis/OPPP network. Three groups of metabolically adjacent metabolites are considered to be in isotopic equilibrium with one another, and these groups are treated as single metabolic pools in the model. These are shown in boxes: hexose phosphate (HP), pentose phosphate (PP), and triose phosphate (TP). A shows the reactions modeled: 1, hexokinase; 2, glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase; 3, transketolase; 3, transaldolase; 6, phosphofructokinase/aldolase; and aldolase. The reaction numbers are the same as in Table I. B shows the net fluxes through each of these reactions relative to the rate of uptake of Glc which is defined as 1. The forward, reverse, and net fluxes for these reactions are listed in Table I.

### Table I

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Reaction</th>
<th>Net flux</th>
<th>Forward rate</th>
<th>Reverse rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glc</td>
<td>→ Glc-6-P</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Glc-6-P</td>
<td>→ Ru-5-P + CO₂</td>
<td>3X</td>
<td>X + Vₜₖ</td>
</tr>
<tr>
<td>3(TK₁)</td>
<td>Rib-5-P + Xu-5-P</td>
<td>→ Sh-7-P + GAP</td>
<td>X + Vₜ₆</td>
<td>Vₜ₆</td>
</tr>
<tr>
<td>4(TK₁)</td>
<td>E-4-P + Xu-5-P</td>
<td>→ Fru-6-P + GAP</td>
<td>X + Vₜ₆</td>
<td>Vₜ₆</td>
</tr>
<tr>
<td>5</td>
<td>Sh-7-P + GAP</td>
<td>→ E-4-P + Fru-6-P</td>
<td>X + Vₜ₆</td>
<td>Vₜ₆</td>
</tr>
<tr>
<td>6</td>
<td>Glc-6-P</td>
<td>→ GAP + CO₂</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Fru-6-P</td>
<td>→ DHAP + GAP</td>
<td>1 - X</td>
<td>1 - X + Vₜ₆</td>
</tr>
<tr>
<td>8</td>
<td>GAP</td>
<td>→ PGA</td>
<td>2 - X</td>
<td></td>
</tr>
</tbody>
</table>

### RESULTS

Developing embryos of *B. napus* (rapeseed) were cultured for 14 days in liquid medium with Suc (80 mm) and Glc (40 mm), one or both of which was 1³C-labeled in different positions in different labeling experiments. The labeling patterns in sucrose, oil, amino acids of seed protein, and Glc from starch were measured using GC/MS and NMR. The steady state approach was used for the interpretation of the results in order to determine major flux parameters of the glycolysis/OPPP network.
**Flux Model in Developing Plant Embryos**

TABLE II

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Metabolite analyzed</th>
<th>Measured C-1/C-6 ratio</th>
<th>$V_{TPC}$ fitted to C-1/C-6 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[1\text{-}^{13}\text{C}]\text{Glc/Palatinose}$</td>
<td>Suc glucosyl</td>
<td>2.7 (2.6–2.9)</td>
<td>1.0 (0.9–1.1)</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td>2.3 (2.2–2.5)</td>
<td>1.4 (1.3–1.5)</td>
</tr>
<tr>
<td></td>
<td>Starch glucosyl</td>
<td>2.4 (2.3–2.5)</td>
<td>1.3 (1.2–1.5)</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td>0.20 (0.19–0.21)</td>
<td>0.6 (0.5–0.7)</td>
</tr>
<tr>
<td></td>
<td>Starch fructose</td>
<td>0.23 (0.22–0.25)</td>
<td>0.8 (0.7–0.9)</td>
</tr>
<tr>
<td></td>
<td>Starch glucosyl</td>
<td>0.25 (0.23–0.27)</td>
<td>0.8 (0.7–0.9)</td>
</tr>
</tbody>
</table>

TABLE III

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Precursor</th>
<th>$1.2^{13}\text{C}_2$</th>
<th>$1.2^{13}\text{C}_2/1.1^{13}\text{C}$</th>
<th>$1.2^{13}\text{C}_2/6.1^{13}\text{C}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluc$_{1-2}$ of starch</td>
<td>$pl \text{. Glc-6-P}$</td>
<td>ND$^a$</td>
<td>1.16</td>
<td>1.24</td>
</tr>
<tr>
<td>Ala$_{1-3}$</td>
<td>Pyruvate$_{1-3}$</td>
<td>0.46</td>
<td>0.38</td>
<td>1.50</td>
</tr>
<tr>
<td>Glycerol</td>
<td>cyt. DHAP$_{1-3}$</td>
<td>ND</td>
<td>1.31</td>
<td>1.45</td>
</tr>
<tr>
<td>Ala$_{2-3}$</td>
<td>Pyruvate$_{2-3}$</td>
<td>0.28</td>
<td>0.22</td>
<td>1.21</td>
</tr>
<tr>
<td>Val$_{2-5}$</td>
<td>$2\times$ pyruvate$_{2-3}$</td>
<td>0.25</td>
<td>0.22</td>
<td>1.21</td>
</tr>
<tr>
<td>C18:1$_{1-2}$</td>
<td>$pl \text{. acetyl-CoA}$</td>
<td>0.21</td>
<td>0.22</td>
<td>1.24</td>
</tr>
<tr>
<td>H18:1$_{1-6}$</td>
<td>$pl \text{. pentose-P}$</td>
<td>0.43</td>
<td>0.54</td>
<td>1.48</td>
</tr>
</tbody>
</table>

