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The Catabolism of Phosphatidylethanolamine by the Rumen Protozoon *Entodinium caudatum* and its Conversion into the *N*-(1-Carboxyethyl) Derivative

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1. The *N*-(2-hydroxyethyl)alanine esterified to phosphatidic acid in anaerobic ciliate rumen protozoa has the L configuration. 2. Labelling experiments with *Entodinium caudatum* cultures using [^{32}P]P_i [2-^{14}C]ethanolamine and ^{32}P- and ^{14}C-labelled phosphatidylethanolamine show that phosphatidylethanolamine is the direct lipid precursor of the *N*-(2-hydroxyethyl)alanine-containing phospholipid. 3. Labelling experiments with [^{14}C]starch, [^{14}C]lactate and [^{14}C]pyruvate with *E. caudatum* cultures indicate that a three-carbon glycolytic intermediate is probably the precursor of the *N*-(1-carboxyethyl) grouping which substitutes on the amino group of phosphatidylethanolamine. 4. [^{32}P]phosphatidylethanolamine is catabolized by *E. caudatum* forming initially glycerylphosphorylethanolamine and subsequently glycerophosphate and P_i. A little phosphorylethanolamine formed may possibly arise from bacterial enzymes ingested by the protozoa.

In previous work, a new phospholipid was isolated from a mixed sample of rumen protozoa and cultures of *Entodinium caudatum*. It was shown to contain *N*-(2-hydroxyethyl)alanine esterified to phosphatidic acid (Kemp & Dawson, 1969*a*,*b*). This phosphoglyceride has only been detected in anaerobic protozoa where it seems to replace phosphatidylserine in the membranous structure of the animal.

Two possible mechanisms could be involved in its biosynthesis. The first would be a direct utilization of *N*-(2-hydroxyethyl)alanine through a cytidine-nucleotide mediated pathway equivalent to that observed for the biosynthesis of phosphoglycerides in higher animals. The second would involve the substitution of a (1-carboxyethyl) group on the free amino group of a preformed phosphatidylethanolamine molecule. From the evidence obtained in the present investigation with cultures of *E. caudatum* the second alternative seems to be the most likely route of biosynthesis. The use of labelled phosphatidylethanolamine as a precursor of the new phospholipid has demonstrated that these animals can rapidly ingest phospholipid particles breaking down phosphatidylethanolamine into a variety of metabolic products.

EXPERIMENTAL

Culture of organism. Stock cultures of *E. caudatum* were maintained as described by Coleman (1962). 'Inoculum' cultures (100ml) were taken from these and were fed each day with 1.0ml of 1.5% rice-starch suspension and 10mg of ground dried grass. The protozoa were prepared for incubation by the procedure of Coleman (1969). They were used at a final concentration of $1–5 \times 10^6$ protozoa/ml. The incubation with radioactive substrates was carried out at 37°C under $N_2 + CO_2$ (19:1, v/v) in Coleman's (1960) saline B except for experiments with [^{32}P]P_i when a saline solution containing 0.5% NaCl and 0.05% $NaHCO_3$ was used. After incubation, the protozoa were sedimented by centrifugation and washed twice in 10ml of saline B on a bucket-head centrifuge for 20s from starting, the maximum speed was equivalent to 200*g*. The protozoa were then suspended in 2.0ml of saline B and were sometimes broken by immersion of the tube to the depth of the liquid in the tube in a 80kHz 40W ultrasonic cleaning bath (KG 80/1, manufactured by Kerrys, Basildon, Essex, U.K.) for 15s. The whole homogenate was then centrifuged at 7000*g* for 20min to yield a supernatant fluid fraction (membranes) and, after washing once, a pellet fraction that contained cell walls, bacteria, starch grains etc.

