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# Phosphatidylethanolamine is the donor of the terminal phosphoethanolamine group in trypanosome glycosylphosphatidylinositols

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**A variety of eukaryotic cell surface proteins, including the variant surface glycoproteins of African trypanosomes, rely on a covalently attached lipid, glycosylphosphatidylinositol (GPI), for membrane attachment. GPI anchors are synthesized in the endoplasmic reticulum by stepwise glycosylation of phosphatidylinositol (via UDP-GlcNAc and dolichol-P-mannose) followed by the addition of phosphoethanolamine. The experiments described in this paper are aimed at identifying the biosynthetic origin of the terminal phosphoethanolamine group. We show that trypanosome GPIs can be labelled via CDP-[$^3$H]ethanolamine or [$\beta$-$^{32}$P]CDP-ethanolamine in a cell-free system, indicating that phosphoethanolamine is acquired *en bloc*. In pulse-chase experiments with CDP-[$^3$H]ethanolamine we show that the GPI phosphoethanolamine is not derived directly from CDP-ethanolamine, but instead from a relatively stable metabolite, such as phosphatidylethanolamine (PE), generated from CDP-ethanolamine in the cell-free system. To test the possibility that PE is the immediate donor of the GPI phosphoethanolamine moiety, we describe metabolic labelling experiments with [$^3$H]serine and show that GPIs can be labelled in the absence of detectable radiolabelled CDP-ethanolamine, presumably via [$^3$H]PE generated from [$^3$H]phosphatidylserine (PS). The data support the proposal that the terminal phosphoethanolamine group in trypanosome GPIs is derived from PE.**

*Key words:* glycosylphosphatidylinositol/membrane anchor/phospholipid/phosphatidylethanolamine/trypanosome

## Introduction

Numerous proteins from eukaryotic organisms are covalently modified by lipids including fatty acids, isoprenoids or inositol-containing glycophospholipids (Sefton and Buss, 1987; Ferguson and Williams, 1988; Olson, 1988; Towler *et al.*, 1988; Low, 1989; Cross, 1990; Glomset *et al.*, 1990; McIlhinney, 1990; Thomas *et al.*, 1990; Sinensky and Lutz, 1992). Fatty acids or isoprenoids may be directly attached to amino acids in the polypeptide backbone through ester, thioester, thioether or amide linkages via enzyme-mediated reactions that occur in the cytoplasm, or on the cytoplasmic leaflets of cell membrane bilayers. In contrast, current models suggest that the addition of a glycosylphosphatidylinositol (GPI) anchor to protein occurs in the endoplasmic reticulum (ER), soon after translocation of the polypeptide across the ER membrane bilayer and cleavage of the N-terminal signal sequence. GPI addition then proceeds by cleavage of a C-terminal 'signal' sequence and attachment of a GPI precursor (via ethanolamine) to the newly exposed $\alpha$-carboxyl group of the polypeptide.

Ethanolamine-containing GPI anchor precursors have been identified in trypanosomes and in mammalian cells (Krakow *et al.*, 1986; Menon *et al.*, 1988; DeGasperi *et al.*, 1990). Two lipids from bloodstream-form *Trypanosoma brucei* have been characterized in considerable detail. Both lipids contain the evolutionarily conserved GPI backbone sequence ethanolamine phosphate-Man$\alpha$1-2Man$\alpha$1-6Man$\alpha$1-GlcN-inositol (Menon *et al.*, 1988; Mayor *et al.*, 1990a), and in both cases the inositol residue is linked to dimyristylglycerol via a phosphodiester bond. The two structures—termed P2 and P3 [Menon *et al.*, 1988; also known as (glyco)lipids A and C (Krakow *et al.*, 1986; Masterson *et al.*, 1989)]—differ only in the presence or absence of a fatty acid substituent on the inositol. P2 does not contain acylated inositol and can be cleaved by phospholipases C specific for phosphatidylinositol (PI-PLCs). P3 contains a palmitic acid residue ester-linked to a hydroxyl group on the inositol and is therefore resistant to cleavage by PI-PLCs (Krakow *et al.*, 1989; Mayor *et al.*, 1990b).

Recent biochemical and genetic data suggest that GPIs are assembled by stepwise addition of components to PI (Figure 1) (Doering *et al.*, 1989, 1990; Masterson *et al.*, 1989; Menon *et al.*, 1990a,b; Orlean, 1990; Hirose *et al.*, 1991, 1992a,b; Puoti *et al.*, 1991; Stevens and Raetz, 1991; Sugiyama *et al.*, 1991). The first committed step is the transfer of *N*-acetylglucosamine (GlcNAc) to PI to form GlcNAc-PI, followed by the de-acetylation of GlcNAc-PI to give GlcN-PI. Inositol acylation occurs at this stage, and may be a prerequisite for mannose addition since GlcN-(acyl)PI accumulates in cells defective in mannosylation (Orlean, 1990; Costello and Orlean, 1992; Urakaze *et al.*, 1992). The three core mannose residues are transferred sequentially to GlcN-(acyl)PI from mannosylphosphoryldolichol (Menon *et al.*, 1990a; Orlean, 1990; DeGasperi *et al.*, 1990; see also Lemansky *et al.*, 1991), and the core GPI structure is then completed by adding a phosphoethanolamine cap. The entire biosynthetic process has been demonstrated *in vitro* using crude membrane preparations from trypanosomes supplemented with the sugar nucleotides GDP-mannose and UDP-GlcNAc (Masterson *et al.*, 1989; Menon *et al.*, 1990b). Synthesis of early GPI biosynthetic intermediates has also been examined in lysates derived from lymphoma cells and lymphocytes (Hirose *et al.*, 1991; Stevens and Raetz, 1991; Urakaze *et al.*, 1992). Endogenous PI is sufficient to sustain GPI synthesis although synthesis

of GlcN-PI and GlcNAc-PI in thymoma cell lysates is stimulated by adding PI to the incubation mixture (Stevens and Raetz, 1991). Curiously, GPI synthesis in trypanosome lysates proceeds efficiently in the absence of added sources of the terminal phosphoethanolamine group. For example, P2 synthesis is not influenced by including CDP-ethanolamine in the reaction mixture (Masterson *et al.*, 1989), indicating that the phosphoethanolamine group in P2 is derived from an endogenous donor. Since it is unlikely that significant quantities of CDP-ethanolamine are present in the membrane preparations, the inability of exogenously added CDP-ethanolamine to influence GPI assembly suggests that the immediate donor of the GPI phosphoethanolamine is not CDP-ethanolamine. In this paper we present data from both *in vivo* and *in vitro* radiolabelling experiments supporting the proposal that the terminal phosphoethanolamine residue in GPIs is derived from an abundant cellular phospholipid, phosphatidylethanolamine (PE).

