Incorporation of serine into *Paramecium* ethanolamine phospholipid and phosphonolipid head groups

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Ethanolamine phospholipid head groups in *Paramecium* were synthesized directly from ethanolamine. As in other cell types, radioactivity from ethanolamine failed to incorporate significantly into head groups of ethanolamine phosphonolipids, indicating that the phosphonolipids are not derived from their phospholipid analogues. Unlike other systems previously examined, radioactivity from serine is incorporated into both ethanolamine phospholipid and phosphonolipid head groups of glycerolipids and sphingolipids in this ciliate. These observations suggest that synthesis of ethanolamine phosphonolipids involves synthesis *de novo* of free phosphoserine, which is then incorporated into lipids, and then lipid-bound phosphoserine intermediates (glycerolipids or sphingolipids) undergo decarboxylation, forming lipid-bound phosphoethanolamine compounds.

Phosphonolipids have been isolated from various invertebrates, protozoa and mycobacteria (Kittredge & Roberts, 1969; Rosenberg, 1973; Hori & Nozawa, 1982). Formation of free aminoethylphosphonate (phosphonoethylamine, AEP) involves the decarboxylation and amination of phosphoenolpyruvate (Rosenberg, 1973; Hori & Nozawa, 1982).

Although free AEP was found in the ciliate *Tetrahymena*, it was demonstrated that this was not a major precursor of lipid-bound AEP since it was synthesized after lipid-bound AEP (Liang & Rosenberg, 1968; Rosenberg, 1973; Hori & Nozawa, 1982). The 'Kennedy pathway', in which free CMP-AEP and diacylglycerol participate in an exchange reaction, was considered a salvage pathway (Rosenberg, 1973; Hori & Nozawa, 1982). Re-arrangement of phosphoenolpyruvate to phosphonopyruvate, as CMP-AEP, or as phosphatidylphosphonopyruvate, was suggested as the major step *de novo* to lipid-bound 2-amino-3-phosphonopropanate (alanine-3-phosphonate, 3-phosphonoalanine, phosphoserine, APnP) followed by the formation of ethanolamine phosphonolipids by decarboxylation (Rosenberg, 1973). Smith & Law (1970) showed that APnP was incorporated into *Tetrahymena* phosphonolipids more efficiently than was AEP. These workers, however, concluded that APnP was converted into AEP as the free phosphonate. Formation of ethanolamine phosphonolipids by a mechanism

involving decarboxylation of a lipid-bound APnP intermediate has been dismissed by a number of investigators because the lipid intermediate has not been isolated, phosphatidylserine was not detected in *Tetrahymena*, and incorporation of serine into ethanolamine phosphonolipids was not demonstrated (Warren, 1968; Rosenberg, 1973). No unified scheme for explaining the formation of phosphonolipids that receives general acceptance has yet been postulated (Hori & Nozawa, 1982). The processes leading to the formation of phosphoryl bonds in sphingolipids are totally unknown (Hori & Nozawa, 1982). In the present paper we describe metabolic radiolabelling studies with *Paramecium* that implicate the existence of previously hypothesized serine pathways for the synthesis of ethanolamine phosphonolipid head groups (Rosenberg, 1973). This ciliate has three ethanolamine sphingophospholipids and three ethanolamine sphingophosphonolipids, in addition to the ethanolamine glycerophospholipids and glycerophosphonolipids (Rhoads & Kaneshiro, 1979; Andrews & Nelson, 1979).

Experimental

Paramecium tetraurelia 51s was grown in an axenic medium in the presence of 0.02–3.0 μ Ci of radiolabelled precursor/ml as previously described (Rhoads & Kaneshiro, 1979; Kaneshiro *et al.*,

1979). Unless otherwise indicated, all radiolabelled compounds were added at day 3 or 4 of culture growth, and cells were extracted for lipids on day 5 (late exponential phase). Lipids were extracted, purified and fractionated by TEAE- (triethylaminoethyl)-cellulose column chromatography as previously described (Rhoads & Kaneshiro, 1979; Kaneshiro *et al.*, 1979). Individual sphingolipids and glycerolipids in the ethanolamine lipid fraction eluted from TEAE-cellulose columns were separated by two-dimensional t.l.c. on 0.25 mm-thick pre-coated Silica Gel 60 plates (E. Merck, Darmstadt, Germany) with the solvent systems previously described (Rhoads & Kaneshiro, 1979). Fatty acids and water-soluble products from glycerolipids were isolated after hydrolysis under alkaline conditions (Rhoads & Kaneshiro, 1979) or phospholipase C (from *Bacillus cereus*; Sigma Chemical Co., St. Louis, MO, U.S.A.) digestion (Kaneshiro, 1980). Fatty acids, long-chain bases and water-soluble groups were isolated from sphingolipids after acid hydrolysis (Carter & Gaver, 1967) or phospholipase C digestion.

