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METABOLISM OF SERINE AND ETHANOLAMINE PLASMALOGENS IN *MEGASPHAERA ELSDENII*

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SUMMARY

1. *Megasphaera elsdenii* appears to be a very suitable organism for studies on the metabolism of plasmalogens in anaerobic bacteria due to its extremely high content of both serine and ethanolamine plasmalogen.

2. Growth of this organism in the presence of either $^{32}P_i$ or [2-^{3}H]glycerol resulted in similar labelling patterns of the various lipid constituents. The ^{3}H label, incorporated into the plasmalogens, was found to be localized exclusively in the glycerol moiety of these compounds. This implies that, at least in *M. elsdenii*, the synthesis of plasmalogens from glycerol does not, or only partially, proceed via dihydroxyacetone phosphate.

3. After growth in the simultaneous presence of $^{32}P_i$ and [2-^{3}H]glycerol, phosphatidylserine and phosphatidylethanolamine on the one hand, and serine and ethanolamine plasmalogen on the other hand, had similar $^3H/^{32}P$ ratios suggesting that both phosphatidylserine and serine plasmalogen are decarboxylated to form phosphatidylethanolamine and ethanolamine plasmalogen, respectively.

4. Turn-over experiments with ^{32}P-labelled cells also showed a precursor–product relation between serine and ethanolamine plasmalogen, and between phosphatidylserine and phosphatidylethanolamine.

5. Growth of *M. elsdenii* in the presence of hydroxylamine resulted in a disappearance of ethanolamine plasmalogen and a concomitant accumulation of serine plasmalogen, strongly supporting the concept that, at least in this organism, ethanolamine plasmalogen is formed from serine plasmalogen. This observation suggests that growth of this organism or other bacteria in the presence of hydroxylamine may provide an important tool to modify the polar phospholipid composition of bacterial membranes.

INTRODUCTION

Phosphatidylserine, phosphatidylethanolamine and their plasmalogen analogs were found to be the principal phospholipids in *Megasphaera elsdenii*, a strictly

anaerobic rumen bacterium [1]. The presence of phosphatidylserine as a major lipid constituent in this organism is unique, since this phospholipid has been found in trace amounts only in all other bacteria investigated so far. (See excellent recent reviews by Op den Kamp et al. [2], Lennarz [3], Kates and Wassef [4], Cronan and Vagelos [5] and Goldfine [6].) Similarly, significant amounts of serine plasmalogen have never been detected in bacteria. On the other hand, ethanolamine plasmalogens are abundant in many anaerobic bacteria [7].

During the past few years considerable progress has been made in the elucidation of the metabolic pathways leading to the biosynthesis of plasmalogens in mammalian tissues [8]. However, our knowledge of the mechanisms involved in the formation of plasmalogens in bacteria is very scarce [7]. *M. elsdenii* appeared to be a very suitable model to investigate these mechanisms due to its unique phospholipid composition. In this paper conclusive evidence will be presented that, at least in this organism, serine plasmalogen is the immediate precursor in the biosynthesis of ethanolamine plasmalogen.

During these studies it was found that the phospholipid composition of *M. elsdenii* could be changed drastically by growing the organism in the presence of hydroxylamine. This may provide an important tool in future studies on the effect of alterations in the polar phospholipids on the properties of biological membranes.

MATERIALS AND METHODS

Culture and growth of M. elsdenii

M. elsdenii strain B 159 was obtained from Dr M. P. Bryant, Department of Dairy Science, University of Illinois, Urbana, Ill., U.S.A.

The organism was grown at 39 °C in 16 mm × 150 mm roll-tubes (Bellco Glass, Vineland, N.J., U.S.A.) fitted with recessed butyl rubber stoppers, under an atmosphere of pure CO_2 in a medium described in details previously [1]. Growth was followed by measurement of the absorbance at 660 nm in a Spectronic-20 colorimeter (Bausch and Lomb, Rochester, N.Y., U.S.A.). An absorbance of 0.500 corresponded to $4.4 \cdot 10^8$ cells/ml.