## Results

### *Transfer of phosphoethanolamine to GPIs in a cell-free system*

The main biosynthetic steps involved in the construction of the GPI anchor core were originally delineated using cell-free preparations from African trypanosomes. The preparations were capable of using radioactive sugar nucleotides (GDP-[$^3$H]mannose or UDP-[$^3$H]GlcNAc) and endogenous substrates to synthesize complete phosphoethanolamine-containing GPI anchor precursors (Masterson *et al.*, 1989; Menon *et al.*, 1990b). As a first step towards identifying the route by which the terminal phosphoethanolamine residue is incorporated into the GPI structure, we incubated trypanosome membrane preparations with CDP-[$^3$H]ethanolamine or [$\beta$-$^{32}$P]CDP-ethanolamine (synthesized as described in Materials and methods), and extracted and analyzed the labelled lipids. Figure 2 shows that $^3$H- or $^{32}$P-labelled phosphoethanolamine is readily incorporated into phosphatidylethanolamine (PE), *lyso* PE, and into the trypanosome GPIs P2 and P3.

The labelled GPIs were identified by their characteristic mobility in two TLC systems (silica 60, solvent system B and silica 50 000 HPTLC, solvent system C), and by their susceptibility to phospholipases. [$^3$H] and [$^{32}$P]phosphoethanolamine-labelled P2 and P3 co-migrated with [$^3$H]myristic acid or [$^3$H]mannose-labelled P2 and P3 standards in both TLC systems; both lipids were susceptible to phospholipase $A_2$ treatment, giving rise to *lyso* products. P2, but not P3, could be cleaved by PI-PLC (data not shown). The TLC profile of the $^{32}$P-labelled lipids (Figure 2B) shows broader peaks than the TLC profile of the corresponding $^3$H-labelled material because of the lower resolution of the TLC scanner for $^{32}$P. The ability to incorporate both $^3$H and $^{32}$P into the GPIs from appropriately labelled CDP-ethanolamine donors *in vitro* indicates that phosphoethanolamine addition to GPI acceptors occurs *en bloc* rather than by sequential transfer of phosphate and ethanolamine.

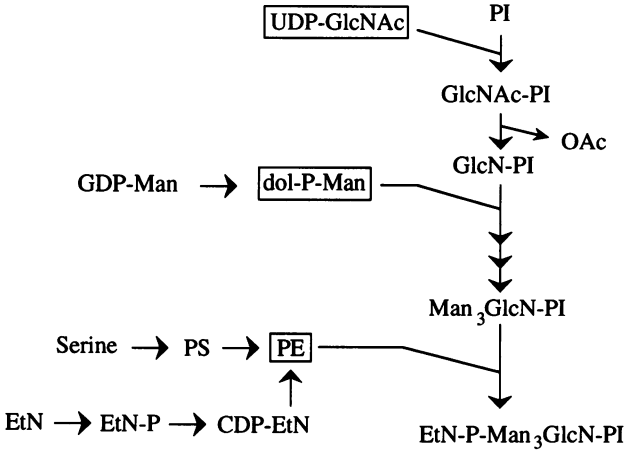


**Fig. 1.** Biosynthesis of GPI. The figure shows an outline of GPI biosynthesis. Inositol acylated structures (see Introduction) are omitted for simplicity. GPI assembly is initiated by transfer of GlcNAc from UDP-GlcNAc to PI to form GlcNAc-PI. GlcNAc-PI is de-acetylated to give GlcN-PI. Three mannose residues are transferred successively from dolichol-*P*-mannose, and the $Man_3$-GPI structure is completed by the addition of a phosphoethanolamine cap. The data described in this paper indicate that the immediate donor of the phosphoethanolamine moiety is PE, which can be synthesized from CDP-ethanolamine and diacylglycerol, or by decarboxylation of PS. Dol-P-man, dolichol-phosphomannose; EtN, ethanolamine; EtN-P, ethanolamine phosphate; GDP-mannose, guanosine diphosphomannose; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; UDP-GlcNAc, uridine diphospho-*N*-acetylglucosamine.

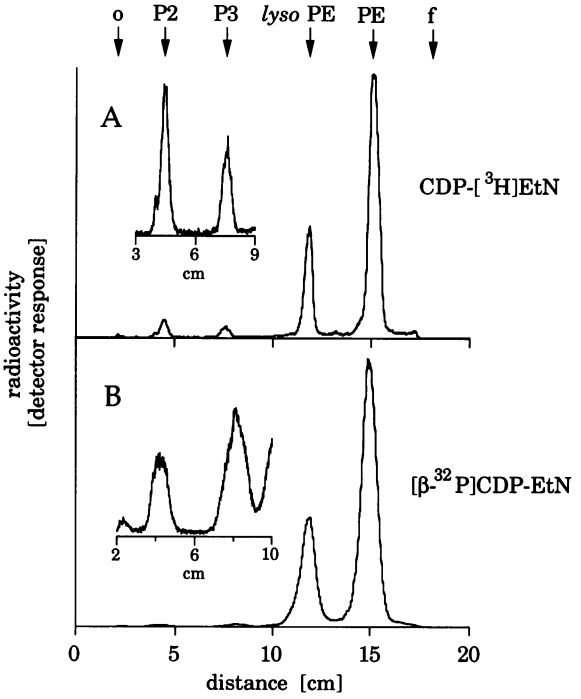


**Fig. 2.** Labelling of P2 and P3 via CDP-[$^3$H]ethanolamine and [$\beta$-$^{32}$P]CDP-ethanolamine *in vitro*. Trypanosome membranes ($2\times10^8$ cell equivalents) were incubated with CDP-[$^3$H]ethanolamine (18 $\mu$Ci) or [$\beta$-$^{32}$P]CDP-ethanolamine (0.5 $\mu$Ci) as described in Materials and methods, and lipids were extracted and analyzed by TLC (silica 60, solvent system B). The top panel shows the TLC radioactivity profile of lipids labelled via CDP-[$^3$H]ethanolamine. The bottom panel shows the TLC radioactivity profile of lipids labelled via [$\beta$-$^{32}$P]CDP-ethanolamine. Insets in both panels show amplified views of the region of the chromatogram corresponding to the GPIs P2 and P3. The migration of standards is indicated at the top (o, origin, f, front). The incorporation of radioactivity was as follows (c.p.m. per $10^8$ cell equivalents). [$^3$H]: PE, $\sim10^6$ c.p.m.; *lyso* PE, $\sim0.4\times10^6$ c.p.m.; P2, $\sim 6\times10^4$ c.p.m.; P3, $\sim4\times10^4$ c.p.m.; [$^{32}$P]: PE, $\sim3.5\times10^4$ c.p.m.; *lyso* PE, $\sim2\times10^4$ c.p.m.; P2, $\sim$ 300 c.p.m.; P3 $\leq$ 800 c.p.m..