Examination of phospholipids. The washed protozoal pellet suspended in 2ml of saline B was treated for 1h or longer with 40ml of chloroform–methanol (2:1, v/v). After centrifuging, the residue was re-extracted with 10ml of the same solvent. The combined extracts were shaken with 0.2vol. of 0.9% NaCl and the separated lower phases washed twice with an equal volume of a theoretical upper phase (chloroform–methanol–water; 1:15:16, by vol.) containing 0.1% of the inactive 'carrier' depending on the radioactive substrate used. The phospholipids were deacylated with alkali and the P-containing products separated on paper by chromatography and ionophoresis (Dawson & Kemp, 1967). The spots were located either

by spraying with ninhydrin or reagents to detect phosphorus or, where further hydrolysis was to be carried out, by radioautography. In the latter experiments the radioactive spot was cut out and eluted with 1M-HCl. The acid extract was heated at 100°C for 20min and after the hydrolysis products were taken to dryness they were separated by ionophoresis on paper by using a buffer of pyridine–acetic acid–water (1:10:89, by vol., pH3.6). The glycerylphosphoryl ester of *N*-(2-hydroxyethyl)-alanine was split by the acid hydrolysis into glycerophosphoric acid which migrated towards the anode and *N*-(2-hydroxyethyl)alanine which was zwitterionic and remained at the origin. On hydrolysis of the combined glycerylphosphorylethanolamine and glycerylaminoethylphosphonate spot, the former gave glycerophosphoric acid (anodic mobility) and ethanolamine (cathodic mobility) whereas the latter was unchanged (zwitterionic).

Radioactivity counting. The radioactivities of some chromatographic spots were counted after spraying by a multiple-flow counter chromatogram scanner (Baird and Tatlock Ltd., London E.C.1, U.K.). Other spots were assayed for ^{14}C radioactivity by immersing in a scintillation fluid and counting in a liquid-scintillation counter (Nuclear–Chicago) (Jungalwala, Freinkel & Dawson, 1971). ^{32}P-labelled spots were digested in $HClO_4$ and the digests diluted with water and assayed directly in a liquid-scintillation counter by Čerenkov counting without the use of a wavelength shifter (Läuchli, 1969; Jungalwala & Dawson, 1970). Phosphorus determinations for calculating specific radioactivities were by the method of Bartlett (1959).

Radioactive substrates. Primary radioactive substrates such as ^{14}C-labelled amino acids, [^{14}C]starch, and ^{14}C-labelled algal protein were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. ^{14}C-labelled L-*N*-(2-hydroxyethyl)alanine was prepared from L-[U-^{14}C]-alanine in a manner similar to that described below for D-*N*-(2-hydroxyethyl)alanine. Phosphatidylethanolamine doubly labelled with ^{32}P and ^{14}C was prepared by incubating rat liver slices (500mg) with 1.3mCi of [^{32}P]P_i and 20μCi of [2-^{14}C]ethanolamine for 3h in a saline medium as described by Jungalwala *et al.* (1971). After extraction of the lipids and washing to remove water-soluble radioactive impurities as described, the phosphatidylethanolamine was separated by two t.l.c. runs with acid and alkaline solvents (silica gel F254 plates; E. Merck A.-G.); chloroform–methanol–acetic acid–water (65:50:1:4, by vol.) chloroform–methanol–aq. NH_3 (sp. gr. 0.88)–water (110:50:3:8, by vol.). After each run, the phosphatidylethanolamine strips located by radioautography and the use of markers were eluted from the chromatogram (Scott & Dawson, 1968).

Examination of water-soluble breakdown products of [^{32}P]*phosphatidylethanolamine.* In these experiments the protozoal incubate was centrifuged and both the pellet (resuspended in 2ml of saline B) and the supernatant were extracted with chloroform–methanol as described above. This solvent appeared to solubilize the water-soluble phosphate esters produced since a quantitative recovery of the added radioactivity was obtained. To the extract was added 0.2vol. of water and after shaking, the lower phase was separated and was used to examine the phospholipids after further washing as described. A portion (3ml) of the upper phase was spotted on to paper and the phosphate esters were separated in two dimensions by chromatography and ionophoresis as described by Dawson, Hemington & Davenport (1962). Glycerylphosphorylethanolamine, phosphorylethanolamine, glycerophosphoric acid and P_i were localized by the use of the appropriate markers and by spraying with ninhydrin and reagents to detect phosphorus.