Radiochemicals

Sodium [^{32}P]orthophosphate, spec. act. > 165 Ci/mole, was purchased from Philips–Duphar, Petten, The Netherlands. [2-^{3}H]Glycerol, spec. act. 496 Ci/mole, was obtained from the Radiochemical Centre, Amersham, Great Britain.

Pulse experiments with $^{32}P_i$ and [2-^{3}H]glycerol

A series of 10-ml cultures of *M. elsdenii* were grown to an *A* of 0.15. At this point, 100 μCi $^{32}P_i$ or [2-^{3}H]glycerol were added to each tube, after which growth was continued. At 15, 30, 60 and 90 min after the addition of the radioactive tracer, cells from triplicate 10-ml cultures were collected by filtration through Gelman Metrical α-6 filters (pore size 0.45 μm). The cell pellets were immediately taken up in 3 ml of methanol and the lipids were extracted as described in detail by Van Golde et al. [9]. In some experiments, *M. elsdenii* was grown in the presence of both [2-^{3}H]glycerol and $^{32}P_i$ to follow simultaneously the incorporation of both isotopes into the lipids of this organism.

Turn-over of phospholipids of M. elsdenii

A 40-ml culture of *M. elsdenii* was grown in the presence of 0.5 mCi of $^{32}P_i$. After an *A* of 0.8 was reached, the cells were harvested by Millipore filtration. The cell pellet was immediately resuspended in 10 ml of non-labelled medium. 0.5-ml aliquots were taken from this suspension and added to a series of tubes containing 10 ml of non-labelled medium to give an initial *A* of approx. 0.15. After 15, 30 and 60 min of growth in the presence of non-labelled phosphate, cells from triplicate 10-ml cultures were collected by Millipore filtration and the lipids were extracted as described before [9].

Growth of M. elsdenii in the presence of NH_2OH

10-ml cultures of *M. elsdenii* were grown until an *A* of 0.15 was reached. At this point NH_2OH was added to the cultures in the following concentrations: 0; 0.5; 1.0; and 2.0 mM. After 10 min 100 μCi of $^{32}P_i$ were added and growth was continued for 2 h. The cells were then harvested by Millipore filtration and the lipids were extracted [9].

Analysis of phospholipids and their plasmalogen analogues

The incorporation of $^{32}P_i$ and/or [2-^{3}H]glycerol into the diacylphospholipids and their plasmalogen analogs could be determined basically by the method of Singh et al [10]: 1.5 μmoles of lipid-P were applied as a single spot in the left lower corner of a 20 cm × 20 cm silica HR (Merck, Germany) plate impregnated with 2% magnesium silicate. The thin-layer plates were prepared and activated exactly as described by Broekhuyse [11]. Chloroform–methanol–conc.NH_3–water (90:54:5.5:5.5, by vol.) was used as solvent for development into the first direction. After removal of the solvent under a stream of N_2, the plate was covered with a sheet of paper except for a 3-cm track at the left side of the plate, which was sprayed with a 12% (v/v) solution of conc.HCl in methanol. After 2 min the methanolic HCl was evaporated under N_2 and the chromatogram was developed in the second direction in chloroform–methanol –acetic acid–water (90:40:12:2, by vol.). The diacylphospholipids and the monoacyl derivatives of the plasmalogens, originally present, were detected by radiochromatogramscanning and autoradiography as described in detail previously [1]. Phosphatidylserine and phosphatidylethanolamine reference compounds were obtained from Applied Science (U.S.A.) and Koch-Light (Great Britain), respectively. The various radioactive spots were scraped from the plate and transferred into scintillation vials, to which 1 ml of water and 10 ml of Instagel (Packard, U.S.A.) were added. Scintillation counting was performed in a Packard Tricarb 2425 B. In some experiments an alternative method has been used to follow the incorporation of radioactivity into the diacyl phospholipids and their plasmalogen analogs. This method [12], which is based on a selective hydrolysis of the diacyl phospholipids and plasmalogens, yielded identical results to those obtained by the method of Singh et al. [10] used routinely in this study.