$^a$ ND, not determined.

**Model Validation**

If a steady state flux model of metabolism of this type is to be useful, several criteria must be met; its underlying assumptions need to be tested, its results should be consistent and reproducible, and the flux parameters derived should be sensitive to the labeling data used. We examined these criteria as follows.

**Isotopic Steady State and Metabolic Homogeneity**—To establish steady state, nutrient concentrations were kept constant during growth of embryos in culture by providing nutrients in more than 10-fold surplus to the expected uptake during the growth period. The concentrations of sugars in growth media were measured after 14 days of growth and found to be only minimally altered (data not shown). Embryos were grown for 3 days and for 14 days on $[U\text{-}^{13}\text{C}]\text{Glc}/[U\text{-}^{13}\text{C}]\text{Sucrose}$. During the 14-day culture period, the biomass increased more than 10-fold. With a 3-day labeling period, it was found by GC/MS analysis that about one-third of the fatty acid molecules of seed oil were labeled, whereas two-thirds were unlabeled preexisting biomass, whereas after 14 days, the oil was uniformly labeled. By contrast, the labeling pattern in sucrose, free amino acids, and starch was the same after 3 days as after 14 days of labeling. From this it can be concluded that there is the same fractional labeling in intermediate metabolic pools after 3 days and after 14 days, which indicates that both metabolic and isotopic labeling steady state were maintained during the experimental growth period and that metabolic pools that are turned over (sucrose, free amino acids, and starch) can be used for analysis under the steady state assumption after labeling for shorter periods.

**Equilibration of DHAP and GAP**—Whereas fatty acids are derived from pyruvate and hence from plastidic GAP, the glycol part of TAG molecules is derived from cytosolic DHAP (Fig. 1). Thus, measuring labeling in glycerol and fatty acids allows a comparison of DHAP and GAP pools. Labeling was analyzed in TAG extracted from embryos that had been labeled with $[1\text{-}^{13}\text{C}]\text{Glc}, [6\text{-}^{13}\text{C}]\text{Glc}$, or $[1,2\text{-}^{13}\text{C}]\text{Glc}$, and the findings indicated that in both cytosol and plastids, the pools of GAP and DHAP are isotopically equilibrated (data not shown). Accordingly, the flux model unifies DHAP and GAP as one triose phosphate pool (Fig. 2).

**Interconversion of Hexose Phosphates**—Synthesis of Fru-6-P from triose phosphate causes the exchange of $^{13}\text{C}$ label between C-1 and C-6 in C-6-P (Table II). To the extent that Glc-6-phosphate isomerase (EC 5.3.1.9) interconverts Fru-6-P and Glc-6-P, this exchange can also be found in Glc-6-P. We measured the extent of randomization of label between C-1 and C-6 in Glc derived from starch and in both hexose moieties of sucrose (Table II). The same degree of C-1/C-6 randomization was seen in both hexose moieties of sucrose, indicating that cytosolic glucose-6-phosphate isomerase equilibrates the Glc-6-P and Fru-6-P pools rapidly compared with the other fluxes of the network being modeled. The same C-1/C-6 randomization was also found in starch (Table II), suggesting that the plastidic and cytosolic pools of hexose phosphates have the same
metabolic imprints. In the flux model, the hexose phosphate pools were treated as one pool.