The extent of incorporation of [$^3$H]ethanolamine into P2 and P3 was not influenced by adding the sugar nucleotides GDP-mannose and UDP-GlcNAc (1 mM each, final concentration) to the cell-free system, indicating that a large pool of $Man_3$-GPI acceptors was available in the membrane preparation and that the amount of *de novo* GPI synthesis (via UDP-GlcNAc, GDP-mannose and endogenous substrates) was comparatively small (data not shown). These $Man_3$-GPI acceptors appeared to have already undergone fatty acid re-modelling (Masterson *et al.*, 1989) as the [$^3$H]ethanolamine-labelled products P2 and P3 chromatographed on thin layer plates as sharp peaks corresponding to dimyristyl species (Figure 2A). This observation is consistent with the recently described fatty acid re-modelling of trypanosome GPIs prior to phosphoethanolamine addition (Masterson and Ferguson, 1991).

Curiously, [$^3$H]ethanolamine could be used to label PE, P2 and P3 *in vitro* in the presence of 1–10 mM $Ca^{2+}$ (data not shown); no labelling was seen when $Ca^{2+}$ was omitted from the incubation buffer. The most likely explanation for this observation is that PE, labelled via base-exchange (Borkenhagen *et al.*, 1961), donates [$^3$H]ethanolamine to GPI acceptors to form [$^3$H]P2 and [$^3$H]P3. It is worth noting that only ethanolamine, and not phosphoethanolamine, can be transferred via base-exchange, and that the alternative possibility that [$^3$H]ethanolamine is directly exchanged into pre-existing P2 and P3 in the membrane preparation cannot be strictly ruled out.

### *CDP-ethanolamine is not the immediate donor of the GPI terminal phosphoethanolamine moiety*

Pulse–chase experiments were performed to determine whether phosphoethanolamine was transferred directly from CDP-ethanolamine to GPI, or whether an intermediate was involved. Membranes were pulse-labelled for 1 min with CDP-[$^3$H]ethanolamine before adding a 4000-fold excess of non-radioactive CDP-ethanolamine to initiate the chase. These chase conditions were effective since the total radioactivity incorporated into the lipid fraction did not increase during the chase. As shown in Figure 3, incorporation of radioactivity into P2 increased steadily during the chase period investigated (P2 synthesized during the short pulse period was not detectable); PE levels fell slightly, and *lyso* PE (presumably synthesized from PE) accumulated during the chase. Similar data for P3 could not be obtained from TLC as chromatographic tailing of the radiolabelled phospholipid material routinely obscured the P3 peak making TLC quantification unreliable. The synthesis of P2 during the chase period clearly demonstrates that CDP-ethanolamine is not the immediate donor of the GPI terminal phosphoethanolamine moiety. The data imply that the direct donor must be a radiolabelled product such as PE, synthesized from CDP-ethanolamine during the radiolabelling pulse.

### *Metabolic labelling with [$^3$H]serine*

To test the possibility that PE is the donor of the GPI terminal phosphoethanolamine moiety, we took advantage of the fact that PE can be generated metabolically via decarboxylation of phosphatidylserine (PS) without synthesis of hydrophilic ethanolamine metabolites such as ethanolamine phosphate and CDP-ethanolamine (Kennedy, 1961). Trypanosomes were metabolically labelled with [$^3$H]serine and subjected to a two-step lipid extraction procedure [extraction with chloroform/methanol (CM) and chloroform/methanol/water (CMW)] as described in Materials and methods. The extracts were analyzed by TLC as shown in Figure 4.

TLC analysis of the CM extract (Figure 4A) showed a major peak of radioactivity corresponding to a PS standard, as well as a faster moving labelled product co-migrating with a PE standard. Both the radiolabelled lipids were susceptible to cleavage by *Bacillus cereus* phospholipase C (>85%). Amine analysis showed that the radioactivity in the material

