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-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 overestimated 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 of *B. napus* are oil (triacylglycerols) and storage proteins, which are derived from sugars and amino acids taken up from the surrounding endosperm liquid (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) 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
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 division, mutation, 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 3–5% 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-13C]Glc, [1,2-13C]Glc, [1-13C]Glc, [6-13C]Glc, and [2-13C]Glc (all 99% 13C abundance) were purchased from Isotec (Miamisburg, OH) and Omicron (South Bend, IN). Methoxamine hydrochloride, α-amylace (EC 3.2.1.1) and Aspergillus niger amyloglucosidase (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 of fresh weight) and were grown for 14 days in 5 ml of growth medium under low light conditions (continuous light, 50 μmol m−2 s−1) under aseptic conditions. The growth medium contained Suc (80 mM), Glc (40 mM) and amino acids as carbon sources in concentrations that closely mimic in planta conditions during maximal oil synthesis (22). For different labeling experiments, part of the Glc or Suc was replaced by 13C-labeled sugars. A 1:10 isotopic dilution of 13C-labeled Glc was achieved by a mixture of, for example, [1,2-13C]Glc/Glc/Suc (10:10:80) (mol % hexose units) or, in the case of uniformly 13C-labeled sugars, by a mixture of [U-13C]Glc/Glc/[U-13C]Suc/Suc (2:18:8:72) (mol % hexose units). Experiments with Glc labeled in different positions were also performed using, for example, [1-13C]Glc/[1,2-13C]Suc (10:19:80) (mol % hexose units).

In some experiments aimed at analysis of intermediates and starch, embryos were labeled for 3 days. In one such experiment, embryos were cultured with [U-13C]Glc/Glc/[1-13C]Suc/Suc (2:18:8:72) (mol % hexose units), and after 3 days labeled Suc, free amino acids, and starch were extracted and analyzed by GC/MS methods. In other experiments aimed at labeling free Suc and starch, 50 embryos in the early stage of oil accumulation (2–3 mg fresh weight) were grown for 3 days in 20 ml of growth medium with either [1-13C]Glc or [6-13C]Glc (99% 13C enrichment, 20 mM) (23). Since labeled Suc at C-1 or C-6 of hexose units was not available, Suc in the growth medium was substituted by its analog palatinitose (6-O-α-D-gluco-pyranosyl-β-D-fructofuranosan, 80 mM), which is not taken up or metabolized in plants but which appears to have similar signal functions to Suc (38). Therefore, in these experiments, the main carbohydrate carbon source was the labeled Glc, and the starch and seed oil in the embryos were substantially labeled. These experiments yielded 1–10 mg of free Suc, Glc (from starch), and seed oil for analysis by NMR spectroscopy. To ensure that the palatinose in the growth medium has no major artificial influence on the results, analogous experiments using [1-13C]Glc with unlabeled sucrose were also performed, which confirmed the experimental results with palatinose although with inferior accuracy due to the isotopic dilution of label from the unlabeled sucrose.

**Extraction of Lipids and Proteins—**Labeled embryos were ground, and lipids were extracted with hexane/diethylether (1:1, v/v). Proteins were extracted in a buffer containing sodium phosphate, pH 7.5 (10 mM), and NaCl (500 mM) as described by Schwender and Ohlrogge (22). Extracted soluble proteins were precipitated by the addition of tenfold volume of 50% trichloroacetic acid.

**Extraction of Sucrose—**Embryos labeled for 3 days were ground in a glass homogenizer in methanol/H2O (1:1) (v/v) and extracted three times at 50 °C. The combined extracts were separated into a water-soluble and a lipid fraction by adding chloroform to a final ratio close to CHCl3/methanol/H2O (8:4:3). The aqueous phase containing mainly Suc was freeze-dried and dissolved in D2O for NMR analysis.

**Starch Degradation—**After extraction of lipids and water/methanol-soluble compounds, the cell residue (equivalent to 50–100 mg fresh weight tissue) was washed three times with 5 ml of 80% (v/v) aqueous methanol and dried under vacuum. After the addition of 1 ml of H2O and sealing and heating at 110 °C for 1 h, starch was degraded to Glc by the addition of 1 ml of 0.1 acetate buffer (pH 4.8), 20 units α-amylase, and 20 units amyloglucosidase with heating to 55 °C for 3 h. Proteins were precipitated by the addition of 1 volume of ethanol, sealing and heating to 100 °C for 5 min, and centrifugation. The supernatant was reduced in volume by evaporation under nitrogen, freeze-dried, and dissolved in D2O for 13C NMR spectroscopy or derivatized for GC/MS analysis.

