

Phospholipid Biosynthesis in the Anaerobic Protozoon *Entodinium caudatum*

By T. E. BROAD and R. M. C. DAWSON

Department of Biochemistry, Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

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1. The anaerobic rumen protozoon *Entodinium caudatum* was incubated either intact or with various radioactive precursors of phospholipids after ultrasonication. 2. Pulse-chase experiments showed a rapid turnover of phosphatidylinositol and much slower turnovers of phosphatidylethanolamine and phosphatidylcholine. 3. *E. caudatum* imbibed choline very rapidly; this was immediately and exclusively converted into phosphatidylcholine which was shown by radioautography after 10 min to be distributed throughout the cell membranes. 4. Phosphatidylcholine was synthesized through a phosphorylcholine-CDP-choline pathway, the methylation or base-exchange pathways not being present. 5. Under suitable conditions [$Me-^{14}C$]choline can be substantially (50–60%) converted into CDP-choline by sonicated *E. caudatum* and this provides an excellent method of preparing this biosynthetic intermediary. 6. [$2-^{14}C$]ethanolamine was taken up much less readily than choline. The former was incorporated into phosphatidylethanolamine by the CDP-ethanolamine pathway. 7. Doubly labelled [^{32}P]phosphatidyl[$2-^3H$]ethanolamine was converted into ceramide phosphorylethanolamine and *N*-(1-carboxyethyl)phosphatidylethanolamine, without change in the isotopic ratio. Ceramide phosphoryl [$2-^{14}C$]ethanolamine was converted into phosphatidylethanolamine. 8. Palmitic acid, oleic acid and linoleic acid were taken up by *E. caudatum* cells and incorporated into phospholipids. By contrast, although stearic acid was taken up it was hardly incorporated into phospholipids.

Fairly extensive studies have been made on the way membrane phospholipids are synthesized in aerobic protozoa and especially by *Tetrahymena pyriformis* (Lust & Daniel, 1966; Thompson, 1967; Smith & Law, 1970; Dennis & Kennedy, 1970; Chlapowski & Band, 1971). By contrast the metabolism of these substances in anaerobic protozoa has been almost completely neglected presumably because of the difficulty of maintaining them in culture. The successful culture of the rumen protozoal anaerobe *Entodinium caudatum* in this laboratory over many years (Coleman, 1962) gave us the opportunity to obtain information in this field. We were also encouraged to do this by the rather unusual lipid composition of this organism which was shown by previous studies. It contains a high proportion of phosphonolipids of various types, of ceramide phosphorylethanolamine and a unique lipid *N*-(1-carboxyethyl)phosphatidylethanolamine, which has so far only been reported to occur in anaerobic protozoa (Kemp & Dawson, 1969).

The present paper reports the avidity with which a number of phospholipid precursors are taken up by the intact protozoon and the rates and pathways by which certain phospholipids are synthesized. It is shown that the organism takes up choline extremely

rapidly. It then converts this quantitatively and exclusively by a cytidine-mediated pathway into membrane phosphatidylcholine, the labelling being distributed throughout the cell within minutes. However, in contrast with many aerobic protozoa there is no capacity to convert phosphatidylethanolamine into phosphatidylcholine by methylation.

Experimental

Growth and harvesting of E. caudatum

E. caudatum cultures were kindly supplied by Dr. G. S. Coleman and were maintained by a slight modification of his method (Coleman, 1960, 1971). In this the cultures were fed on 4% by volume of 1.5% (w/v) rice starch every 2 days and the cultures were divided twice a week. The protozoa were harvested from the medium and washed three times with protozoal saline (Coleman, 1969) to give a final light-green pellet. The yield of cells from 20 culture bottles each containing 100 ml of culture medium was 1×10^7 – 5×10^7 cells or approximately 0.1–0.2 g dry weight. The cells were used for experiments immediately after harvesting.

T. pyriformis (strain W)

This was obtained from the N.E.R.C. collection of algae and protozoa (Storeys Way, Cambridge, U.K.) and grown aseptically by the method of Munn (1970).

Incubation conditions

E. caudatum cells were ultrasonicated when appropriate by suspending them in protozoal saline at room temperature and immersing them in an ultrasonic water bath (Kerry's Ultrasonics Ltd., Basildon, Essex, U.K.) at 80kHz. After 90s virtually 100% disruption had occurred at a density of 10^7 cells/ml. Washed suspensions of intact *E. caudatum* cells in protozoal salt solution (Coleman, 1971) gassed with N_2+CO_2 (19:1) and containing cysteine and chloramphenicol were pipetted into stoppered tubes. These were gassed and stoppered and continually gassed when withdrawals or additions were made. The incubation was at 39°C with slow shaking under N_2+CO_2 (19:1). In experiments with ^{32}P , the phosphate buffer in the protozoal salt solution was replaced with 0.5% (w/v) $NaHCO_3+0.05\%$ (w/v) NaCl. In some experiments a suspension of polystyrene latex beads (diam. 1.3 μm ; Dow Chemical Co., Midland, Mich., U.S.A.) was included (2×10^{11} beads/ 10^7 cells). These were ingested by the protozoa and the formation of food vacuoles was stimulated. Other incubation conditions are given in the legends to the Figures and Tables.

Uptake of labelled phospholipid precursors by E. caudatum

After incubation, the protozoa were centrifuged at 1000g for 2min and then washed three times with 10ml of ice-cold protozoal salt solution. The cells were suspended in 0.5ml of distilled water and a portion was transferred to vials and the radioactivity determined by liquid-scintillation spectrometry.

Determination of incorporation of radioactivity into phospholipids

The washed cell suspension was mixed with 20 vol. of chloroform-methanol (2:1, v/v): the mixture was left for 2h at room temperature then heated for 15min at 55°C, centrifuged and the supernatant shaken with 0.2vol. of 0.9% NaCl. The separated lower phase was washed three times with theoretical upper phase (0.9% NaCl-methanol-chloroform, 16:15:1, by vol.) containing 0.1% non-radioactive carrier equivalent to the radioactive precursor added. Suitable portions were taken for liquid-scintillation spectrometry and for further analysis.