Synthesis of D-N-(2-*hydroxyethyl*)*alanine.* D-Alanine (0.89g) and $NaHCO_3$ (1.619g) were dissolved in water and after the addition of ethylene chlorhydrin (0.67ml) the mixture was heated at 90°C for 18h. The faintly yellow reaction mixture was diluted to 200ml and passed down a column (25cm×1.5cm) of Dowex 1 (OH^- form). After eluting with water to neutrality the unchanged amino acid and *N*-(2-hydroxyethyl)alanine were removed with 0.5M-HCl. The acid was largely removed by taking the solution to dryness. The residue was dissolved in 100ml of water and adjusted to pH2 and the solution passed through a column (25cm×1.5cm) of Dowex 50 (H^+ form). The column was washed to neutrality with water, and the adsorbed alanine and *N*-(2-hydroxyethyl)-alanine eluted with 5M-NH_3. The eluate was taken to dryness and the residue extracted with boiling ethanol. The material (250mg) obtained on cooling the extract for 1h was subjected to preparative paper chromatography (Whatman no. 1) in a water-saturated phenol–acetic acid–ethanol solvent (50:5:6, by vol.), using a loading of 0.8mg/cm. The *N*-(2-hydroxyethyl)alanine (R_F 0.87) was well separated from the alanine (R_F 0.65) and after removal of the solvent and location by spraying marker lanes either side with ninhydrin, the strips were eluted chromatographically with water. The combined eluates were taken to dryness and the D-*N*-(2-hydroxyethyl)-alanine was recrystallized three times from methanol (yield 25mg). The product had i.r. and n.m.r. spectra which were identical with those obtained for the natural product and to those of the DL-*N*-(2-hydroxyethyl)alanine previously synthesized from chloropropionic acid and ethanolamine (Kemp & Dawson, 1969*a*).

Optical rotations. These were measured in a Bendix Automatic polarimeter (by courtesy of the Department of Organic Chemistry, University of Cambridge, Cambridge, U.K.) with L-alanine as a standard, assuming this to have $[\alpha]_D^{20}$ + 14.6° in 5M-HCl (2mg/ml).

RESULTS

Configuration of N-(2-*hydroxyethyl*)*alanine isolated from naturally occurring phospholipid.* A sample of *N*-(2-hydroxyethyl)alanine isolated from the new protozoal phospholipid (Kemp & Dawson, 1969*a*) has a measured $[\alpha]_D^{20}$ of $+31\pm2.5°$ (1mg/ml in 5M-HCl). The synthesized D-*N*-(2-hydroxyethyl)-alanine gave an $[\alpha]_D^{20}$ of $-28\pm2.5°$ (2mg/ml in 5M-HCl). It is concluded therefore that the naturally occurring form is the enantiomorph of the synthetic compound and therefore has the L configuration.

Formation of N-(2-*hydroxyethyl*)*alanine-containing phospholipid from various precursors.* When protozoa were incubated for 3.5h with 1μCi of ^{14}C-labelled L-*N*-(2-hydroxyethyl)alanine they con-

tained after washing with saline B, only 1% of the radioactivity, and of this uptake, 95% was soluble in cold 5% trichloroacetic-acid solution. It was not possible to detect any significant incorporation of radioactivity into the *N*-(2-hydroxyethyl)alanine-containing phospholipid. Nor could any synthesis of the phospholipid from 10 μCi each of L-[U-^{14}C]-proline, DL-hydroxy[2-^{14}C]proline or L-[U-^{14}C]-serine be detected, and L-[U-^{14}C]alanine (6.5 μCi) only donated a trace of radioactivity. On incubating ^{14}C-labelled crude algal protein (50 μCi) with protozoa, some incorporation of ^{14}C into all the phospholipids was observed and the deacylation products of phosphatidylglycerol and the *N*-(2-hydroxyethyl)alanine-containing phospholipid had the maximum specific radioactivity. With the latter substance, all the radioactivity was in the *N*-(2-hydroxyethyl)alanine part of the molecule and none was found in the glycerophosphoric acid liberated by acid hydrolysis. When strains of *Escherichia coli* labelled with [U-^{14}C]glucose or L-[U-^{14}C]serine were fed to the protozoa there was also a limited incorporation of radioactivity into the *N*-(2-hydroxyethyl)alanine-containing phospholipid: no labelled phospholipid of this nature being in the original bacteria. These preliminary observations showed that cultures of *E. caudatum* could synthesize the new phospholipid and further, that they could form the *N*-(2-hydroxyethyl)alanine part of the molecule rather than 'scavenge' for this in the rumen liquor added as part of the nutrient medium.