**Measurement of Glucose Labeling—**For analysis by GC/MS, Glc was derivatized to Glc methoxime penta-acetate. 1 ml of methoxamine 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 acid anhydride was added, and the sample was again heated to 50 °C for 1 h. Finally, the derivative was extracted with toluene by adding 1 ml of H2O to the reaction. The ions m/z 386, m/z 288, and m/z 89 (C10H12O5N, Glc; C10H12O4, and C8H6O2N, respectively) were monitored by GC/MS.

**Measurement of Lipid Labeling—**For analysis by GC/MS or 13C NMR, the lipid fraction consisting mainly of TAG was hydrogenated (40). For analysis of fatty acids and glycerol by GC/MS, lipids were transesterified by heating to 90°C in 5% (w/v) HCl in methanol for 1 h. After cooling to room temperature, 1 volume of H2O was added, and fatty acid methyl esters were extracted with hexane (41). The aqueous phase was freeze-dried, and the residue, containing glycerol, was derivatized with trifluoroacetic acid anhydride for 1 h at room temperature to obtain glycerol trifluoroacetate. Residual derivatization reagent was removed with a stream of nitrogen, and the derivatives were dissolved in hexane.

**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,O-tetra-butyl-methylisilyl derivatives by adding 100 μl of N-methyl-N-(tert-butyldimethylsilyl)- trifluoroaceticamide/acetoniitrile (1:1) to 100 μg of amino acids and heating at 120 °C for 1 h (36, 42). The identities of NMR fragments of tert-BDMS amino acid derivatives in mass spectrometry spectra were derived from the literature (36, 42).

**GC Conditions—**One microliter of each derivatized sample (100–500 ng/ml) 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 × 0.25 mm, 0.25 μm) was used (J&W Scientific, Folsom, CA). For N,O-tetra-butyl-methylisilyl derivatives of amino acids, Glc methoxime penta-acetae, 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-tetra-butyl-methylisilyl derivatives of amino acids were as previously described (22). For glycerol trifluoroacetate, the injector temperature was 250 °C. Initial temperature was 60 °C for fractionation of TAG (30 m, 0.25 mm) was used (J&W Scientific, Folsom, CA).

2 Carbon atoms in different molecules are denoted as subscripts. For example, 1-13C refers to the part of the molecule comprising carbons 1 and 2, and 3 of Glc, and pyruvate, to carbons 1 and 2 of pyruvate.
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 characterized. 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 vitro and in vivo labeling studies. Since *Brassica* and *Arabidopsis*

**TheorY**

**Defining the Metabolic Network**

The molar abundances of molecule fragments containing i labeled carbons are referred to as m_i. The isotopomer clusters defined by the presence of one or more 13C-labeled carbons are referred to as m_i. The ion clusters were corrected for natural isotopic abundance in heteronuclei and in derivative residues as well as in the labeled molecule (43). The molar abundances of molecule fragments containing i labeled carbons are 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 isotopic 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 13C-labeled amino acids (hydrolysis of U-13C-labeled protein, 99% 13C, Isotec) and Glc (11-13C[Glc, 1-13C[Glc, [1,2-13C2]Glc, and [U-13C6]Glc), respectively, which leads to mass shifts of one or more 13C-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, 1-3. In the mass spectra of saturated fatty acid methyl esters, the ion m/z 74 can be used to measured labeling in C18 (22). Since in the extracted TAG, C18:1 dominated over C18 and since fatty acids were hydroge-

**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 plastids (3, 9, 14). Resynthesis of Fru-6-P from triose phosphate is possible by plastidic fructose-1,6-bisphosphatase (EC 3.1.3.11) or cytosolic pyrophosphate-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 OPP**—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 OPP 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, PEP, 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 (plasticid pyruvate kinase (EC 2.7.1.40) and plastidic pyruvate dehydrogenase (EC 1.1.1.40)), 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.

**Plasticid 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 phosphorolyated serine biosynthetic pathway (60), in which serine is derived from 3-phosphoglyceric acid. Aspartate 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). In 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 OPPP, 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 $^{13}$C 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 isomomper patterns in the cytosol and plastid (see “Results”) and were thus considered to function as single pools (Fig. 2). In addition, the pentose
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”).

