Sinapine as a Supply of Choline for the Biosynthesis of Phosphatidylcholine in *Raphanus sativus* Seedlings

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Biosynthesis of phosphatidylcholine in young seedlings of *Raphanus sativus* is supplied with choline from degradation of the seed constituent sinapine (sinapoylcholine). This conclusion has been deduced from the following results:

(1) *Raphanus sativus* seedlings accumulate approx. 70 nmol phosphatidylcholine which may be relevant for the consumption of choline, liberated during hydrolysis of approx. 130 nmol sinapine.

(2) [^{14}C]choline and [^{14}C]ethanolamine, taken up by germinating *Raphanus* seeds, are exclusively consumed in the biosynthesis of phosphatidylcholine and phosphatidylethanolamine, respectively.

(3) Pulse-chase experiments with seedlings at different germination stages show a reduced [^{14}C]choline incorporation into phosphatidylcholine at the time when degradation of sinapine occurs, obviously as a result of an isotope dilution by an increase of the endogenous choline pool.

(4) After [^{14}C]choline pulse to immature seeds, during the process of seed maturation most of the activity taken up is incorporated into accumulating sinapine and approx. 50% compared to this into phosphatidylcholine. During seedling development the quantity of labelled sinapine rapidly decreases as a result of sinapine degradation with a concomitant label increase in free choline, phosphorylcholine, and phosphatidylcholine. Approx. 50% of the choline liberated from sinapine, is consumed in the biosynthesis of phosphatidylcholine.

High performance liquid chromatographic analyses of phosphatidylcholine during *Raphanus* germination revealed that this phospholipid might be a metabolically active compound. Changes in the absorptivity of this compound at 210 nm indicate changes in the degree of unsaturation in the fatty acyl groups.

Introduction

During development of *Raphanus sativus* seedlings the seed constituent sinapine (sinapoylcholine) is interconverted to 1-sinapoylglucose [1–3]. This interconversion seems to be widespread among members of the Brassicaceae [2, 4]. So far the fate of choline, liberated in this interconversion, remained obscure. This choline merely could accumulate, it could be metabolized *via* betaine and subsequent demethylation reactions [5, 6], or it could play a role in the phospholipid metabolism of *Raphanus* as a building block in the biosynthesis of phosphatidylcholine [7, 8].

Tzagoloff, who discovered the degradation of sinapine in *Sinapis* [9, 10], tried to approach this problem by attempts to find a choline oxidase activity at the germination stage when sinapine is degraded. Since he neither could detect this enzymatic activity nor free choline at any stage of *Sinapis* seedling development, we assumed that this choline might be involved in the phospholipid metabolism.

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Pulse-chase experiments, reported in the present paper, suggest, that sinapine serves as a ready-stored supply of choline for the biosynthesis of phosphatidylcholine in young seedlings of *Raphanus sativus*.

Materials and Methods

Plant material and growing conditions

Seeds of *Raphanus sativus* L. var. *sativus* cv. Saxa were purchased from Zwaan u. Co's u. Komp., Delfter Marktgärtner-Samenzucht GmbH, Netherlands.

Seedlings were grown in a phytotron under fluorescent light (ca. 7000 lx) at 21–22 °C and 60–70% relative humidity. Plants to be harvested on or before the 3rd day of germination were grown on moist filter paper in petri dishes, the others in defined soil (type T, Keller, Cologne) mixed 1:1 with peat.

In order to harvest immature seeds, adult plants were cultivated in the field of the botanical garden of the university of Cologne. Developing pods (2–3 weeks after pollination) were harvested and seeds isolated, which were dark green or in the stage of

beginning of browning. In the experiments these seeds were put on dry sheets of filter paper for 1–2 weeks in the phytotron until they became dark brown and could not be distinguished from mature seeds. Subsequent seedling development was under the conditions as described above.

Chemicals

Choline chloride was purchased from Fluka, Neu-Ulm; choline phosphate chloride (calcium salt) from Sigma, Taufkirchen. L-α-Lecithins (dipalmitoyl, 1-palmitoyl-2-oleoyl, dioleoyl, dilinoleoyl) were obtained from Serva, Heidelberg.

[2-^{14}C]Ethanolamine hydrochloride (44 mCi/mmol) and [methyl-^{14}C]choline chloride (59.8 mCi/mmol) were supplied by Amersham Buchler, Braunschweig.