Lipids were radiolabelled *in vitro* with $0.6\mu\text{Ci}$ of phosphoenol[1- ^{14}C]pyruvic acid/ml and a crude sonicated *Paramecium* whole-cell preparation. Cells were centrifuged as described previously (Rhoads & Kaneshiro, 1979; Kaneshiro *et al.*, 1979) and washed in a solution containing 150 mM-sucrose and 20 mM-Tris/HCl buffer, pH 7.4. A cell suspension of 10^6 cells in 8 ml of buffer solution containing either 10 mM- CaCl_2 or 10 mM- MgCl_2 was maintained at 4°C . The cells were sonicated with three 10 s pulses. The radiolabelled compound was added to this sonicated whole-cell preparation and the reaction was left to proceed at room temperature for 30 min. The reaction was terminated by the extraction of lipids with 30 ml of chloroform/methanol (1:2, v/v).

Results

Incubation of *Paramecium* cells with $0.7\text{--}1.0\mu\text{Ci}$ of $\text{H}_3^{32}\text{PO}_4/\text{ml}$ for 5 days under isotope-equilibrium conditions resulted in the distribution of radioactivity in lipids that reflected the quantity of the various phospholipid classes present in the cell (Rhoads & Kaneshiro, 1979). However, when cells were grown with $0.6\text{--}3.0\mu\text{Ci}$ of $\text{H}_3^{32}\text{PO}_4/\text{ml}$ for shorter periods, the rate of ^{32}P incorporation into phosphonolipids was different from that of the incorporation into phospholipids. Ethanolamine phospholipids from cells grown for 9 h (at day 5) with the [^{32}P]phosphate were more radioactive than the ethanolamine phosphonolipids. The relative specific radioactivities (% of radioactivity of ethanolamine lipid fraction/% of mass of ethanolamine lipid fraction $\pm$ S.E.M., $n = 9$) were: *N*-

acyl-*trans*-4-hydroxysphinganine-1-phosphonoethanolamine (*N*-acylphytosphingosine-1-phosphonoethanolamine, PPnE), 0.58 ± 0.08 ; *N*-acylsphingenine-1-phosphonoethanolamine (*N*-acylsphingosine-1-phosphonoethanolamine, SPnE), 0.89 ± 0.11 ; *N*-acylsphinganine-1-phosphonoethanolamine (*N*-acyldihydrosphingosine-1-phosphonoethanolamine, DPnE), 4.47 ± 0.82 ; 1,2-diacyl-*sn*-glycero-3-(2-aminoethyl)phosphonate and 1-alkyl-2-acyl-*sn*-glycero-3-(2-aminoethyl)phosphonate (PnE), 0.86 ± 0.04 ; *N*-acyl-*trans*-4-hydroxysphinganine-1-phosphoethanolamine (*N*-acylphytosphingosine-1-phosphoethanolamine, PPsE), 0.99 ± 0.20 ; *N*-acylsphingenine-1-phosphoethanolamine (*N*-acylsphingosine-1-phosphoethanolamine, SPsE), 1.66 ± 0.45 ; *N*-acylsphinganine-1-phosphoethanolamine (*N*-acyldihydrosphingosine-1-phosphoethanolamine, DPsE), 8.68 ± 2.68 ; 1,2-diacyl-*sn*-glycero-3-(2-aminoethyl) phosphate and 1-alkyl-2-acyl-*sn*-glycero-3-(2-aminoethyl) phosphate (PsE), 1.09 ± 0.03 . Radioactivity appeared faster in head groups of phospholipids compared with the respective phosphonolipid analogues. Furthermore, in these relatively short exposures to $\text{H}_3^{32}\text{PO}_4$ *in vivo*, the relative specific radioactivities of sphingolipids were greatest for those containing the long-chain base, dihydrosphingosine > sphingosine > phytosphingosine.

