Phosphatidylcholine Biosynthesis and Choline Transport in the Anaerobic Protozoon *Entodinium caudatum*

By F. L. BYGRAVE* and R. M. C. DAWSON

Biochemistry Department, A.R.C. Institute of Animal Physiology, Babraham, Cambridge CB24AT, U.K.

(Received 10 June 1976)

1. Choline accumulation and phosphatidylcholine biosynthesis were investigated in the choline-requiring anaerobic protozoon *Entodinium caudatum* by incubating whole cells or subcellular fractions with [^{14}C]choline, phosphoryl[^{14}C]choline and CDP-[^{14}C]choline. 2. All membrane fractions contained choline kinase (EC 2.7.1.32) and CDP-choline-1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2), although the specific activities were less in the cell-envelope fraction. Choline phosphate cytidyltransferase (EC 2.7.7.15) was limited to the supernatant, and this enzyme was rate-limiting for phosphatidylcholine synthesis in the whole cell. 3. Synthesis of phosphatidylcholine from free choline by membranes was only possible in the presence of supernatant. Such reconstituted systems required ATP (2.5 mM), CTP (1 mM) and Mg^{2+} (5 mM) for maximum synthesis of the phospholipid. CTP and Mg^{2+} were absolute requirements. 4. Hemicholinium-3 prevented choline uptake by the cells and was strongly inhibitory towards choline kinase; the other enzymes involved in phosphatidylcholine synthesis were minimally affected. 5. Ca^{2+} ions (0.5 mM) substantially inhibited CDP-choline-1,2-diacylglycerol cholinephosphotransferase in the presence of 15 mM- Mg^{2+} , but choline phosphate cytidyltransferase and choline kinase were less affected. 6. No free choline could be detected in intact cells even after short (10–180 s) incubations or at temperatures down to 10°C. The [^{14}C]choline entering was mainly present as phosphorylcholine and to a lesser extent as phosphatidylcholine. 7. It is suggested that choline kinase effectively traps any choline within the cell, thus ensuring a supply of the base for future growth. At low choline concentrations the activity of choline kinase is rate-limiting for choline uptake, and the enzyme might possibly play an active role in the transport phenomenon. Thus the choline uptake by intact cells and choline kinase have similar K_m values and show similar responses to temperature and hemicholinium-3.

The anaerobic protozoon *Entodinium caudatum* has phosphatidylcholine as a major component of the phospholipids present in its membranes (Dawson & Kemp, 1967). It has been shown that, unlike many aerobic protozoa, it cannot synthesize phosphatidylcholine by the methylation of phosphatidylethanolamine (Broad & Dawson, 1975). These observations suggested that growth of the organism would depend on the availability of choline, and this has been confirmed in nutritional experiments (Broad & Dawson, 1976).

In the present experiments we have investigated the enzymes involved in phosphatidylcholine synthesis with regard to their subcellular distribution, their requirement for cofactors and the effect of inhibitors. In addition, the rapid choline uptake and retention by the cell has been shown to be highly dependent on choline kinase, in that only phosphorylcholine and no free choline could be detected in

the cell under various conditions of choline entry. Such an effective method of trapping choline entering the cell is probably essential to an organism whose growth would be dependent on a limited and transient availability of the base in its normal environment (Broad & Dawson, 1976). Some of the present results have been briefly reported (Bygrave & Dawson, 1975).

Experimental

Growth and harvesting of E. caudatum

Cultures of *E. caudatum* were those supplied originally by Dr. G. S. Coleman of this department. They were maintained by the procedures previously outlined (Coleman, 1960, 1971; Broad & Dawson, 1975). Harvesting of cells was carried out as described (Broad & Dawson, 1975). The cells (approx. 1 ml packed volume from 20 culture bottles each containing 100 ml of culture medium) were washed in Coleman's (1971) protozoan salt solution

* Permanent address: Department of Biochemistry, Faculty of Science, Australian National University, Canberra, Australia.

(well gassed with $N_2 + CO_2$, 95:5) until essentially free from contaminating debris.