Thin layer chromatography (TLC)

Phospholipids were separated on pre-coated silica gel 60 plates (Merck, Darmstadt) in CMW (chloroform-methanol-water, 35:15:2), ABW (acetone-benzene-water, 91:30:8), and two dimensional in CMW and CMA (chloroform-methanol-*gl.* acetic acid, 62:25:8). Choline and phosphorylcholine were chromatographed on microcrystalline cellulose (Avicel) in BAW (*n*-butanol-*gl.* acetic acid-water, 6:1:2) or two dimensional in three solvent systems according to ref. [11]. Chromatography of crude extracts and cochromatography with reference compounds in order to identify ^{14}C-labelled constituents were done after separation into a water soluble and lipid fraction in the system described in ref. [11] and in CMW, ABW, and CMW/CMA. Unfractionated extracts were resolved in BAW (see Fig. 3).

Phospholipids and phosphorylcholine were visualized by a phospholipid spray reagent [12], sinapine and choline by a Dragendorff's reagent (Merck, Darmstadt). Lipids separated in CMW/CMA in order to quantify phosphatidylcholine by means of P_i determination [13] were revealed by I_2 vapour. Identifications were made by comparison of R_f values of known standards.

High performance liquid chromatography (HPLC)

HPLC analyses of sinapine were carried out on Spherisorb Silica (Spectra-Physics, Darmstadt) with dichloromethane-methanol-sulfuric acid (85:15:1) according to ref. [14]. Phosphatidylcholine was analyzed by a modified method of Hax *et al.* [15] with two elution systems. Resolution into individual phospholipids (phosphatidylethanolamine, $t_R = 769$ sec; phosphatidylcholine, $t_R = 1413$ sec) was achieved by a linear gradient elution with 1 ml/min from solvent A (2-propanol-*n*-hexane-*ortho* phosphoric acid, 250:150:4) to solvent B (2-propanol-*n*-hexane-water-*ortho* phosphoric acid, 250:75:75:4) within 20 min. A rapid analysis of phosphatidylcholine ($t_R = 432$ sec) was accomplished with the isocratic system 2-propanol-*n*-hexane-water-*ortho* phosphoric acid (250:75:40:4) (1 ml/min). Phospholipids were detected at 210 nm (1.0 a. u. f. s.). The chromatograph, detector, and the computing integrator are described in ref. [16].

Administration of labelled compounds

Experiment *a.* 600 Germinated seeds (24 h old) were placed into 20 ml of water, containing 10 μCi [^{14}C]choline and 10 μCi [^{14}C]ethanolamine, respectively. After 3 h of incubation seeds were removed, rinsed with water, and seedlings were grown on moist filter paper or defined soil. Sampling was done in 24 h-intervals up to the fourth day of germination.

Experiment *b.* At different developmental stages 100 seedlings each were incubated with 2 μCi [^{14}C]-choline in various amounts of water, depending on seedling size. After 3 h the seedlings were removed, rinsed with water, and were placed on moist filter paper for 24 h.

Experiment *c.* 600 Immature seeds were submerged into 22 ml of water, containing 50 μCi [^{14}C]-choline. After 5 h the seeds were removed, rinsed with water, and placed on dry filter paper until they reached maturity. During subsequent seedling development on moist filter paper seedlings were harvested at two representative germination stages (see legend of Fig. 4).

All incubations were done in 50 ml-erlenmeyers at ambient room temperature and light.

Preparation of extracts

For extraction of sinapine 20 individuals were treated with an Ultra Turrax homogenizer for 2 min in methanol and the homogenate was centrifuged at $3000 \times g$ for 5 min. The supernatant was directly analyzed by HPLC.

Other extractions were done depending on the experiments *a*, *b*, or *c* (see previous chapter).

