Intermolecular Transacylation of Phosphatidylethanolamine by a *Butyrivibrio* sp.

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1. Washed cells and supernatant from a culture of a *Butyrivibrio* sp. carry out the intermolecular transacylation reaction 2 phosphatidylethanolamine $\rightarrow$ *N*-acylphosphatidylethanolamine + lysophosphatidylethanolamine. 2. Washed cells can catalyse the intramolecular transacylation of *N*-(acyl)glycerylphosphorylethanolamine to lysophosphatidylethanolamine; the culture supernatant is largely devoid of activity.

Recently we have isolated a *Butyrivibrio* sp. from ovine rumen fluid which we have tentatively identified as *Butyrivibrio fibrisolvens*. This organism possesses a potent phospholipase activity which can bring about a rapid and complete deacylation of phosphatidylcholine (Hazlewood & Dawson, 1975). On examining the action of this organism's enzymes using phosphatidylethanolamine as substrate we have observed the accumulation of *N*-acylphosphatidylethanolamine and *N*-acyl-lysophosphatidylethanolamine in substantial amounts. Evidence is presented that the organism catalyses an intermolecular transacylation with one phosphatidylethanolamine molecule acting as acyl donor and another as acyl receptor.

N-acylphosphatidylethanolamine has previously been described as a minor constituent of seed lipids (Bomstein, 1965; Dawson *et al.*, 1969) which rapidly disappears during early germination (Dawson *et al.*, 1969). Attempts made during the latter investigation to obtain from immature or mature seeds an enzyme preparation which was capable of bringing about the *N*-acylation of phosphatidylethanolamine were unsuccessful.

Methods

Incubation details

The isolation of the *Butyrivibrio* sp. and the method of growing the shake-flask batch cultures is described in Hazlewood (1974) and Hazlewood & Dawson (1975). Cells or culture supernatant were taken 4–6 h after the culture had achieved maximum turbidity. For each incubation, the cells from a portion of culture medium were separated by centrifugation (2000*g*_{av}), washed once with 0.1 M-Na₂HPO₄–NaH₂PO₄ buffer, pH 6.8, containing cysteine (0.1 M) and EDTA (1 mM) and then suspended to a total volume of 4 ml in the same buffer solution. After equilibrium at 39°C the cell suspen-

sions were transferred to tubes containing labelled phosphatidylethanolamine (25–50 µg of P) or various isolated lipid intermediaries which had been dispersed in 1 ml of the same buffer by vortexing for 30 s with a glass bead. All incubations were at 39°C under a gas phase of O₂-free CO₂.

Examination of lipid and water-soluble products

The reaction was stopped by the addition of 80 ml of chloroform-methanol (2:1, v/v). After 30 min, the mixture was shaken with 0.2 vol. of water, and the two phases were separated by centrifugation. The phospholipids in the lower phase were examined by t.l.c. on silica gel (Kieselgel F₂₅₄; Merck, Darmstadt, Germany) in a solvent containing chloroform-methanol-18 M-NH₃-water (110:50:3:5, by vol.).

Water-soluble phosphorus compounds were examined by paper ionophoresis at pH 3.6 or by descending paper chromatography in phenol saturated with water-acetic acid-ethanol (100:10:12, by vol.). Details of the location of the compounds by radioautography and the determination of their radioactivity have been described elsewhere (Hazlewood & Dawson, 1975).

Isolation of ³²P-labelled lipid intermediaries

The ³²P-labelled *N*-acyl derivatives of phosphatidylethanolamine, lysophosphatidylethanolamine and glycerylphosphorylethanolamine as well as lyso-[³²P]phosphatidylethanolamine were prepared from suitable incubates of [³²P]phosphatidylethanolamine with the washed bacterial cells or culture supernatants. The lipids were separated by preparative strip t.l.c. (silica gel H) in first a solvent of diethyl ether to remove neutral lipids and pigments to the end of the plate and then the chloroform-methanol-NH₃-water solvent described above. The products were located by radioautography, the band of silica gel was scraped off and the phospholipid was eluted with chloroform-methanol-water (10:10:1, by vol.). The

extracts were evaporated to dryness at 40°C and the products stored in chloroform under N₂ at -16°C. For most preparations of *N*-acylphosphatidylethanolamine, the t.l.c. was usually repeated to remove minor impurities.