Synthesis of phospholipids from inorganic [^{32}P]*phosphate.* As particulate matter is taken up into *E. caudatum* by the formation of membrane-lined vesicles (Coleman & Hall, 1969) it is possible that the relative rates of incorporation of ^{32}P into individual phosphoglycerides may be altered by the simultaneous uptake of polystyrene beads (diameter 1.3 μm). Although it has been shown (Coleman, 1969) that the uptake of substances that are not incorporated directly into phospholipids such as glucose and glutamic acid was not greatly stimulated by the presence of these beads, the uptake of ^{32}P was increased 3.5 times under these conditions. Table 1 shows incorporation into the individual phosphoglycerides. Phosphatidic acid, an intermediary in the synthesis of all phosphoglycerides in animal tissue, was labelled most rapidly. On the other hand, the incorporation of ^{32}P into the *N*-(2-hydroxyethyl)alanine-containing phospholipid was very limited, suggesting either a slow rate of synthesis from an immediate water-soluble P-containing precursor or alternatively that it was formed from another phospholipid (e.g. phosphatidylethanolamine) that itself needed to be labelled before incorporation of the isotope could occur.

Synthesis of phospholipids from [2-^{14}C]*ethanolamine.* Because of the possibility that the *N*-(2-hydroxyethyl)alanine containing phospholipid could be formed from phosphatidylethanolamine, the

Table 1. *Incorporation of* ^{32}P *into the phosphoglycerides of* E. caudatum

A portion (4 ml) of washed *E. caudatum* suspension (2.7×10^6 cells/ml) was incubated for 1 h with 1 mCi of [^{32}P]P_i in a medium containing 0.5% NaCl + 0.05% $NaHCO_3$. The gas phase was $N_2 + CO_2$ (19:1) at 39°C. Polystyrene latex beads suspension (1.3 μm) (Dow, Corning Ltd.), 0.006 ml, was added to one incubation.

Phosphoglyceride	Sp. radioactivity (c.p.m./μg of P)	
	No beads added	+ beads
Phosphatidic acid	2160	3550
Phosphatidylinositol	332	910
Phosphatidylcholine	245	675
Phosphatidylglycerol	360	502
Phosphatidylethanolamine + diglyceride aminoethylphosphonate	143	294
N-(2-Hydroxyethyl)alanine-phosphoglyceride	3	9

Table 2. *Incorporation of radioactivity from* [2-^{14}C]*ethanolamine into the phospholipids of* E. caudatum

A portion (6 ml) of protozoal suspension (3×10^6 cells/ml) was incubated for 80 min at 39°C with 10 μCi of [2-^{14}C]ethanolamine. The protozoa after washing were sonicated and centrifuged to produce a pellet and a supernatant fraction.

	Phosphorus (μg)		Sp. radioactivity (c.p.m./μg of P)	
	Pellet	Supernatant	Pellet	Supernatant
Phosphatidylethanolamine	9.9	15.6	13200	10900
Ethanolamine plasmalogen	7.3	5.0	2900	3260
Ceramide phosphorylethanolamine	11.9	14.5	880	920
Diglyceride aminoethylphosphonate	16.9	17.1	v. low	v. low
N-(2-Hydroxyethyl)alanine phosphoglyceride	3.9	5.2	430	270

incorporation of label from [2-^{14}C]ethanolamine into the phospholipids was measured. The protozoa were sonicated and centrifuged to separate them into a pellet and supernatant fraction before examination of the phospholipids. With the exception of ethanolamine plasmalogen the supernatant fraction contained more of each phospholipid (Table 2). It is of note that the pellet containing the bulk of the protozoal cell walls showed no enrichment of the diglyceride aminoethylphosphonate in view of findings that the surface membrane sheath of the cilia of *Tetrahymena* is enriched with this phospholipid (Kennedy & Thompson, 1970; Smith, Snyder & Law, 1970). The radioactivity from the [^{14}C]-ethanolamine had been incorporated in significant amounts only into the known ethanolamine-containing phospholipids, i.e. phosphatidylethanolamine, ethanolamine plasmalogen and ceramide phosphorylethanolamine, and to a lesser extent into the *N*-(2-hydroxyethyl)alanine-containing phospholipid. The incorporation into diglyceride aminoethylphosphonate was scarcely detectable. Liang & Rosenberg (1968) likewise found that ethanolamine was not a precursor of this phospholipid in the protozoon *T. pyriformis*.