Experiment *a* and *b*. 20 or 50 seedlings and their detached organs, respectively, were ground in a mortar, containing 2 ml of MCF (methanol-chloroform-formic acid, 12:5:3). The homogenate was transferred into a centrifuge tube. The mortar was rinsed three times with 2 ml MCF each and this MCF for rinsing was combined with the homogenate. This was allowed to stand for 18–24 h. After centrifugation at 3000 × *g* for 5 min the supernatant was removed and the pellet extracted a second time with 4 ml MCF. The supernatant of this second extract was combined with that obtained from the first extraction. Extracts were separated into a water soluble and lipid fraction by emulsifying with a mixture of chloroform (5 ml) and water (2 ml). This emulsion was centrifuged at 4000 rpm for 60 min and the resulting two phases were separated. The lipid fraction was taken to dryness under a cold and the water soluble one under a hot airstream and were redissolved in 2 ml tetrahydrofurane and 20% aq. ethanol, respectively. These fractions were stored at −18 °C.

Experiment *c*. 20 individuals were treated as described above except for extract fractionation.

HPLC of phosphatidylcholine was done from the lipid fractions.

Determination of radioactivity

Radioactivity was measured by scintillation counting (Tricarb, Model 3380, Packard) using Unisolve I (Zinsser). Data were corrected for quenching by the external standard channels ratio method. Localization of radioactive compounds on thin layer plates was either achieved by means of a scanner (Berthold) or using Kodak X-ray paper.

Results and Discussion

[^{14}C]choline and [^{14}C]ethanolamine, taken up by 24 h germinated *Raphanus* seeds (experiment *a*), are exclusively consumed in the biosynthesis of phosphatidylcholine and phosphatidylethanolamine, respectively. Sampling was done in 24 h-intervals up to the fourth day of germination. Over the first 24 h 53% of total ethanolamine uptake (29589 dpm/seed) were recovered in phosphatidylethanolamine and 25% of total choline uptake (31 399 dpm/seed) in phosphatidylcholine. During subsequent growth relative activity decreased to approx. 34 and 10%, respectively. The only other compound labelled from [^{14}C]choline was found in the water soluble fractions and was identified as phosphorylcholine (approx. 7% incorporation). The remaining radioactivities were recovered in the administered precursors. There was no loss of the radioactivity taken up.

Provided that the label reached the *in situ* pools of choline and ethanolamine, these results exclude firstly that phosphatidylcholine is formed in young *Raphanus* seedlings in a methylation pathway and secondly that choline might be degraded *via* betaine and subsequent demethylation reactions.

The involvement of choline in the biosynthesis of phosphatidylcholine (CDP-base pathway) seems to

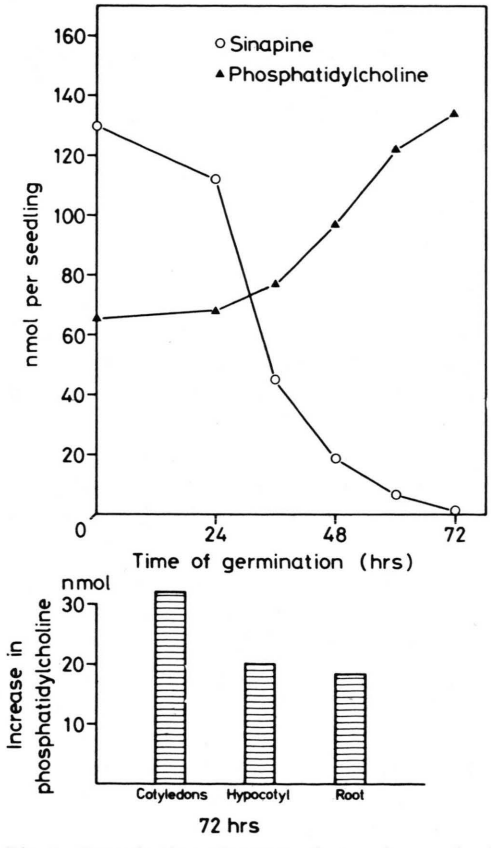

Fig. 1. Quantitative changes of sinapine and phosphatidylcholine during early stages of seedling development. Each point represents the mean of three independent determinations. (Depending on the seed batch the quantity of sinapine per seed varies between approx. 110 and more than 160 nmol.) The distribution of newly accumulated phosphatidylcholine in the organs of the 72 h old seedling is illustrated underneath.