Since *N*-(acyl)glycerylphosphorylethanolamine was only formed in small amounts compared with the other products, additional quantities were sometimes prepared by the mild alkaline alcoholysis of the more abundant *N*-acyl-lysophosphatidylethanolamine (Dawson *et al.*, 1969).

Materials

[³²P]Phosphatidylethanolamine was prepared from *Saccharomyces cerevisiae* grown in the presence of ³²P_i and unlabelled phosphatidylethanolamine from a commercial preparation of baker's yeast (Hazlewood & Dawson, 1975).

1-[³H]Stearoyl phosphatidylethanolamine and 2-[¹⁴C]linoleoyl phosphatidylethanolamine were kindly given by the late Professor G. R. Webster and were prepared from rat liver by the method of Cooper & Webster (1970). Samples of authentic *N*-acylphosphatidylethanolamine, *N*-acyl-lysophosphatidylethanolamine and *N*-(acyl)glycerylphosphorylethanolamine were prepared from pea lipids (Dawson *et al.*, 1969). *N*-Oleoylethanolamine was synthesized by the method of Roe *et al.* (1949). Lysophosphatidylethanolamine was obtained from Lipid Products, Epsom, Surrey, U.K. Glycerylphosphorylethanolamine was prepared by the alkaline deacylation of phosphatidylethanolamine (Dawson *et al.*, 1962).

Results

Phospholipids formed from [³²P]phosphatidylethanolamine

T.l.c. and radioautography of the phospholipids formed on incubating [³²P]phosphatidylethanolamine with the washed stationary-phase cells of the *Butyrivibrio* sp. gave consistently in at least ten experiments a distribution of products illustrated in Plate 1. The phosphatidylethanolamine was converted into three radioactive products A (*R_F* 0.80), B (*R_F* 0.69) and C (*R_F* 0.11). If the culture supernatant was incubated with the substrate, the same three products were observed, but in addition there was a fourth (E) migrating a little slower (*R_F* 0.23) than the substrate (spot D).

Products A and B migrated in similar t.l.c. runs with authentic *N*-(acyl)phosphatidylethanolamine and *N*-acyl-lysophosphatidylethanolamine markers respectively. On mild alkaline degradation (Dawson *et al.*, 1962) they were both completely converted into a further lipid substance which migrated to the same position as spot E and also co-chromatographed with an *N*-(acyl)glycerylphosphorylethanolamine marker. Spot E was itself stable to mild alkaline degradation

which was also true of *N*-(acyl)glycerylphosphorylethanolamine. After acid hydrolysis of samples of spots A and B (2M-HCl, 100°C, 12 min) followed by distribution between water (1 ml) and chloroform (2 ml) the sole water-soluble product identified by paper ionophoresis was glycerophosphoric acid. The chloroform-soluble materials formed after acid hydrolysis were separated by t.l.c. (silica gel H) in chloroform-methanol-acetic acid-water (130:50:4:1, by vol.) and gave evidence on iodine vapour staining for the presence of *N*-acylethanolamine; *N*-oleoylethanolamine was used as a marker (*R_F* 0.73). These substances are known to be produced on the acid hydrolysis of both *N*-acylphosphatidylethanolamine and *N*-acyl-lysophosphatidylethanolamine (Dawson *et al.*, 1969). From such results it was therefore concluded that products A, B and E formed from the phosphatidylethanolamine substrate are respectively *N*-acylphosphatidylethanolamine, *N*-acyl-lysophosphatidylethanolamine and *N*-acylglycerylphosphorylethanolamine.

Product C was entirely labile when subjected to mild alkaline degradation and the sole water-soluble product identified after two-dimensional paper chromatography/ionophoresis (Dawson *et al.*, 1962) was glycerylphosphorylethanolamine. This suggested that it was probably lysophosphatidylethanolamine which was confirmed by thin-layer co-chromatography of product C with an authentic sample of this phospholipid.