Fluxes of Glucose 6-Phosphate, Pentose Phosphate, and Erythrose 4-Phosphate into Cell Wall Polymers and Protein—Mature *B. napus* embryos contain ~50% oil, 30% protein, 8% water, and 7% sugars and cell wall polymers (67). From this, it can be estimated that at most 5% of the total Glc influx is used for cell wall synthesis. The seed protein consists of 60% (w/w) storage protein, 30% (w/w) embryo protein, 8% (w/w) seed oil, and 7% sugars and cell wall polymers (67). From this, it can be estimated that at most 5% of the total Glc influx is used for cell wall synthesis. The seed protein consists of 60% (w/w) storage protein, 30% (w/w) embryo protein, 8% (w/w) seed oil, and 7% sugars and cell wall polymers (67). The total fluxes through 2 X 5-P and Ru-5-P (via Xu-5-P), relative to the flux through erythrose 4-phosphate into Phe, Tyr, and Trp is about 1% of the total Glc influx. These small fluxes into cell wall polymers and into Phe, Tyr, Trp, and His were not included in the model (Fig. 2).

**Defining the Proportion of Glucose Metabolized via the OPPP and the Reversible Fluxes**—In the OPPP Glc-6-P is oxidized to pentose phosphate and CO2, with production of 2 NADPH/mol of Glc-6-P oxidized (Fig. 2). A cyclic flux is established by regeneration of Fru-6-P from pentose phosphate and by the isomerization of Fru-6-P to Glc-6-P. In the first flux model for glycolysis and OPPP, Katz and Wood (70) defined the flux parameter X for the proportion of Glc-6-P that is “degraded to smaller units” (i.e., into CO2 and triose phosphate) by the action of the OPPP. Thus, X defines the split of net Glc utilization between glycolysis and OPPP as being 1 - X and X, respectively (Table I). According to this convention, the flux through Glc-6-P DH is X (Table I), although in some studies (e.g. Ref. 31), the OPPP flux is defined as the total molar flux through Glc-6-P DH, which corresponds to 3X in our notation. The reversibility fluxes (fluxes in both forward and reverse directions that act in addition to the net fluxes) at TK, at TA, and between hexose P and triose phosphate are designated V_{TK}, V_{TA}, and V_{TPC}, respectively (Table I). These three model parameters together with X were determined by a recursive fitting procedure that minimizes the sum of squared differences between measured and simulated labeling levels in metabolites (X42).

**Computer Program**—We developed a computer program (Microsoft Visual Basic/Excel™ Macro Language), which can predict the steady state distribution of 13C-labeled Glc in the glycolysis/OPPP network (see Appendix). It has an interface with an Excel™ spreadsheet for input of parameters and for the output of calculated steady state isotopomer enrichments in metabolite pools (hexose 6-phosphate, pentose 5-phosphate, sedoheptulose 7-phosphate, erythrose 4-phosphate, triose phosphate, and acetyl-CoA). The program also calculates positional enrichments (percentage of 13C at each carbon position) for comparison with NMR spectra and the abundances of mass isotopomers for simulating mass spectra. Labeling experiments with singly 13C-labeled sugars as well as [1,2-13C2]Glc or [U-13C6]Glc or mixtures of labeled and/or unlabeled sugars can be simulated. Input parameters required by the simulation program are the relative flux rates (X, V_{TK}, V_{TA}, and V_{TPC}; see Fig. 2) and the labeling levels of the supplied Glc. The software is available from the authors on request.