Dimethylacetals and methylesters derived from the plasmalogens

The dimethylacetals and methylesters derived from the alk-1-enyl and acyl-moieties of the plasmalogens were obtained as follows: the serine and ethanolamine-containing phospholipids were isolated via two-dimensional thin-layer chromato-

graphy and treated with 2 ml of a 1 M methanolic solution of H_2SO_4 in closed tubes at 100 °C for 12 min. After cooling in ice, 4 ml of hexane were added followed by careful addition of 2 ml of 2 M NaOH. The tubes were then centrifuged at 0 °C and the hexane layer was removed. The aqueous layer was extracted once more with 4 ml of hexane and the hexane layers were combined. The dimethylacetals and methylesters produced in this way [13] could then be separated on silica plates which were run in benzene [14]. After detection by iodine, the methylesters (derived from the diacyl phospholipids and plasmalogens) and dimethylacetals were scraped from the plate and assayed for radioactivity by scintillation counting as described above. A dimethylacetal reference compound was generously donated by Dr F. Snyder, Oak Ridge Associated Universities, Oak Ridge, Tenn.

RESULTS AND DISCUSSION

Experiments with $^{32}P_i$

In a previous paper [1] it has been shown by three independent methods that both the serine- and ethanolamine-containing phospholipids of *M. elsdenii* comprise a high proportion of plasmalogens, viz. 72 and 90%, respectively. As a first approach to investigate the biosynthesis of the plasmalogens in this organism, *M. elsdenii* was grown in the presence of $^{32}P_i$ for different periods. The incorporation of radioactivity into phosphatidylserine, phosphatidylethanolamine and their plasmalogen analogs as a function of time is presented in Fig. 1. Phosphatidylserine is the component which is labelled most rapidly. This phospholipid is known to be the precursor of phosphatidylethanolamine, at least in all bacteria investigated so far [15, 2–6]. In agreement with this concept, labelling of phosphatidylethanolamine occurs at a later stage. After prolonged growth (240 min) in the presence of $^{32}P_i$, the percentage of ^{32}P incorporated into phosphatidylserine and phosphatidylethanolamine approaches the relative amounts of these phospholipids as determined by phosphorus analysis [1].

Serine plasmalogen was found to be labelled prior to ethanolamine plasmalogen. This is in line with a formation of ethanolamine plasmalogen by decarboxylation of serine plasmalogen. Such a decarboxylation could be catalyzed by the same enzyme responsible for the decarboxylation of phosphatidylserine. In this respect, it may be relevant to mention that phosphatidylserine decarboxylase from *E. coli* is able to decarboxylate both phosphatidylserine and serine plasmalogen [1]. However, a conversion of phosphatidylethanolamine into ethanolamine plasmalogen, as suggested by Goldfine and Hagen [7] for *C. butyricum*, cannot yet be ruled out by the results presented in Fig. 1.

Experiments with [2-^3H]glycerols

A similar labelling pattern of the diacyl phospholipids and their plasmalogen analogs was obtained after growth in the presence of [2-^{3}H]glycerol instead of $^{32}P_i$ (data not shown).

Strong evidence has been presented by Wykle and Snyder [16] and by Hajra [17] that dihydroxyacetone phosphate is the precursor in the biosynthesis of alkyl- and alk-1-enyl lipids in mammalian tissues. If this were true for bacteria as well, no labelling should have been observed in serine and ethanolamine plasmalogen after growth in the presence of [2-^{3}H]glycerol, since the ^{3}H label would have been lost upon con-