Incorporation of labelled precursors into intermediaries of phospholipid biosynthesis

The sonicated protozoal preparation after incubation with labelled precursor was mixed with an

equal volume of ice-cold 10% (w/v) trichloroacetic acid. After being left for 30min at 0°C the mixture was centrifuged (1000g_{av.}, 5min) and the pellet extracted with chloroform-methanol (2:1, v/v) to prepare the phospholipids for radioactivity determinations as above. Little hydrolysis of the phospholipids was apparent. The trichloroacetic acid extract was washed four times with an equal volume of diethyl ether to remove most of the acid and neutralized with NH_3 ; a suitable portion was then spotted on to paper for high-voltage ionophoresis. This was carried out at 55 V/cm at pH 3.6 in water-acetic acid-pyridine buffer (89:10:1, by vol.) for 5h under white spirit (high-flash-point petroleum fraction). Phosphorylated bases and CDP bases were located by radioautography and by the use of appropriate markers. Although both classes of intermediaries are virtually zwitterionic they were well separated after this long period of ionophoresis. The CDP bases showed a slight movement towards the anode relative to the phosphorylated bases. The free bases used as precursors completely ran off the paper. Confirmation of the identity of the products was provided by their elution from the paper followed by paper chromatography with markers in the following systems: ([Me- ^{14}C]choline labelling used ethanol-conc. aq. NH_3 -water (7:3:1, by vol.); [2- ^{14}C]ethanolamine labelling used phenol saturated with water-ethanol-acetic acid (75:9:7.5, by vol.).

Separation of phospholipids

The separation of radioactive lipids was performed by two-dimensional t.l.c. in the system described by Kemp *et al.* (1972). The lipids were located by radioautography and, when not required for radioactivity determinations, by spraying with 0.25% (w/v) ninhydrin in acetone to localize aminolipids and with Vaskovsky's & Kostetsky's (1968) reagent to detect phosphorus. For ^{14}C determination the spots were scraped off into scintillation vials and 10ml of Unisolve (Koch-Light, Colnbrook, Bucks., U.K.) was added. ^{32}P was determined by the use of Cerenkov radiation after total oxidation with $HClO_4$ (Jungalwala *et al.*, 1971); ^{31}P in the solution was determined by the method of Bartlett (1959).

In certain experiments the phospholipid components in the 'phosphatidylethanolamine' and ceramide phosphorylethanolamine spots were determined by elution of the radioactive spots and examination of these by the hydrolytic and ionophoretic procedures of Dawson & Kemp (1967) and Coleman *et al.* (1971).

Fatty acids and long-chain bases

Fatty acids were cleaved from glycerophospholipids by the Brockerhoff (1963) procedure and from sphingophospholipids by the method of Gaver & Sweeley (1965). In the latter instance, the chloroform-

soluble materials consisting of fatty acid methyl esters and long-chain bases were separated on a silicic acid column (1cm×5cm) of Silic A.R. CC4 (Mallinckrodt, St. Louis, Mo., U.S.A.). The fatty acid esters were eluted with 10ml of chloroform and the long-chain bases with 40ml of methanol. Fatty acids were examined by g.l.c. on a Pye series 104 chromatograph with radioscan attachment (James & Piper, 1961) and in some experiments by argentation t.l.c. and radioautography (Kemp & Dawson, 1968). In experiments where fatty acids were labelled after incubation with [*Me*-¹⁴C]methionine the retention time on columns of polyethyleneglycol adipate and E-30 (Pye Unicam, Cambridge, U.K.) of the radioactive C₁₉ cyclopropane fatty acid methyl ester was identical with that of an authentic C₁₉ cyclopropane fatty acid methyl ester prepared from oleic acid by the procedure of Christie & Holman (1966). The retention time of the radioactive C₁₇ cyclopropane fatty acid methyl ester was similar to that predicted theoretically by plotting log (retention time) against carbon number for saturated straight-chain and cyclopropane fatty acids.

Radioautography

Cells were fixed in 5% (w/v) glutaraldehyde for 1 day. They were then washed at least four times over the next 4 days in Ringer solution containing 1% (w/v) choline chloride and 1% (w/v) phosphorylcholine. The cells were post-fixed in OsO₄ (1%, w/v), rinsed in water, dehydrated in acetone and embedded in araldite. Sections (1 μm) were cut, coated with a film of carbon particles (3 μm) and then with a layer

of photographic emulsion; they were developed and fixed after 5 days' exposure. After staining with Fuchsin Red the sections were examined by light microscopy. Although approx. 50% of the radioactive phosphatidylcholine was lost during the dehydration and embedding procedures about 99% of the radioactivity remaining in the slice was present in the lipid fraction.

Preparation of [³²P]phosphatidyl[2-³H]ethanolamine

Doubly labelled phosphatidylethanolamine was prepared by growing *Saccharomyces cerevisiae* in 2 litres of culture medium in the presence of ³²P_i (10mCi) and [2-³H]ethanolamine (1mCi) by the method of Quarles & Dawson (1969). After removal of the phosphatidylcholine from the alumina column as stated, the phosphatidylethanolamine plus phosphatidylinositol was eluted with 350ml of chloroform-ethanol-water (2:5:2, by vol.). The phosphatidylethanolamine was purified by preparative t.l.c. (Kieselgel F254, Merck, Darmstadt, Germany) in chloroform-methanol-acetic acid-water (65:50:1:4, by vol.) and then, after localization by radioautography and elution, in a second run in chloroform-methanol-concn. NH₃ (70:20:2, by vol.).

Results

Phospholipid and fatty acid composition of *E. caudatum*

A bulk preparation of *E. caudatum* contained 210 μg of P/10⁷ cells or 1.62mg of lipid P/mg dry weight of cells. The percentage composition of phospholipids obtained by two-dimensional t.l.c.

Table 1. *Phospholipid and fatty acid composition of E. caudatum*

For details see the text. Each analysis was performed in duplicate.

Phospholipid	Amount (% distribution of lipid P)	
	Present results	Dawson & Kemp (1967)
Phosphoglycerides:		
*Phosphatidylcholine	83.9	83.6
Phosphatidylserine	23	26.1
Phosphatidylinositol	0	0
Phosphatidic acid	4	3.9
†(Phosphatidylethanolamine+glyceryl ether phospholipids)	—	3.0
Diglyceride aminoethylphosphonate	27	25.1
Ethanolamine plasmalogen+aminoethylphosphonate plasmalogen	21.5	18.0
Choline plasmalogen	1.7	1.2
<i>N</i> -(1-Carboxyethyl)phosphatidylethanolamine	—	0
	6.7	5.3
Sphingophospholipids:		
Ceramide phosphorylethanolamine	16.1	16.8
Ceramide aminoethylphosphonate	9.6	6.4
Sphingomyelin	6.5	4.3
Unidentified	0	5.3
	—	0.8

* Fatty acid composition (wt. %) 16:0, 39%; 16:1, 12%; 18:0, trace; 18:1, 34%; 18:2, 4%.

† Fatty acid composition (wt. %) 16:0, 47%; 16:1, 0%; 18:0, 18:1, 39%; 18:2, 7%.