Subcellular fractionation of cells

Cells were washed in ice-cold 0.1 M-Tris/HCl buffer (pH 6.5) and finally resuspended in 10 ml of this buffer at 0°C. The suspension of intact cells was completely disrupted by immersion in an ultrasonic water bath (Kerry's Ultrasonic Ltd., Basildon, Essex, U.K.) at 80 kHz for 60 s. An equal volume of the same Tris buffer was added to the homogenate, and the mixture centrifuged at 500g for 2 min at 4°C. The white-coloured sediment was washed four times by centrifugation at 700g for 2 min with 100 mM-Tris/HCl buffer and finally resuspended in approx. 1 ml of this buffer. This suspension was placed on a column approx. 6 cm high made by packing washed no. 12 Ballotini glass beads into a Pasteur pipette, the outlet of which was plugged with five or six larger glass beads. The suspension was drawn through the column with gentle suction and eluted with approx. 10 ml of cold buffer. The upper two-thirds of the beads were drawn off and diluted with approx. 2 ml of 100 mM-Tris/HCl buffer and vortexed to detach the cell envelope from the glass beads. This method of separating the cell envelope was developed by Professor G. Thompson (University of Texas) while working in our laboratories.

The (500g, 2 min) supernatant (approx. 20 ml) was centrifuged at 6000g for 10 min in a Sorvall bench centrifuge (SS-34 head) maintained at 0–4°C. The packed sediment consisted of two distinct layers. The upper layer, coloured dark-brown, was carefully removed with a glass rod and resuspended in approx. 1 ml of the same Tris/HCl buffer (fraction A). The lower layer, coloured white, was also resuspended in 1 ml of Tris/HCl buffer (fraction B). The supernatant (6000g, 10 min) was centrifuged at 144000g for 60 min. The transparent-looking sediment was resuspended in approx. 1 ml of Tris/HCl buffer (fraction C). The supernatant is presumed to contain the soluble components of the cell. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Incubation of subcellular fractions

Reaction vessels usually contained a medium consisting of 20 mM-Tris/HCl buffer (pH 6.5), 0.5 mM-EGTA [ethanedioxybis(ethylamine)tetra-acetate], 1 mM-CTP, 2.5 mM-ATP, 15 mM-MgSO₄, approx. 1 mg of protein, 0.2 μ Ci of [*Me*-¹⁴C]choline and other additions or variations as indicated in the legends to Figures or Tables. The final volume was 1.0 ml. Incubations were usually carried out for 40 min at 39°C and terminated by the addition of 5 ml of chloroform/methanol (2:1, v/v).

Determination of incorporation of [¹⁴C]choline into phosphatidylcholine

The solvent-treated incubation mixtures were left at room temperature (17–22°C) for approx. 1–2 h, with occasional shaking. Subsequent separation of the phases was facilitated by the addition of 0.1 ml of 100 mM-KCl. After a brief centrifugation the upper phase was removed and the chloroform-rich lower phase evaporated to dryness *in vacuo*. The dry phospholipid was taken up in 200 μ l of chloroform/methanol, and 50 μ l spotted on to t.l.c. plates (Merck silica gel 60, F254), dried and run in the solvent chloroform/methanol/water (19:7:1, by vol.). The iodine-staining areas that chromatographed with the same *R_F* value as authentic phosphatidylcholine were scraped off the plate into vials and dispersed in 15 ml of Unisolve scintillation fluid (Koch-Light Ltd., Colnbrook, Bucks., U.K.).

Determination of radioactivity in choline, phosphorylcholine and CDP-choline

A portion (usually 50 or 100 μ l) of the aq.-methanol upper phase was subjected to long-term high-voltage paper ionophoresis (Broad & Dawson, 1975). Areas containing phosphorylcholine and CDP-choline were located by using carrier markers as described by Broad & Dawson (1975), cut out and put into vials containing 10 ml of Unisolve scintillation fluid. In some experiments with intact cells, further samples were ionophoresed for shorter periods (30 min) under similar conditions in the presence of 50 μ g of choline as a carrier marker. After drying of the paper, the choline spot was detected by spraying with 0.5% Bromocresol Green in 95% (v/v) ethanol, cut out, and its radioactivity assessed by scintillation counting.