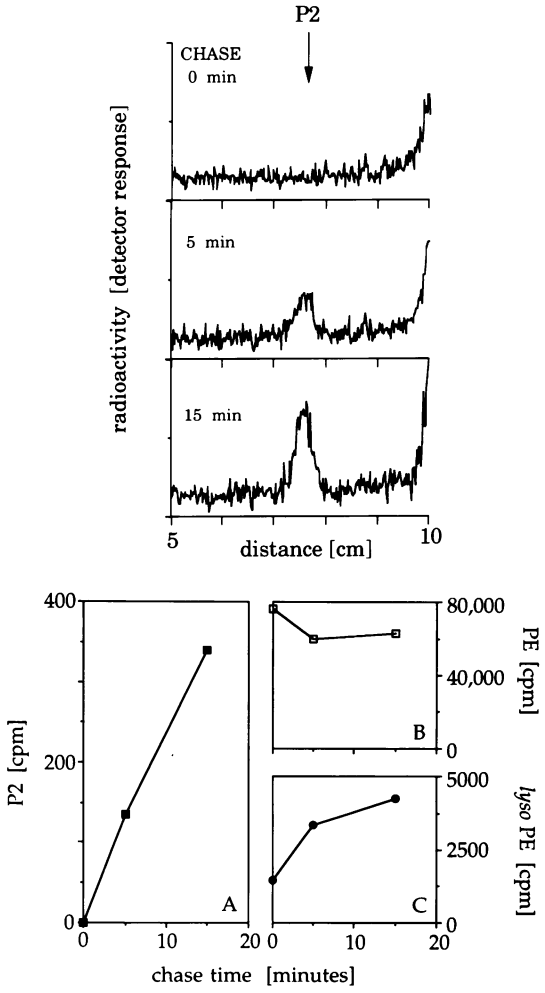


**Fig. 3.** Pulse–chase labelling with CDP-[$^3$H]ethanolamine. Three samples of trypanosome membranes (~$10^8$ cell equivalents per sample) were incubated with CDP-[$^3$H]ethanolamine ($6.5\times10^6$ c.p.m. per sample) at 37°C. After 1 min of incubation, CDP-ethanolamine (10 μl, 100 mM) was added to each sample and the incubation was continued: the samples were incubated for different periods of time (0, 5 or 15 min). Each reaction was stopped by placing on ice and adding 1.7 ml of ice-cold chloroform: methanol (1:1, v/v). Extracted lipids were processed as described, and analyzed by TLC (silica 60, solvent system B). The top panels show the 5–10 cm region of the thin layer chromatogram (origin: 2 cm; front, 18 cm). The migration of a P2 standard is indicated. The results are also illustrated graphically in the lower panels (**A**: P2, **B**: PE, **C**: *lyso* PE; data obtained by integration of the TLC profiles as described in Materials and methods). Data for P3 are not shown since chromatographic tailing of the radiolabelled phospholipids (PE, *lyso* PE and unidentified material) precluded reliable quantification. In separate experiments where chase times up to 30 min were examined, it was found that incorporation of radioactivity into P2 ceased by ~20 min and decreased slightly thereafter.

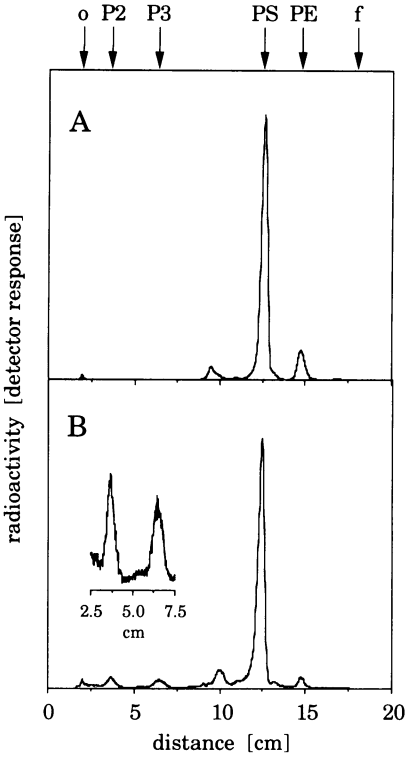


**Fig. 4.** TLC analysis of lipid extracts from [$^3$H]serine-labelled trypanosomes. Trypanosomes ($5 \times 10^7$/ml, 16 ml) were incubated with [$^3$H]serine (58 $\mu$Ci/ml) for 2 h at 37°C. At the end of the incubation, the cells were pelletted and lipids were extracted in two steps (CM and CMW) as described in Materials and methods. The extracts were processed as described and analyzed by TLC (silica 60, solvent system B). Panel **A** shows the TLC radioactivity profile of the Folch-washed CM extract. Panel **B** shows the TLC radioactivity profile of the CMW-derived butanol extract. The inset in panel B is an 8× amplified view of the 2.5−7.5 cm region of the chromatogram. Incorporation of radioactivity was estimated as described in Materials and methods: PS, $\sim 10^6$ c.p.m./$10^8$ cells; PE, $\sim 1.4 \times 10^5$ c.p.m./$10^8$ cells; P2, ~1500 c.p.m./$10^8$ cells. P3 ≤ 1400 c.p.m./$10^8$ cells.

co-migrating with the PS standard was 99% [$^3$H]serine. A similar analysis showed that the radioactivity in the material co-migrating with the PE standard was 96% [$^3$H]ethanolamine (data not shown). The CM extract also contained a third radiolabelled product migrating more slowly than PS that was resistant to *B.cereus* phospholipase C. Although this material was not further analyzed, its TLC mobility and resistance to *B.cereus* phospholipase C are consistent with it being a *lyso* lipid (Little, 1981), probably *lyso* PS.

The most prominent labelled species in the CMW extract (Figure 4B) were residual CM-soluble lipids (PS and PE). However, the polar section of the chromatogram (Figure 4B, see inset) contained two radiolabelled products not seen in the CM extract, which comigrated with authentic P2 and P3. These lipids were isolated from the thin layer plate and chromatographed in a second TLC system to confirm their identity. Re-chromatography in different TLC systems (Si60 HPTLC or Si50000 HPTLC plates, solvent system C) showed that both species were homogeneous and co-chromatographed with authentic P2 and P3 respectively (data not shown). In addition, the radiolabelled material corresponding to P2 was susceptible to cleavage by PI-PLC, whereas the material corresponding to the P3 standard was resistant to cleavage by the enzyme (Mayor *et al.*, 1990b).

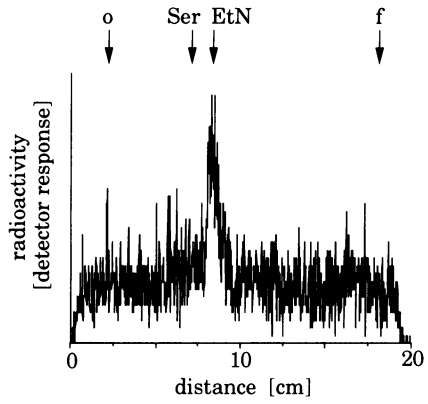


**Fig. 5.** Amine analysis of P2 labelled via [$^3$H]serine. P2 (900 c.p.m.) was hydrolyzed as described in Materials and methods, and the hydrolysate was analyzed by TLC (cellulose, solvent system A). The figure shows the TLC radioactivity profile of the hydrolysate. The positions of non-radioactive serine and ethanolamine standards (included in the hydrolysis) are indicated. o, origin; f, front.

Material corresponding to P2 was pooled from several [$^3$H]serine-labelling experiments, re-purified by phase separation (see Materials and methods), and subjected to acid hydrolysis to determine the identity of the incorporated radioactivity. Figure 5 shows that the primary radioactive product recovered from the hydrolysis of [$^3$H]serine-labelled P2 was [$^3$H]ethanolamine. The sample contained essentially no [$^3$H]serine; however, trace amounts of unidentified material running ahead of the [$^3$H]ethanolamine standard could be seen (Figure 5). The corresponding P3 sample was not analyzed as the amount of radioactivity recovered in the pure sample was inadequate for the purpose. The data demonstrate that P2 and P3 can be metabolically labelled via [$^3$H]serine, consistent with a radiolabelling pathway involving decarboxylation of [$^3$H]serine-labelled PS to [$^3$H]ethanolamine-labelled PE, and subsequent transfer of [$^3$H]ethanolamine from [$^3$H]PE to a GPI acceptor.