(Table 1) was similar to that previously reported (Dawson & Kemp, 1967) for the same organism deduced by successive chemical degradations except for the sphingophospholipids. Here it became apparent that the organism contains no sphingomyelin. It was shown that the methanolic HCl treatment of the alkali-stable phospholipids in the degradative procedure produced a compound from ceramide phosphorylethanolamine and ceramide phosphorylaminoethylphosphonate (probably sphingosylphosphorylethanolamine + its phosphono analogue) which mimicked phosphorylcholine in its behaviour during the chromatographic procedure used and was thus previously reported as sphingomyelin. The present results therefore give a truer picture of the sphingophospholipid composition of the organism. Although phosphatidylcholine and phosphatidylethanolamine are the major phospholipids, the presence of *N*-(1-carboxyethyl)phosphatidylethanolamine and a high proportion of phosphonolipids (both diglyceride and ceramide types) is confirmed, as is the absence of phosphatidylserine.

The fatty acids esterified in the main phospholipid groups, i.e. phosphatidylcholine and phosphatidylethanolamine + phosphonolipid, were predominantly 18:1 and palmitic acid. Stearic acid was virtually absent (Table 1).

Uptake of $^{32}\text{P}_i$ and its incorporation into phospholipids

Even in an incubation medium containing low concentrations of phosphate, 10^8 washed cells of

E. caudatum took up only 5% of added $^{32}\text{P}_i$ in 10h and only 10% of this uptake was incorporated into its phospholipids. The uptake into all individual phospholipids was approximately linear over the time-period; in terms of the total mass of phospholipid synthesized the ethanolamine phosphoglycerides and their phosphono analogues showed maximum active synthesis (Fig. 1*a*), although phosphatidylinositol showed by far the most active synthesis when compared with the mass present (Fig. 1*b*). The rate of labelling of *N*-(1-carboxyethyl)phosphatidylethanolamine and ceramide phosphorylethanolamine was slow.

An equivalent number of cells was pulse-labelled with $^{32}\text{P}_i$ for 1h and then chased with non-radioactive phosphate. An active turnover of the phosphatidylinositol occurred, whereas the loss of radioactivity from ethanolamine phosphoglycerides and choline phosphoglycerides was negligible, suggesting at the most a very slow turnover rate (Fig. 2). The specific radioactivities of *N*-(1-carboxyethyl)phosphatidylethanolamine and ceramide phosphorylethanolamine continued to increase throughout the chase period indicating that these were being formed from some ^{32}P -labelled precursor pool of high specific radioactivity in the cell.

Uptake of $[2-^{14}\text{C}]$ ethanolamine and its incorporation into phospholipids

E. caudatum cells (10^8) took up a similar percentage (5–7%) of radioactive ethanolamine from the

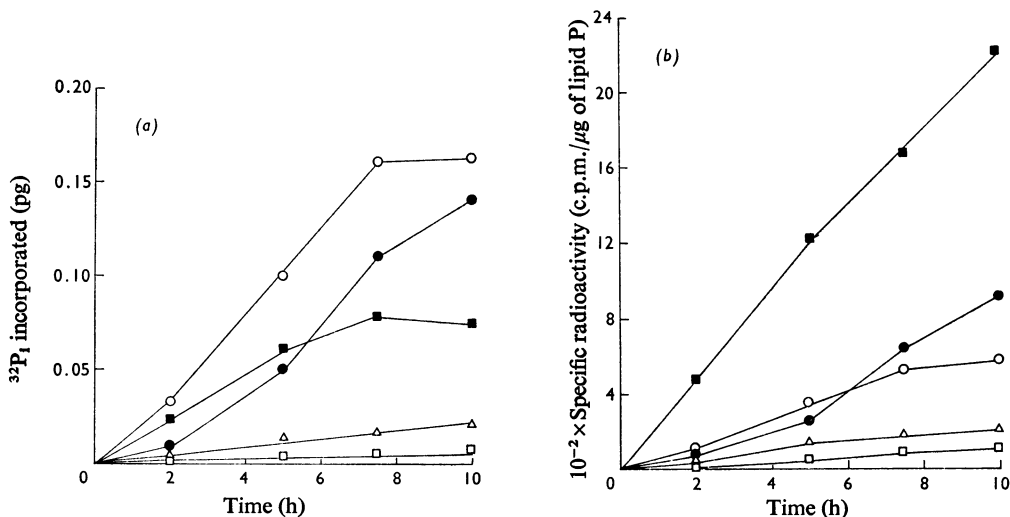


Fig. 1. Incorporation of $^{32}\text{P}_i$ into the phospholipids of *E. caudatum*

E. caudatum cells (10^8) were incubated anaerobically ($\text{N}_2 + \text{CO}_2$, 19:1) at 39°C with $15 \mu\text{Ci}$, $0.014 \mu\text{g}$ of $^{32}\text{P}_i$ in 4 ml of NaCl (0.5%) + NaHCO_3 (0.05%). The incorporations observed are representative of four similar experiments. (a) Mass of phosphate incorporated (minimum, i.e. assuming no dilution of radioactive isotope with intracellular phosphate). (b) Specific radioactivity of phospholipids. ○, Phosphatidylethanolamine + phosphonolipid; ●, phosphatidylcholine; ■, phosphatidylinositol; △, ceramide phosphorylethanolamine + phosphonolipid; □, *N*-(1-carboxyethyl)phosphatidylethanolamine.

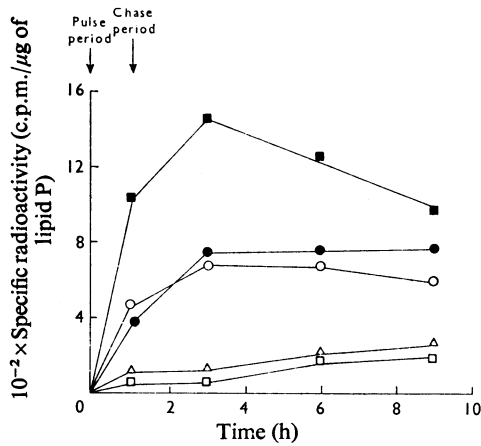


Fig. 2. Pulse-labelling of phospholipids in *E. caudatum* with $^{32}\text{P}_1$

E. caudatum cells (10^8) cells were incubated for 1 h anaerobically ($\text{N}_2 + \text{CO}_2$, 19:1) at 39°C with $80\ \mu\text{Ci}$ ($0.75\ \mu\text{g}$) of $^{32}\text{P}_1$ in 6 ml of NaCl (0.5%) + NaHCO_3 (0.05%). The cells were washed twice with normal protozoal salt solution containing sodium phosphate buffer and then resuspended in 10 ml of this protozoal salt solution and incubated at 39°C under the same conditions for a further 8 h. Results are representative of three independent experiments. ○, Phosphatidylethanolamine+phosphonolipid; ●, phosphatidylcholine; ■, phosphatidylinositol; △, ceramide phosphorylethanolamine+phosphonolipid; □, *N*-(1-carboxyethyl)phosphatidylethanolamine.

incubation medium in 10h as P_1 and this was not increased by adding Latex beads for the organism to ingest. In contrast with P_1 , however, nearly all of the radioactivity (97%) was incorporated into the phospholipid fraction and the remainder was water-soluble. This situation of almost complete conversion of the radioactivity into phospholipid applied at all time-points. Fractionation of the phospholipids showed heavy labelling of phosphatidylethanolamine whose specific radioactivity consistently fell between the eighth and tenth hours of incubation. There was somewhat less active labelling of ceramide phosphorylethanolamine and comparatively slow labelling of *N*-(1-carboxyethyl)phosphatidylethanolamine (Fig. 3). All of the radioactivity was associated with the base moieties of these phospholipids; the glycerol and the fatty acids were unlabelled. The phosphonolipids, diglyceride aminoethylphosphonate and ceramide aminoethylphosphonate were unlabelled, confirming previous observations that ethanolamine does not act as a precursor of these phosphonolipids (Liang & Rosenberg, 1968; Coleman *et al.*, 1971). Phosphatidylcholine was

unlabelled even when the incubation period was extended to 28h.