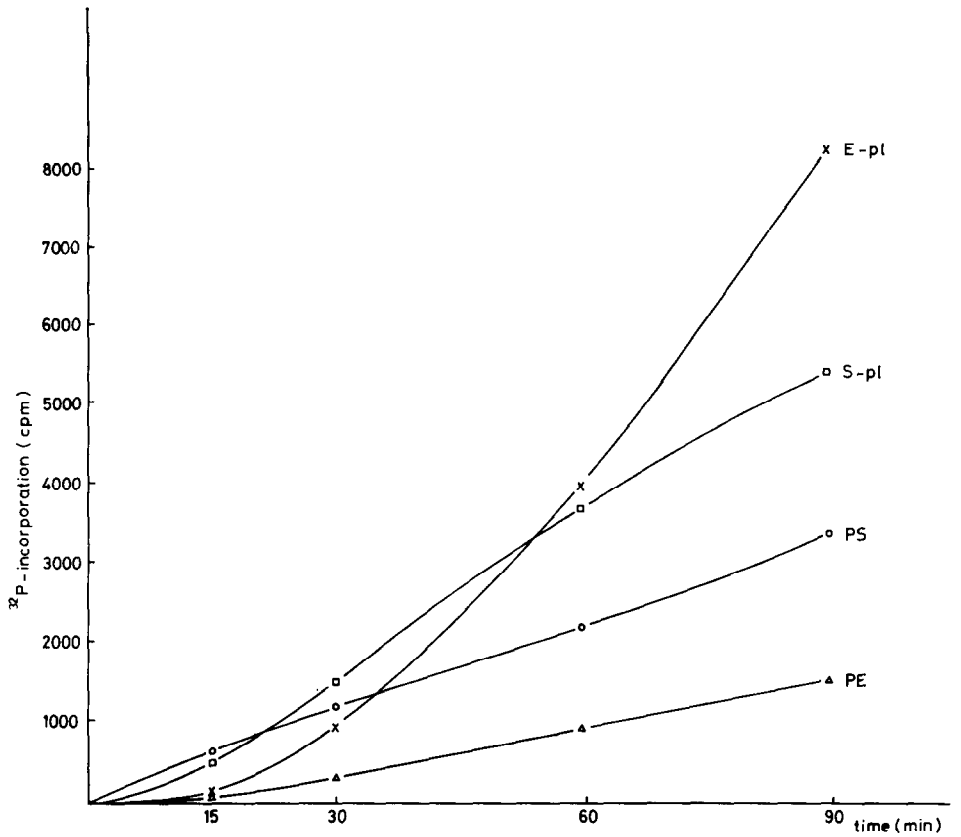

Fig. 1. Growth of *M. elsdenii* in the presence of $^{32}P_i$. 10-ml cultures of *M. elsdenii* were grown until an *A* of 0.15 was reached. At this point 100 μCi of $^{32}P_i$ were added after which growth was continued. At the indicated times, cells from triplicate 10-ml cultures were collected by Millipore filtration and the lipids extracted. The separation of diacyl phospholipids and their plasmalogen analogs and the assay of radioactivity incorporated into these compounds, were accomplished as described in details in Materials and Methods. ○—○, phosphatidylserine (PS); □—□, serine plasmalogen (S-pl); △—△, phosphatidylethanolamine (PE); ×—×, ethanolamine plasmalogen (E-pl).

version of [2-^{3}H]glycerol into dihydroxyacetone phosphate. Since both serine and ethanolamine plasmalogen became labelled during growth in the presence of [2-^{3}H]-glycerol, it was extremely important to investigate in which part of the molecule the ^{3}H label was localized. Both in serine and in ethanolamine plasmalogen, only $2^0/_{00}$ of the label was found in the acyl groups and even less in the alk-1-enyl moieties, whereas over 99% was recovered in the glycerol moiety of both plasmalogens. The fact that the ^{3}H label is recovered almost exclusively in the glycerol moiety of the plasmalogens must imply that dihydroxyacetone phosphate is certainly not the only compound via which glycerol enters into plasmalogens in this organism. A similar finding has been reported by Hill and Lands [18] for *C. butyricum*. No significant amounts of labelled O-alkyl analogues of phosphatidylserine or phosphatidylethanolamine could be detected after growth of *M. elsdenii* in the presence of [2-^{3}H]glycerol.