We next attempted to rule out the formal possibility that labelling of P2 and P3 via [$^3$H]serine was due to the presence of CDP-[$^3$H]ethanolamine, formed via degradation of [$^3$H]PE. We therefore extracted water-soluble metabolites from [$^3$H]serine-labelled trypanosomes by the perchloric acid method, and analyzed the extract by paper chromatography (solvent system A). The main radioactive products in the perchloric acid extract co-migrated with serine and serine phosphate (Figure 6). The radioactive material chromatographing as serine phosphate contained [$^3$H]serine, but could not be unequivocally identified (see Materials and methods). Less than 0.4% of the total PCA-soluble radioactivity was found in the ethanolamine region of the chromatogram. Hydrolysis of the CDP-ethanolamine region of the chromatogram yielded no detectable [$^3$H]ethanolamine. Similarly, hydrolysis of the ethanolamine phosphate region of the chromatogram yielded insignificant amounts of [$^3$H]ethanolamine (0.07% of the radioactivity in the perchloric acid extract, data not shown). Thus, since both CDP-[$^3$H]ethanolamine and [$^3$H]ethanolamine phosphate are negligible in [$^3$H]serine-labelled trypanosomes, it can be concluded that GPI labelling in this situation occurs via [$^3$H]PE, synthesized from [$^3$H]PS, rather than via a water-soluble [$^3$H]ethanolamine-labelled donor.

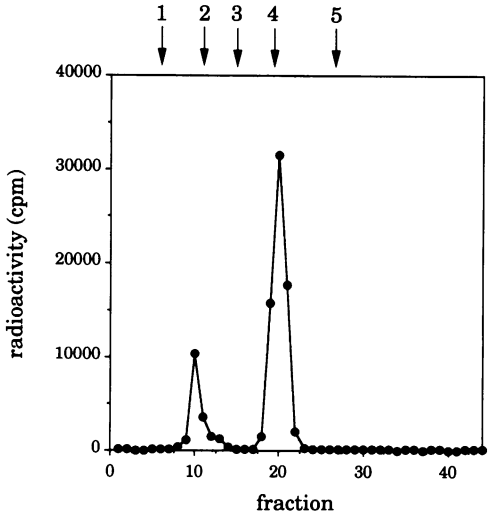


**Fig. 6.** Analysis of a perchloric acid extract of [$^3$H]serine-labelled trypanosomes. Trypanosomes ($5\times10^7$/ml, 75 ml total) were incubated with [$^3$H]serine (58 $\mu$Ci/ml) for 2.5 h at 37°C. A fraction (1/15) of the total volume was used for perchloric acid extraction as described in Materials and methods. The perchloric acid extract (280 000 c.p.m.) was applied to Whatman 3MM paper and subjected to descending paper chromatography for 17 h using solvent system A. Standards (1, CDP-ethanolamine; 2, serine phosphate; 3, ethanolamine phosphate; 4, serine; 5, ethanolamine) were run in parallel and visualized by spraying with ninhydrin reagent. The middle section of the sample strip was cut into 1 cm sections. The sections were taken for liquid scintillation counting to give the profile illustrated in the figure. The distribution of radioactivity was as follows: serine region, 78.1%, ethanolamine phosphate region, 3.4%, serine phosphate region, 17.4% (total 98.9%).

## Discussion

Glycerophospholipids have been shown to be donors of the phosphoethanolamine and phosphoglycerol substituents on the membrane-derived oligosaccharides of *Escherichia coli* (Miller and Kennedy, 1987; Jackson and Kennedy, 1983). In mammalian cells phosphatidylcholine donates phosphocholine to ceramide to form sphingomyelin (Ullman and Radin, 1974; Voelker and Kennedy, 1982), and PE donates phosphoethanolamine to ceramide to form ceramide-phosphoethanolamine (Bennert and Ullman, 1981; Malgat *et al.*, 1986). In this paper we present data consistent with the proposal that a glycerophospholipid, PE, is the donor of the terminal phosphoethanolamine group in trypanosome GPIs.

*In vitro* labelling using CDP-[$^3$H]ethanolamine and [$\beta$-$^{32}$P]CDP-ethanolamine showed clearly that the phosphoethanolamine moiety was transferred *en bloc* from CDP-ethanolamine to GPI acceptors ($Man_3GlcN$-PI and $Man_3GlcN$-acyl PI), ruling out other conceivable but elaborate mechanisms in which the phosphate and ethanolamine residues are transferred in separate steps. Further insight was obtained from *in vitro* pulse–chase experiments with CDP-[$^3$H]ethanolamine. The pulse–chase data indicated that the immediate donor of the phosphoethanolamine moiety was not CDP-ethanolamine itself, but another molecule synthesized from CDP-ethanolamine during the labelling pulse. The most abundant radiolabelled species synthesized during the labelling pulse was PE, and since the PE pool is large and relatively metabolically stable (i.e. stable to the chase conditions) we considered the possibility that PE is the immediate donor of the GPI phosphoethanolamine moiety. Experimental support for this hypothesis came from metabolic labelling experiments with [$^3$H]serine. In these experiments we exploited the alternative pathway for PE biosynthesis in which PE is synthesized via decarboxylation of PS without concomitant synthesis of any water-soluble ethanolamine metabolites. The data showed clearly that the GPIs P2 and P3 could be labelled via [$^3$H]serine, the radioactivity in these molecules being present as [$^3$H]ethanolamine. The only detectable [$^3$H]ethanolamine metabolite was PE; no CDP-[$^3$H]ethanolamine was detected. The cumulative data provide clear support for the proposal that the terminal GPI phosphoethanolamine moiety is derived from PE. However, these data do not indicate whether phosphoethanolamine is transferred directly from PE to GPI acceptors, and it remains possible that other PE-derived intermediates may be involved in the transfer process.

We made several unsuccessful attempts to provide additional tests of this proposal through experiments in which trypanosome membranes were incubated with [$^3$H]PE. However, we could not circumvent the problem that the [$^3$H]PE had to be delivered to the reaction mixture in detergent solution or in ethanol, conditions which inhibited transfer of phosphoethanolamine (data not shown). It may be possible to resolve this problem by developing a short-chain analogue of [$^3$H]PE which, because of its water solubility, can be used in aqueous media in the absence of detergent.