Possible role of bacteria in phospholipid labelling from $^{32}\text{P}_1$ and $[2\text{-}^{14}\text{C}]\text{ethanolamine}$

Since *E. caudatum* cannot be cultured in the absence of associated bacteria the question arises as to whether the washed protozoa would possess either adhering or recently ingested viable bacteria in sufficient amounts to affect significantly the incorporations observed. It has been shown by White (1969) that washed *E. caudatum* preparations contained up to 20–30 viable bacteria per protozoal cell. Portions (5% of the volume) of the supernatant obtained from the initial low-speed centrifugation of the protozoal culture medium were incubated with $^{32}\text{P}_1$ under the conditions stated (legend of Fig. 1). About 0.5–0.8% of the radioactive isotope was incorporated into the lipid fraction in 10h. However, the distribution of incorporation was entirely different from that observed with the protozoal fraction. The phosphatidylethanolamine component was the only phospholipid labelled. When similar experiments were repeated with $[2\text{-}^{14}\text{C}]\text{ethanolamine}$ a small incorporation (0.5%) of radioactivity into the lipids of the bacterial control was observed. This incorporation was again entirely into phosphatidylethanolamine; no labelled ceramide phosphorylethanolamine or *N*-(1-carboxyethyl)phosphatidylethanolamine was

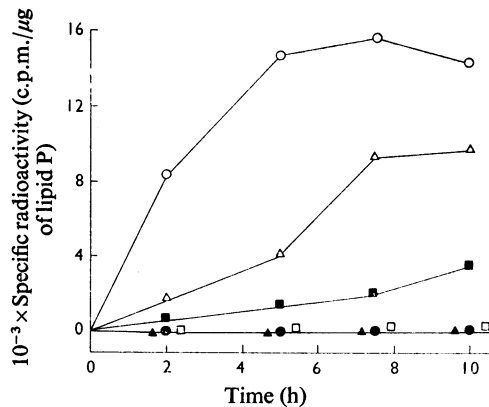


Fig. 3. Incorporation of $[2\text{-}^{14}\text{C}]\text{ethanolamine}$ into *E. caudatum* phospholipids

E. caudatum cells (10^8) were incubated anaerobically ($\text{N}_2 + \text{CO}_2$, 19:1) at 39°C with $10\ \mu\text{Ci}$ ($0.33\ \mu\text{mol}$) of $[2\text{-}^{14}\text{C}]\text{ethanolamine}$ in 4 ml of protozoal saline. Results are representative of three independent experiments. ○, Phosphatidylethanolamine; △, ceramide phosphorylethanolamine; ■, *N*-(1-carboxyethyl)phosphatidylethanolamine; ●, diglyceride aminoethylphosphonate; ◐, ceramide aminoethylphosphonate; □, phosphatidylcholine.

formed. It is concluded therefore that contaminating bacteria have a minimal effect on the phospholipid labelling pattern observed with *E. caudatum*. Any interference is likely to be confined to phosphatidylethanolamine labelling. Approximate calculation based on the numbers of bacteria involved suggest that this would be at the most amount to less than 10% of the phosphatidylethanolamine labelling observed.

Conversion of phosphatidylethanolamine into ceramide phosphorylethanolamine and N-(1-carboxyethyl)phosphatidylethanolamine

The pulse-labelling experiments with $^{32}\text{P}_i$ and the results of $[2-^{14}\text{C}]$ ethanolamine labelling suggested that ceramide phosphorylethanolamine could be formed in *E. caudatum* from phosphatidylethanolamine. Incubation of *E. caudatum* cells with $^{32}\text{P}_i$ caused an accumulation of label in ceramide phosphorylethanolamine (Broad & Dawson, 1973). Consequently we incubated doubly labelled $[^{32}\text{P}]$ -phosphatidyl $[2-^3\text{H}]$ ethanolamine with *E. caudatum* and compared the ratio of label in this and the labelled ceramide phosphorylethanolamine formed. After 5 and 10h of incubation when 3.0 and 5.2% respectively of the labelled phosphatidylethanolamine had been converted into ceramide phosphorylethanolamine the ratio of $^3\text{H}/^{32}\text{P}$ in the latter was the same as in the original substrate (Table 2). The same was true for the *N*-(1-carboxyethyl)phosphatidylethanol-

amine formed although the percentage conversion was somewhat less. During these incubations there was a substantial hydrolysis of phosphatidylethanolamine with the formation of lysophosphatidylethanolamine and glycerylphosphorylethanolamine, as had been observed earlier (Coleman *et al.*, 1971).

When ceramide phosphoryl $[2-^{14}\text{C}]$ ethanolamine was prepared from *E. caudatum* after being labelled with $[2-^{14}\text{C}]$ ethanolamine and incubated with protozoa there was a substantial conversion of this into phosphatidylethanolamine (Table 3); no other phospholipids such as *N*-(1-carboxyethyl)phosphatidylethanolamine were labelled.

No success was obtained in effecting a conversion of $[^{32}\text{P}]$ phosphatidylethanolamine into ceramide phosphorylethanolamine in a cell-free system prepared by disruption of the protozoa by ultrasonication or other means, with or without supplementation of the medium by various factors such as ceramide.