Over 75% of the radioactivity from [^{14}C]ethanol-1-ol-2-amine hydrochloride ($0.2\mu\text{Ci}/\text{ml}$ for 24 h) that was incorporated into whole-cell lipids was associated with the ethanolamine phospholipids. Of the fraction isolated by TEAE-cellulose column chromatography, 0.4% of the label was in the neutral lipids and free ceramides, 12% was in the choline lipid fraction and 20% was associated with the serine lipids ($n = 2$). [^{14}C]Ethanolamine was incorporated readily into both ethanolamine sphingophospholipids and glycerophospholipids and only slightly into the phosphonolipid analogues (Table 1). These results were confirmed by experiments performed *in vitro* (results not shown). This suggests that the head group of ethanolamine glycerophospholipids and sphingophospholipids in *Paramecium* can be synthesized *de novo* by direct incorporation of ethanolamine.

Phosphoenol[1- ^{14}C]pyruvic acid ($0.4\mu\text{Ci}/\text{ml}$ of culture for 48 h) that was tested as a precursor of phosphonolipids did not label cellular lipids, probably owing to decarboxylation of phosphoenol[1- ^{14}C]pyruvic acid, although 10% of the label was taken up by the cells after 24 h. However, when crude sonicated whole-cell preparations were used for assays of incorporation *in vitro* radioactivity from phosphoenol[^{14}C]pyruvate ($0.6\mu\text{Ci}/\text{ml}$ of incubation mixture) was incorporated into the neutral (14%), choline (21%), ethanolamine (53%) and minor (12%) lipid frac-

Table 1. *Incorporation of radiolabelled precursors into water-soluble head-group fractions of Paramecium ethanolamine lipids*
Radiolabelled compounds were added to cultures for 1–2 days (day 3 or 4 to day 5 of culture growth) in the studies performed *in vivo*, and in experiments performed *in vitro* phosphoenol[^{14}C]pyruvate was added to the incubation mixtures for 30 min at room temperature. Water-soluble products after phospholipase C, acid (sphingolipids) or alkaline (glycerolipids) hydrolysis were isolated and the radioactivities determined. For experimental details see the text. Polar head groups are water-soluble products from sphingolipids; polar head groups and glycerol backbones are water-soluble products from glycerolipids. Relative specific radioactivities (% of radioactivity of the total ethanolamine lipid fraction/% of mass of the total ethanolamine lipid fraction) are given in square brackets. The composition of the ethanolamine lipid fraction at day 5 was 4.9% sphingophosphonolipids, 1.6% sphingophospholipids, 34.2% PnE and 59.3% PsE ($n = 9$). For definition of abbreviations see the text.

	% of radioactivity of total ethanolamine phospholipids ($\pm$ s.d.)		
	[2- ^{14}C]Ethan-1-ol-2-amine hydrochloride (<i>in vivo</i> ; $n = 3$)	L-[U- ^{14}C]Serine (<i>in vivo</i> ; $n = 3$)	Phosphoenol[1- ^{14}C]pyruvic acid (<i>in vitro</i> ; $n = 2$)
Sphingophosphonolipids	Trace [—]	10.3 $\pm$ 3.3 [2.10]	20.6, 28.3 [4.99]
Sphingophospholipids	5.0 $\pm$ 2.2 [3.13]	6.3 $\pm$ 1.8 [3.94]	0.0, 0.0 [—]
PnE	1.5 $\pm$ 1.0 [0.04]	19.7 $\pm$ 4.8 [0.58]	79.3, 71.8 [1.27]
PsE	92.4 $\pm$ 5.8 [1.56]	64.5 $\pm$ 5.7 [1.09]	Trace [—]

Table 2. *Incorporation of radiolabelled precursors into Paramecium sphingolipid moieties*
Sphingolipid moieties were isolated after phospholipase C digestion or acid hydrolysis, and the radioactivities were determined. For experimental details see the text.