Recently described experiments with a yeast mutant also suggest that PE is the donor of the GPI terminal ethanolamine moiety (Menon, 1991; Menon and Stevens, 1992). The mutant used in these experiments was obtained by deleting the genes *EPT1* and *CPT1*, encoding the ethanolamine-phosphotransferases (Hjelmstad and Bell, 1991). The mutant was unable to synthesize [$^3$H]PE from [$^3$H]ethanolamine (via CDP-[$^3$H]ethanolamine, which was synthesized normally) and was also unable to incorporate [$^3$H]ethanolamine into GPI-anchored proteins. However, PE levels in the mutant were found to be the same as in the wild-type strain (synthesis was via PS decarboxylation) and GPI anchoring, assayed by [$^3$H]inositol incorporation, was normal. The inability to incorporate radioactivity from [$^3$H]ethanolamine into GPIs in mutant cells specifically unable to synthesize PE via the CDP-ethanolamine route indicated that PE was an obligatory intermediate in the transfer of ethanolamine to GPI.

Similar conclusions were reached via metabolic radiolabelling of mammalian cells. [$^3$H]Serine-labelling experiments with murine thymoma cells (M.Dhalla and A.K.Menon, unpublished observations) showed incorporation of radioactivity into the most polar non-protein-linked GPI (termed 'core', Kamitani *et al.*, 1992), a species containing three phosphoethanolamine substituents. Analysis of the purified lipid showed that the radioactivity was in the form of [$^3$H]ethanolamine. The data suggest that at least one of the three phosphoethanolamine substituents in 'core' is donated by PE.

Recent observations indicate that early biosynthetic intermediates (GlcNAc-PI and GlcN-PI) in GPI assembly are predominantly distributed in the cytoplasmic leaflet of the ER membrane bilayer (J.Vidugiriene and A.K.Menon,

manuscript submitted). The topology of the remaining biosynthetic steps is not known, although the mature phosphoethanolamine-containing GPI anchor precursor is presumed to be lumenally oriented in order to be available for transfer to newly translocated polypeptides. Since known glycosylation reactions involving dolichol-*P*-mannose occur in the ER lumen (Tanner and Lehle, 1987; Hirschberg and Snider, 1987), it has been suggested that the dolichol-*P*-mannose-dependent mannosylation steps in GPI assembly also occur in the lumenal leaflet of the ER (Menon *et al.*, 1990a; Orlean, 1990). The identity of the GPI phosphoethanolamine donor provides no clues as to the transbilayer location of the phosphoethanolamine transfer step in GPI assembly since PE is available in both leaflets of the ER membrane. PE is synthesized from CDP-ethanolamine and diacylglycerol in the cytosolic leaflet of the ER membrane bilayer, or delivered to the ER from the mitochondria where it is synthesized in the mitochondrial inner membrane by decarboxylation of PS (van Meer, 1989; Voelker, 1991). Some PE is subsequently flipped into the inner leaflet of the ER membrane bilayer, providing a source of phosphoethanolamine in both leaflets of the ER membrane.

As mentioned above, GPI structures have been described that contain up to three phosphoethanolamine substituents on the trimannose GPI core. The structures include the GPI anchors of human erythrocyte acetylcholinesterase (Deeg *et al.*, 1992), and rat brain Thy-1 (Homans *et al.*, 1988), as well as a profusion of non-protein-linked GPIs in mammalian cells (Hirose *et al.*, 1992a,b; Kamitani *et al.*, 1992; Puoti and Conzelmann, 1992). The immediate precursor of these extra phosphoethanolamine moieties is not known, nor is it clear whether these modifications are acquired in the ER, or at a later point in the secretory pathway. If the transfer occurs late, it may occur in the lumen of an intracellular organelle. Since CDP-ethanolamine is not known to be transported across organellar membranes, these additional phosphoethanolamine moieties may also be donated by PE.

## Materials and methods

### Materials

[1-$^3$H]Serine (32 Ci/mmol), [1-$^3$H]ethan-1-ol-2-amine hydrochloride (29.5 Ci/mmol) and [$\gamma$-$^{32}$P]adenosine 5′-triphosphate, triethylammonium salt (5000 Ci/mmol) were purchased from Amersham Corp. [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol) was purchased from NEN-Dupont. The [$^3$H]serine used in metabolic labelling studies was routinely purified by descending paper chromatography on Whatman 3MM paper using solvent system A to eliminate minor radioactive impurities that co-chromatographed with [$^3$H]ethanolamine. [$^3$H]Mannose and [$^3$H]myristic acid-labelled GPI standards were prepared from metabolically labelled trypanosomes as described previously (Mayor *et al.*, 1990b). *B.cereus* PI-specific phospholipase C (PI-PLC) (20 U/400 $\mu$l), *B.cereus* phospholipase C, bee venom phospholipase A2 (2400 U/mg lyophilisate), *Crotalus durissus* phosphodiesterase, and calf intestine alkaline phosphatase were purchased from Boehringer Mannheim. *Bacillus thuringiensis* PI-PLC was a gift from Dr Martin Low (Columbia University College of Physicians and Surgeons, New York).

### Trypanosomes

Trypanosomes of the Molteno Institute trypanozoon antigenic type 1.4 (variant clone 118) of *Trypanosoma brucei* strain 427 were isolated from infected rat blood by centrifugation (Field and Menon, 1992). The upper part of the buffy coat layer was removed, taking care to avoid contamination with blood cells. 'Buffy-coat' trypanosomes (>99% pure) were used directly or purified on DEAE-cellulose (Field and Menon, 1992) before metabolic labelling or preparation of lysates for *in vitro* labelling. Lysates were prepared from tunicamycin-treated trypanosomes as described by Masterson *et al.* (1989).

### Enzyme preparations for the synthesis of [$^3$H]ethanolamine phosphate and CDP-[$^3$H]ethanolamine

Liver homogenates were prepared according to the procedures of Schneider (1969) with some modifications. Pig liver (150 g) obtained from the abbatoir of the city of Marburg immediately after slaughter, was cut into small strips, cooled on ice, and homogenized (10 s) in 4 vol of 145 mM NaCl in a 'Starmix' blender. Small portions (30 ml) were re-homogenized on ice using a Potter S homogenizer equipped with a Teflon pestle (Braun Melsungen, Melsungen, Germany). The resulting homogenate was centrifuged in an SW 28 rotor (Beckman, Palo Alto, CA, USA) at 27 000 r.p.m. (100 000 *g*) for 1 h at 2°C. A portion (90 ml) of the resulting supernatant (total 140 ml) was dialyzed for 24 h against 145 mM NaCl, 2 mM Tris−succinate, pH 7.5 at 4°C. Aliquots of this preparation (referred to as Enzyme 1) were stored at −80°C. Ammonium sulfate was slowly added to the remainder of the supernatant (50 ml) on ice to give 25% saturation. The resulting suspension was cleared by centrifugation (2000 *g*, 10 min) at 4°C. The pellet was discarded and the solution was adjusted with ammonium sulfate to 50% saturation. The precipitate was resuspended in 20 mM Tris−HCl pH 7.5 and dialyzed against the same buffer at 4°C. Aliquots (referred to as Enzyme 2) were stored at −80°C.