Growth of M. elsdenii in the simultaneous presence of [2-^{3}H]glycerol and $^{32}P_i$

In order to investigate whether in *M. elsdenii* serine plasmalogen is decarboxylated to form ethanolamine plasmalogen, just like phosphatidylserine is decarboxylated to phosphatidylethanolamine, double-label experiments using [2-^{3}H]glycerol and $^{32}P_i$ were conducted. The $^3H/^{32}P$ ratios of phosphatidylserine, phosphatidylethanolamine, and their plasmalogen analogs after several periods of growth in the presence of both labelled substrates are presented in Table I. It is very interesting that

TABLE I

$^3H/^{32}P$ RATIOS OF PHOSPHOLIPIDS OF *M. elsdenii* AFTER SIMULTANEOUS LABELLING WITH [2-^{3}H]GLYCEROL AND $^{32}P_i$

10-ml cultures of *M. elsdenii* were grown until an *A* of 0.15 was reached. At this point 100 μCi of [2-^{3}H]-glycerol and 100 μCi of $^{32}P_i$ were added after which growth was continued. At the indicated times, the cells were collected and the lipids were extracted. The various diacylphospholipids and their plasmalogen analogs were separated as described in Materials and Methods, and assayed for radioactivity.

	$^3H/^{32}P$ ratio		
	60 min	90 min	120 min
Phosphatidylserine	12.95	14.03	13.98
Phosphatidylethanolamine	14.02	14.52	14.14
Serine plasmalogen	9.08	10.36	11.40
Ethanolamine plasmalogen	8.17	9.23	9.95

at all time points the isotope ratios of serine and ethanolamine plasmalogen on the one hand and those of phosphatidylserine and phosphatidylethanolamine on the other hand, closely parallel each other. This strongly suggests that serine plasmalogen and phosphatidylserine are the precursors of ethanolamine plasmalogen and phosphatidylethanolamine, respectively. Furthermore, it is interesting to note that the isotope ratios of serine plasmalogen and phosphatidylserine are different. This might indicate that serine plasmalogen is not synthesised from phosphatidylserine.

Turn-over of the phospholipids of M. elsdenii

Cells of *M. elsdenii* were grown in the presence of $^{32}P_i$ until an *A* of 0.8 was reached, which corresponds to the late-logarithmic phase of growth. The cells were then transferred to a medium containing non-labelled phosphate after which growth was continued for 0, 30 and 60 min, respectively. The amount of radioactivity retained in the individual phospholipid constituents during growth in the presence of non-labelled phosphate was expressed (Fig. 2) as the percentage of the total amount of ^{32}P incorporated into the total phospholipids. Though specific radioactivity data is not available, it is clear from Fig. 2 that the radioactivity of phosphatidylserine decreases after transfer of the cells to a non-labelled medium. A concomitant increase can be observed in the radioactivity of phosphatidylethanolamine. This is in line with the concept that phosphatidylserine is the precursor of phosphatidylethanolamine in bacteria [15, 2–6].

More interesting is the similar reciprocity of the curves representing the percentage of radioactivity retained in serine and ethanolamine plasmalogen, respectively. These results strongly endorse the finding described above that ethanolamine plasma-