Radiolabelled precursor (n)	% of radioactivity ($\pm$ s.d.)			
	Sphingolipid fraction	Fatty acid	Long-chain base	Polar head group
Incorporation <i>in vivo</i> [2- ^{14}C]Ethan-1-ol-2-amine hydrochloride (3)	Total	0.0, 0.0	0.0, 0.0	100, 100
	Phospholipids	0.0, 0.0	0.0, 0.0	93.6, 96.1
	Phosphonolipids	0.0, 0.0	0.0, 0.0	3.1, 7.5
L-[U- ^{14}C]Serine (3)	Total	Trace	43.7 $\pm$ 5.0	57.3 $\pm$ 7.1
	Phospholipids	0.0	36.4 $\pm$ 6.3	63.6 $\pm$ 2.8
	Phosphonolipids	Trace	42.9 $\pm$ 2.5	57.2 $\pm$ 5.5
Incorporation <i>in vitro</i> Phosphoenol[1- ^{14}C]pyruvic acid (2)	Total*	9.6, 15.1	20.1, 24.5	70.0, 58.1

* Radioactivity was not detected in sphingophospholipids, and therefore values represent radioactivities in sphingophosphonolipids.

tions ($n = 2$). Radioactivity was detected in the water-soluble fraction of hydrolysed sphingolipids (Table 2), and all label in the ethanolamine lipid fraction was associated with the phosphonolipids (Table 1). In these experiments performed *in vitro* the incorporation of radiolabel from phosphoenol[^{14}C]pyruvate into lipids in the presence of Mg^{2+} was only 20–25% of that obtained with Ca^{2+} .

Radiolabel from L-[U- ^{14}C]serine (0.02–0.05 $\mu\text{Ci/ml}$ of culture for 1–2 days) was incorporated into all lipid fractions; 5% was associated with neutral lipids and free ceramides, 30% with choline lipids, 52% with ethanolamine lipids and 18% with serine lipids ($n = 5$). Furthermore, radioactivity from [^{14}C]serine was incorporated into PsE, PnE and all six ethanolamine sphingolipids (Fig. 1). Incorporation of label from [^{14}C]serine

into long-chain bases of all sphingolipids is consistent with the known initial condensation reaction of serine and palmitoyl-CoA, forming 3-dehydrosphinganine (Stoffel, 1977). More than half of the radioactivity in all sphingolipids was in the water-soluble fraction isolated after acid hydrolysis or phospholipase C digestion (Table 2). Radiolabel from [^{14}C]serine was also readily detected in the water-soluble products of the glycerolipids, PsE and PnE (Table 1). It was considered unlikely that $^{14}\text{CO}_2$ derived from decarboxylation of [U- ^{14}C]serine was subsequently incorporated into glucose and glycerol and thereby labelled the water-soluble products of glycerolipids. In other studies, $^{14}\text{CO}_2$ was not detected when *Paramecium* cells were grown with 0.034 μCi of [U- ^{14}C]serine/ml for 48 h of expo-

Fig. 1. Autoradiogram of a two-dimensional t.l.c. separation of ethanolamine phospholipids and phosphonolipids. Lipids of *Paramecium* were labelled by growth of the cells with $0.4 \mu\text{Ci}$ of $\text{L}[\text{U-}^{14}\text{C}]$ serine/ml for 5 days. For experimental details see the text. The solvent system used for the first dimension was chloroform/methanol/water (13:7:1, by vol.) and that used for the second dimension was chloroform/acetic acid/water (13:7:1, by vol.). The origin is indicated by 0; X is an unknown component that was rarely present in these analyses. For definition of abbreviations see the text.

quential growth (E. S. Kaneshiro, S. F. Reuter & D. F. Matesic, unpublished work). The results of the present studies on incorporation *in vivo* of radiolabel from $[\text{C}^{14}]$ serine into ethanolamine lipids of *Paramecium* indicate that serine was a precursor of ethanolamine glycerophospholipid, glycerophosphonolipid, sphingophospholipid and sphingophosphonolipid head groups.