Relative rates of production of lipid and water-soluble phosphorus-containing reaction products

Fig. 1 shows results of a typical experiment in which [³²P]phosphatidylethanolamine was incubated with washed bacterial cells, and measurements were made of the radioactivity in the reaction products. At very short periods of incubation (up to 5 min) the major products were *N*-acylphosphatidylethanolamine and lysophosphatidylethanolamine. Subsequently water-soluble phosphorus and *N*-acyl-lysophosphatidylethanolamine were produced in substantial amounts. In further experiments examination of the water-soluble products by paper chromatography in phenol saturated with water-ethanol-acetic acid (100:12:10, by vol.) indicated that glycerylphosphorylethanolamine (*R_F* 0.64) was the main component initially (Fig. 2). The accumulation and subsequent decrease in the proportion of glycerophosphoric acid (*R_F* 0.29) and the increase in the proportion of P_i (*R_F* 0.14) suggested that the glycerylphosphorylethanolamine formed was rapidly attacked by a phosphodiesterase giving glycerophosphate followed by phosphomonoesterase action on the latter.

A small amount of water-soluble radioactivity accumulated which migrated in the position of a phosphorylethanolamine marker (*R_F* 0.48). The

EXPLANATION OF PLATE I

Radioautogram of a t.l.c. plate showing the lipid products formed during the metabolism of [32 P]phosphatidylethanolamine by washed stationary-phase cells and culture supernatant

Each incubation (5 ml) was carried out for the time-period indicated by the method stated in the Methods section by using 0.57 mg dry weight of cells/ml and 10 μ g of [32 P]phosphatidylethanolamine P/ml. The lipids were extracted with chloroform-methanol (2:1, v/v) and the purified lipids were separated by t.l.c. (chloroform-methanol-18M-NH₃-water; 110:50:3:5, by vol.). The plates were radioautographed for 48 h.

Fig. 1. Appearance of radioactive products during the incubation of [32 P]phosphatidylethanolamine with washed bacterial cells

The washed cells (2.85 mg dry weight) were incubated in 5 ml of medium containing 50 μ g of [32 P]phosphatidylethanolamine P for each time-point. The reaction was stopped with chloroform-methanol (2:1, v/v) and the extract separated into water-soluble and lipid products. The latter were separated by t.l.c. and the radioactivity in the individual phospholipids and water-soluble [32 P] was determined by Cerenkov radiation after oxidation. Similar results were obtained in three other experiments. ●, Water-soluble 32 P; ○, *N*-acylphosphatidylethanolamine; ▲, *N*-acyl-lysophosphatidylethanolamine; ■, lysophosphatidylethanolamine.

origin of this component is obscure; it could be derived by low-activity phospholipase C hydrolysis of the substrate or rephosphorylation of the unesterified ethanolamine formed with 32 P. In addition some radioactivity was observed migrating near the solvent front (R_F 0.89). This consisted of a mixture of *N*-(acyl)glycerylphosphorylethanolamine and lysophosphatidylethanolamine, lipid decomposition products which, because of their polarity and single acyl group, had partially distributed in the upper phase of the chloroform-methanol-water system used for separating lipids and non-lipids.

Incubation of cells and culture supernatant with various phospholipid intermediates

To obtain further information about the reactions involved in the metabolism of phosphatidylethanolamine certain 32 P-labelled lipid intermediates were isolated and were incubated with the washed bacterial cells or culture supernatant. T.l.c. of the

lipid products obtained or paper chromatography of the water-soluble substances formed gave a semi-quantitative picture of the decomposition of these intermediates.

N-Acyl[32 P]phosphatidylethanolamine was, after 2 h incubation with washed cells, substantially (38%) broken down into water-soluble products (28%), *N*-acyl-lysophosphatidylethanolamine (8%) and lysophosphatidylethanolamine (1%). The culture supernatant gave the same products and in addition there was minimal radioactivity in the *N*-(acyl)glycerylphosphorylethanolamine fraction.