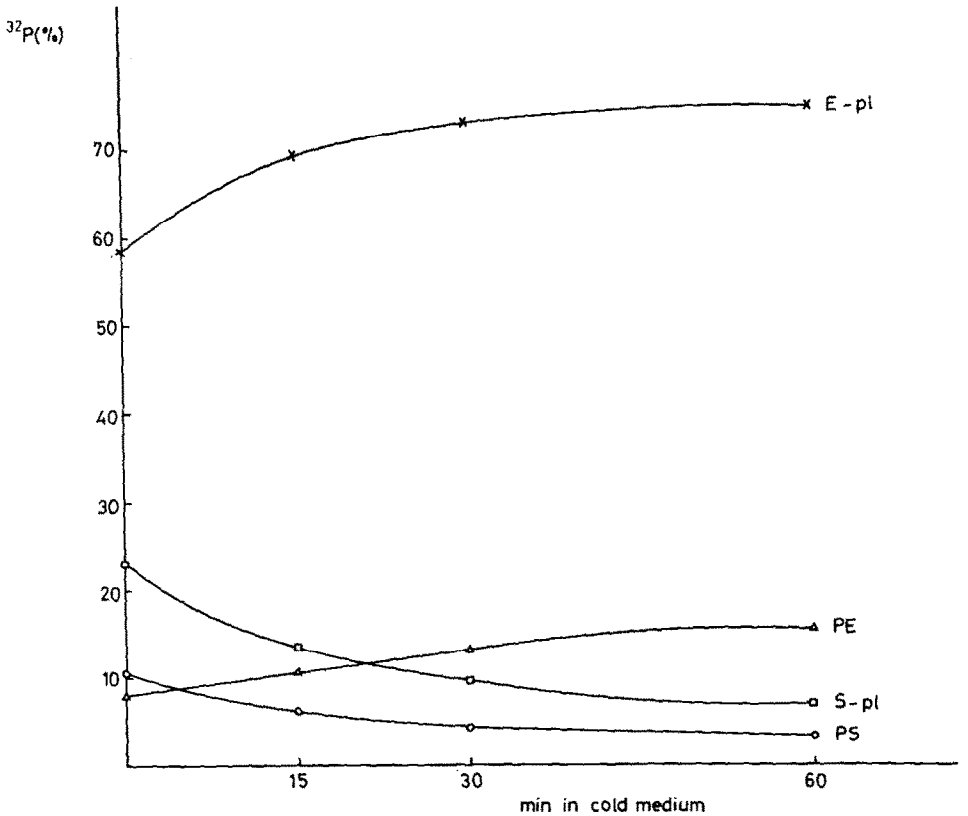

Fig. 2. Turnover of phospholipids of *M. elsdenii*. Cells of *M. elsdenii* were grown in the presence of $^{32}P_i$ until an *A* of 0.8 was reached. The cells were then transferred to a medium containing unlabelled phosphate after which growth was continued for the indicated periods of time. The amount of radioactivity retained in the individual lipid constituents was expressed as the percentage of the total amount of $^{32}P_i$ incorporated into the total lipids. For further details see Materials and Methods. ○—○, phosphatidylserine (PS); □—□, serine plasmalogen (S-pl); △—△, phosphatidylethanolamine (PE); ×—×, ethanolamine plasmalogen (E-pl).

logen is produced by decarboxylation of serine plasmalogen. Formation of ethanolamine plasmalogen from its diacyl analog, as suggested by Goldfine and Hagen [7], seems rather unlikely from the results presented in Fig. 2.

Growth of M. elsdenii in the presence of NH_2OH

It is known [19] that phosphatidylserine decarboxylase is inhibited by NH_2OH. Therefore, it was decided to investigate the possibility of growing *M. elsdenii* in the presence of varying concentrations of NH_2OH in order to corroborate our conclusion that serine plasmalogen is the precursor of ethanolamine plasmalogen.

Cells of *M. elsdenii* were grown until an *A* of 0.15 was reached. At this point NH_2OH was added at the concentrations indicated in Fig. 3. After 10 min 100 μCi of $^{32}P_i$ were added after which growth was continued. 0.5 and 1.0 mM NH_2OH hardly affected growth of the organism, whereas growth was 50% reduced in the presence of 2 mM NH_2OH. It is clear that addition of NH_2OH strongly reduces the percentage of