Discussion

Biosynthesis of nitrogen bases, or polar head groups, of ethanolamine glycerolipids and sphingolipids in *Paramecium* involves a *de novo* pathway in which ethanolamine is directly utilized for the formation of phospholipids (Scheme 1). Only small amounts of label from ethanolamine were detected in ethanolamine phosphonolipid fractions, which was similar to observations on *Tetrahymena* (Thompson, 1969), indicating that phosphonolipids are not directly derived from ethanolamine phospholipids and that synthesis of the P-C bond involves slower or more reaction steps than does the addition of phosphorus to phosphonolipid head groups. The observation that ^{32}P

incorporation into ethanolamine phospholipids appeared earlier than into the phosphonolipids is in agreement with this conclusion. The observations that the relative specific radioactivities were highest in sphingolipids containing dihydrosphingosine and lowest in those containing phytosphingosine during the 9h ^{32}P -incorporation studies suggest that (1) the head groups, if not the entire molecules, of dihydrosphingosine-containing sphingolipids have turnover rates greater than those of sphingolipids containing sphingosine, which in turn are greater than those of sphingolipids containing phytosphingosine, or (2) long-chain base moieties can be modified after dihydrosphingosine is incorporated into phospholipids and that the conversion of the long-chain base moiety in *Paramecium* is sequential from dihydrosphingosine to sphingosine to phytosphingosine.

The possibility of synthesis of phosphonolipids via alternative phosphoenolpyruvate pathways in *Paramecium*, either by CMP-phosphoenolpyruvate or through lipid-bound APnP intermediates (Scheme 1), was suggested by experiments performed *in vitro*. We are presently unable to conclude whether or not these are major or minor pathways, or that both of these pathways are present in this organism. Furthermore, the results with phosphoenol[1- ^{14}C]pyruvate indicate that biosynthetic reactions leading from phosphoenolpyruvate to the formation of phosphonolipids 'may be more complex than originally envisaged' (Rosenberg, 1973). The inability to detect incorporation of radiolabel from phosphoenol[^{14}C]pyruvate into phosphonolipids in experiments performed *in vivo* is interpreted as either the result of a loss of radiolabel from the C-1 position and/or strict cellular compartmentalization of pools that do not exchange. This interpretation is supported by other studies of lipid metabolism in *Paramecium*, e.g. radiolabel from exogenously supplied $[\text{C}^{14}]$ acetate, $[\text{C}^{14}]$ malonate, $[\text{C}^{14}]$ pyruvate and $[\text{C}^{14}]$ glucose was not incorporated into fatty acids of the cell (Rhoads & Kaneshiro, 1980). The presence of enzymes for fatty acid synthesis *de novo* was demonstrated by experiments performed *in vitro* with acetyl-CoA and malonyl-CoA.

The results of the present study provide evidence that pathways for synthesis *de novo* from serine to ethanolamine phospholipids as well as phosphonolipids occur in *Paramecium*. One pathway, as proposed by Dennis & Kennedy (1970) for *Tetrahymena*, is the direct incorporation of serine into phosphatidylserine followed by decarboxylation to phosphatidylethanolamine (Scheme 1). The incorporation of label from $[\text{C}^{14}]$ serine into ethanolamine, choline and serine lipids indicated that pathways for synthesis *de novo* from serine are

Scheme 1. Proposed pathways for the biosynthesis of ethanolamine lipid head groups in *Paramecium* (→) Pathway 1 is the *de novo* pathway known for the synthesis of PsE from ethanolamine. *Paramecium* ethanolamine glycerophospholipids and sphingophospholipids can be synthesized by this pathway. Pathways 2 and 3 are those that have been proposed for the synthesis of *Tetrahymena* glycerophospholipids from phosphoenolpyruvate (PEP). One or both exist in *Paramecium*. Pathways 4 and 5 involve serine and include the decarboxylation of phosphatidylserine to PsE, as proposed for *Tetrahymena*. The proposed serine pathways leading to the formation of ethanolamine glycerophosphonolipids and sphingophosphonolipids involve the lipid-bound intermediates that contain APnP. For definition of abbreviations see the text.

important in phospholipid synthesis in *Paramecium*. Recovery of label from water-soluble head-group fractions from ethanolamine glycerolipids and sphingolipids further suggested the presence of these pathways. Clearly, *Paramecium* differs from *Tetrahymena*, and other cell types examined (Warren, 1968; Rosenberg, 1973) in that label from [¹⁴C]serine was efficiently incorporated into ethanolamine phosphonolipid head groups of this ciliate. The biosynthetic pathways leading to phosphonolipids in *Paramecium* appear to be similar for both glycerolipids and sphingolipids.

Our results support the hypothesis that major pathways in ethanolamine phospholipid and phosphonolipid metabolism in *Paramecium* involve serine lipids.

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