In two experiments [^{14}C]ethanolamine was incubated with a protozoal preparation which was then flooded with unlabelled ethanolamine to prevent the further incorporation of radioactivity by greatly decreasing the specific radioactivity of the precursor. The results of both experiments were essentially similar. The addition of unlabelled ethanolamine prevented the further incorporation of radioactivity into phosphatidylethanolamine, whereas that into the *N*-(2-hydroxyethyl)alanine-containing phospholipid was not noticeably affected (Fig. 1). This suggested that the slow synthesis of the latter lipid proceeds via a labelled pool (possibly phosphatidylethanolamine) that enables incorporation to continue even after the addition of unlabelled ethanolamine.

Formation of N-(2-*hydroxyethyl*)*alanine-containing phospholipid from labelled phosphatidylethanolamine.* To obtain adequate dispersion of the labelled phosphatidylethanolamine at the pH of the incubation mixture, it was mixed with an excess (4.5M) of unlabelled phosphatidylcholine (egg). The mixture readily dispersed on shaking with water and on centrifugation to recover the protozoa it remained in the supernatant unless it had been ingested (Table 3). About two-thirds of the [^{32}P]-phosphatidylethanolamine was removed from the supernatant by the protozoa in 2h; most of the radioactivity appearing in the protozoal lipid fraction. There had clearly also been a catabolism to water-soluble ^{32}P-labelled components (Table 3). On examination of the phospholipid fraction in the protozoa, the labelling occurred primarily in the

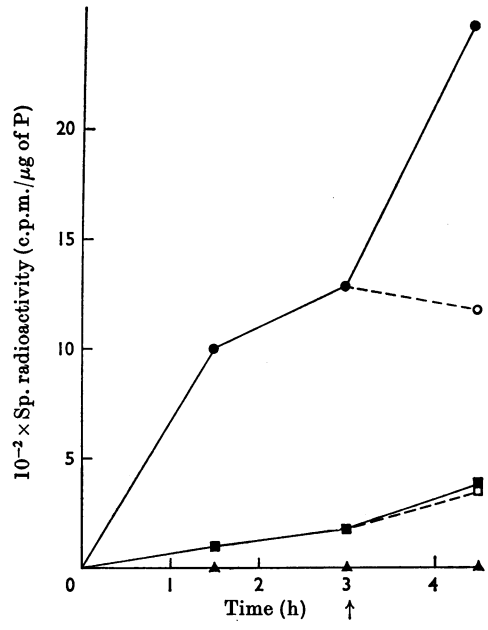

Fig. 1. Incorporation of radioactivity from [2-^{14}C]-ethanolamine into phosphoglycerides of *E. caudatum*. Samples (5ml) of protozoal suspension (5×10^6 cells/ml) were incubated with 10μCi of [2-^{14}C]ethanolamine (22.3μg) for various times. To one incubation (○, □) unlabelled ethanolamine hydrochloride (3.65mg) was added after 3h (at arrow). ●, ○, Phosphatidylethanolamine; ■, □, *N*-(2-hydroxyethyl)alanine-containing phospholipid; ▲, diglyceride aminoethylphosphonate.

Table 3. *Ingestion and catabolism of* [^{32}P]*phosphatidylethanolamine by* E. caudatum

[^{32}P]Phosphatidylethanolamine (0.35μmol) was mixed with 1.6μmol of ovolecithin in chloroform, the solvent was evaporated and the lipid dispersed in 1.5ml of saline B. These mixtures were incubated for various time-periods with 0.5ml of protozoal suspension containing 2.0×10^6 organisms. After centrifugation, the protozoa and supernatants were separated into lipid and water-soluble fractions (see the Experimental section).

Incubation time (min)	10^{-3} × Radioactivity (c.p.m.) Supernatant Lipid	Supernatant Water-soluble	Protozoa Lipid	Protozoa Water-soluble
0	222.2	1.0	0.5	0
60	108.4	6.2	90.3	5.8
120	73.1	17.4	104.9	9.6
120*	204.2	1.1	0.3†	0†

* No protozoa.

† Mixture submitted to the same centrifugation procedure as the protozoa.