**Consistency and Reproducibility of Modeling Results**—Consistency of modeling results is tested in three ways. To test for internal consistency, data output is automatically tested for steady state (summation of influx and efflux into each isotope-pomer = 0) and for conservation of mass (sum of all isotopomers in one pool = 1). Also, arithmetic instability was considered as described in Ref. 66. Second, we tested the modeling results for consistency with the results of equations that have been solved analytically elsewhere. Data output from the model using different sets of values for \( X, V_{TA}, \) and \( V_{TK} \) was compared with the output of steady state equation systems developed by Katz and Rognstad (71). The Katz and Rognstad equations allow the distribution of label in a subset of metabolic pools (hexose phosphate, pentose phosphate, and sedoheptulose 7-phosphate) to be calculated after labeling with either \([1,14C]Glc, [2,14C]Glc, \) or \([6,14C]Glc \) (assuming \( V_{TPC} = 0 \)). In addition, output data of our computer model matched data produced by a steady state equation system from Follstad and Stephanopoulos (65), which yields positional labeling in certain metabolites. Third, the values of flux parameters obtained by fitting the labeling patterns measured in one metabolite were checked for consistency with the label in another metabolite. For example, the value of \( X \) (glycolysis/OPPP split) obtained from analysis of fatty acids (\( m_1/m_2 \) ratio) was found to also explain the observed labeling in Ala, Val, His, and Glc (starch) (Table III).

Reproducibility of modeling results depends on the variation in labeling data both when the same sample is analyzed repeatedly and when different replicate experiments are performed. In general, reproducibility of repeated GC/MS analyses of the same sample was higher than that of replicate experiments. Repeated measurements of \( m_1/m_2 \) ratios resulted in S.D. values of \(< 2.5\% \), whereas triplication of experiments resulted in S.D. values between 2 and 8\% of \( m_1/m_2 \) (Table III). In the data shown in Table III, reproducibility is also given by comparison of experiments with differently labeled substrates. Based on the flux model, the same value for \( X \) explains data from labeling with \([1,2-13C_2]Glc, [1,2-13C_2]Glc/1-13C \)Glc, and \([1,2-13C_2]Glc/6-13C \)Glc (Table III). Due to the cost and time involved in stable isotope labeling experiments, achieving replication is a nontrivial matter and is less often done than is desirable. In this study, we have in some cases replicated reproducible results, and in addition, by performing a number of the experiments with similar substrates as described in Table III, we have achieved additional cross-checks on our conclusions.

**Sensitivity of Model Parameters to Variation in Labeling Experiments**—After embryos were cultured for 14 days with \([U-^{13}C_6]sucrose/[U-^{13}C_6]Glc \), the labeling of Glc was measured by GC/MS in three fragments of Glc. Figs. 3 and 4 illustrate the fitting of model parameters to measured data. Fitting was performed by minimizing the sum of squared differences (\( \Sigma \Delta^2 \)) between measured and simulated labeling patterns (see “Experimental Procedures”). For statistical analysis, a threshold of significance for the fitting results was defined to reflect the level of uncertainty introduced into the derived flux parameter values by the uncertainty in the experimental data. This threshold for the value of \( \Sigma \Delta^2 \) was conservatively set at 100 times the sum of the squared S.D. values of the experimental data (replicate measurements of the same experimental material). This yields confidence limits for the flux parameters (Figs. 3 and 4, Table IV).

Figs. 3 and 4 show that there are clear optima for fitting \( V_{TK}, V_{TA}, \) and \( V_{TPC} \) to experimental data and that the model parameters are sensitive to the experimental data, since changes in any optimized parameter value lead to a significantly worse fitting of model results to measured data. By comparing the shapes of the curves shown in Fig. 3, A and B, one can see that the slopes at the left and the right side of the optima are similar for \( V_{TPC} \) and \( V_{TK} \). This means that the sensitivity of both flux parameters is similar. The optimum for \( V_{TA} \) is close to 0 (Fig. 4B). With increasing \( V_{TA} \), the slope is similar to that found with \( V_{TPC} \) and \( V_{TK} \) (not shown).

After labeling with \([1,2-13C_2]Glc/[1,3C]Glc \), the ratio \( m_i/m_2 \) was determined for three independent experiments. The S.D. of these experimental data translates according to the flux model to an S.D. in the derived value of \( X \) (Fig. 5). Since the two standard deviations in \( m_1/m_2 \) and \( X \) are similar, the flux \( X \) can be described as “well determined” (72).

**Metabolic Fluxes**

**Interconversion of Fru-6-P and Triose Phosphate**—When embryos were labeled with either \([1-13C]Glc \) or \([6-13C]Glc \) for 3 days, the hexose units of sucrose and of starch all showed substantial randomization of label between C-1 and C-6 (Table II), indicative of triose phosphate cycling. By simulation, values for \( V_{TPC} \) between 0.6 and 1.4 were found (Table II). In other experiments, embryos were labeled with \([U-13C]sucrose/[U-^{13}C_6]Glc \), and here the fitting of model parameters to the labeled starch (Fig. 3A) resulted in an optimum for \( V_{TPC} = 1.0 \). Having determined \( V_{TPC} \) by two independent experimental approaches, a value of 1.0 was used for subsequent simulations.