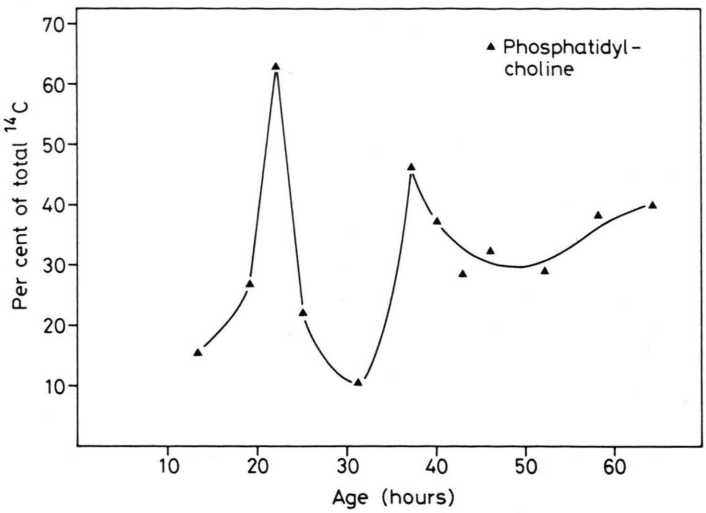

Fig. 2. Effect of seedling age on the relative incorporation of [^{14}C]choline into phosphatidylcholine (experiment *b*). Total uptake was at the average 25998 (± 11%) dpm per seedling.

be the predominant pathway of choline in higher plant tissues [7, 8]. The degradation of choline, however, has also been demonstrated to occur in higher plants [5, 6].

Most members of the Brassicaceae contain the seed constituent sinapine (sinapoylcholine) [17] and it has been shown for various plants of this family that sinapine is hydrolyzed during early stages of seedling development [2, 9, 18, 19]. The liberated sinapic acid is reesterified to 1-sinapoylglucose in cotyledons of *Raphanus sativus* [2, 3, 20] and *Sinapis alba* [4]. It is demonstrated in the present paper that in *Rhaphanus* choline which is released in this metabolism enters the CDP-base pathway in the biosynthesis of phosphatidylcholine. This possibility was anticipated from the initial incorporation studies described above.

Quantitative analyses of phosphatidylcholine (P_i determination) during *Raphanus* germination revealed the accumulation of this phospholipid in a quantity (approx. 70 nmol) which may be relevant for the consumption of choline, liberated during sinapine hydrolysis (Fig. 1). If this is the case, pulse-chase studies (experiment *b*) with seedlings at different germination stages should show a reduced [^{14}C]choline incorporation into phosphatidylcholine at the time, when degradation of sinapine occurs. As expected this result was obtained and is illustrated in Fig. 2.

Between the first and second day of germination there is the most rapid hydrolysis of high amounts of sinapine. 31 h old seedlings showed a relatively low [^{14}C]choline incorporation into phosphatidylcholine of 10% of total uptake, obviously as a result of an isotope dilution by an increase of the endogenous choline pool. In 9 h younger seedlings this incorporation was 63% and those which degraded already most of the sinapine showed again a higher incorporation. Total label uptake was not affected by the seedling age and was found to be at the average 25998 (± 11%) dpm per seedling.

In *Raphanus*, grown at lower temperatures, sinapine degradation was retarded and much slower. Consequently the kinetics of the isotope dilutions were markedly different. At 16 °C sinapine degradation occurs between 35 and 90 h of germination with a concomitant drop of the [^{14}C]choline incorporation into phosphatidylcholine from 45 to 7% of total uptake. At 10 °C sinapine degradation was observed between 60 and 120 h and at that time the ^{14}C incorporation was reduced from 50 to 6%. Thereafter in both experiments incorporation increased again.

The most informative data were obtained by [^{14}C]choline pulse-chase experiments with detached immature seeds.

Sinapis alba accumulates sinapine during the main growth phase of developing embryos [4, 21]. Depending on the age, detached *Sinapis* embryos continue to accumulate sinapine. Taking advantage of this phenomenon it was possible to specifically label the choline moiety of sinapine during maturation of detached *Raphanus* seeds (experiment *c*).