Table 4. *Labelling of phosphoglycerides after ingestion of* [^{32}P]*phosphatidylethanolamine by* E. caudatum

Experimental conditions as in Table 3.

Incubation time (min)	Sp. radioactivity (c.p.m./μg of P)	
	Phosphatidylethanolamine	*N*-(2-hydroxyethyl)alanine phospholipid
0	17690*	—
60	3878	299
120	4080	502

* Phospholipid in original dispersion before protozoal ingestion.

phosphatidylethanolamine component; however, there was a significant incorporation of radioactivity into the *N*-2(hydroxyethyl)alanine-containing phospholipid which increased between the 60 min and 120 min incubation time-periods (Table 4). No labelling of other phospholipids, including the diglyceride aminoethylphosphonate was detected.

In a further experiment, doubly labelled phosphatidylethanolamine (^{32}P and ^{14}C in the ethanolamine moiety) was incubated for 190 min with *E. caudatum* under conditions similar to those given in Table 3 except that the saline contained 5 mM-ethanolamine to minimize any secondary labelling after breakdown to [^{14}C]ethanolamine. The protozoal phospholipids were degraded with alkali and after paper chromatography and ionophoresis the glycerylphosphoryl ester of *N*-(2-hydroxyethyl)-alanine spot and the combined glycerylphosphoryl-ethanolamine plus glycerylaminoethylphosphonate spot were located by radioautography. After elution, the two spots were hydrolysed with acid (1 M-HCl, 20 min, 100°C) and the glycerophosphate spots and the respective *N*-(2-hydroxyethyl)-alanine and ethanolamine spots were located and their radioactivities counted. The ^{32}P/^{14}C counting ratio was 1.9 in the protozoal phosphatidylethanolamine and 1.8 in the *N*-(2-hydroxyethyl(alanine-containing phospholipid.

Labelling of phosphoglycerides on incubation of E. caudatum *with* [^{14}C]*starch*, [^{14}C]*pyruvate and* [^{14}C]*lactate.* When *E. caudatum* was incubated with [U-^{14}C]starch there was only a small incorporation into the phosphoglycerides, but in contrast with the experiments with [^{32}P]phosphate and [^{14}C]-ethanolamine the *N*-(2-hydroxyethyl)alanine-containing phospholipid now had the highest specific radioactivity. Degradation of the phosphoglyceride showed that the activity was localized entirely in the *N*-(2-hydroxyethyl)alanine portion of the

Table 5. *Incorporation of radioactivity into phosphoglycerides of* E. caudatum *on incubation with* [^{14}C]*starch*, [^{14}C]*pyruvate and* [^{14}C]*lactate*

Radioactive substrate and addition	Total no. of protozoa	Incubation time (h)	Sp. radioactivity of phosphoglycerides (c.p.m./μg of P)					
			Phosphatidylcholine	Phosphatidylethanolamine+diglyceride aminoethylphosphonate	Phosphatidic acid	*N*-(2-hydroxyethyl)-alanine phospholipid	Phosphatidylinositol
2.8 mg of [U-^{14}C]starch (100 μCi) + 0.08 M-ethanolamine	6.8×10^6	4.5	1.8	2.0	4.5	4.6	4.2
0.26 mM-[2-^{14}C]pyruvate (15 μCi) + 13 mM-Li_2SO_4	2.0×10^6	3	14.6	46.7		80.2	
+ 13 mM-Lithium L-lactate	2.0×10^6	3	16.6	48.3		102.0	
+ 13 mM-Lithium D-lactate	2.0×10^6	3	19.1	46.0		98.5	
0.17 mM-D-[U-^{14}C]lactate (5 μCi)	1.5×10^6	2.5	8.8	13.0		50.2	
+ 17 mM-Sodium pyruvate	1.5×10^6	2.5	6.1	15.6		47.2	
0.069 mM-L-[U-^{14}C]lactate (10 μCi)	1.5×10^6	2.5	15.2	29.9		111.8	
+ 6.9 mM-Sodium pyruvate	1.5×10^6	2.5	13.1	26.3		99.8	

Table 6. *Distribution of* ^{14}C*-labelled phosphoglycerides produced on incubating* E. caudatum *with* DL-[2-^{14}C]*-lactate*

DL-[2-^{14}C]Lactate (0.46 mM, 100 μCi) was incubated with protozoa (6.8×10^6) for 5 h in saline B containing 67 mM-ethanolamine. Specific radioactivities of glycerol, inositol and the aminoethyl group are based on direct P assays on the glycerophosphate, inositol monophosphate and aminoethylphosphonate liberated on acid hydrolysis of the alkaline deacylation products obtained from each phosphoglyceride (see the Experimental section). Those of choline, ethanolamine and *N*-(2-hydroxyethyl)alanine are based on the equivalent release of glycerophosphate-P during acid hydrolysis.