**Reversible Reactions of the Pentose Phosphate Pathway**—After labeling of \( B. napus \) embryos with \([U-^{13}C_6]sucrose/[U-^{13}C_6]Glc \), the fractional labeling of Glc isolated from starch was used to fit the reversible fluxes through TK and TA with the parameters \( V_{PA}, V_{TA} \) (Fig. 3B) and \( V_{TK} \) (Fig. 4A), respectively. To determine whether the labeling experiments were capable of yielding information on possible differences between reversibility constants for the two different reactions of transketolase (as indicated in Table I), experimental data were simulated in two ways. In the first analysis, the two TK reactions had one value of \( TK \) for both reactions (\( V_{TK} \)); in the second set of simulations, the reversible fluxes of the two TK reactions had two independent values (\( V_{TK1} \) and \( V_{TK2} \)). There was no significant difference in the goodness of fit between the two analyses, and in the second analysis there was no clear optimum combination of \( V_{TK1} \) and \( TK2 \). Therefore, one parameter, \( V_{TK} \), was used for both TK reactions for all subsequent simulations.

Fig. 4A shows the best fit value for \( V_{TK} \) and \( V_{TA} \) with \( X = 0.12 \). If \( X \) decreases, \( V_{TK} \) and \( V_{TA} \) change (Fig. 4A). The best fit value of \( V_{TA} \) is rather sensitive to the exact value of \( X \), whereas the value of \( V_{TK} \) is not. The independent determination of \( X \) by labeling with \([1,2-13C_2]Glc \) (see below) allows a global optimum for \( X, V_{TPC}, V_{TK}, \) and \( V_{TA} \) to be found, since optimal fit for the labeling in Glc, labeled from \([U-13C_6]sucrose/[U-^{13}C_6]Glc \) and for the ratio \( m_1/m_2 \) in C18:1(1\( \sim \)6) (labeling with \([1,2-13C_2]Glc \)) across \( V_{TPC} \) (Fig. 4B).

As shown in Fig. 6, using the above optimal values, the model calculates mass distributions that agree very well with the fractional labeling measured in Glc (from starch), glycerol, and histidine, representing Glc-6-P, cytosolic DHAP, and pentose phosphate, respectively. The fact that parameters obtained by fitting the labeling in one set of metabolites yield simulations that agree well with labeling in different metabolites supports the validity of the model and the metabolic network (Fig. 2). Since histidine includes the carbon chain of pentose phosphate plus one carbon from C-1 metabolism, the difference in \( m_1 \) can be explained by the labeling in this extra carbon (Fig. 6).
Because the flux through the OPPP is low \((X = 0.12; \text{ see below})\), the value for the reversible flux \(V_{\text{TPC}} (0.95)\) is almost 10 times higher than the net flux through TK (which is equal to \(X\); Table I). The reversible flux, \(V_{\text{TPC}} (1.0)\), is similar to the net flux through glycolysis \((1 – X = 0.88)\). For the reversible TA flux, the value \(V_{\text{TA}} = 0.01\) was obtained, which is negligible compared with the net forward flux through OPPP (Table I). Since \(V_{\text{TK}}\) and \(V_{\text{TPC}}\) are large reversible fluxes, the impact of TK and triose/hexose cycling on the labeling pattern shown in Fig. 6 can be qualitatively explained. TK reversibly exchanges two-carbon units between Fru-6-P, Xu-5-P, and other ketose phosphates, so that the abundance of [1,2-\(^{13}\)C\(_2\)]Fru-6-P increases at the expense of [U-\(^{13}\)C\(_6\)]Fru-6-P, contributing to the abundances of \(m_2\) isotomers in Glc(1-6) (Fig. 6). Using the computer simulation, the same effect can be seen for \(m_3\) of the triose phosphate and pentose phosphate derivatives. By contrast, the abundance of \(m_3\) isotomers (Fig. 6) of Glc(1-6), is largely attributed to triose/hexose cycling. Thus, the labeling patterns of all of the metabolites shown in Fig. 6 reveal the signature of reversible TK and of triose/hexose cycling.