### Synthesis of [$^3$H]ethanolamine phosphate

A 500 $\mu$Ci sample of [1-$^3$H]ethan-1-ol-2-amine hydrochloride was dried in a Speed-Vac evaporator. The sample was resuspended (on ice) in 30 $\mu$l water, 20 $\mu$l 250 mM Tris−succinate (pH 7.5), 2.5 $\mu$l 300 mM $MgCl_2$, 10 $\mu$l 60 mM ATP (pH 7.0), 20 $\mu$l 200 mM 3-phosphoglyceric acid, sodium salt (pH 7.0), 167.5$\mu$l Enzyme 1 (see above), and incubated for 1 h at 37°C. The reaction was stopped with 250 $\mu$l 100% ethanol and the reaction mixture was centrifuged for 5 min at 10 000 *g* in a Biofuge A (Heraeus, Osterrode, Germany). The supernatant was applied to strips of Whatman 3MM chromatographic paper (Whatman, Maidstone, UK) and subjected to descending paper chromatography for 15−18 h using solvent system A. Markers (ethanolamine, ethanolamine phosphate and CDP-ethanolamine) were run in parallel and visualized by inspection of the chromatograms under 254 nm transillumination, and by spraying with a ninhydrin reagent [0.2% (w/v) ninhydrin in *n*-butanol]. Strips were scanned for radioactivity using an LB-280 paper chromatogram scanner (Berthold, Wildbad, Germany) and the area containing [$^3$H]ethanolamine phosphate was cut out. Radioactivity was eluted with water and aliquots were stored at −20°C. The yield of [$^3$H]ethanolamine phosphate was 55%.

### Synthesis of CDP-[$^3$H]ethanolamine

[$^3$H]Ethanolamine phosphate (100 $\mu$Ci) was dried in a Speed-Vac evaporator and resuspended (on ice) in 25 $\mu$l water, 20 $\mu$l 250 mM Tris−succinate (pH 7.5), 2.5 $\mu$l 300 mM $MgCl_2$, 10 $\mu$l 60 mM ATP (pH 7.0), 20 $\mu$l 200 mM 3-phosphoglyceric acid, sodium salt (pH 7.0), 5 $\mu$l 100 mM CTP (pH 7.0), 167.5 $\mu$l Enzyme 2 (see above), and incubated for 2 h at 37°C. The reaction was stopped with 250 $\mu$l 100% ethanol and centrifuged for 5 min at 10 000 *g* in a Biofuge A. The supernatant was applied to Whatman 3MM paper and subjected to descending chromatography for 14−18 h using solvent system A. Standard compounds were analyzed in parallel and detected as described above. Radioactivity was detected as described above. The area of the chromatogram containing CDP-ethanolamine was cut out and radioactivity was eluted with water. The product yield was 64%. Aliquots were stored at −20°C. The quality of the material was checked by phosphodiesterase treatment and paper chromatography in solvent systems A or E.

### Synthesis of [$^{32}$P]ethanolamine phosphate

400 $\mu$Ci [$\gamma$-$^{32}$P]ATP was dried, taken up in 3 $\mu$l water and added to 59.5 $\mu$l of a reaction mixture containing 100 mM ethanolamine, 16 mM 3-phosphoglyceric acid (sodium salt), 20 mM Tris−succinate (adjusted to pH 7.5 with HCl), 3 mM $MgCl_2$, 2.4 mM ATP and 42 $\mu$l Enzyme 1 (the ethanolamine, 3-phosphoglyceric acid and ATP were added from neutralized stock solutions). The mixture was incubated for 1 h at 37°C, then stopped with 62.5 $\mu$l 100% ethanol and centrifuged for 5 min at 10 000 *g* in a Biofuge A. The supernatant was chromatographed as above on Whatman 3MM paper using solvent system A (run time 14−19 h). The chromatogram was exposed to Kodak X-OMat film for 45 s to locate the radiolabelled material, and the product was eluted with water (yield 14%).

### Synthesis of [$\beta$-$^{32}$P]CDP-ethanolamine

54 $\mu$Ci [$^{32}$P]ethanolamine phosphate (specific activity 2.7 Ci/mmol) was dried, resuspended in 2.5 $\mu$l water and added to 60 $\mu$l of a reaction mixture containing 16 mM 3-phosphoglyceric acid (sodium salt), 20 mM Tris−succinate (adjusted to pH 7.5 with HCl), 3 mM $MgCl_2$, 2.4 mM ATP, 8 mM CTP and 40 $\mu$l Enzyme 2. After an incubation of 2 h at 37°C, the reaction was stopped with 62.5 $\mu$l 100% ethanol and centrifuged as

described above. The supernatant was chromatographed on paper as described above, and autoradiographed for 35 min. The product (identified by autoradiography and comparison with markers) was eluted with water (yield 0.9%).

### *Radiolabelling of lipids and glycolipids in a cell-free system*

Trypanosome lysates ($2.5 \times 10^8$ cell equivalents) were incubated with CDP-[$^3$H]ethanolamine or [$\beta$-$^{32}$P]CDP-ethanolamine in the presence of 0.2 $\mu$g/ml tunicamycin. ATP, CoA, UDP-GlcNAc and GDP-mannose (1 mM each) were also included in some incubations but were subsequently found to be unnecessary. The total reaction volume was 250 $\mu$l. After incubation (typically 30 min) at 37°C, lipids were extracted by adding 1.7 ml chloroform:methanol (1:1, v/v) and mixing with a glass Pasteur pipette. Cell debris was pelletted by centrifugation and the supernatant extract was transferred to a fresh tube, dried in a Speed-Vac evaporator and partitioned between water and *n*-butanol (Field and Menon, 1992). Lipids were quantitatively recovered in the upper, butanol-rich phase.