Short-term uptake of [¹⁴C]choline by intact cells

Cells (approx. 1 ml packed volume) were finally resuspended in about 1 ml of the protozoan salt solution and maintained at room temperature in a stoppered tube and gassed with the $N_2 + CO_2$. The cell concentration was approx. 10⁷/ml. Intact cell suspension (0.1 ml) was added to 1.9 ml of 0.1 M-Tris/HCl buffer (pH 6.5) in a small 3 ml incubation vessel kept at 40°C unless otherwise indicated. The contents were continuously mixed by means of a small magnetic stirrer. After a 1 min preincubation, choline of various specific radioactivities (see legends) was added. At short time-intervals 0.3 ml portions were rapidly transferred to Eppendorf centrifuge tubes kept on ice and containing 0.3 ml of 20 mM-hemicholinium-3. The contents were rapidly mixed and centrifuged (12000g for 2 min) in an Eppendorf Microfuge. The cell pellets were washed with

2×0.6ml of 10mM-hemicholinium solution and then resuspended in 0.5ml of chloroform/methanol (2:1, v/v) plus 0.2ml of 0.1M-Tris/HCl buffer (pH6.5). The tubes were left for at least 1h. Portions of the upper phase were analysed for radioactive choline, phosphorylcholine and CDP-choline, and portions of the lower phase were analysed for radioactive phosphatidylcholine as described above. In some experiments, cells in 0.1ml portions of the incubation medium were collected by rapid filtration on Oxoid membrane filters. The cells on the filter were quickly washed with Tris/HCl buffer (0.1M, pH6.5), dried, and total cell radioactivity was determined by direct scintillation counting after the dried disc had been arranged around the inside circumference of a counting vial. This agreed well with that computed from the measurements of phosphatidylcholine and water-soluble choline components.

Materials

[methyl-¹⁴C]Choline (specific radioactivity, 57 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Only the choline spot was radioactive when a portion was subjected to high-voltage ionophoresis. Phosphoryl[methyl-¹⁴C]-choline and CDP-[methyl-¹⁴C]choline were prepared as described previously (Broad & Dawson, 1975). All other reagents used were of analytical-reagent grade.

Reproducibility

Each type of experiment described was carried out three times with minor variations in the experimental

procedure, and the results agreed in all essential details.

Results

Subcellular location of enzymes involved in phosphatidylcholine biosynthesis

The subcellular distribution of enzymes involved in phosphatidylcholine biosynthesis is shown in Table 1. The incorporations of choline, phosphorylcholine and CDP-choline into intermediates and products of phosphatidylcholine biosynthesis by various cell fractions were determined. Choline kinase (EC 2.7.1.32) was present in all cell fractions, with the lowest specific activities found in the cell envelope.

In contrast with the ubiquitous distribution of choline kinase activity, choline phosphate cytidylyl-transferase (EC 2.7.7.15) activity was present only in the soluble fraction of the cell. However, CDP-choline-1,2-diacylglycerol cholinephosphotransferase activity (EC 2.7.8.2) was located in all membrane fractions, with only very low activity in the supernatant fraction. As with choline kinase, the lowest specific activity in the membrane fractions was in the cell envelope.

Reconstitution of phosphatidylcholine-biosynthesis activity

Since the three enzymes involved in the incorporation *de novo* of choline into phosphatidylcholine are located in different cell fractions, it seemed likely that, unless some other pathway was operative (e.g. choline exchange), then incorporation into the phospholipid will only occur when the soluble

Table 1. *Incorporation of precursors of phosphatidylcholine biosynthesis by various cell fractions of E. caudatum*

Cell fractions were prepared from washed *E. caudatum* cells as described in the Experimental section. Incubation vessels contained the basic reaction mixture (see the Experimental section) with choline (10⁶d.p.m.), phosphorylcholine (10⁵d.p.m.) or CDP-choline (10⁵d.p.m.) as indicated. Approx. 0.5mg of protein of each cell fraction was present. Incubations were for 40min at 39°C. All results are expressed as d.p.m./mg of protein.