**Equilibration of Pentose Phosphate Pools**—Histidine is derived from Rib-5-P, which can be synthesized by two metabolic routes. The first route is the oxidative decarboxylation of Glc-C-6; in the second route, TK forms Xu-5-P, from which ribulose-5-phosphate-3-epimerase makes Ru-5-P, and this in turn is acted upon by ribose-5-phosphate isomerase to form Rib-5-P. After labeling with [U-\(^{13}\)C\(_6\)]sucrose/[U-\(^{13}\)C\(_6\)]Glc, flux through the first route produces the \(m_5\) isomer of His(1-6), and flux through the TK route produces \(m_2\) and \(m_3\) isotomers. Since the \(m_5\) isomer is only about one-quarter as abundant as the

**Fig. 3.** Least square fitting of the model parameters \(V_{\text{TK}} (A)\) and \(V_{\text{TPC}} (B)\) to experimental data. Embryos were labeled for 14 days with [U-\(^{13}\)C\(_6\)]sucrose/[U-\(^{13}\)C\(_6\)]Glc (see "Experimental Procedures"). Labeling in the glucosyl units of starch was measured by GC/MS (see "Experimental Procedures"). The measured fractional enrichment in fragments Glc(1-2), Glc(3-4), and Glc(1-6) was compared with fractional enrichments predicted by the model. The sum of squared differences \((\Sigma \Delta^2; \text{ see "Experimental Procedures"})\) was calculated for a range of values for \(V_{\text{TPC}}\) while keeping the other parameters constant \((X = 0.12, V_{\text{TA}} = 0.01, V_{\text{TK}} = 0.95)\). \(B\), the sum of squared differences was calculated for a range of values of \(V_{\text{TK}} (X = 0.12, V_{\text{TA}} = 0.01, V_{\text{TK}} = 1)\). The \(2 \times 10^{-4}\) level for the sum of squared differences was used as a threshold well above the sum of squared S.D. values of the GC/MS measurements for all isotomers. This threshold was used to estimate confidence intervals for the values (Table IV). \(V_{\text{TK}}\) was also determined independently to be between 0.6 and 1.4 by measuring redistribution of label between C-1 and C-6 of hexose phosphate (Table II).

**Fig. 4.** Optimized flux parameter values and obtaining a global solution by experimental over determination. Embryos were labeled for 14 days with [U-\(^{13}\)C\(_1\)]sucrose/[U-\(^{13}\)C\(_6\)]Glc. Glc obtained by starch hydrolysis was derivatized and analyzed by GC/MS (see "Experimental Procedures"). The measured fractional enrichment in fragments Glc(1-2), Glc(3-4), and Glc(1-6) was compared with the fractional enrichments predicted by the model. The sum of squared differences \((\Sigma \Delta^2; \text{ see "Experimental Procedures"})\) was calculated for a range of values in the \(V_{\text{TK}}/V_{\text{TA}}\) plane, with \(V_{\text{TA}} = 1.0\). \(A\), contour map showing lines of equal \(\Sigma \Delta^2\) in the \(V_{\text{TK}}/V_{\text{TA}}\) plane. These contour lines were calculated using \(V_{\text{TK}} = 1\) and \(X = 0.12\). The contour line of \(\Sigma \Delta^2 = 2 \times 10^{-4}\) surrounds an area (shaded) that corresponds to the limits of confidence that we place around the combination of \(V_{\text{TK}}\) and \(V_{\text{TA}}\) values for the best fit to the experimental data (where \(\Sigma \Delta^2\) has its minimum). Depending on the value of \(X\), the best fit combination of \(V_{\text{TK}}\) and \(V_{\text{TA}}\) changes (dashed arrow). Two such additional points are shown that correspond to \(V_{\text{TK}}/V_{\text{TA}}\) values that yield minima in \(\Sigma \Delta^2\) when \(X = 0.05\) and \(X = 0\) (open circles). \(B\) shows the results of optimization of the fit for \(V_{\text{TK}}\) and \(X\) for the same experiment and for \(A\). In this case, there is no single minimum for \(\Sigma \Delta^2\) but instead a set of points indicated by the dashed line with a confidence area again based on a \(2 \times 10^{-4}\) threshold for \(\Sigma \Delta^2\) shaded in gray. The results of optimizing the fit for \(V_{\text{TK}}\) and \(X\) for an experiment with [1,2-\(^{13}\)C\(_2\)]Glc/[1,2-\(^{13}\)C\(_2\)]Glc (1:1) are also shown in \(B\). In this case, mass spectroscopic data from C18:1-\(^{13}\)C\(_{18}\), showing a \(m_5/m_3\) ratio of 1.24 \pm 0.04 \((n = 3\) independent experiments) was measured. Two additional points are shown that correspond to \(V_{\text{TK}}/V_{\text{TA}}\) values that yield minima in \(\Sigma \Delta^2\) when \(X = 0.05\) and \(X = 0\) (open circles).
TABLE IV