### *Metabolic labelling of trypanosomes*

Trypanosomes ($\sim 10^8$/ml in HEPES-buffered serine-free RPMI-1640 supplemented as described by Mayor *et al.* (1990b) and Field and Menon (1992) were incubated with [$^3$H]serine (30–60 $\mu$Ci/ml) for 1–2 h at 37°C. At the end of the labelling period, the cells were pelletted and lipids were extracted sequentially using chloroform:methanol (CM, 2:1, v/v) and chloroform:methanol:water (CMW, 10:10:3, v/v/v) as described by Mayor *et al.* (1990b) and Menon *et al.* (1990a). The CM extract was subjected to Folch-washing (Menon *et al.*, 1990a) to remove water-soluble contaminants. The CMW extract was dried and the residue partitioned between water and *n*-butanol as described above.

### *Thin layer chromatography*

Radiolabelled lipids and water-soluble metabolites were analyzed by TLC on silica (Merck) or cellulose (Kodak Chromagram Sheet 13256) thin layer plates. Radioactivity was detected using a Berthold LB 2842 Automatic TLC Linear Analyzer and quantified using the integration software supplied with the analyzer in conjunction with liquid scintillation counting. In some cases minor peaks were quantified by cutting and weighing enlarged traces of the radioactivity profile.

### *Solvent systems for thin layer and paper chromatography*

Solvent systems are given as volume ratios. System A: *n*-butanol:acetic acid:water (5:2:3); B, chloroform:methanol:water (4:4:1); C, chloroform:methanol:35% ammonia:1 M ammonium acetate:water (180:140:9:8:23); D, *n*-propanol: 8.8% ammonium hydroxide (4:1); E, *n*-butanol:acetic acid:water (12:3:5).

### *Lipid purification*

Lipids were purified after silica TLC by scraping the relevant regions of the thin layer plate, sonicating the silica with chloroform:methanol:water (10:10:3, v/v/v), drying the extract and partitioning the residue between water and *n*-butanol as described above. An alternative approach was sometimes used for separating P2 and P3 in CMW-derived butanol extracts from metabolically labelled cells. Since the cells had been first extracted extensively with CM (see above), the CMW-derived butanol extract contained primarily radiolabelled P2 and P3. The extract was dried, taken up in 1 ml CM + 200 $\mu$l water and vortexed vigorously to mix. Phases were separated by centrifugation: P2 was recovered in the water-rich upper phase, P3 and any contaminating PE were recovered in the chloroform-rich lower phase. Aliquots of all purified lipids were analyzed by TLC to assess purity.

### *Perchloric acid extraction*

Water-soluble [$^3$H]serine metabolites were recovered in perchloric acid extracts of metabolically labelled cells. Labelled cells were washed twice with phosphate-buffered saline, then resuspended in 2 ml ice-cold 0.9 N perchloric acid. The extract (still on ice) was neutralized with KOH (a few drops of phenol red indicator dye were added to the extract to monitor the neutralization process). Cell debris was removed by centrifugation and the supernatant stored at −20°C until required for analysis (Field and Menon, 1992).

Analysis of the PCA extract by paper chromatography (Figure 6) showed two major peaks corresponding to serine and serine phosphate. The radiolabelled material corresponding to the serine standard co-migrated with serine in two TLC systems (cellulose, solvent systems A and D). The material co-migrating with serine phosphate yielded [$^3$H]serine after acid hydrolysis, but was resistant to alkaline phosphatase treatment, suggesting that it was not serine phosphate. It was highly charged since 0.49 N formic acid was required to elute it from a Dowex AG 1X8 (formate form) column; in contrast, ethanolamine phosphate could be eluted with 0.049 N formic acid and CDP-ethanolamine with 0.147 N formic acid (Field and Menon, 1992). Also, since it was unaffected by exposure to pH 9.1 for 1 h at 37°C, a procedure known to cleave activated amino acids (Glabe *et al.*, 1980), it is unlikely to be serine-tRNA. Furthermore, the chromatographic properties of the material were unchanged after phosphodiesterase treatment. Thus, beyond the fact that it contains [$^3$H]serine, the identity of this material remains unknown at present.

Regions of the paper chromatogram (Figure 6) corresponding to CDP-ethanolamine and ethanolamine phosphate were also eluted to examine the extent to which these ethanolamine-containing compounds were present in radiolabelled form. The small amount of radioactive material eluted from the CDP-ethanolamine and ethanolamine phosphate regions was acid hydrolysed, and the hydrolysates were analyzed by TLC (cellulose plate, solvent system D). In both cases, insignificant amounts of [$^3$H]ethanolamine were recovered (see Results).

### *Phospholipase treatments*

Lipid extracts or purified lipids were treated with *B.cereus* phospholipase C [PC, PE, PS (Little, 1981)], *B.cereus* PI-PLC, or *B.thuringiensis* PI-PLC using the conditions described by Mayor *et al.* (1990b). Cleavage products were extracted into *n*-butanol as described, and analyzed by TLC using solvent systems B and C.

### *Phosphodiesterase treatment*

Water-soluble radiolabelled samples were treated with *C.durissus* phosphodiesterase in 11 $\mu$l 25 mM Tris–HCl (pH 8.9), 0.5 mM $(CH_3COO)_2Mg$, 0.27 U/ml enzyme. After an incubation of 20 min at 37°C, the reaction was stopped by heating briefly to 100°C. Products were analyzed on cellulose thin layer sheets using solvent system A. Markers (CDP-ethanolamine and ethanolamine phosphate) were run in parallel and visualized by inspection of the chromatogram under 254 nm illumination and by spraying with ninhydrin (see above).

### *Alkaline phosphatase treatment*

Dried perchloric acid extracts were treated with calf intestinal alkaline phosphatase in 50 $\mu$l 0.5 M Tris–HCl, 1 mM EDTA (pH 8.5) containing 200 U/ml enzyme. The reaction was carried out for 4 h at 37°C before paper chromatography analysis (solvent system A or D).

### *Amine analysis*

Radiolabelled metabolites from [$^3$H]serine-labelled cells were extracted and purified as described above. The identity of the incorporated radioactivity in each case was determined by acid hydrolysis and TLC as follows. The samples were dried in a 0.3 ml Reacti-Vials™ (Pierce Chemical Co.) along with 150 nmol each of non-radioactive ethanolamine and serine, 100 $\mu$l 6 N HCl (Pierce) was added, and the vials were incubated for 6 h at 100°C. The samples were then dried in a Speed-Vac evaporator. In each case the residue was resuspended in 20 $\mu$l water and analyzed by TLC on cellulose sheets using solvent system A or D, or by descending paper chromatography on Whatman 3MM paper using solvent system A or D. Radioactivity was detected with an automatic scanner as described above. Non-radioactive standards were located by spraying the chromatograms with ninhydrin reagent and heating briefly in a 100°C oven.

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