Cell fraction	Incorporation of [¹⁴ C]choline into:			Incorporation of phosphoryl[¹⁴ C]choline into:		Incorporation of CDP-[¹⁴ C]choline into: Phosphatidylcholine
	Phosphorylcholine	CDP-choline	Phosphatidylcholine	CDP-choline	Phosphatidylcholine	
Whole homogenate	238 000	1770	2012	253	935	22 450
Cell envelope	27 800	0	0	0	0	7190
Membrane fraction A (10min, 6000g)	107 600	0	120	0	0	23 440
Membrane fraction B (10min, 6000g)	155 700	0	0	0	0	9395
Membrane fraction C (60min, 144 000g)	209 400	0	180	0	0	13 160
Supernatant	231 750	4280	0	1440	0	1290

Fig. 1. Influence of the soluble fraction of the cell on the ability of the membrane fractions to synthesize phosphatidylcholine *de novo*

The composition of the reaction mixture was as described in the Experimental section, with [methyl- ^{14}C]choline (10^6 d.p.m.) as precursor. Approx. 0.5 mg of membrane protein was present and the concentration of soluble protein varied as indicated. Incubations were for 40 min at 39°C . The membrane fractions were A (●), B (□), C (○) and cell envelope (▲).

fraction is added back to the membrane fractions. In further experiments a fixed concentration of each membrane fraction was incubated in the presence of various amounts of supernatant fraction (Fig. 1). In the absence of supernatant fraction no labelled phosphatidylcholine was formed. The capacity for phosphatidylcholine biosynthesis *de novo* by the membrane fractions increased approximately linearly as the amount of supernatant fraction was increased. The degree of stimulation between the membrane fractions A, B and C was somewhat variable between experiments but was always greater than that of the cell envelope.

Further experiments showed that the addition of supernatant fraction did not stimulate the incorporation of labelled CDP-choline into phosphatidylcholine by membrane fractions.

Cofactor requirements for synthesis de novo of phosphatidylcholine by a reconstituted system from E. caudatum

The work of Kennedy & Weiss (1956) established the requirements for CTP, ATP and Mg^{2+} in the incorporation of choline into phosphatidylcholine by liver cell fractions. Fig. 2 shows the requirement for CTP and ATP in a reconstituted membrane plus supernatant system from *E. caudatum*. The requirement for CTP was absolute; maximum incorporation was obtained at 1 mM-CTP, with half-maximum stimulation at about 0.4 mM-CTP. Increasing concentrations of ATP also stimulated phosphatidylcholine synthesis. Optimum stimulation occurred at approx. 2.5 mM-ATP and half-maximum stimulation at about 1 mM-ATP. Similar effects of CTP and ATP were obtained when a homogenate was used in place of the reconstituted system.

Fig. 3 shows the effect of increasing Mg^{2+} concentrations on the incorporation of labelled choline, phosphorylcholine and CDP-choline into products on the synthetic pathway. Each of the enzymic steps has an absolute requirement for Mg^{2+} . Near-optimum rates of activity were obtained with Mg^{2+} concentrations of 5–10 mM. Higher concentrations of Mg^{2+} inhibited each of the enzymes involved except for choline kinase.

In confirmation of previous experiments (Broad & Dawson, 1975), 1,2-diacyl-*sn*-glycerol did not enhance the incorporation of choline into phosphatidylcholine by reconstituted systems.

Inhibitors of phosphatidylcholine synthesis in E. caudatum

Ca^{2+} ions at low concentrations are potent inhibitors of synthesis of phosphatidylcholine *de novo* in mammalian systems by their action on CDP-choline – diacylglycerol cholinephosphotransferase (Borkenhagen & Kennedy, 1957; McCaman & Cook, 1966; Kanoh & Ohno, 1973). It was therefore clearly of interest to examine their effect on phosphatidylcholine biosynthesis in the *E. caudatum* reconstituted system.

Fig. 4(a) shows the effect of increasing concentrations of added Ca^{2+} on the incorporation of choline into phosphorylcholine, CDP-choline and phosphatidylcholine at a fixed concentration (15 mM) of Mg^{2+} . The incorporation of choline into phosphorylcholine was usually slightly stimulated by Ca^{2+} , whereas the incorporation into CDP-choline was slightly inhibited. The incorporation

Fig. 2. Effect of ATP and CTP concentration on the incorporation of choline into phosphatidylcholine by the reconstituted system

The composition of the reaction mixture was as described in the Experimental section with [*methyl*- ^{14}C]choline (10^6 d.p.m.) as precursor. Each reaction vessel contained 1 mg of protein of membrane fraction B and 0.3 mg of supernatant protein. In (a) the CTP concentration was varied while the ATP concentration was kept at 5 mM; in (b) ATP concentration changed and CTP kept at 0.5 mM. Incubations were for 40 min at 39°C .

into phosphatidylcholine was very sensitive to Ca^{2+} , with almost total inhibition occurring at 1 mM. The incorporation of labelled CDP-choline into phosphatidylcholine was likewise virtually stopped by 1 mM- Ca^{2+} (Fig. 4b).