<table>
<thead>
<tr>
<th>Flux parameter</th>
<th>Labeled substrate supplied</th>
<th>Most informative metabolites</th>
<th>Value (confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X$</td>
<td>[1,2-13C]Glc</td>
<td>Starch, glycerol, Ala, Val, C18:1</td>
<td>0.12 (0.07–0.14)</td>
</tr>
<tr>
<td>$V_{pX}$</td>
<td>[U-13C]Glc</td>
<td>Starch</td>
<td>0.95 (0.5–1.4)</td>
</tr>
<tr>
<td>$V_{pA}$</td>
<td>[U-13C]Glc</td>
<td>Starch</td>
<td>0.01 (0–0.17)</td>
</tr>
<tr>
<td>$V_{pTPC}$</td>
<td>[1-13C]Glc, [6-13C]Glc</td>
<td>Suc, starch</td>
<td>0.6–1.4</td>
</tr>
<tr>
<td>$V_{pTPC}$</td>
<td>[U-13C]Glc</td>
<td>Starch</td>
<td>1.0 (0.7–1.4)</td>
</tr>
</tbody>
</table>

DISCUSSION

The construction of a metabolic network (Fig. 1) for developing *B. napus* embryos was possible because extensive literature allowed the assumptions of pathways and subcellular localiza-
tion to be justified. On this basis, a flux model for glycolysis/OPPP was constructed, which was used to interpret the results of labeling experiments (Fig. 2) and to determine relative flux of carbon through a number of key reactions and intermediates. The flux model was implemented as a computer program that simulates labeling patterns in intermediates based on given flux parameters. Glc labeled in different positions was used for the steady state labeling experiments (Table IV). Most of the combined metabolic pools and the connections between them incorporated in the flux model could be verified based on the results of the labeling experiments. Flux parameters for reversibility of TK ($V_{TK}$), TA ($V_{TA}$), and the cleavage of hexose phosphate into, and resynthesis from, triose phosphate ($V_{TPC}$) were determined by optimizing the agreement between model-generated output data and labeling patterns measured in different biosynthetic products (Figs. 3–5). The proportion of the Glc taken up that was metabolized through OPPP versus glycolysis (model parameter $X$) was then determined as a measure of net flux through OPPP.

The interdependence of the values obtained for $X$ and $V_{TPC}$ as shown in Fig. 4, emphasizes that in the metabolic network glycolysis/OPPP, the contribution of the OPPP cannot be determined without taking account of the reversibilities of the nonoxidative reactions. The problem becomes that of finding a global optimum in a four-dimensional parameter space ($X$, $V_{TPC}$, $V_{TA}$, $V_{TPC}$). This was achieved by overdetermination of the flux model both by analysis of multiple metabolic products and by labeling embryos in different experiments with Glc labeled in different positions.

Several previous studies have attempted to address the contributions of OPPP to metabolism in plant and other cells. Early methods based on differential release of $^{14}CO_2$ from [1-14C]Glc and [6-14C]Glc suffer from limitations due to (a) refixation of CO$_2$, (b) failure to account for the effects of TK and TA reversibility, (c) the effects of cyclic flux, and (d) the contributions of mitochondrial respiration to CO$_2$ release. For example, $^{14}CO_2$ release from $^{14}C$ Glc by castor bean endosperm (73) suggested that 50–70% of NADPH needed for fatty acid synthesis can be provided by OPPP. However, that study applied a simplified flux model without consideration of resynthesis of Fru-6-P from triose phosphate. Without modeling the OPPP/glycolysis network, the determination of OPPP flux is prone to major errors (30). Other studies based on the distribution of activity from [1-14C]Glc-6-P into starch, CO$_2$, and fatty acids by isolated plastids also suggested substantial OPPP activity of developing $B. napus$ embryos (3). However, since there is evidence that the major carbon influx into the plastid in vivo occurs at the level of PEP or pyruvate or triose phosphate (3, 9, 14, 46), the fate of label supplied to isolated plastids as Glc-6-P will not accurately reflect the OPPP flux in vivo.

The use of [1,2-13C]Glc in vivo, alone and in combination with other $^{13}C$ substrates, and the use of computer modeling addresses the above limitations. In particular, the impact of TA and TK reversibilities can be assessed.

In developing $B. napus$ seeds, most of the carbon entering the OPPP/glycolysis network is metabolized to pyruvate, acetyl-CoA, and finally fatty acids. Based on the flux model used in this study (Fig. 2, Table I) one can determine how much of the NADPH needed for fatty acid synthesis is provided by the OPPP. The glycolysis/OPPP split was determined as $X = 0.12$ (0.07–0.14) (Table IV). According to the flux model (Fig. 2, Table I), the efflux of C$_3$ units into fatty acid synthesis is 2 – $X$ (equal to 1.88 (1.86–1.93)), whereas the amount of NADPH produced by glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase is 6X (equal to 0.72 (0.42–0.84)). Because the elongation of a fatty acid chain by one C$_2$ unit uses two reduction equivalents (one NADPH by ketoacyl-ACP reductase and one NADH by enoyl-ACP reductase), there is demand for the rate of production of NADPH to be 1.88 and the same for NADH production. The pyruvate dehydrogenase reaction meets the NADH demand. OPPP produces 0.72/1.88 (38%) of the NADPH required for fatty acid synthesis (confidence range is 22–45%). To produce all the NADPH required, $X$ would have to be 0.286, which is incompatible with the labeling patterns (see, for example, Fig. 5).