Uptake of $[Me-^{14}\text{C}]$ choline and its incorporation into phospholipids

The rate of uptake of $[Me-^{14}\text{C}]$ choline into *E. caudatum* cells was extremely rapid compared with that of $[2-^{14}\text{C}]$ ethanolamine (Fig. 4). This uptake was linear with the cell concentration up to the maximum cell density examined (6.25×10^7 cells/ml). It was not affected by the presence of betaine or ethanolamine added at 425 times the molar concentration of choline

Table 2. Incubation of *E. caudatum* with $[^{32}\text{P}]$ phosphatidyl $[2-^3\text{H}]$ ethanolamine

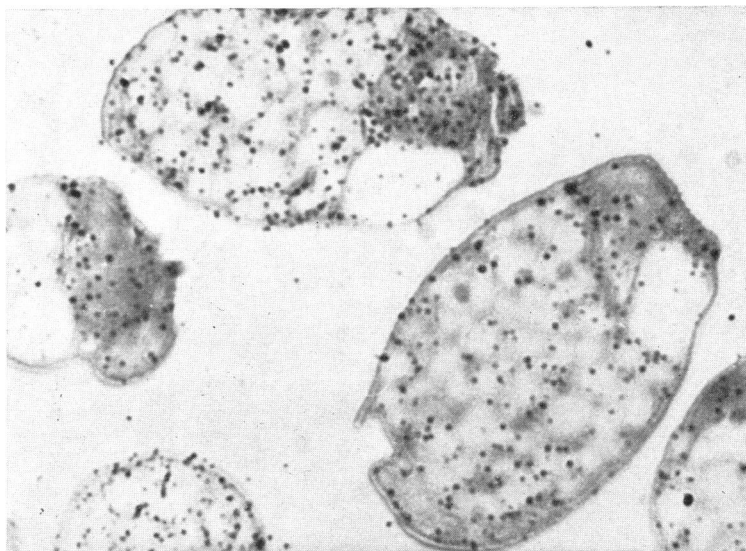
Cells (2×10^7) were incubated with $[^{32}\text{P}]$ phosphatidyl $[2-^3\text{H}]$ ethanolamine ($20 \mu\text{g}$ of P) in protozoal salt solution (2.5 ml) at 39°C . The cells were washed, their lipids extracted and subjected to two-dimensional t.l.c. radioautography. The radioactive spots were scraped off and the radioactivity was determined by liquid-scintillation spectrometry. Results are representative of two similar experiments.

Time incubated (h)	Phospholipid	Percentage conversion	Ratio of $^3\text{H}/^{32}\text{P}$
0	Phosphatidylethanolamine (original substrate)	—	0.95
5	Phosphatidylethanolamine	—	0.95
5	Ceramide phosphorylethanolamine	3.0	0.95
5	<i>N</i> -(1-Carboxyethyl)phosphatidylethanolamine	1.0	0.99
10	Phosphatidylethanolamine	—	0.94
10	Ceramide phosphorylethanolamine	5.2	0.96
10	<i>N</i> -(1-Carboxyethyl)phosphatidylethanolamine	2.0	0.96

Table 3. Incubation of ceramide phosphoryl $[2-^{14}\text{C}]$ ethanolamine with *E. caudatum*

E. caudatum cells (5×10^6) were incubated anaerobically at 39°C with $0.1 \mu\text{Ci}$ of ceramide phosphorylethanolamine ($20 \mu\text{g}$ of P) in 2.5 ml of protozoal saline. At the end of the incubation period the cells were recovered by centrifuging and washed with 5 ml of protozoal saline before radioactive assay and lipid extraction, etc.

Time incubated (h)	Radioactivity recovered with cells (% of total)	Radioactivity (% of total in cell) in	
		Phosphatidylethanolamine	Ceramide phosphorylethanolamine
4.5	29.5	16.5	83.5
9.0	22.8	26.3	73.5



EXPLANATION OF PLATE I

Light-microscopic radioautogram of E. caudatum showing silver grains over the phosphatidyl[³H]choline-containing structures of the protozoon

E. caudatum cells (10^4) were incubated with $50\mu\text{Ci}$ ($0.1\mu\text{mol}$) of [$\text{Me-}^3\text{H}$]choline in 1 ml of protozoal saline for 10 min anaerobically at 39°C .

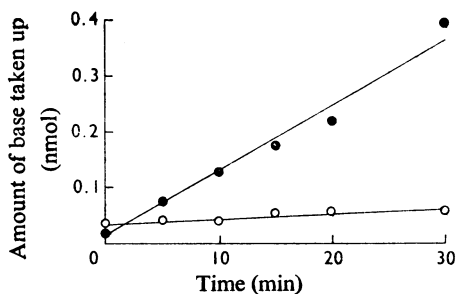


Fig. 4. Comparison of the rates of uptake of [2-¹⁴C]-ethanolamine and [Me-¹⁴C]choline by *E. caudatum* cells

Cells (4.8×10^6) were incubated anaerobically at 39°C in 6 ml of protozoal saline with 4.6 μ Ci (0.06 μ mol) of [2-¹⁴C]-ethanolamine (○) or 3.5 μ Ci (0.06 μ mol) of [Me-¹⁴C]choline (●); at various intervals 0.1 ml portions were withdrawn, filtered rapidly on a Millipore filter, washed well with protozoal saline and the radioactivity in the cells was determined by liquid-scintillation spectrometry. Similar results were obtained in three experiments.

or when the protozoa were given Latex beads to ingest. On the other hand, the choline transport inhibitor hemicholinium-3 was highly effective at preventing the uptake of choline by the organism (50% inhibition at 0.05 mM, 100% inhibition at 10 mM); chlorocholine chloride was much less effective.

At an added choline concentration of 1.3 mM, 10^7 *E. caudatum* cells/ml had taken up 86% of the [¹⁴C]choline after 2 h of incubation and 95% by 10 h. The choline taken up was rapidly and almost quantitatively incorporated into a single phospholipid which was identified as phosphatidylcholine. None was found in the sphingophospholipid fraction which is consistent with the absence of sphingomyelin from the protozoal membranes as determined by analysis. On pulse-labelling the phosphatidylcholine of *E. caudatum* by incubation with [¹⁴C]choline for 2 h and subsequently chasing with a 1000-fold molar excess of non-radioactive choline there was a slow loss of label amounting to 2% of the radioactivity per h (Fig. 5).

Radioautography of *E. caudatum* after 10 min of incubation with radioactive choline showed that radioactive phosphatidylcholine was widely distributed throughout the membranes of the cell (Plate 1). More radioactivity appeared to be concentrated at the head end but this may be dependent on the greater concentration of membranes in this area rather than a greater specific radioactivity of the phosphatidylcholine. Similar results were obtained after the organism had been incubated with choline for 1 h.

Incorporation of L-[U-¹⁴C]serine radioactivity into phospholipids

The rate of uptake of L-serine by *E. caudatum* was very low. After 10 h of incubation, 10^8 cells had only taken up 2% of the added L-[U-¹⁴C]serine (5 μ Ci, 0.003 μ mol in 10 ml) radioactivity and of this only 15% had been incorporated into phospholipid. The only phospholipid labelled was phosphatidylethanolamine with trace labelling of ceramide phosphorylethanolamine and *N*-(1-carboxyethyl)phosphatidylethanolamine which can be formed from it; no labelled phosphatidylserine or phosphatidylcholine could be detected. After 10 h incubation 75% of the radioactivity in the total lipid fraction was present in ethanolamine, 24% was present in the fatty acid moieties, and less than 1% in long-chain bases. The addition of hydroxylamine, which is an inhibitor of the enzyme which decarboxylates phosphatidylserine (Dennis & Kennedy, 1970), to either an intact or a sonicated preparation of *E. caudatum* decreased the rate of incorporation of L-serine to less than 30% of the previous value but no accumulation of phosphatidylserine could be detected.