**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.

**Table I. Steady state equations of the reactions of the network glycolysis/OPPP (see also Fig. 2)**

Reactions with net fluxes and definition of forward and reverse fluxes are shown. Reaction 1 represents the carbon influx into the system, which is normalized to 1. Reactions 2–5 form the OPPP network. The net fluxes through 2–5 add up to reaction 6, which is the net flux through OPPP and which has a rate of X. Note that the net flux through Glc-6-P oxidation (reaction 2) is 3X. Since the influx of Glc into the system is 1 and Glc-6-P is degraded via OPPP with the rate X, the rate of glycolytic degradation of Fru-6-P is 1 - X (reaction 7). Thus, X describes the split of carbon flux between OPPP and glycolysis. Reactions are as follows (see Fig. 2): 1, hexokinase; 2, glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase; 3, transketolase; 4, transaldolase; 5, transaldolase; 6, phosphofructokinase 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>
<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>$\rightarrow$ Glc-6-P</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Glc-6-P</td>
<td>$\rightarrow$ Ru-5-P + CO2</td>
<td>3X</td>
<td>X</td>
</tr>
<tr>
<td>3(TK)</td>
<td>Rib-5-P + Xu-5-P $\rightarrow$ Sh-7-P + GAP</td>
<td>X</td>
<td>$X + V_{TK}$</td>
<td>V_{TA}</td>
</tr>
<tr>
<td>4(TK)</td>
<td>E-4-P + Xu-5-P $\rightarrow$ Fru-6-P + GAP</td>
<td>X</td>
<td>$X + V_{TK}$</td>
<td>V_{TA}</td>
</tr>
<tr>
<td>5</td>
<td>Sh-7-P + GAP $\rightarrow$ E-4-P + Fru-6-P</td>
<td>X</td>
<td>$X + V_{TA}$</td>
<td>V_{TPC}</td>
</tr>
<tr>
<td>6</td>
<td>Glc-6-P</td>
<td>$\rightarrow$ GAP + 3CO2</td>
<td>X</td>
<td>1 - X</td>
</tr>
<tr>
<td>7</td>
<td>Fru-6-P</td>
<td>$\rightarrow$ DHAP + GAP</td>
<td>1 - X</td>
<td>2 - X</td>
</tr>
<tr>
<td>8</td>
<td>GAP</td>
<td>$\rightarrow$ PGA</td>
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</table>
Flux Model in Developing Plant Embryos

TABLE II
Randomization of 13C label between the upper and lower three carbons in starch (glucose) and in the glucosyl and fructosyl moieties of sucrose from labeled embryos

Embryos were grown for 3 days on [1-13C]Glc or [6-13C]Glc. In order to obtain maximal 13C label in Suc and starch, Suc was substituted by palatinose, which is not metabolized (see “Experimental Procedures”). 13C label in C-1 and C-6 of hexose units was determined by 13C NMR. Using the ratio C-1/C-6, the flux parameter for reversible interconversion of triose phosphate and Fru-6-P (V_{TPC}) was determined assuming X = 0.12, V_{GA} = 0.01, and V_{X} = 0.95. X, V_{GA}, and V_{X} were obtained by other labeling experiments (see “Metabolic Fluxes”). By simulation with different values for V_{TPC}, X, V_{TA}, and V_{TPC}, it was found that V_{TPC} is well determined by the ratio C-1/C-6 and largely independent from the values for X, V_{TA}, and V_{TPC} in particular for the labeling with [6-13C]Glc. Confidence intervals are given based on ±1% error in measured 13C contents of C-1 and C-6.

<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>[1-13C]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>Suc fructosyl</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>Suc glucosyl</td>
<td>0.20 (0.19–0.21)</td>
<td>0.6 (0.5–0.7)</td>
</tr>
<tr>
<td></td>
<td>Suc fructosyl</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>
<tr>
<td>[6-13C]Glc/Palatinose</td>
<td>Suc glucosyl</td>
<td>2.5 (2.4–2.6)</td>
<td>1.0 (0.9–1.1)</td>
</tr>
<tr>
<td></td>
<td>Suc fructosyl</td>
<td>2.5 (2.4–2.6)</td>
<td>1.0 (0.9–1.1)</td>
</tr>
<tr>
<td></td>
<td>Starch glucosyl</td>
<td>2.5 (2.4–2.6)</td>
<td>1.0 (0.9–1.1)</td>
</tr>
</tbody>
</table>

TABLE III
Comparison of the measured and simulated m_{j/m_{x}} ratios for different metabolates

The m_{j/m_{x}} ratio was measured by GC/MS in different metabolites after labeling with [1,2-13C_{2}]Glc or with mixtures of [1,2-13C_{2}]Glc with [1-13C]Glc or with [6-13C]Glc. Observed values (Obs.) are compared with values simulated (Sim.) using the parameters X = 0.12, V_{GA} = 0.01; V_{X} = 0.95; V_{TPC} = 1.0 (10% enrichment of the supplied Glc). In each experiment, 3–5 embryos were grown separately, each in 5 ml of medium. The embryos were extracted, and the experimental value of m_{j/m_{x}} is an average of three GC/MS measurements with S.D. < 2.5% (for C18:1_14:0).

For the experiments with a 1–13C,2–13C ratio of 1:1, the values represent the average of triplication of the experiment, resulting in S.D. values of 2–8% of m_{j/m_{x}}. pl., plastidic; cyt., cytosolic.