Phosphoglyceride	Component	Sp. radioactivity (c.p.m./μg of P)
Phosphatidylcholine	Glycerol	3.7
	Choline	0
Phosphatidylethanolamine	Glycerol	2.4
	Ethanolamine	0.5
Diglyceride aminoethylphosphonate	Aminoethyl group	6.9
Phosphatidylinositol	Glycerol	15.4
	Inositol	14.8
N-(2-Hydroxyethyl)alanine phosphoglyceride	Glycerol	0
	N-(2-Hydroxyethyl)alanine	39.5

Table 7. *Water-soluble phosphate esters formed after the ingestion of* [^{32}P]*phosphatidylethanolamine by* E. caudatum

Conditions of incubation as Table 3. The protozoal concentration was 0.95×10^6 cells/ml. [^{32}P]phosphatidylethanolamine (0.32 μmol; 10^5 c.p.m. per incubation flask).

Fraction	Incubation time (min)	$10^{-3} \times$ Total water-soluble radioactivity (c.p.m.)	Distribution of radioactivity (% of recovered counts)			
			P_i	Glycero-phosphate	Phosphoryl-ethanolamine	Glycerylphos-phorylethanol-amine
Supernatant	90	153.9	23.0	35.6	10.6	18.8
	190	252.1	39.2	22.9	14.7	12.7
Protozoa	90	38.2	3.7	4.0	3.8	72.6
	190	58.4	7.7	4.0	4.3	76.4

molecule. This suggested that the *N*-(2-hydroxyethyl)alanine-containing phospholipid might be formed by the reaction of phosphatidylethanolamine with a glycolytic intermediate. On incubation of *E. caudatum* with labelled pyruvate and lactate, the specific radioactivity of the *N*-(2-hydroxyethyl)-alanine phosphoglyceride became much higher than those of the other phospholipids (Tables 5 and 6). L-Lactate appeared to label all the phospholipids better than D-lactate but rather surprisingly neither the introduction of a mass of unlabelled lactate when [^{14}C]pyruvate was the substrate or unlabelled pyruvate when [^{14}C]lactate was the substrate appeared to have any inhibitory effect. The labelling was entirely in the *N*-(2-hydroxyethyl)alanine portion of the new phosphoglyceride (Table 6) and since unlabelled ethanolamine had been included in the incubation medium to limit secondary labelling through any formation of [^{14}C]ethanolamine it seems reasonable to assume that the radioactivity was in the *N*-(1-carboxyethyl) group. The lack of labelling of the glycerol moiety is in contrast with the labelling of this component in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (Table 6). Unexpectedly there appeared to be a significant amount of label incorporated into the inositol moiety of phosphatidylinositol. On the other hand, the incorporation of radioactivity into the aminoethyl group of diglyceride aminoethylphosphonate was not unexpected because of the suggested biosynthesis of this phosphoglyceride from the glycolytic intermediate, phosphoenolpyruvate (Trebst & Geike, 1967; Warren, 1968; Liang & Rosenberg, 1968).

Water-soluble products formed from [^{32}P]*phosphatidylethanolamine by* E. caudatum. Since the experiments with [^{32}P]phosphatidylethanolamine had clearly indicated that part of the ingested

phospholipid was broken down into water-soluble substances, further experiments were performed to ascertain the nature of these products. Paper chromatography and the use of marker substances established that the four main P-containing substances present in the aqueous phase prepared from chloroform–methanol–water extracts of the protozoa and supernatant were mainly P_i, glycerophosphoric acid, phosphorylethanolamine and glycerylphosphorylethanolamine. The latter phosphate ester seems to be the primary product within the protozoa but clearly before this is released from the animal it is broken down into glycerophosphate and eventually P_i (Table 7). However, substantial amounts of phosphorylethanolamine are also slowly released.