Fig. 2. Appearance of individual water-soluble phosphates during the incubation of [32 P]phosphatidylethanolamine with washed bacterial cells

The washed cells (8.5 mg dry weight) were incubated in 5 ml of medium containing 38 μ g of [32 P]phosphatidylethanolamine P for each time-point. The reaction was stopped with chloroform-methanol (2:1, v/v) and the extract treated with 0.2 vol. of water. A portion (3%) of the upper aqueous phase was chromatographed on paper in phenol saturated with water-acetic acid-ethanol (100:10:12, by vol.). After drying and radioautography (7 days) the radioactive spots were oxidized and assayed by scintillation counting (Cerenkov). Similar results were obtained in three other experiments. ●, P_i ; □, glycerylphosphorylethanolamine; ▲, glycerophosphate; ■, phosphorylethanolamine.

N-Acyl-lyso[^{32}P]phosphatidylethanolamine when incubated for 1 h with washed bacterial cells gave 16% of water-soluble products, a trace (<1%) of *N*-(acyl)glycerylphosphorylethanolamine and 4% of *N*-acylphosphatidylethanolamine.

Washed cells incubated with *N*-(acyl)glyceryl-[^{32}P]phosphorylethanolamine for 2 h converted this substrate almost completely into lysophosphatidylethanolamine and thence to water-soluble products. By contrast, the culture supernatant had little action on this substrate. When lyso[^{32}P]phosphatidylethanolamine was incubated with washed cells or with the culture supernatant the main ^{32}P -containing products were water-soluble. However, a few percent of *N*-acylphosphatidylethanolamine and *N*-acyl-lysophosphatidylethanolamine were formed suggesting that some of the degradative enzymes involved may be acting in an anabolic reversible manner.

Origin of the *N*-acyl group

The *N*-acyl group inserted on to a phosphatidylethanolamine molecule could have been derived from the substrate itself or from endogenous fatty acids in the bacterial cells. Incubation of the cells with either $1\text{-}^{14}\text{C}$ -labelled stearic acid or linoleic acid plus yeast phosphatidylethanolamine resulted in no detectable incorporation of radioactivity into the *N*-acyl group of the *N*-acylphosphatidylethanolamine formed. On the other hand, incubation of the cells with $1\text{-}[9,10\text{-}^3\text{H}]\text{stearoylphosphatidylethanolamine}$ resulted in 11–14% of the radioactivity incorporated appearing in the *N*-acyl group. With $2\text{-}[1\text{-}^{14}\text{C}]\text{linoleoylphosphatidylethanolamine}$ as substrate a lesser amount (4–6%) was incorporated. The mass yield of *N*-acylphosphatidylethanolamine in such experiments performed on four occasions was low compared with when yeast phosphatidylethanolamine was used as substrate. This could be due to the different fatty acid composition of the specifically labelled liver phospholipids used.

Stoichiometry of acyl donors and acceptors

Since the evidence from the previous section had indicated that an *O*-acyl group of phosphatidylethanolamine was the donor of the *N*-acyl group an attempt was made to relate *O*-acyl loss to *N*-acyl gain. On the assumption that *N*-acyl-lysophosphatidylethanolamine and *N*-(acyl)glycerylphosphorylethanolamine are largely formed in the system from *N*-acylphosphatidylethanolamine, the total phosphorus present in these compounds was compared with that in lysophosphatidylethanolamine and the glycerylphosphorylethanolamine and other water-soluble compounds which can be rapidly formed from it. Table 1 shows that for the first 30 min of incubation there was very close parity between the radioactivity present in each of these fractions. After this time the radioactivity in the lysophosphatidyl-

Table 1. *Distribution of radioactivity after the incubation of washed stationary-phase Butyrivibrio cells with [^{32}P]phosphatidylethanolamine*

Time (min)	Percentage of radioactivity added to incubation medium which is recovered in	
	Lysophosphatidylethanolamine + water-soluble products	<i>N</i> -Acyl-phospholipids
0.5	1.3	1.3
1	2.0	1.9
2	2.2	2.2
5	5.5	6.4
10	6.6	6.8
20	14.7	16.7
30	17.3	17.4
60	23.2	16.2
120	28.9	18.5

ethanolamine plus water-soluble phosphorus became greater than that in the *N*-acylphospholipids; this is presumably because whole bacterial cells can slowly convert *N*-(acyl)glycerylphosphorylethanolamine into lysophosphatidylethanolamine.