<table>
<thead>
<tr>
<th>Fragment/Precur</th>
<th>Labeled substrate supplied (Glc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1,2-13C_{2}</td>
</tr>
<tr>
<td>Glucose-1,2</td>
<td>1.16</td>
</tr>
<tr>
<td>Glyceraldehyde-1,2</td>
<td>0.46</td>
</tr>
<tr>
<td>Acetyl CoA-4,5</td>
<td>0.89</td>
</tr>
<tr>
<td>Palmitate-1,2</td>
<td>0.25</td>
</tr>
<tr>
<td>Acetyl-CoA-1,2</td>
<td>0.21</td>
</tr>
<tr>
<td>Palmitate-1,2</td>
<td>0.43</td>
</tr>
</tbody>
</table>

* 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-13C]Glc/[U-13C]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 glyceral part of TAG molecules is derived from cytosolic DHAP (Fig. 1). Thus, measuring labeling in glycerol and fatty acids allows determination of the extent of randomization of label between C-1 and C-6 of the network being modeled. The same C-1/C-6 randomization of label between C-1 and C-6 of sucrose from labeled embryos was determined by 13C NMR. Confidence intervals are given based on ±1% error in measured 13C contents of C-1 and C-6.

Interconversion of Hexose Phosphates—Synthesis of Fru-6-P from triose phosphate causes the exchange of 13C label between C-1 and C-6 in Fru-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 isotope-pomers 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 systems 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]Glc, [1,3-13C]Glc, and [1,2-13C]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 reproductive experiments, 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-13C]sucrose/[U-13C]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 (ΣΔ^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 ΣΔ^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_{TPC}, V_{TA}, and V_{TK} 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]Glc/[1,3-13C]Glc, the ratio m_{1}/m_{2} 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-13C]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-13C]sucrose/[U-13C]Glc, the fractional labeling of Glc isolated from starch was used to fit the reversible fluxes through TK and TA with the parameters V_{TK} (Fig. 3B) and V_{TA} (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]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]sucrose/[U-13C]Glc and for the ratio m_{1}/m_{2} in C18:1 (1,2-13C)Glc (labeling with [1,2-13C]Glc) cross (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; see below), the value for the reversible flux \( V_{\text{TK}} \) (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 \([1,2^{-13}\text{C}_2]\text{Fru-6-P}\) increases at the expense of \([\text{U-}^{13}\text{C}_6]\text{Fru-6-P}\), contributing to the abundances of \( m_3 \) isotopomers 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 \( m_3 \) isotopomers (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 Glic-6-P; 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 \([\text{U-}^{13}\text{C}_1]\text{sucrose/}[^{13}\text{C}_6]\text{Glc}, \) flux through the first route produces the \( m_1 \) isotopomer of His(1,6)-, and flux through the TK route produces \( m_2 \) and \( m_3 \) isotopomers. Since the \( m_1 \) isotopomer is only about one-quarter as abundant as the

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**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 \([\text{U-}^{13}\text{C}_1]\text{sucrose/}[^{13}\text{C}_6]\text{Glc} \) (see “Experimental Procedures”). Labeling in the glucose units of starch was measured by GC/MS (see “Experimental Procedures”). The measured fractional enrichment in fragments Glc(1-2), Glc(3-6), and Glc(1-6) was compared with fractional enrichments predicted by the model. The sum of squared differences \( \Sigma \Delta^2 \); 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 × 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 isotopomers. This threshold was used to estimate confidence intervals for the values (Table IV). \( V_{\text{TPC}} \) 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).

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**Fig. 4.** Optimized flux parameter values and obtaining a global solution by experimental over determination. Embryos were labeled for 14 days with \([\text{U-}^{13}\text{C}_1]\text{sucrose/}[^{13}\text{C}_6]\text{Glc} \) (see “Experimental Procedures”). The measured fractional enrichment in fragments Glc(1-2), Glc(3-6), and Glc(1-6) was compared with the fractional enrichments predicted by the model. The sum of squared differences \( \Sigma \Delta^2 \); see “Experimental Procedures”) was calculated for a range of values in the \( V_{\text{TK}}/V_{\text{TA}} \) plane, with \( V_{\text{TK}} = 1.0 \) 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 (black point), which gives 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 \( V_{\text{TA}} \) 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 × 10⁻⁴ threshold for \( \Sigma \Delta^2 \) shaded in gray. The results of optimizing the fit for \( V_{\text{TA}} \) and \( X \) for an experiment with \([1,2^{-13}\text{C}_2]\text{Glc/}[^{13}\text{C}_6]\text{Glc} \) (1:1) are also shown in B. In this case, mass spectroscopic data from C18:1 (1-2) to Glc(1,2) was also shown in B. In this case, mass spectroscopic data from C18:1 (1-2) to Glc(1,2) was also shown in B. In this case, mass spectroscopic data from C18:1 (1-2) to Glc(1,2) was also shown in B.
 Flux Model in Developing Plant Embryos

The flux parameters as described in Fig. 2 and Table I were determined by using different labeled substrates and by analyzing different metabolites. Confidence intervals are estimated as described in Figs. 3 and 4.