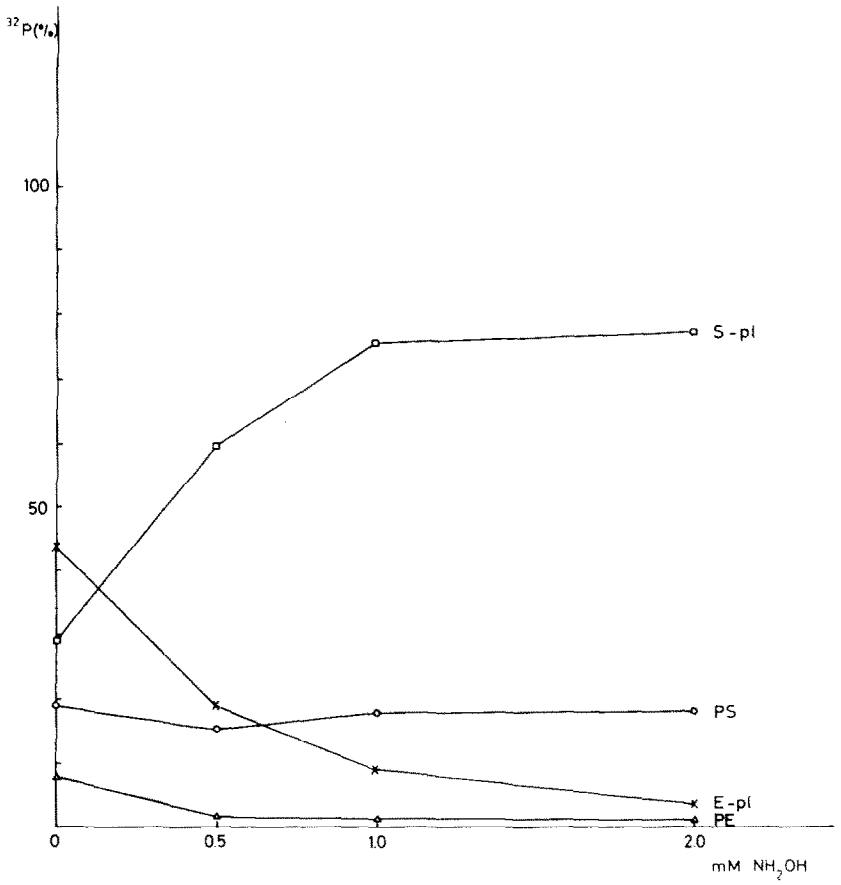

Fig. 3. *M. elsdenii* grown in the presence of [^{32}P]-phosphate and varying concentrations of NH_2OH. Cells of *M. elsdenii* were grown until an *A* of approx. 0.15 was reached. At this point, varying amounts of NH_2OH were added and, 10 min later, 100 μCi of $^{32}P_i$. Growth was then continued for 2 additional h. The amount of radioactivity incorporated into the individual lipids was expressed as described for Fig. 2.

radioactivity incorporated into phosphatidylethanolamine and even more pronounced that into ethanolamine plasmalogen. A corresponding accumulation of radioactivity takes place in phosphatidylserine and especially in serine plasmalogen. The reciprocity of the curves representing the radioactivity of serine and ethanolamine plasmalogen strongly supports the precursor–product relationship between these plasmalogens.

It is very interesting to note that the phospholipids of this organism, after growth in the presence of 1 or 2 mM NH_2OH, consist almost exclusively of phosphatidylserine and serine plasmalogen. This may provide a tool to investigate the effect of these alterations in polar phospholipid composition on the physico-chemical properties of the membrane of this organism.

In this paper conclusive evidence has been presented that, at least in this organism, ethanolamine plasmalogen is formed by decarboxylation of serine plasma-

logen, rather than from diacyl phosphatidylethanolamine as suggested by Goldfine and Hagen [7] for *C. butyricum*. Whether formation of ethanolamine plasmalogen from serine plasmalogen is a pathway generally occurring in anaerobic bacteria remains to be established. Studies on the nature of the alk-1-enyl moieties of both serine and ethanolamine plasmalogen and on the mechanisms via which the alk-1-enyl moiety is introduced into serine plasmalogen are in progress.

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