Under the conditions in this experiment approx. 60% of the administered [^{14}C]choline was taken up

by 600 immature seeds. This activity was chased during seed and seedling development. Table I lists the data of one representative experiment and Fig. 3 depicts profiles of radioactivity from TLC of total extracts, derived from immature seeds (embryos), immediately after incubation, from matured seeds (1–2 weeks later), and from developed seedlings at stage II. It can be seen that most of the activity, taken up, was incorporated into sinapine during the process of seed maturation. Starting with approx. 25 nmol per immature seed, a level of approx. 115 nmol was reached in matured seeds. During seedling development labelled sinapine decreased as a results of sinapine degradation with a concomitant label increase predominantly in phosphatidylcholine. Fig. 4 shows the time course of the relative [^{14}C]cho-

Table I. Incorporation of [^{14}C]choline into phosphorylcholine, sinapine, and phosphatidylcholine during seed and seedling development. For details see experimental section. Values are expressed as dpm × 10^{-3} per individual and per cent of total uptake.

Compound	Radioactivity					
	Immature seed		Mature seed		Seedling at stage II	
	[dpm]	[%]	[dpm]	[%]	[dpm]	[%]
Choline	100.3	82	33.5	25	34.0	31
Phosphoryl-choline	11.5	10	9.1	8	15.9	14
Sinapine	11.1	8	54.7	41	6.4	6
Phosphatidyl-choline	4.7	4	20.8	16	46.0	41

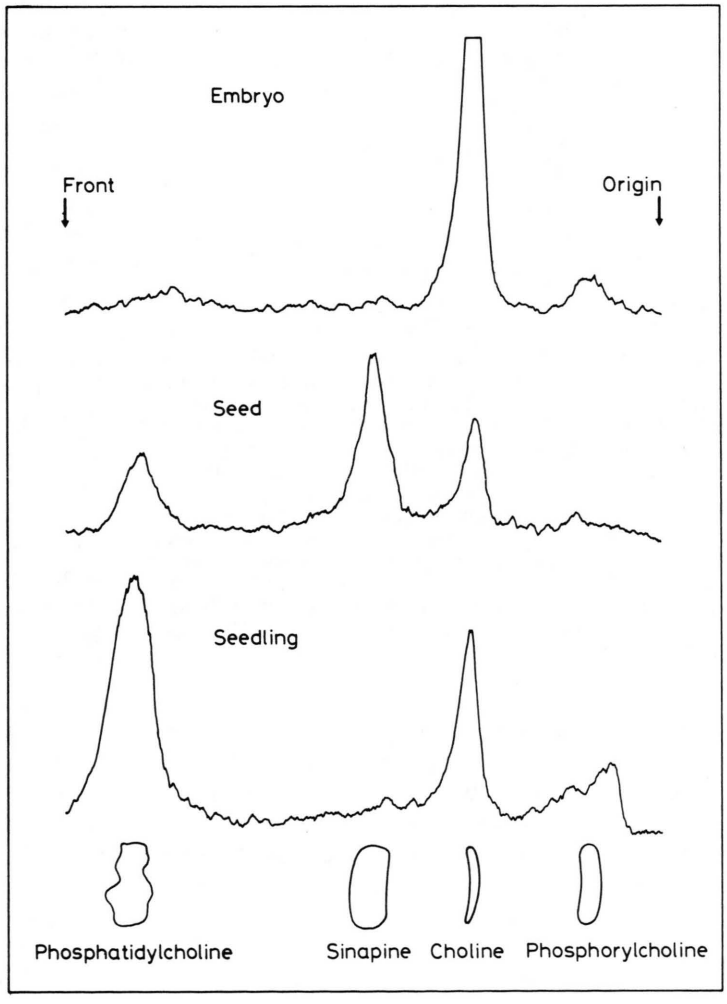

Fig. 3. Radiochromatograms of extracts from immature seeds (embryos), seeds, and seedlings at stage II, defined in legend of Fig. 4. TLC was developed with *n*-butanol-*gl.* acetic acid-water, 6:1:2. The positions of the identified ^{14}C-labelled compounds are shown beneath the profiles of radioactivity. Extracts were derived from experiment *c*.