Hemicholinium-3 is a potent inhibitor of phosphatidylcholine biosynthesis, and it has been shown to inhibit choline kinase in brain (Spanner & Ansell, 1974) and in the soluble fraction from *E. caudatum* (Broad & Dawson, 1974). Fig. 5 shows the effect of hemicholinium-3 concentration on the incorporation of labelled choline, phosphorylcholine and CDP-choline into phosphatidylcholine by the reconstituted system. Of the three precursors used, only free choline incorporation is inhibited by very low concentrations of hemicholinium-3. An inhibition of 98% occurred at about 0.5 mM-hemicholinium-3, and half-maximal inhibition was at about $40\text{ }\mu\text{M}$. Concentrations of the inhibitor up to 3 mM had no effect on the CDP-choline-diacylglycerol choline-phosphotransferase reaction. These results show that the site of inhibitory action of hemicholinium-3

on phosphatidylcholine synthesis is primarily through inhibition of choline kinase. Hemicholinium-15 had little effect on the choline kinase of *E. caudatum* when tested at similar concentrations.

Uptake of choline into intact E. caudatum cells

It has been shown that the uptake of choline by intact *E. caudatum* cells was extremely rapid compared with the uptake of ethanolamine (Broad & Dawson, 1975). In contrast, when labelled phosphorylcholine or CDP-choline was incubated with cells virtually no uptake of radioactivity could be detected (Fig. 6) compared with that of choline, nor did the addition of excess of unlabelled CDP-choline or phosphorylcholine interfere with the uptake of radioactive choline by the organism. The total uptake of choline over a 15 min period exhibited Michaelis-Menten kinetics, with an apparent K_m of $20\text{ }\mu\text{M}$. This was of the same order as that observed for choline incorporation into

Fig. 3. Effect of Mg^{2+} concentration on phosphatidylcholine biosynthesis from labelled choline, phosphorylcholine and CDP-choline by the reconstituted system

The composition of the reaction mixture was as described in the Experimental section, with [*methyl*- ^{14}C]choline (10^6 d.p.m.), phosphorylcholine (10^5 d.p.m.) or CDP-choline (10^5 d.p.m.) present as indicated. Each reaction mixture contained 1 mg of protein of membrane fraction B and 0.3 mg of supernatant protein fraction. Incubations were for 40 min at $39^\circ C$. (a) Incorporation of choline into phosphorylcholine ($\bullet$), CDP-choline (Δ) and phosphatidylcholine ($\circ$); (b) incorporation of phosphorylcholine into CDP-choline (Δ) and phosphatidylcholine ($\circ$); (c) incorporation of CDP-choline into phosphatidylcholine ($\circ$).

phosphorylcholine catalysed by a supernatant fraction of the organism (K_m $25 \mu M$).

The uptake of choline into the cells was substantially inhibited by hemicholinium-3 (Fig. 7), as has been observed for many other systems in which choline transport into cells and vesicles has been studied (Diamond & Milfay, 1972). The concentration range of hemicholinium-3 producing inhibition of choline transport into the intact *E. caudatum* cells was very similar to that producing inhibition of choline kinase in both membrane and supernatant fractions from sonicated cells (Fig. 7).

In further experiments, the short-term uptake of [^{14}C]choline into intact cells was determined as a function of temperature and measurements were made of the distribution of the radioactivity between the free choline, phosphorylcholine, CDP-choline and phosphatidylcholine components. At no time or temperature, including determinations made with less than 1 min incubation, could radioactivity be detected in the free choline fraction or in CDP-choline. At $0^\circ C$ there was no uptake of choline into the cells, whereas at $10^\circ C$ the small uptake of radioactivity could be almost entirely accounted for as phosphorylcholine (Fig. 8). At higher temperatures

(20° , 30° , $40^\circ C$) a linear increase in the labelling of phosphatidylcholine with time was apparent, but phosphorylcholine still remained the predominant reservoir for label for times up to 3 min. The initial rate of phosphorylcholine and phosphatidylcholine formation as a percentage of the rate obtained at $40^\circ C$ was compared with the rate of total choline uptake determined by filtration of the cells on a membrane, rather than by centrifugation. Such experiments show that the variation in the rate of choline phosphorylation with temperature closely correlates with the total uptake of choline into the cells.