<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>$V_{TK}$</td>
<td>[U-13C]Glc</td>
<td>Starch</td>
<td>0.95 (0.5–1.4)</td>
</tr>
<tr>
<td>$V_{TA}$</td>
<td>[U-13C]Glc</td>
<td>Starch</td>
<td>0.01 (0–0.17)</td>
</tr>
<tr>
<td>$V_{TPC}$</td>
<td>[1-13C]Glc, [6-13C]Glc</td>
<td>Suc, starch</td>
<td>0.6–1.4</td>
</tr>
<tr>
<td>$V_{TPC}$</td>
<td>[U-13C]Glc</td>
<td>Starch</td>
<td>1.0 (0.7–1.4)</td>
</tr>
</tbody>
</table>

**TABLE IV**

Table of flux parameters and their corresponding values.

**FIG. 5.** Relation between the $m_1/m_2$ ratio in C18:1–3 and $X$, after labeling with [1,13C]Glc/[1-13C]Glc (1:1). After labeling with [1,13C]Glc/[1,2-13C]Glc (1:1), the ratio $m_1/m_2$ in C18:1–3 was determined by GC/MS. The ratio $m_1/m_2$ was simulated dependent on $X$, with the parameters $V_{TK} = 0.95$, $V_{TPC} = 1.0$, and $V_{TA} = 0.01$. The horizontal line with the gray bar denotes the measured value with the S.D. (1.24 ± 0.04, $n = 3$ independent experiments), and the vertical gray bar denotes the uncertainty in the derived value of $X$ that corresponds to this experimental range.

**FIG. 6.** Comparison of measured and simulated MS data. Embryos were grown for 14 days with [U-13C]glucose/[U-13C]Glc (see “Experimental Procedures”). Labeling in glycolytic units of starch, in glycerol from TAG, and in amino acids from seed protein was analyzed by GC/MS (see “Experimental Procedures”). The measured isotopomer abundances for the fragments Glc(1-1), Glc(1-6), Glc(3-6), glycerol(1-3), and Histidine were shown as black bars. With the flux parameters $X = 0.1$, $V_{TPC} = 1.0$, $V_{TK} = 0.95$, and $V_{TA} = 0.01$ (see Fig. 4), the model yields simulated fractional labeling for hexose phosphate, triose phosphate, and pentose phosphate (gray bars). Good agreement between experimental and simulated isotopomer levels is seen except for histidine $m_1$. Since histidine comprises 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.

$m_0$ isotopomer in Glc(1-6) (Fig. 6) and is much less abundant than the $m_2$ and $m_4$ isotopomers, most of the histidine must be synthesized via the TK route. Since the TK signature is produced first in Xu-5-P molecules, the flux from Xu-5-P → Ru-5-P → Rib-5-P must be much larger than the flux from Glc-6-P → Ru-5-P → Rib-5-P. This implies that the reversible ribulose-5-phosphate-3-epimerase and ribose-5-phosphate isomerase fluxes are substantially higher than the flux through oxidative decarboxylation, which has a magnitude of 3X in the notation of our model. This supports the model assumption (Fig. 2) that the pentose phosphates are in isotopic equilibrium and may be treated as one pool.