Discussion

Although *N*-acylphosphatidylethanolamine has previously been recognized as a constituent of seed oils (Bomstein, 1965; Dawson *et al.*, 1969) no information has been available about the mechanism of its biosynthesis. The present results indicate that cultures of a *Butyrivibrio* sp. (probably *B. fibrisolvens*) can bring about an intermolecular transacylation between two molecules of phosphatidylethanolamine producing a molecule each of *N*-acylphosphatidylethanolamine and lysophosphatidylethanolamine. This is demonstrated by the identification of these phospholipids as the main initial reaction products, the conversion of label in the *O*-acyl group of phosphatidylethanolamine into the *N*-acyl group of *N*-acylphosphatidylethanolamine, and the correct stoichiometry in the appearance of the products. The reaction is analogous to the intermolecular transacylation between two molecules of lysophosphatidylcholine producing phosphatidylcholine and glycerylphosphorylcholine (Erbland & Marinetti, 1965; van Bosch *et al.*, 1965) except that here an hydroxy rather than an amino group acts as the acyl acceptor. It has been shown that *N*-acylphosphatidylethanolamine is a component of the membrane phospholipids of this bacterium (G. P. Hazlewood & R. M. C. Dawson, unpublished work).

Since it has been shown that the organism contains an active deacylating phospholipase (Hazlewood & Dawson, 1975) there must be a strong possibility that this enzyme catalyses the reaction by acting as a transferase. This would be analogous to phosphatidyl transfer brought about by phospholipase D

Scheme 1. Main pathways of metabolism of phosphatidylethanolamine by bacterial culture

(Dawson, 1967) or acyl transfer from diacylglycerol, phosphatidylethanolamine and lysophosphatidylethanolamine to a monoacylglycerol acceptor catalysed by a phospholipase A of the plasma membrane of rat hepatocytes (Waite & Sisson, 1973). However, this latter phospholipase did not bring about the formation of *N*-acylphosphatidylethanolamine from phosphatidylethanolamine, and in addition we have tested the phospholipase A of snake venom (*Naja naja*) and of sheep pancreatic secretion and found these to be ineffective.

It seems certain that both the cells and the culture supernatant fraction can bring about the *O*-deacylation of *N*-acylphosphatidylethanolamine and *N*-acylphosphatidylethanolamine producing *N*-(acyl)-glycerylphosphorylethanolamine. Since the subsequent *N*-deacylation of this is almost exclusively carried out by the cells with minimal action by the supernatant this would indicate that a different enzyme or co-factors are involved. The mechanism of this *N*-deacylation seems to be through a conversion of the *N*-(acyl)glycerylphosphorylethanolamine to lysophosphatidylethanolamine which is subsequently *O*-deacylated. Since *N*-acyl-lysophosphatidylethanolamine can be converted into *N*-acylphosphatidylethanolamine it is clear many types of acyl transfers with an hydroxyl group acting as the acceptor are catalysed by the bacterial cells. The evidence presented suggests that the main pathways of metabolism of phosphatidylethanolamine by the bacterial culture are as shown in Scheme 1. As to whether one or both of the *O*-acyl groups of the phosphatidylethanolamine substrate act as donors to form the *N*-acyl

group cannot be decided from the present results. Although more labelled *N*-acyl phospholipids seemed to be formed from the 1-[³H]acylphosphatidylethanolamine it is possible that the transfer reaction could show specificity for the type of fatty acyl group available. Moreover, the unpurified system is obviously so adept at deacylation and inter- and intra-molecular fatty acyl transfer of phospholipids that the possibilities of labelled acyl group recycling are high.

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