Discussion

The subcellular fractionation of *E. caudatum* used in the present investigation cannot be compared with that of a typical mammalian cell such as the hepatocyte. These protozoa, being strictly anaerobic, contain no mitochondria and under the electron microscope there is nothing resembling an endoplasmic reticulum, as is seen with many other protozoan species. The cell-envelope fraction isolated

Fig. 4. Effect of Ca^{2+} concentration on the incorporation of $[^{14}C]$ choline and CDP- $[^{14}C]$ choline into phosphatidylcholine and intermediates in its synthesis

The composition of the reaction mixture was as described in the Experimental section, with [*methyl*- ^{14}C]choline (10^6 d.p.m.) (a) or CDP-choline (10^6 d.p.m.) (b). EGTA was omitted from the reaction medium and the concentration of Ca^{2+} varied as indicated. Mg^{2+} was present at a concentration of 15 mM. Each reaction vessel contained 1 mg of protein of membrane fraction B and supernatant fraction (0.3 mg). Incubations were for 40 min at $39^\circ C$. Incorporation into phosphorylcholine ($\bullet$), CDP-choline (Δ) and phosphatidylcholine ($\circ$) was measured.

appears to be pure on electron-microscopic examination, but in the absence of defined marker enzymes it is difficult to be entirely sure that it is uncontaminated with other membrane fragments. The separation of the other membrane fractions may be described as purely operational, with little evidence from electron-microscopic examination of their morphological origin. Nevertheless examination of these fractions does yield information about the location of the enzymes involved in phosphatidylcholine synthesis within the organism.

The location of the choline phosphate cytidyltransferase activity solely in the soluble fraction of the cell and of CDP-choline-1,2-diacylglycerol cholinephosphotransferase in the membrane fractions is in line with previous studies on other types of cell (Fiscus & Schneider, 1966; Stoffel & Schiefer, 1968; McMurray & Dawson, 1969). However, it is

perhaps surprising that significant amounts of choline kinase activity were found in all membrane fractions even after washing, since in many other cell systems this activity is confined to the soluble fraction of the cell (Weinhold & Rethy, 1974). It is possible that this may be connected with a role of the enzyme in trapping and concentrating choline within the cell, which will be discussed below. That none of the membrane fractions on their own could carry out phosphatidylcholine synthesis from free choline is undoubtedly due to the exclusive presence of choline phosphate cytidyltransferase in the supernatant fraction. Thus all of the membrane fractions, including the cell envelope, were able to synthesize phosphatidyl $[^{14}C]$ choline from free $[^{14}C]$ choline if supernatant fraction was added or if they were supplied with CDP- $[^{14}C]$ choline in the absence of supernatant. Roberts & Bygrave (1973) have shown

Fig. 5. Effect of hemicholinium-3 concentration on the incorporation of [^{14}C]choline, phosphoryl[^{14}C]choline and CDP-[^{14}C]choline into phosphatidylcholine by the reconstituted system

The composition of the reaction medium was as described in the Experimental section with radioactive choline (●), phosphorylcholine (○) or CDP-choline (▲) as precursor. The cell fractions present were membrane B (1mg of protein) and supernatant fraction (0.3mg of protein). Incubations were for 40min at 39°C.

Fig. 6. Inability of intact cells to take up labelled CDP-choline or phosphorylcholine

Experiments were carried out as described in Fig. 8, except that the cells were collected and washed on membrane filters at the times indicated after the addition of radioactive choline (●), CDP-choline or phosphorylcholine (○).

Fig. 7. Effect of hemicholinium-3 concentration on uptake of choline by intact cells and choline phosphorylation by cell fractions

The reactions were carried out as described in the Experimental section, with the indicated concentrations of hemicholinium-3 present during the preincubation and reaction periods. A cell sonicate, prepared as described in the Experimental section, was centrifuged for 60min at 144000g to prepare total membrane and supernatant fractions. Δ, Choline uptake into whole cells; ●, choline phosphorylation by total membrane fraction; ○, choline phosphorylation by supernatant.

that a somewhat similar situation exists with the membrane fractions of rat liver, where no phosphatidylethanolamine synthesis from [^{14}C]ethanolamine can take place in the absence of supernatant fraction.