**Quantification of the Split of Carbon Flux between Glycolysis and OPPP (X) —** The results of labeling with [1,2-13C]Glc are particularly sensitive to the OPPP flux (Fig. 5), and this substrate was therefore used (Table III) in addition to the experiments described above using uniformly 13C-labeled sugars. When metabolized by glycolysis, double-labeled [1,2-13C]Glc produces the double-labeled intermediates [2,3-13C]triose phosphate, [2,3,4,5,6-13C]pyruvate, and [1,2,3,4,5,6-13C]acetyl-CoA, resulting in $m_2$ mass peaks (e.g. in the fragment C18:1–3). However, oxidative decarboxylation of [1,2-13C]Glc produces [1-13C]pentose phosphate, which is then converted via TK and TA reactions to singly labeled fructose ([1-13C]Fru-6-P, [2,3-13C]Fru-6-P, and [3-13C]Fru-6-P) and subsequently to other singly labeled intermediates that contribute to $m_1$ abundance in mass spectra. The more [1,2-13C]Glc is converted to single labeled hexose phosphate by the OPPP, the higher the ratio $m_1/m_2$ is measured by mass spectrometry. In addition to labeling with [1,2-13C]Glc alone, mixtures of [1,2-13C]Glc with [1-13C]Glc or [6-13C]Glc were used. The action of glycolysis on a 1:1 mixture of [1,2-13C]Glc and [1-13C]Glc will produce an $m_1/m_2$ ratio of 1, whereas OPPP flux will result in an $m_1/m_2$ ratio of >1 (Fig. 5), which was indeed observed (Table III). The results of using a mixture of [1,2-13C]Glc and [6-13C]Glc (1:1) would be sensitive to any disequilibrium at triose phosphate isomerase and provide information on triose/hexose cycling. For this experiment, a $m_1/m_2$ ratio lower than 1 was found for Glc(1-6) (Table III), which is to be expected, because only about 20% of the label in C-6 of hexose phosphate is redistributed to C-1 of hexose phosphate by triose cycling (Table III).

After labeling with [1,2-13C]Glc/[1-13C]Glc (1:1), the fragment C18:1–3 was measured by GC/MS, and an average value for $m_1/m_2$ of 1.24 ± 0.04 was found ($n = 3$). As described in the legend to Fig. 4B, this value corresponds to $X = 0.12$ (0.07–0.14). The $m_1/m_2$ ratios were also measured for several metabolites derived from intermediates of the pathway of Glc breakdown (Table III) after labeling with [1,2-13C]Glc or with [1,2-13C]Glc/[6-13C]Glc (1:1). Again, the computer simulation predicted $m_1/m_2$ ratios for different intermediates from the different substrate labeling experiments that were similar to the measured values (Table III).

**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 bioisothetic 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_{TA}, 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_{TK}, V_{TA}, V_{TPC}). This was achieved by overdetermination of the flux model both by analysis of multiple metabolic products and by labeling experiments 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 14CO2 from [1,14C]Glc and [6-14C]Glc suffer from limitations due to (a) reoxidation of CO2, (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 CO2 release. For example, 14CO2 release from 14C Glc by castor bean endosperm (73) suggested that 50–70% of NADPH needed for fatty acid synthesis can be provided by OPPP. However, this 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, CO2, 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 13C 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 C3 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 C2 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 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 NADPH 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), sedohexulose bisphosphatase, and TK (sedohexulose 7-phosphate + GAP → Rib-5-P + Xu-5-P). By adding TA to this reaction network, sedohexulose 7-phosphate can be cyclically removed (sedohexulose 7-phosphate + GAP → erythrose 4-phosphate + Fru-6-P) and regenerated from sedohexulose 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-
1,5-bisphosphate carboxylase/oxygenase-specific isotopomer patterns and in vivo activation status.

Potential Limitations of This Study—By closely mimicking in vivo growth conditions, we have aimed to obtain information on metabolic fluxes relevant to the developing embryo in a plant. The culture conditions are such that an embryonic mode of growth is preserved, and the embryos keep in culture achieve similar oil and protein content as well as similar fatty acid composition to in planta seeds (22). In addition, the embryos from culture are viable and able to germinate if transferred to a different culture medium. Under the low light conditions used in the labeling experiments, the embryos remain green during culture, which indicates that they maintain their principally chloroplast-like plastid structure. Nevertheless, growth in culture and any flux model of metabolism must represent an approximation of true in vivo conditions. We present some of the possible limitations to the model presented in this study and efforts to address them.

First, the assumptions of isotopic steady state and metabolic homogeneity are at the heart of flux analyses based on steady state labeling patterns, and they are far more difficult to achieve in plant tissues than in microorganisms. In this study, developing B. napus embryos were dissected from siliques at 20 days after flowering and cultured during the main phase of oil accumulation under conditions close to in planta growth. It is reported that the Suc/hexose ratio changes in the lipid endosperm surrounding the developing embryos during development (22, 23), and therefore embryo growth may not be achieved within 3 days for inter-

mediate pools (sucrose, free amino acids) and also for starch. Furthermore, although the embryos when placed in culture already have some storage TAG and protein that is not turned over, after the full 14-day labeling period, TAG and protein increased over 10-fold, resulting in essentially complete labeling of accumulated end products. No evidence of multiple pools in any of the storage products (starch, protein, oil, sucrose) was observed, which again probably reflects the fact that the embryo is dominated by cotyledon cells and that exposure to constant labeling conditions in the external medium allowed the principal metabolic fluxes to be investigated.