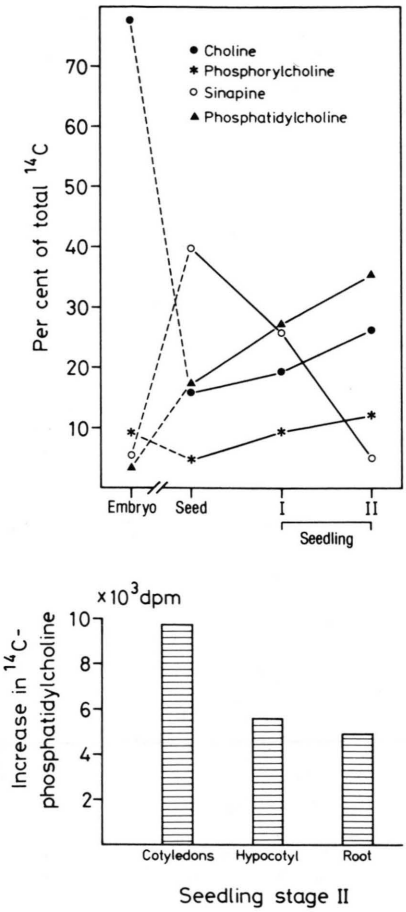

Fig. 4. Incorporation of radioactivity into choline derivatives during seed maturation and seedling development after a [^{14}C]choline pulse to immature seeds (experiment *c*). Due to a limited number of matured seeds and unequal rates of seedling development, seedlings were harvested at two representative stages, defined from germination of seeds matured *in situ* (*i.e.* in pods). Stage I: primary root 2–3 cm, beginning of anthocyanin synthesis in the hypocotyl and chlorophyll synthesis in the cotyledons (approx. 36 h stage of "normal" development); stage II: primary root approx. 5 cm, strongly growing hypocotyl and cotyledons (approx. 3 day-stage). The distribution of newly accumulated [^{14}C]phosphatidylcholine in the seedling organs at stage II is illustrated underneath.

line incorporation into phosphorylcholine, sinapine, and phosphatidylcholine, derived from three independent experiments. This figure shows also the distribution of newly accumulated [^{14}C]phosphatidylcholine in the seedling organs, which correlates well with the molar distribution of newly accumulated phosphatidylcholine (Fig. 1).

Comparative TLC revealed that during sinapine degradation an appreciable amount of free choline accumulates, which was deduced from an increase in color intensity by spraying with Dragendorff's reagent. This indicates a transient pool of choline, liberated from sinapine. Half of this pool will enter the phospholipid metabolism in the subsequent biosynthesis of phosphatidylcholine (refer to Figs 1 and 4). This assumption might explain the sinapine degradation and phosphatidylcholine accumulation to be somewhat out of line. In later stages of *Raphanus* germination most of the remaining [^{14}C]choline will be mainly consumed in the phosphatidylcholine synthesis in hypocotyl and root.

Since Tzagoloff [9] could not detect free choline at the stage of sinapine degradation in *Sinapis alba* and was unsuccessful to find a choline oxidase activity, we might assume that in this plant sinapine also serves as a supply of choline for the biosynthesis of phosphatidylcholine. Compared to *Raphanus* the sinapine degradation in *Sinapis* is much slower [2, 18] and may be there is a linear correlation between sinapine degradation and phosphatidylcholine accumulation.

Quantitative HPLC data of *Raphanus* phosphatidylcholine, detected at 210 nm, strikingly differ from those obtained by P_i determinations, since the results obtained by HPLC would suggest a 3-fold higher increase in comparison with the results obtained by P_i determinations. This increase in absorptivity was mainly observed in phosphatidylcholine, extracted from the radicle and hypocotyl between 24 and 36 h of germination. At 48 h the absorptivity has decreased to approx. 20% compared to that at 36 h. This discrepancy can be explained by different intensities of the "end absorption" of phosphatidylcholine, depending on unsaturated centres [15]. This is confirmed by the following correlation between absorbance at 210 nm and degree of unsaturation of the fatty acid moieties. Taking dipalmitoyl-lecithin as reference, the absorbance of the oleoyl-palmitoyl species increases by a factor of 9.3, dioleoyl by 22.3, and dilinoleoyl by 57.2 (sample load, 100 µg each in 20 µl of solvent). Thus the observed changes in the absorptivity of this phospholipid indicate changes in the degree of unsaturation in the fatty acyl groups.

It cannot be decided whether there is a fatty acid exchange, phosphatidate turnover, or a direct desaturation and saturation of phosphatidylcholine-bound acyl groups. This question is a matter of controversy [22].

Conclusion

The results described in this paper suggest that sinapine serves as a ready-stored supply of choline for the biosynthesis of phosphatidylcholine in young seedlings of *Raphanus sativus*. This phosphatidylcholine seems to be a metabolically active compound, since there are changes in the degree of unsaturation of the fatty acyl groups.

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