The synthesis of phosphatidylcholine by the cell envelope can be contrasted with the plasma membranes of the hepatocyte (Stahl & Trams, 1968; Victoria *et al.*, 1971) and neurons (Miller & Dawson, 1972), which are devoid of CDP-choline-1,2-diacylglycerol cholinephosphotransferase.

The inhibitory effect of Ca^{2+} on phosphatidylcholine synthesis from free choline agrees with that observed in equivalent mammalian systems (McCaman & Cook, 1966; Kanoh & Ohno, 1973; see also Roberts & Bygrave, 1973), and this inhibition is again largely confined to CDP-choline-1,2-diacylglycerol cholinephosphotransferase (Borkenhagen & Kennedy, 1957). Ca^{2+} did not inhibit choline kinase, even though this enzyme, like the others on the synthetic chain, is Mg^{2+} -dependent. Hemicholinium-3, on the other hand, inhibits phosphatidylcholine synthesis through its action on choline kinase: its effects on the other enzymes involved in phosphatidylcholine synthesis are minimal.

Our observations suggest that choline kinase plays a central role in the rapid uptake and accumulation of choline in the cells. On incubation of cells

Fig. 8. Influence of temperature on initial rates of uptake and incorporation of choline into phosphorylcholine and phosphatidylcholine by intact cells

The reaction vessel contained the basic incubation medium (see the Experimental section). After a 1 min preincubation at 10° (a), 20° (b), 30° (c) or 40°C (d), the reaction was started by the addition of 14 μ M-choline containing 5×10^6 d.p.m. of [methyl- 14 C]choline. At the times indicated 0.3 ml portions were removed and rapidly transferred to Eppendorf microfuge tubes standing on ice and containing 0.3 ml of 20 mM-hemicholinium-3. The cells were washed by centrifugation and the distribution of radioactivity was determined. ●, Phosphorylcholine; ○, phosphatidylcholine.

with [14 C]choline, even for short periods or at low temperatures, no free choline could be detected within the cell: all of it had become phosphorylated and then partially converted into phosphatidylcholine. No radioactive CDP-choline could be detected, suggesting that in the living cell choline phosphate cytidylyl-transferase is the rate-limiting step in phosphatidylcholine biosynthesis. This rapid quantitative phosphorylation of the choline entering the cell can be contrasted with uptake of choline into synaptosomes, where within 10 min very little metabolism of the free choline entering the nerve ending has occurred (Diamond & Kennedy, 1969). It is more akin to the situation in rat hepatoma cells grown in suspension culture, where the bulk of the choline entering is converted into phosphorylcholine (Plagemann, 1968).

In nervous tissue and erythrocytes a specific carrier-mediated transport system for choline uptake has been described (Diamond & Milfar, 1972; Martin, 1972). In ascites tumour (Haeflner, 1975) or hepatoma cells (Plagemann, 1971) it is suggested that a similar facilitated diffusion system appertains at low concentrations of choline (below 30 μ M) and exhibits Michaelis-Menten kinetics. At higher

choline concentrations simple diffusion appears to be the principal mode of uptake.

The present observations, showing that choline entry into the *E. caudatum* cell is substrate-saturable (K_m 20 μ M), indicates that the uptake is not by simple diffusion through the cell envelope, at least at low choline concentrations. It is possible therefore that a carrier exists in the cell envelope of *E. caudatum* similar to that in other cells, so that the choline transported through the membrane is almost instantly converted into phosphorylcholine by the excess of choline kinase present within the cell. However, it could be that the choline kinase is more directly involved in the entry process. Thus the uptake of choline cannot be distinguished from choline phosphorylation even by lowering the temperature to a point at which choline uptake by the cell is negligible. The apparent K_m of choline uptake at 39°C by *E. caudatum* cells is of the same order as that observed for choline phosphorylation in sonicates of the same cells. This can be contrasted with the situation in hepatoma cells, where the apparent K_m for choline uptake is at least one order of magnitude lower than that for the phosphorylation of the base by cell-free extracts (Plagemann & Roth,