DISCUSSION

The results presented indicate that the new phospholipid, containing *N*-(2-hydroxyethyl)alanine, discovered in anaerobic ciliate protozoa is not likely to be formed by a phosphorylated derivative of *N*-(2-hydroxyethyl)alanine forming the phosphoglyceride through a cytidine-nucleotide-mediated pathway. Rather it suggests that phosphatidylethanolamine is the direct lipid precursor of the molecule. The latter conclusion would fit in with the very slow incorporation of ^{32}P into the phosphoglyceride, the negligible incorporation of *N*-(2-hydroxyethyl)[^{14}C]alanine and of [^{14}C]lactate into the glycerol portion of the molecule. The exclusive labelling of the ethanolamine-containing phospholipids and the new phospholipid from [^{14}C]ethanolamine and the continuance of the incorporation into the latter phospholipid on flooding the system with unlabelled ethanolamine are again indicative of phosphatidylethanolamine acting as precursor. The feeding of doubly labelled phosphatidylethanolamine to protozoa and its conversion into the *N*-(2-hydroxyethyl)alanine phosphoglyceride without a change, outside the limits of experimental error, in the ratio between the two isotopes gives good evidence that the whole phosphatidylethanolamine molecule is used as a unit in the synthesis. Either the synthesis occurs in the food vesicle formed after the engulfing of the phosphatidylethanolamine particle by the protozoa or more likely the ingested substrate phospholipid becomes incorporated as a complete molecular unit into the membranes of the animal and is then used as a precursor. This could occur by an incorporation into the lipoprotein walls of the vesicle followed by a distribution by exchange diffusion through the animal cell (Dawson, 1966).

The rapid labelling of the *N*-(2-hydroxyethyl)-alanine part of the phosphoglyceride from starch, lactate and pyruvate compared with the labelling of other phospholipids suggests that the *N*-(1-carboxyethyl) group added on to the phosphatidylethanolamine precursor is probably a three-carbon intermediary in the glycolytic pathway. The lack of any effect on addition of unlabelled lactate and unlabelled pyruvate to whole protozoa incorporating ^{14}C into phospholipids from [^{14}C]pyruvate and [^{14}C]lactate respectively allowed no positive conclusions to be reached about the relative roles of these glycolytic intermediaries in the substitution of the amino group. This result could be caused by the uptake of lactate and pyruvate into the organism being passive rather than active so that the metabolizing systems are never saturated with substrate. This would mean that the rate-limiting step for the incorporation would be the entry of isotope into the animal cell. A satisfactory answer to the question of the direct precursor of the *N*-(1-carboxyethyl) group is likely to require the attainment of a functional cell-free system *in vitro* for synthesis which is devoid of the permeability barriers appertaining in the whole cell system *in vivo*. Preliminary experiments to achieve this end have not yet been successful. Since the *N*-(2-hydroxyethyl)alanine isolated from the phospholipid has the L configuration, this must mean that either the donor for the *N*-(1-carboxyethyl) group also has the L structure which is retained or alternatively the addition itself is stereospecific.

As well as incorporating phosphatidylethanolamine as a whole unit into its membranes, it is apparent that some catabolism of this phospholipid occurs. This could take place either in the internal economy of the cell but perhaps more likely in the food vesicle formed on ingestion of the phospholipid particle. The main catabolic process appears to be a complete deacylation of the phosphoglyceride (phospholipase A+B) with a further degradation of the glycerylphosphorylethanolamine formed by the successive action of a phosphodiesterase and phosphomonoesterase so that glycerophosphate and P_i are released into the external medium. The origin of the phosphorylethanolamine identified is uncertain since the specific phosphodiesterase which attacks glycerylphosphorylethanolamine seems to operate exclusively between the phosphate and ethanolamine moieties (Dawson, 1956). Phospholipase C which would directly liberate phosphorylethanolamine from the phosphatidylethanolamine substrate appears to be largely confined to a limited number of bacterial species (Kushner & Feldman, 1958; Takahashi & Schmid, 1968). Since the protozoal cultures contain a mixed culture of bacteria as a food source it is possible that the ingestion of these by the protozoa provides a source of enzyme for this type of degradation.

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