Our conclusion of a limited role for OPPP in oilseed NADPH production is perhaps surprising, considering the general conclusions of most previous studies on oilseed metabolism (3, 73). However, other sources of reductant such as glycolysis, light reactions of photosynthesis, and the mitochondrial metabolism may supply this need. Glycolysis, together with the subsequent formation of acetyl-CoA by pyruvate dehydrogenase complex (PDH), produces 2 mol of reductant for each mol of acetyl-CoA (during the steps catalyzed by GAPDH and PDH) and 1 mol of ATP (at GAPDH). Thus, glycolysis could in principle provide all of the carbon and cofactors for fatty acid synthesis without the need for additional production of NADPH. Plastid GAPDH can produce NADPH, or cytosolic NADH could either be converted to NADPH or used directly for fatty acid synthesis. Indeed, there is evidence for different transhydrogenase and transport systems for NAD(P)H across the inner plastid membrane and for NADH utilization in plastidial anabolism (74, 75).

Although glycolysis can meet the demands of fatty acid synthesis for cofactors, the consumption of ATP and NAD(P)H by transport processes or other cell “maintenance” functions or futile cycles must demand additional production of cofactors. In this regard, another potential source of plastid NADPH is photosynthesis. The labeling experiments described here were performed with a low light intensity (continuous light, 50 μmol m$^{-2}$ s$^{-1}$) to simulate the degree of penetration of sunlight through the silique wall and the seed coat. Under these conditions, the embryos are green during growth, as they are in planta. Indeed, calculations indicate that this amount of light could substantially contribute to NADPH production via the photosynthetic light reactions (10).

The low value for $V_{TA}$ (Table IV) may also be associated with the photosynthetic potential of $B. napus$ embryos (76). One can assume that in chloroplasts TA activity is limited to the minimum needed for OPPP flux, because the TA reaction interferes with the regenerative phase of the photosynthetic reduction cycle and produces a futile cycle. In photosynthesis, recycling of pentose phosphate is in part provided by a reaction sequence comprising aldolase (erythrose-4-phosphate + GAP → sedoheptulose-1,7-bisphosphate), sedoheptulose bisphosphatase, and TK (sedoheptulose 7-phosphate + GAP → Rib-5-P + Xy-5-P). By adding TA to this reaction network, sedoheptulose 7-phosphate can be cyclically removed (sedoheptulose 7-phosphate + GAP → erythrose 4-phosphate + Fru-6-P) and regenerated from sedoheptulose 1,7-bisphosphate, resulting in a futile cycle that hydrolyzes sugar-phosphates. The assumption of relatively low TA activity in chloroplasts is supported by Thom et al. (77), who report a major increase of TA activity during the transition from chloroplasts to chromoplasts in ripening red pepper fruits. Inhibition of TA activity in chloroplasts could be mediated via light (78). The action of the full Calvin cycle is not consistent with the labeling patterns we observed, but ribulose-1,5-bisphosphate carboxylase/oxygenase activity is present in $B. napus$ embryos (10), which could produce a flux between Ru-5-P and 3-phosphoglyceric acid. However, none of the parameters derived in this study would be affected by such a flux. In order to test this possibility, labeling and other experiments are under way, which have the sensitivity to detect ribulose-
model uses the flux parameters $X_{\text{PP}}, V_{\text{PP}}, V_{\text{TP}}$, and the isotopomer abundances derived from the biochemical reactions outlined in Table 1 (e.g. HP_TA, abundance of hexose-phosphate isotopomer $i$ from transaldolase reaction). $Glc_{\text{in}},$ input of labeled Glc; HP_TK2, hexose phosphate from transketolase 2, respectively; HP_TA, hexose phosphate from TA. $PP_{\text{TK2back}},$ pentose phosphate from transketolase 2, back-reaction; $PP_{\text{TK2back}},$ pentose phosphate from HP by oxidative decarboxylation. $EP_{\text{TA}},$ erythrose 4-phosphate from transaldolase; $EP_{\text{TK2back}},$ erythrose-4-phosphate from transketolase 2, back-reaction; $SP_{\text{Tk}},$ sedoheptulose-7-phosphate from transaldolase, back-reaction. $TP_{\text{kini}},$ triose-P from hexose-P by aldolase; $TP_{\text{Tk2}},$ triose phosphate from transketolase 2; $TP_{\text{Tk2back}},$ triose phosphate from transketolase 1; $TP_{\text{Tkback}},$ triose-P from transaldolase, back-reaction.