Incorporation of radioactivity from L-[Me-¹⁴C]-methionine into lipids

The rate of lipid labelling from [Me-¹⁴C]methionine by intact *E. caudatum* cells was extremely low; even after 24 h less than 1% of the radioactivity had been

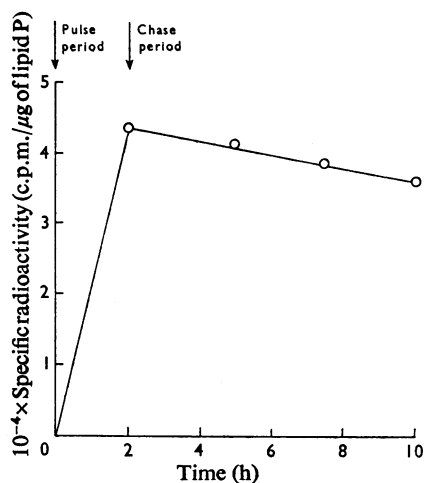


Fig. 5. [Me-¹⁴C]Choline pulse-chase labelling of phosphatidylcholine in *E. caudatum* cells

Cells (10^8) were incubated with 8 μ Ci (0.13 μ mol) of [Me-¹⁴C] choline in 10 ml of protozoal saline for 2 h anaerobically at 39°C. A 1000-fold molar excess of non-radioactive choline was then added and the incubation continued. The experiment was done in duplicate.

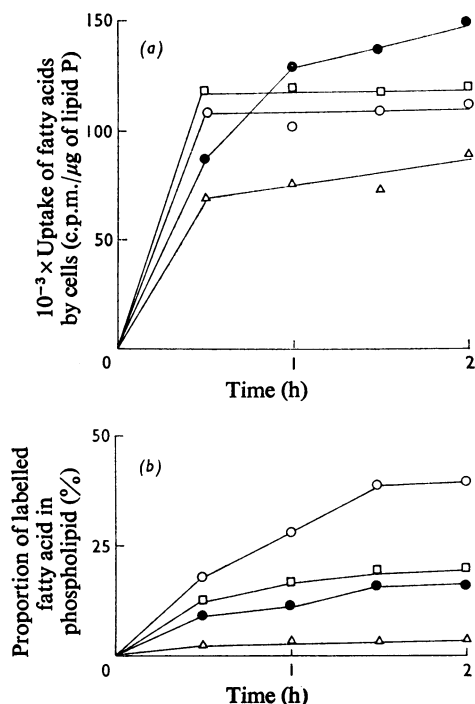


Fig. 6. Uptake (a) of radioactive fatty acids into the total lipids of *E. caudatum* cells and (b) the percentage esterified in phospholipids

Cells (2×10^7) were incubated anaerobically at 39°C separately with each of the radioactive [$1-^{14}\text{C}$]fatty acids shown ($5 \mu\text{Ci}$, $0.08 \mu\text{mol}$). The fatty acids were dispersed by ultrasonication (30 s at 80 kHz in an ultrasonic water bath) in 2.4 ml of protozoal saline. After withdrawal of portions and washing of the cells the radioactivity in the total lipids and in the phospholipids was determined. ●, Palmitic acid; ○, oleic acid; Δ, stearic acid; □, linoleic acid.

incorporated into the lipid fraction. No phosphatidylcholine labelling could be detected. All of the label was present in unesterified fatty acids and in fatty acids esterified in phosphatidylethanolamine. Examination by g.l.c. and argentation t.l.c. (details in the Experimental section) of the radioactive fatty acids produced showed that these consisted mainly (75%) of a C_{17} cyclopropane fatty acid together with a C_{19} cyclopropane fatty acid. The slowness of the labelling and the nature of the products suggests that the L-methionine is largely metabolized by contaminating bacteria. Cyclopropane fatty acids are only formed by bacteria and occasionally by higher plants (Smith, 1971). In these, monoenoic fatty acids esterified in a phospholipid, which is usually phosphatidylethanolamine, have a methylene bridge

inserted across the double bond, by donation from the methyl group of S-adenosylmethionine.

Uptake of long-chain fatty acids and incorporation into phospholipids

The rate at which *E. caudatum* incorporated long-chain fatty acids into their total lipid fraction is shown in Fig. 6(a). The uptake is expressed as radioactivity incorporated/ μg of lipid P since the amount of phospholipid present did not change during the incubation. A small amount of the added radioactivity was incorporated in the first 30 min. Subsequently the organism took up stearic acid, oleic acid and linoleic acid very slowly although palmitic acid was taken up at rather a higher rate. At all time-periods the major proportion of radioactivity remained in the unesterified fatty acid fraction; no mono-, di- or tri-glycerides could be detected. However, there was a progressive proportion converted into phospholipids; that most actively esterified was oleic acid, whereas palmitic acid and linoleic acid were less so, with stearic acid being hardly esterified at all (Fig. 6b). It would appear therefore that stearic acid can be taken up by *E. caudatum* almost as readily as the other fatty acids but, in contrast, is scarcely used at all for phospholipid formation. This deficiency is perhaps reflected in the almost total absence of stearic acid from *E. caudatum* lipids.

Further examination of the palmitic acid labelling of the phospholipids showed this to be predominantly in the phosphatidylcholine and phosphatidylethanolamine fractions. This is in marked contrast with the distribution of fatty acid labelling on incubation with L-[$U-^{14}\text{C}$]serine where phosphatidylcholine fatty acids were unlabelled.

Cytidine diphosphate bases as intermediaries in phosphatidylcholine and phosphatidylethanolamine biosynthesis

When *E. caudatum* was incubated for 2 h with [$\text{Me}-^{14}\text{C}$]choline and the cells were then extracted with 10% (w/v) trichloroacetic acid, only a few per cent of the radioactivity was extracted and of this 85% was as phosphorylcholine and the remainder as free choline. Incubation of ultrasonicated *E. caudatum* with [$\text{Me}-^{14}\text{C}$]choline resulted in a small labelling of phosphatidylcholine with limited incorporation into phosphorylcholine and CDP-choline (Table 4). The addition of $\text{ATP} + \text{Mg}^{2+}$ resulted in a large increase in the radioactivity in phosphorylcholine and a 55-fold increase in the rate of incorporation of radioactive choline into phosphatidylcholine. The addition of CTP plus Mg^{2+} resulted in a huge increase in radioactivity in CDP-choline and a 118-fold increase in the incorporation into phosphatidylcholine. Surprisingly, the addition of L-1,2-diglyceride dispersed in Tween 20 did not enhance the incorporation into phosphatidylcholine. In the

Table 4. Incorporation of [*Me*-¹⁴C]choline into lipids and 'cytidine' pathway intermediates by *E. caudatum*

Each tube contained 10⁷ cells ultrasonicated for 2 min, and incubated with 2.5 μCi (0.4 μmol) of [*Me*-¹⁴C]choline in Tris-HCl buffer (0.1 M, pH 8.3), volume 1.0 ml at 39°C for 1 h. The amounts of the additives were ATP (5.4 μmol), CTP (5.7 μmol), MgSO₄ (20.2 μmol) unless otherwise stated. At the end of the incubation the intermediaries were extracted with trichloroacetic acid and the lipids were then extracted with chloroform-methanol (2:1, v/v). Results are representative of three experiments.