1969). In addition, in *E. caudatum* cells the concentration of hemicholinium-3 producing a given inhibition of choline uptake into the cells is similar to that giving an equivalent inhibition of choline kinase in cell-free preparations. Whether or not choline kinase is involved in the actual entry of choline into the cell, the rapid quantitative phosphorylation of choline probably provides a mechanism for trapping the base within the cell in a form that ensures that its loss from the cell is minimal. Our observations suggest that phosphorylcholine is not taken up by the cell, and this is likely to be true for its permeability in the reverse direction. An efficient mechanism for trapping choline in this manner would be essential for an organism that has an obligatory nutritional requirement for choline, and where the presence of this base in its normal environment, i.e. the rumen, is likely to be at very low concentration and transitory (Broad & Dawson, 1976). The high activity of choline kinase in this organism and its presence in cell-membrane fractions as well as cytoplasm could account for the cell's extraordinary ability to concentrate choline (Broad & Dawson, 1975). Sonicates of *Tetrahymena pyriformis*, a protozoon that in contrast with *E. caudatum* can synthesize choline by the methylation pathway (Smith & Law, 1970), phosphorylates choline at no more than a few per cent of the rate observed in *E. caudatum* (Broad & Dawson, 1975).

F. L. B. is indebted to the Royal Society and to the Wellcome Trust for travel grants. Mr. A. Letcher is thanked for very valuable assistance.

References

- Borkenhagen, L. F. & Kennedy, E. P. (1957) *J. Biol. Chem.* **227**, 951-962
- Broad, T. E. & Dawson, R. M. C. (1974) *Biochem. Soc. Trans.* **2**, 1272-1274
- Broad, T. E. & Dawson, R. M. C. (1975) *Biochem. J.* **146**, 317-328
- Broad, T. E. & Dawson, R. M. C. (1976) *J. Gen. Microbiol.* **92**, 391-397
- Bygrave, F. & Dawson, R. M. C. (1975) *Biochem. Soc. Trans.* **3**, 740-741
- Coleman, G. S. (1960) *J. Gen. Microbiol.* **22**, 555-563
- Coleman, G. S. (1971) in *Isolation of Anaerobes* (Shapton, D. A. & Board, R. G., eds.), pp. 159-176, Academic Press, London
- Dawson, R. M. C. & Kemp, P. (1967) *Biochem. J.* **105**, 837-842
- Diamond, I. & Kennedy, E. P. (1969) *J. Biol. Chem.* **244**, 3258-3263
- Diamond, I. & Milfay, D. (1972) *J. Neurochem.* **19**, 1899-1909
- Fiscus, W. G. & Schneider, W. C. (1966) *J. Biol. Chem.* **241**, 3324-3330
- Haeflner, E. W. (1975) *Eur. J. Biochem.* **51**, 219-228
- Kanoh, H. & Ohno, K. (1973) *Biochim. Biophys. Acta* **306**, 203-217
- Kennedy, E. P. & Weiss, S. B. (1956) *J. Biol. Chem.* **222**, 193-214
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Martin, K. (1972) *J. Physiol. (London)* **224**, 207-230
- McCaman, R. E. & Cook, K. (1966) *J. Biol. Chem.* **241**, 3390-3394
- McMurray, W. C. & Dawson, R. M. C. (1969) *Biochem. J.* **112**, 91-108
- Miller, E. & Dawson, R. M. C. (1972) *Biochem. J.* **126**, 805-823
- Plagemann, P. G. W. (1968) *Arch. Biochem. Biophys.* **128**, 70-87
- Plagemann, P. G. W. (1971) *J. Lipid Res.* **12**, 715-724
- Plagemann, P. G. W. & Roth, M. F. (1969) *Biochemistry* **8**, 4782-4789
- Roberts, J. B. & Bygrave, F. L. (1973) *Biochem. J.* **136**, 467-475
- Smith, J. D. & Law, J. H. (1970) *Biochim. Biophys. Acta* **202**, 141-152
- Spanner, S. & Ansell, G. B. (1974) *J. Neurochem.* **22**, 1153-1155
- Stahl, W. L. & Trams, E. G. (1968) *Biochim. Biophys. Acta* **163**, 459-471
- Stoffel, W. & Schiefer, H. G. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 1017-1026
- Victoria, E. J., van Golde, L. M. G., Hostetler, K. Y., Scherphof, G. L. & van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta* **239**, 443-457
- Weinhold, P. A. & Rethy, V. B. (1974) *Biochemistry* **13**, 5135-5140