Second, for developing seeds of B. napus, oil accumulation overlaps with but slightly precedes storage protein accumulation. Thus, the isotopomer imprint accumulated in seed protein could in part reflect metabolism after the peak phase of oil synthesis. However, the 14-day culture of our experiments covers the main period of oil accumulation and not the later phase that is more dominated by storage protein formation (22). Thus, the isotopic imprint in protein represents metabolism during the phase of maximal oil synthesis. Accordingly, we observed that the acetate units in fatty acids (from seed oil) and homologue C\textsubscript{2} units in amino acids (from storage protein) were almost identically labeled (see Table III).

Finally, the OPPP can operate in a cyclic manner in root plastids, probably without net contribution of the upper part of hexose phosphate molecules to further metabolism (79). This could distort the derived value of X if [\textsuperscript{1-13}C\textsubscript{2}]

pentose phosphate, produced by oxidative decarboxylation of [1,2,13C\textsubscript{3}]Glc-6-P, is produced to recycle single 13C-labeled Fru-6-P that does not enter the glycolytic route leading to fatty acids and therefore would not contribute to the measured isotopomer pattern in glycolytic products. This kind of “sequestered” cyclic OPPP is not supported by the labeling pattern of histidine, which is derived from plastidic pentose phosphate or by the similar m\textsubscript{1}/m\textsubscript{2} ratios between Glc\textsubscript{1-2}, Ala\textsubscript{2-3}, and C18:1\textsubscript{1-2} (Table III).

Conclusions—Plant seeds provide the major food and economic value of most crops, and therefore a quantitative understanding of their metabolic networks is an important goal for plant biochemists. Using steady-state stable isotope labeling, we have developed and tested a model describing flux through B. napus embryo glycolysis and OPPP. Computer simulation allowed the complex interaction of reversible fluxes to be fit to experimental data. The reliability of the determination of OPP and other fluxes in this study was enhanced by using a well studied system that allows the achievement of steady state labeling, through the use of multiple substrates, with the analysis of multiple products, and by the testing of underlying assumptions inherent in such models. Although future im-

### Table V

Steady state equations for the isotopomers of metabolic pools

<table>
<thead>
<tr>
<th>Influx</th>
<th>Efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔHp</td>
<td>Glc\textsubscript{in} + HP\textsubscript{TK2*} (X + V\textsubscript{TP}) + HP\textsubscript{TA} (X + V\textsubscript{TA}) + HP\textsubscript{Aldo} V\textsubscript{TPC}</td>
</tr>
<tr>
<td>ΔPp</td>
<td>PP\textsubscript{TK1Back} (2V\textsubscript{TP} + PP\textsubscript{TK2Back} * V\textsubscript{TP}) + PP\textsubscript{Decarb} * 3X</td>
</tr>
<tr>
<td>ΔEp</td>
<td>EP\textsubscript{TA} (X + V\textsubscript{TA}) + EP\textsubscript{TK2Back} V\textsubscript{TP}</td>
</tr>
<tr>
<td>ΔSp</td>
<td>SP\textsubscript{TK1} (X + V\textsubscript{TP}) + SP\textsubscript{TA Back} V\textsubscript{TP}</td>
</tr>
<tr>
<td>ΔTkp</td>
<td>TP\textsubscript{kin} (2(1 - X + V\textsubscript{TP})) + TP\textsubscript{TK2} (X + V\textsubscript{TP}) + TP\textsubscript{TK1} (X + V\textsubscript{TP}) + TP\textsubscript{TA Back} V\textsubscript{TP}</td>
</tr>
</tbody>
</table>

\(\Delta X\) is the change in the isotopomer abundance relative to the initial abundance.
provenances can be anticipated, we believe this to be the most reliable analysis to date of the OPPP/glycolysis network in a plant system.

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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 reactant isotopomers are combined according to probabilistic state of the intermediate pools (starting with all intermediate pools unlabeled and proceeding by guest on January 5, 2018 http://www.jbc.org/ Downloaded from

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