### TABLE V

<table>
<thead>
<tr>
<th>Influx</th>
<th>Efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta HP_{\text{i}} = G_{\text{in}} + HP_{\text{TK2}}, (X + V_{\text{TP}}) + HP_{\text{TA}}, (X + V_{\text{TP}}) + HP_{\text{Aldo}}, (X + V_{\text{TP}})$</td>
<td>$-HP_{\text{i}}^* (1 + 2X + V_{\text{TP}} + V_{\text{TA}} + V_{\text{TPC}})$</td>
</tr>
<tr>
<td>$\Delta PP_{\text{i}} = PP_{\text{TK2back}}, (2V_{\text{TP}} + PP_{\text{TK2back}}, V_{\text{TP}}) + PP_{\text{decarb}}, V_{\text{TP}}$</td>
<td>$-PP_{\text{i}}^* (3X + 3V_{\text{TP}} + V_{\text{TA}})$</td>
</tr>
<tr>
<td>$\Delta EP_{\text{i}} = EP_{\text{TA}}, (X + V_{\text{TA}}) + EP_{\text{TK2back}}, V_{\text{TP}}$</td>
<td>$-EP_{\text{i}}^* (2X + V_{\text{TA}} + V_{\text{TP})}$</td>
</tr>
<tr>
<td>$\Delta SP_{\text{i}} = SP_{\text{TK1}}, (X + V_{\text{TA}}) + SP_{\text{Tkback}}, V_{\text{TA}}$</td>
<td>$-SP_{\text{i}}^* (X + V_{\text{TP}} + V_{\text{TA}})$</td>
</tr>
<tr>
<td>$\Delta TP_{\text{i}} = TP_{\text{kini}}, (2(1 - X + V_{\text{TP})}) + TP_{\text{TK2}}, (X + V_{\text{TP}}) + TP_{\text{TK1}}, (X + V_{\text{TP}}) + TP_{\text{Tkback}}, V_{\text{TA}}$</td>
<td>$-TP_{\text{i}}^* (2V_{\text{TP}} + V_{\text{TA}} + 2 + 2 V_{\text{TPC}})$</td>
</tr>
</tbody>
</table>
proven methods can be anticipated, we believe this to be the most reliable analysis to date of the OPPP/glycolysis network in a plant system.

Acknowledgments—We are indebted to Dr. Mike Pollard and Dr. Sari Ruuska (Michigan State University) for helpful discussions.

APPENDIX

A computer program was used to simulate the steady state distribution of isotopomers in the intermediate metabolite pools of the flux model (Fig. 2) using a linear equation system that employs the dimensionless flux parameters (Table V). The isotopomers of the intermediate pools are represented as “isotopomer distribution vectors” as described by Schmidt et al. (66). For each biochemical reaction (Table I), the rate of synthesis of each possible isotopomer of the products of that reaction is calculated by multiplication of the vectors representing a subset of the isotopomers of the reactants by “isotopomer mapping matrices” (66). For each product isotopomer, the relevant isotopomers are combined according to probabilistic relations. The carbon transitions that underlie the different reactions can be found in standard biochemistry textbooks.

An iterative calculation process is used by the program, starting with all intermediate pools unlabeled and proceeding to calculate the effects of the influx of labeled Glc into the system. Each cycle of the iterative process consists of 3 steps: (a) calculation of the contributions to the isotopomer abundances from each biochemical reaction, based on the current state of the intermediate pools (e.g. HP_TA represents hexose phosphate from TA); (b) calculation of the net change in the abundance of each isotopomer (e.g. ΔHR represents sum of influx and efflux into isotopomer HP); see Table V; and (c) calculation of new isotopomer values for the intermediate pools (e.g. HP = HP + fΔHP; typically f < 0.001). The iterative process typically requires 2000–5000 iterative cycles to reach steady state, which is defined as being achieved when the sum of all influxes and effluxes into all 248 isotopomers is below an arbitrary threshold (typically 0.01).

REFERENCES
A Flux Model of Glycolysis and the Oxidative Pentosephosphate Pathway in Developing *Brassica napus* Embryos
Jörg Schwender, John B. Ohlrogge and Yair Shachar-Hill

doi: 10.1074/jbc.M303432200 originally published online May 20, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303432200

Alerts:
- When this article is cited  
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 65 references, 24 of which can be accessed free at http://www.jbc.org/content/278/32/29442.full.html#ref-list-1