Additions	[¹⁴ C]Choline (c.p.m.) in:			
	Phosphorylcholine	CDP-choline	Phosphatidylcholine	Lysophosphatidylcholine
None	35 546	13 631	1068	68
ATP, MgSO ₄	3 703 700	17 657	55 874	2 774
CTP, MgSO ₄	898 203	2 727 270	118 750	5 677
ATP, CTP, MgSO ₄	1 831 850	2 189 779	67 063	35 29
ATP, CTP, MgSO ₄ , L-1,2-diglyceride (3 mg)	1 345 290	1 986 753	62 647	3513
ATP, CTP, MgSO ₄ , CaCl ₂ (20 μM)	114 425	61 372	1 569	130
ATP, CTP, MgSO ₄ (8.1 μM)	2 083 331	1 595 743	8 921	456
ATP, CTP, MgSO ₄ (40.5 μM)	1 421 800	2 290 074	229 000	14 812

Table 5. Incorporation of [*2*-¹⁴C]ethanolamine into lipids and 'cytidine' pathway intermediaries by *E. caudatum*

Each tube contained 10⁷ cells ultrasonicated for 1.5 min in 0.1 M Tris-HCl buffer, pH 8.3, (volume 1.0 ml). The amounts of the additives were: ATP (5.4 μmol), MgSO₄ (20.2 μmol), CTP (5.7 μmol) unless otherwise stated. L-1,2-Diglyceride was prepared by the method of Miller & Dawson (1972). After incubation for 1 h with [*2*-¹⁴C]ethanolamine (1.5 μCi, 0.038 μmol) at 39°C, 2 ml of 10% trichloroacetic acid was added and the tubes were left for 30 min at 2°C. The lipids were extracted from the precipitate and the intermediaries isolated from the trichloroacetic acid extract. Results are representative of two experiments.

Additions	[¹⁴ C]Ethanolamine (c.p.m.) in:			
	Phosphoryl-ethanolamine	CDP-ethanolamine	Phosphatidyl-ethanolamine	Lysophosphatidyl-ethanolamine
None	281 023	494	307	202
ATP, MgSO ₄	274 272	731	1 928	449
CTP, MgSO ₄	148 889	102 434	5 656	5 233
ATP, CTP, MgSO ₄	207 202	65 094	4 240	2 804
ATP, CTP, MgSO ₄ , L-1,2-diglyceride (3 mg)	155 107	82 031	6 025	4 530
ATP, CTP, MgSO ₄ , CaCl (20 μM)	124 509	20 773	262	93
ATP, CTP, MgSO ₄ (8.1 μM)	210 101	129 884	919	517
ATP, CTP, MgSO ₄ (40.5 μM)	132 682	69 545	7 227	6 036

presence of CaCl₂ (20 mM) the incorporation into phospholipid was almost totally inhibited and decreased to the control value. This latter observation suggests that a base-exchange pathway for phosphatidylcholine formation is not operative in *E. caudatum*, and this has also been reported for *T. pyriformis* (Dennis & Kennedy, 1970).

It must be emphasized that the capacity of sonicated *E. caudatum* to convert radioactive choline into phosphorylcholine and CDP-choline is extremely high; under appropriate conditions 60–70% of the total radioactivity was incorporated into these intermediates. We have used such sonicates to prepare CDP-[¹⁴C]choline since the yield and simplicity of the procedure compares extremely favourably with the standard biosynthetic method developed by Schneider *et al.* (1966). When an equivalent number of *T. pyriformis* cells were ultrasonicated and incubated

with [*Me*-¹⁴C]choline under the same conditions as given in Table 4 the amounts of radioactivity incorporated into the phosphorylcholine and CDP-choline intermediaries were less than 1% of that seen with *E. caudatum*.

Similar results were obtained when *E. caudatum*, both intact and ultrasonicated, was incubated with radioactive ethanolamine except that here the incorporation into phosphorylethanolamine and CDP-ethanolamine was on a far more limited scale. With intact *E. caudatum* cells incubated with [*2*-¹⁴C]-ethanolamine only phosphorylethanolamine and free ethanolamine were found in the few per cent of total radioactivity removed by trichloroacetic acid extraction. On incubating ultrasonicated preparations with [*2*-¹⁴C]ethanolamine much radioactive phosphorylethanolamine was formed (Table 5) and the addition of ATP plus Mg²⁺ did not increase this. However,

the addition of CTP plus Mg^{2+} resulted in a dramatic increase in the radioactivity in CDP-ethanolamine and an 18-fold increase in the rate of incorporation into phosphatidylethanolamine. As with phosphatidylcholine formation the addition of L-1,2-diglyceride produced no significant enhancement of phosphatidylethanolamine synthesis. The addition of 20 mM- $CaCl_2$ decreased the rate of incorporation into phosphatidylethanolamine. By contrast the presence of a calcium-stimulated ethanolamine-exchange reaction has been well established in *T. pyriformis* (Dennis & Kennedy, 1970).

Discussion

It is impossible to be completely certain whether the synthesis of phospholipids measured in the present investigation is associated with the turnover of phospholipids in existing membranes or whether any part of it is dependent on the formation of new membrane material during growth. The protozoa on being harvested are certainly dividing with a doubling time of about 30h, but they are, after being washed, incubated in a medium which would be entirely unsuitable for growth and would result in their death within a few days. It is possible that they might contain sufficient food reserves for maintaining growth for a limited period. However, the rather steady increase in the specific radioactivity of the ^{32}P label in all phospholipids during the incubation period suggests that the phospholipid biosynthesis measured is primarily associated with turnover rather than with new membrane formation during growth. The rapid turnover of the phosphatidylinositol component and the much slower turnover of phosphatidylcholine and phosphatidylethanolamine revealed by direct and pulse-labelling experiments is characteristic of that seen in higher animals (Dawson, 1955; Ansell & Dohmen, 1957). The absence of any additional labelling from choline or ethanolamine when the protozoa were given Latex beads to ingest would suggest that no part of the phospholipid synthesis is associated with the formation of new membrane material during the creation of food vacuoles.

No-one has yet been able to grow *E. caudatum* successfully in the absence of bacteria. This means that although the organisms are washed on harvesting inevitably they must contain some viable bacteria which have been ingested a short time previously (White, 1969). The phospholipid labelling of these does not seem to be a major factor in the observed labelling of *E. caudatum* phospholipids at least as far as [$Me-^{14}C$]choline, [$2-^{14}C$]ethanolamine and $^{32}P_i$ are concerned. Apart from some rare exceptions bacteria do not form phosphatidylcholine, and the pattern of phospholipid labelling from labelled ethanolamine and phosphate is completely different from that seen with a 'bacterial control'. The incorporation from [$Me-^{14}C$]methionine does appear,

however, to be primarily of bacterial origin because of the very low rate of labelling and the nature of the products, namely cyclopropane fatty acids which are considered to be of bacterial origin. It is also likely that the incorporation of L-[$U-^{14}C$]serine radioactivity into the ethanolamine and fatty acid moieties of phosphatidylethanolamine could be largely of bacterial origin. Both *T. pyriformis* and many bacteria (Dennis & Kennedy, 1970) can form phosphatidylethanolamine by the decarboxylation of phosphatidylserine, a pathway which must be suspected in spite of our failure to demonstrate the latter phospholipid as an intermediary. The radioactivity incorporated into fatty acids suggests that some of the serine is deaminated and converted into acetate which is subsequently incorporated into fatty acids. However, if the slow metabolism of the L-serine observed occurred mainly in the associated bacteria this would explain the absence of fatty acid incorporation in phosphatidylcholine, a phospholipid which is generally absent from bacteria, and also the low rate of conversion into long-chain base since sphingolipids are only found in a few species of bacteria. In animal tissues long-chain bases can be formed by a condensation reaction between serine and long-chain fatty acids.

It is apparent that *E. caudatum* contains the necessary enzymic equipment for synthesizing phosphatidylcholine and phosphatidylethanolamine through the phosphorylated base-CDP-base pathway similar to that found in the tissues of higher animals. This is shown by the detection of phosphorylcholine, phosphorylethanolamine, CDP-choline and CDP-ethanolamine after incubation with radioactive bases, and the responses of these intermediaries as well as the synthesized phospholipids to the addition of cofactors such as Mg^{2+} , ATP and CTP. The failure to increase the rate of phospholipid synthesis by the addition of L-1,2-diglyceride could mean either that the system already has sufficient diglyceride available or alternatively that added diglyceride is rapidly broken down by powerful digestive lipases released on ultrasonication of the organism.

The failure to detect phosphatidylcholine after [$2-^{14}C$]ethanolamine or [$Me-^{14}C$]methionine labelling indicates that the organism cannot synthesize phosphatidylcholine by the donation of methyl groups from *S*-adenosylmethionine to phosphatidylethanolamine. This sharply contrasts with the situation in the tissues of higher animals and in many aerobic protozoa such as *T. pyriformis* (Smith & Law, 1970), *Euglena gracilis* (Tipton & Swords, 1966) and *Ochromonas malhamensis* (Lust & Daniel, 1966) in which the presence of a methylation pathway for phosphatidylcholine synthesis has been well established. However, *E. caudatum* is certainly not unique in this respect, as it has been reported that the methylation pathway does not exist in the parasitic proto-

zoon *Plasmodium knowlesi* (Rock, 1971) and in mammalian brain (Ansell, 1973). The absence of this pathway in *E. caudatum* suggested that the organism would have an absolute nutritional requirement for choline.

The avidity with which *E. caudatum* takes up free choline from the incubation medium and converts this into phosphatidylcholine is also unusual. *T. pyriformis* takes up choline very slowly (Thompson, 1967) and in *Acanthamoeba palestinensis* (Chlapowski & Band, 1971) choline uptake was proportional to the external concentration of choline so that equilibrium was rapidly reached between the internal and external concentrations, with 50% of the internal choline existing as the free base. It seems possible that the ready uptake of choline by *E. caudatum* may be due to the rapid conversion of this into phosphatidylcholine, producing an extracellular-intracellular concentration gradient. Hemicholinium-3 was shown to inhibit the uptake of choline into *E. caudatum* cells but it could do this not by inhibiting any specific transport mechanism for choline, but simply by blocking phosphatidylcholine synthesis owing to its potent inhibition of the organism's choline kinase (EC 2.7.1.32) (Broad & Dawson, 1974). The rapid uptake of choline compared with ethanolamine perhaps fulfils a physiological role in ensuring the growth and survival of the organism in a rumen environment. Whereas the protozoon would be continually able to ingest bacteria containing ethanolamine combined in phosphatidylethanolamine, its imbibing of choline would be limited to brief periods after the ruminant had ingested choline-containing foodstuffs.

The labelling patterns of phosphatidylethanolamine, *N*-(1-carboxyethyl)phosphatidylethanolamine and ceramide phosphorylethanolamine on incubation of *E. caudatum* with [2-¹⁴C]ethanolamine confirm and extend previous observations, suggesting that the former phospholipid is the precursor of the latter two (Coleman *et al.*, 1971; Broad & Dawson, 1973). The incubation of [³²P]phosphatidyl[2-¹⁴C]ethanolamine with the protozoon suggests that a phosphorylethanolamine unit can be incorporated intact into *N*-(1-carboxyethyl)phosphatidylethanolamine and ceramide phosphorylethanolamine and that with the latter phospholipid this reaction is reversible. It is possible that CDP-ethanolamine could be an intermediary in this process, although the absence of ceramide phosphorylethanolamine labelling on stimulating CDP-ethanolamine labelling by CTP addition in cell-free homogenates suggests this may not be so. Unfortunately labelling from phosphatidylethanolamine could not be demonstrated in a cell-free system, which would have allowed further exploration of this point. The analogous transfer of phosphorylcholine from phosphatidylcholine to sphingomyelin has been recently reported to occur in intact and

disrupted mouse fibroblasts which had been transformed with SV-40 virus (Diringer *et al.*, 1972; Diringer & Koch, 1973). This reaction seems to occur generally in mammalian tissues and CDP-choline does not appear to be an intermediary (Ullman & Radin, 1974).

In spite of being surrounded in its normal rumen environment with a very high concentration of stearic acid (Ward *et al.*, 1964), *E. caudatum* makes no use of this fatty acid for phospholipid synthesis as it does with palmitic acid, linoleic acid or oleic acid. Apparently all of the unesterified fatty acids, including stearic acid, are taken up by the protozoon, but the enzymes involved in the incorporation of fatty acids into phospholipids show a distinct specificity against stearic acid. Thus stearic acid is not incorporated into the phospholipid fraction, and the isolated phospholipids from the protozoa contain negligible amounts of stearate. It is rather surprising that palmitic acid with only two fewer methylene groups is readily used by the same organism.

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